Unusual Chemokine Receptor Antagonism Involving a Mitogen-Activated Protein Kinase Pathway

Patricia Ogilvie, Sylvia Thelen, Barbara Moepps, Peter Gierschik, Ana Claudia da Silva Campos, Marco Baggiolini and Marcus Thelen

*J Immunol* 2004; 172:6715-6722; doi: 10.4049/jimmunol.172.11.6715
http://www.jimmunol.org/content/172/11/6715

References
This article cites 66 articles, 36 of which you can access for free at:
http://www.jimmunol.org/content/172/11/6715.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Antagonism of chemokines on chemokine receptors constitutes a new regulatory principle in inflammation. Eotaxin (CCL11), an agonist for CCR3 and an attractant of eosinophils, basophils, and Th2 lymphocytes, was shown to act as an antagonist for CCR2, which is widely expressed on leukocytes and is essential for inflammatory responses. In this report we provide direct evidence for a novel mechanism how chemokine receptor function can be arrested by endogenous ligands. We show that binding of eotaxin to CCR2 stimulates the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 (ERK1/2). Activation of the mitogen-activated protein kinase kinase 1/2-ERK pathway is indispensable for eotaxin-mediated attenuation of CCR2 function, as inhibition of ERK phosphorylation abolishes the arresting effect. ERK is also activated by CCR2 agonists, e.g., monocyte chemotactic protein-1 (MCP-1). However, the involved pathways are different, although in either case coupling of CCR2 to pertussis toxin-sensitive heterotrimeric G proteins is necessary. The results are in agreement with the view that CCR2 could assume different activation states depending on the ligand it encounters. With respect to actin polymerization and calcium mobilization, the different activation states lead to agonistic and antagonistic responses. It is conceivable that the intracellular signal transduction pathway that is activated by eotaxin could cause an attenuation of proinflammatory responses mediated by CCR2.

Eotaxin (CCL11), a major mediator of allergic reactions, is a partial agonist of CCR2 (6). In the presence of eotaxin, MCP-1-stimulated secretion of lysosomal N-acetyl-β-D-glucosaminidase from monocytes and chemotaxis is markedly diminished (6). Eotaxin itself induces these functional responses only marginally, if at all, and does not trigger CCR2 internalization. Eotaxin further competes with MCP-1 for binding to CCR2 (6). Activation of CCR2 is essential for the recruitment and activation of monocytes, T cells, and dendritic cells (15–17). It was concluded that during allergic inflammations eotaxin can desensitize CCR2 for subsequent stimulation with the agonist MCP-1 and thus contributes to the fine-tuning of the immune response.

Activation of GPCR requires their functional coupling to G proteins (4, 18). After ligand binding the G protein rapidly exchanges GDP for GTP, and the GTP-loaded Gα and βγ subunit dissociate from the receptor to activate downstream effectors. Pertussis toxin treatment causes uncoupling of the G protein from the receptor (19–21) and abolishes most receptor-mediated responses, but does not prevent receptor phosphorylation and internalization (22, 23). The latter are considered the main mechanisms for termination of receptor-mediated responses (24, 25). Downstream of G proteins, chemokines induce activation of kinases, including phosphoinositide 3-kinase (PI 3-kinase) and extracellular signal-regulated kinase (ERK) (26, 27).

The mechanism by which eotaxin inhibits MCP-1-mediated CCR2 activation involves neither receptor phosphorylation nor receptor internalization (6), but depends on eotaxin-stimulated PI 3-kinase and mitogen-activated protein kinase kinase (MEK)/ERK activation. We suggest that CCR2 could adopt two activation states depending on the triggering chemokine. The different conformations induce agonistic and antagonistic responses with respect to actin polymerization and calcium mobilization.

Materials and Methods

Cells

Monocyte isolation from donor buffy coats and stable transfection of human CCR2 in murine pre-B 300.19 cells were performed as described previously (6). Influenza virus hemagglutinin (HA) eptope-tagged human CCR2 (HA-CCR2) was transiently expressed in HEK 293 cells using Lipofectamine (TransFast; Promega, Madison, WI) and in 300.19 cells by
Electroporation. CCR2 surface expression was assessed by FACS analysis (FAB151P; R&D Systems, Abingdon, U.K.).

**Chemokines**

All chemokines were provided by Prof. I. Clark-Lewis (Vancouver, Canada).

**Calcium transients**

Changes in the intracellular Ca²⁺ concentration ([Ca²⁺]), were measured in fura-2-loaded cells as previously described (28). Before stimulation, cells were incubated in buffer with or without the indicated MEK1/2 inhibitors (PD 98059 or U0126; both from Alexis, Lausen, Switzerland) or hydroxyworthmann (29) for 20 min at 37°C. The rate of rise in [Ca²⁺] was calculated from the ratio of the mean fluorescence intensity of stimulus-induced and basal phalloidin binding.

**Actin polymerization**

Filamentous actin (F-actin) formation was determined as follows. The incubations were stopped 10 s after the final stimulation, and cells were fixed with cold paraformaldehyde (4%) in PBS. Cells were permeabilized with 0.1% Triton X-100, stained with 6 μg/ml FITC-conjugated phalloidin (Sigma-Aldrich, Buchs, Switzerland), and analyzed by FACS. PD98059 pre-treatment was performed as described above. F-actin formation is calculated from the ratio of the mean fluorescence intensity of stimulus-induced and basal actin binding.

**Cell stimulation and Western blot analysis**

Transfected cells were serum-starved in RPMI 1640 containing 25 mM HEPES (RPMI/HEPES; Invitrogen, Switzerland) for 6 h at 37°C. CCR2 surface expression was assessed by FACS analysis (FAB151P; R&D Systems, Abingdon, U.K.) in RMPI/HEPES culture medium. An aliquot of the cells was used in the experiment immediately after isolation. Aliquots of 10⁶ cells were incubated in RPMI/HEPES for 10 min at 37°C, stimulated for the indicated times. Reactions were terminated by addition of TCA. Whole-cell lysates were separated by SDS-PAGE and transferred to polyvinylidene di fluoride (PVDF) membranes. Activated ERK was detected with an anti-diphospho-ERK Ab (M8159; Sigma-Aldrich); for all experiments equal loading was confirmed by reprobing with an Ab against ERK2 (C-14; Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (27).

**[^35]S[GTP-guanosine 5′-γ-thio]triphosphate ([^35]S[GTPγS]) binding**

Binding of [35S]GTPγS to membranes of CCR2-transfected HEK 293 cells was assayed as previously described (30). Briefly, cells were homogenized and fractionated into soluble and particulate fractions. Aliquots of the membrane fraction (7.5 μg protein/sample) were incubated with 0.34 nM [35S]GTPγS and buffer, MCP-1 (200 nM), eotaxin (500 nM), or a combination of both chemokines for 60 min at 30°C. The samples were analyzed for bound [35S]GTPγS by rapid filtration and scintillation counting.

**Immunoprecipitation**

300.19 cells (5 × 10⁶ cells/ml) expressing human HA-CCR2 (~20% CCR2+) were incubated for 2 h at 37°C in the absence or the presence of 2 μg/ml Bordetella pertussis toxin (List Biological Laboratories, Campbell, CA) in RPMI/HEPES culture medium. An aliquot of the cells was treated for pertussis toxin-mediated inhibition of ERK activation as described above. The remaining cells were washed in PBS; resuspended in 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, and 10 mM K⁺-PIPES, pH 7.3; and subjected to nitrogen cavitation (30 atm, 20 min) on ice. Homogenates were supplemented with 1.25 mM EGTA, nuclei and cell debris were removed by low speed centrifugation, and a postnuclear pellet was obtained by high speed centrifugation. Pellets (~1 × 10⁶ cell equivalent) were solubilized for 45 min on ice with 200 μl of 100 mM KCl, 20 mM NaCl, 1 mM EGTA, 2 mM MgCl₂, 0.1% BSA, 5 μM GTP containing 1% nonylβ-D-glucopyranoside (Anatrace, Maumee, OH), 0.12% cholesterol hemisuccinate (Sigma-Aldrich), 2% heptanetriol (Fluka, Buchs, Switzerland), and 5% glycerol. After centrifugation (400,000 × g, 10 min), 180 μl of the supernatants were diluted with 540 μl of buffer without detergents. HA-CCR2 was immunoprecipitated with an anti-HA Ab (clone 12CA5; Roche, Basel, Switzerland), and protein complexes were resolved by 8.5% SDS-PAGE and transferred to PVDF membranes. The membranes were first probed with an affinity-purified anti-Gα polyclonal Ab, then reprobed with anti-HA to confirm equal loading.

![FIGURE 1](http://www.jimmunol.org/content/156/9/6716/F1a)

FIGURE 1. Eotaxin and MCP-1 stimulate ERK activation via CCR2. A, Mouse 300.19 pre-B cells stably expressing CCR2 were treated with the indicated concentrations of MCP-1 and eotaxin for 90 s at 37°C. The upper panel shows a typical result obtained by Western blot analysis. Gel lysates separated by SDS-PAGE and transferred to PVDF membranes were first probed with anti-diphospho-ERK (dpERK; upper row), then the membranes were stripped and reprobed for total ERK2 (lower row). The lower panel depicts normalized data from up to six experiments. ECL signals of dpERK were integrated, corrected for total ERK contents, and normalized to maximum ERK activation obtained with 1 μM MCP-1. B, Inhibition of ERK activation in CCR2+ 300.19 cells treated with 100 nM MCP-1 or 300 nM eotaxin. Cells were pretreated for 60 min with the indicated concentrations of U0126, PD98059, or DMSO (0.1%) and then treated for 90 s with the indicated chemokines in the presence of inhibitors. Western blot analysis was performed as described in A. C, Time course of ERK activation in transiently transfected HEK293 cells (upper panels) and freshly isolated human monocytes (lower panel). Western blot analysis was performed as described in A. Cells were treated with 100 nM MCP-1 or 300 nM eotaxin. Untransfected control HEK293 cells were treated with 100 nM MCP-1 for 90 s (control (C)).
Receptor phosphorylation

300.19 cells expressing human HA-CCR2 were serum-starved for 6 h in phosphate-free medium, labeled with 32P, and stimulated with the chemokines with or without PD98059 pretreatment. Cells were lysed in RIPA buffer (25 mM Tris-Cl (pH 8), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS), and HA-CCR2 was immuno-precipitated with an anti-HA Ab (clone 12CA5; Roche). Immunoprecipitates were resolved by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were first exposed on PhosphorImager screens (Amersham Biosciences, Otelingen, Switzerland), then probed with anti- 

 Fluorescence microscopy

300.19 cells expressing CCR2 were transiently transfected with 5 µg of a plasmid encoding green fluorescent protein fused to the pleckstrin homology domain of protein kinase B (GFP-PH-Akt; provided by Dr. T. Meyer, Stanford University Medical Center, Stanford, CA) alone or together with 50 µg of a plasmid coding for a dominant negative form of the regulatory subunit of type I PI 3-kinase p85/p110 (Ap85) (31). As a control, cells were transfected with 5 µg of a plasmid encoding an inactive variant of GFP-PH-AktR25C (32). After 12 h, cells were plated on polylysine (5 µg/ml in PBS)-coated coverslips and serum-starved for 6 h. Where indicated, 100 nM wortmannin was included for the last 30 min. Cells were then treated with chemokine or buffer for 90 s, and the reactions were terminated by rapidly replacing the medium with 4% ice-cold paraformaldehyde in PBS. After fixation for 20 min on ice, coverslips were washed with PBS and mounted in Gelvatol (Air Products and Chemicals, Utrecht, The Netherlands) as previously described (33).

Statistical analysis

Student’s t test was used to determine the significance between the means (error bars represent the SD) of the corresponding data points.

Results

It has been reported that eotaxin antagonizes MCP-1-induced responses on CCR2 (6), but also acts as a partial agonist at this receptor at elevated concentrations (1 µM) (14). Chemokines are known to stimulate the mitogen-activated protein kinase (MAPK) cascade (4) that leads to the dual phosphorylation of ERK1 and ERK2 in their activation loop. We therefore tested the capacity of eotaxin to activate the MAPK cascade via CCR2. Fig. 1A illustrates that both eotaxin and MCP-1 induce, in a concentration-dependent manner, the activation of the MAPKs ERK1/2 in murine pre-B cells (300.19) stably transfected with CCR2. Western blot analysis of doubly phosphorylated ERK shows a weak activation with 10 nM eotaxin and a full response with ~1 µM. Specifically, quantitative analysis from several experiments reveals that maximum eotaxin-stimulated ERK phosphorylation reaches ~30-40% of the maximum level obtained with MCP-1. Thus, eotaxin-mediated ERK activation is less potent, and a right-shifted dose response compared with MCP-1 is consistent with the reported lower affinity of eotaxin for CCR2 (6, 14). Both eotaxin and MCP-1 stimulate transient activation of ERK activation, and maximum phosphorylation is observed after 1–2 min in CCR2+ 300.19 cells (Figs. 1C and 3A). Fig. 1B shows that phosphorylation of ERK1/2 was sensitive to inhibitors of the upstream kinases MEK1/2. Two well-characterized inhibitors, U0126 (34) and PD98059 (35), obliterated eotaxin and MCP-1-stimulated ERK activation in a concentration-dependent manner. The inhibitors also blocked chemokine-induced ERK activation in monocytes (not shown). Thus, eotaxin and MCP-1 trigger the activation of ERK through a MAPK cascade.

Similar activation of the MAPK cascade was obtained in transiently transfected HEK 293 cells upon addition of eotaxin and MCP-1. Fig. 1C (upper panel) depicts the typical time course of CCR2-mediated ERK activation in these cells. Untransfected HEK293 (Fig. 1C, lane C) and parental or mock-transfected 300.19 cells (not shown) did not show any increase in ERK phosphorylation upon stimulation with either chemokine, confirming that eotaxin and MCP-1 signal through CCR2. Also, in freshly isolated human monocytes both chemokines stimulated the transient phosphorylation of ERK1/2 shown in Fig. 1C (lower panel). Eotaxin and MCP-1 induced rapid increases in phosphorylation, which returned to baseline within 3 min.

It has been reported that eotaxin antagonizes MCP-1-induced responses on CCR2 (6). To test this, we used HEK293 transient transfections to directly compare the chemokine responses. MCP-1 stimulation of ERK activation is consistent with the reported lower affinity of eotaxin for CCR2 (6, 14). Both eotaxin and MCP-1 stimulate transient activation of ERK activation, and maximum phosphorylation is observed after 1–2 min in CCR2+ 300.19 cells (Figs. 1C and 3A). Fig. 1B shows that phosphorylation of ERK1/2 was sensitive to inhibitors of the upstream kinases MEK1/2. Two well-characterized inhibitors, U0126 (34) and PD98059 (35), obliterated eotaxin and MCP-1-stimulated ERK activation in a concentration-dependent manner. The inhibitors also blocked chemokine-induced ERK activation in monocytes (not shown). Thus, eotaxin and MCP-1 trigger the activation of ERK through a MAPK cascade.

Similar activation of the MAPK cascade was obtained in transiently transfected HEK 293 cells upon addition of eotaxin and MCP-1. Fig. 1C (upper panel) depicts the typical time course of CCR2-mediated ERK activation in these cells. Untransfected HEK293 (Fig. 1C, lane C) and parental or mock-transfected 300.19 cells (not shown) did not show any increase in ERK phosphorylation upon stimulation with either chemokine, confirming that eotaxin and MCP-1 signal through CCR2. Also, in freshly isolated human monocytes both chemokines stimulated the transient phosphorylation of ERK1/2 shown in Fig. 1C (lower panel). Eotaxin and MCP-1 induced rapid increases in phosphorylation, which returned to baseline within 3 min.
FIGURE 3. MCP-1- and eotaxin-induced ERK activation involves distinct pathways. A, CCR2^+ 300.19 cells were treated with 100 nM MCP-1 or 300 nM eotaxin for the times indicated on the abscissa and then analyzed by Western blotting as described in Fig. 1. Before chemokine treatment, cells were treated for 60 min with 1 μg/ml B. pertussis toxin (PTx) or 50 nM wortmannin. B, Membranes of HEK 293 cells transiently transfected with CCR2 cDNA were incubated with [35S]GTP[S] in the presence of 200 nM MCP-1 (black), 500 nM eotaxin (light gray), 200 nM MCP-1 plus 500 nM eotaxin (dark gray), or buffer (white), and then analyzed for bound [35S]GTP[S]. Each value represents the mean of three independent determinations ± SD. One of three representative experiments is shown. C, The association of Gα with CCR2 is abolished upon treatment with PTx. HA-CCR2 was immunoprecipitated (IP αHA) from solubilized membrane proteins (S100), and coimmunoprecipitated Gα were visualized on Western blots. Blots were reprobed with anti-HA Abs to determine HA-CCR2 in each fraction. Note that pertussis toxin treatment caused a slight reduction in the electrophoretic mobility of Gα (filled arrow), indicating efficient ADP ribosylation of the protein (66). D, MCP-1- and eotaxin-stimulated PI 3-kinase activation in CCR2^+ 300.19 cells transiently transfected with GFP-PH-Akt (green fluorescence) alone or together with a dominant negative form of the regulatory subunit (Figure legend continues)
were pretreated with PD98059, the inhibitory effect of eotaxin on the stimulation of CCR5 by the chemokine (Fig. 2) varied considerably between donors and may be attributed to signaling. The weak actin polymerization in monocytes treated with ERK pathway is necessary for eotaxin-dependent inhibition of CCR2 phosphorylation, but is not required for MCP-1-induced actin polymerization (Fig. 2A). Responses commonly obtained upon stimulation with chemokines. Pretreatment of CCR2 cDNA-transfected cells with eotaxin attenuated subsequent MCP-1-induced actin polymerization (p < 0.001). One possibility to explain this inhibitory effect is that eotaxin occupies and thereby desensitizes CCR2 to further stimulation with MCP-1. Alternatively, eotaxin-stimulated intracellular signaling is necessary for attenuation of CCR2-mediated responses to MCP-1. Therefore, we blocked ERK activation with specific inhibitors. Fig. 2A demonstrates that preventing ERK activation had no effect on MCP-1-stimulated actin polymerization. For contrast, when CCR2* 300.19 cells (Fig. 2A, left panel) or monocytes (Fig. 2A, right panel) were pretreated with PD98059, the inhibitory effect of eotaxin on MCP-1-stimulated actin polymerization was abolished (p < 0.004 and p < 0.01). This observation implies that activation of the MEK/ERK pathway is necessary for eotaxin-dependent inhibition of CCR2 signaling, but is not required for MCP-1-induced actin polymerization. The weak actin polymerization in monocytes treated with eotaxin varied considerably between donors and may be attributed to the stimulation of CCR5 by the chemokine (Fig. 2A, right panel).

The functional relevance of eotaxin-induced ERK activation was further confirmed when calcium mobilization was determined. Fig. 2B shows that pretreatment of CCR2* 300.19 cells with eotaxin results in a concentration-dependent attenuation of MCP-1-induced calcium mobilization. MCP-1-induced calcium fluxes were inhibited by ~25% with 100 nM (p < 0.01) and by up to 70% with 1 μM eotaxin (p < 0.0001). Preincubation of the cells with the MEK1/2 inhibitors (U0126, Fig. 2B; PD98059, not shown) had no effect on MCP-1-induced calcium mobilization. By contrast, the presence of 5 μM U0126 abolished the inhibitory effect of eotaxin, which corroborates the importance of the MEK/ERK pathway in mediating eotaxin-dependent attenuation of CCR2 signaling. We also tested whether the specific Pl 3-kinase inhibitor wortmannin (29) reduced the effect of eotaxin on MCP-1-induced calcium fluxes. Wortmannin did not interfere with GPCR-mediated calcium mobilization (36, 37) and, as expected, did not affect the stimulatory capacity of MCP-1. However, preincubation of cells with wortmannin almost completely abolished the inhibitory effect of eotaxin on MCP-1-induced calcium fluxes (p < 0.0001), further confirming that the outcome of eotaxin treatment depends on CCR2-mediated intracellular signal transduction (Fig. 2B).

We next investigated CCR2-dependent MAPK cascade activation to determine potential differences in eotaxin- and MCP-1-induced signal transduction. Fig. 3A shows that with either chemokine, ERK activation was abrogated in B. pertussis toxin-treated cells, which supposes that CCR2 must couple to heterotrimeric Gi proteins (4, 19–21). Nonetheless, when GDP/GTP exchange of CCR2-associated G proteins was determined in membranes of transiently transfected HEK 293 cells (Fig. 3B) or CCR2* 300.19 cells (not shown), a clear difference was evident: binding of the nonhydrolysable GTP analog GTPγS was enhanced in the presence of MCP-1, whereas it was marginally affected by eotaxin (Fig. 3B). In agreement with the finding that MEK/ERK pathway mediates the inhibitory effect of eotaxin on MCP-1-induced signaling, in isolated membranes, which are devoid of ERK (not shown), eotaxin only moderately affected MCP-1-induced GTPγS binding (Fig. 3B). Immunoprecipitation of CCR2 from control and pertussis toxin-treated membranes revealed that toxin-catalyzed ADP ribosylation led to the physical dissociation of Gα from the receptor (Fig. 3C). Because the immunoprecipitations were performed with cells that were not treated with chemokines, it can be assumed that under resting conditions CCR2 is pre-coupled to the G, protein. Together, these findings suggest that eotaxin-mediated ERK phosphorylation is much less dependent on activation of heterotrimeric G proteins, but requires that the G protein is bound to the receptor. The G protein bound to CCR2 presumably contributes to the formation of a platform that is essential for eotaxin-stimulated signal transduction.

Fig. 3A reveals that wortmannin treatment inhibits eotaxin-stimulated ERK activation. The finding indicates that PI 3-kinase acts upstream of the MAPK cascade and, consistent with the results shown in Fig. 2B, is necessary for eotaxin-dependent ERK phosphorylation. By contrast, MCP-1-stimulated ERK activation was not affected by wortmannin. However, chemokines commonly induce Pl 3-kinase activation (4), which is essential for cell polarization and directional migration (38–40). To measure eotaxin- and MCP-1-induced Pl 3-kinase activation, we transiently expressed GFP-PH-Akt in CCR2* 300.19 cells. The PH domain of protein kinase B (also called Akt) possesses high affinity for 3-phosphoinositides and mediates translocation of the kinase or of a GFP-PH-Akt construct to the plasma membrane upon activation of Pl 3-kinase (32, 41). Replacing arginine 25 with a cystein (R25C) abolishes the affinity of the PH domain of protein kinase B for phosphotyolipidinositol 4.5-trisphosphate (PIP3) (32). When the construct GFP-PH-AktR25C was used in control experiments, no membrane localization was observed. Fig. 3D shows that both eotaxin and MCP-1 induced prominent accumulation of GFP-PH-Akt in the plasma membrane. Wortmannin prevented association of the fusion protein with the membrane, in agreement with the requirement of Pl 3-kinase activity for its translocation. The plasma membranes of cells stimulated with MCP-1 often showed ruffle-like structures (Fig. 3D), suggesting the formation of actin-rich lamellipodia. Such ruffles were absent in eotaxin-stimulated cells. The results are in accord with the data shown in Fig. 2A, which indicate that MCP-1, but not eotaxin, induces actin polymerization. Together the data shown in Fig. 3, A and D, indicate that Pl 3-kinase activation is indispensable for eotaxin-mediated ERK activation, but is not required for MCP-1-induced stimulation of p85/p10 Pl 3-kinase (Δp85; middle panels). Cells shown in the lower left panel were transfected with an inactive GFP-PH-Akt construct (GFP-PH-AktR25C). Cells plated on coverslips were incubated for 30 min with 100 nM wortmannin (WTM) or buffer, then treated for 90 s with buffer (left), 300 nM eotaxin (middle), or 100 nM MCP-1 (right and lower left). Cells were fixed on ice with 4% paraformaldehyde. Fluorescence images were taken at ×1000 magnification, and confocal sections were obtained by computer-assisted deconvolution. E. MCP-1, but not eotaxin, induces receptor phosphorylation in an ERK-independent fashion. 300.19 cells expressing human HA-CCR2 were labeled with 32P and treated for the indicated times with 100 nM MCP-1 (● and ○) or 300 nM eotaxin (● and ○), in the absence (● and ○) or the presence (● and ○) of 50 μM PD98059 as described in Fig. 2. Cells were lysed, HA-CCR2 was immunoprecipitated with an anti-HA Ab, and proteins were resolved by SDS-PAGE (upper panel). Blots were subjected to autoradiography (32P), and CCR2 was detected with an anti-HA Ab (α-HA). Integrated counts were evaluated using ImageQuant software. One of two experiments with similar results is shown.
of the MAPK pathway. The observation that eotaxin only marginally stimulated GTP\(\gamma\)S exchange (Fig. 3B) prompted us to test whether MCP-1 and eotaxin stimulate different PI 3-kinase isoforms. Coexpression of a dominant negative variant of the regulatory subunit of type Ia PI 3-kinase \(\Delta p85\), which lacks the inter Src homology 2 domain that confers binding and trans-activation of the catalytic subunit p110 (42), abrogated eotaxin-induced translocation of GFP-PH-Akt, indicating that eotaxin-induced PIP\(_3\) formation is mediated by a type Ia p85/p110 PI 3-kinase (43). By contrast, MCP-1-stimulated translocation of GFP-PH-Akt and membrane ruffling was not affected when \(\Delta p85\) was expressed. Consistent with the observation that MCP-1 induces the GTP\(\gamma\)S exchange of membrane-bound G proteins (Fig. 3B), the lack of effect of \(\Delta p85\) expression on GFP-PH-Akt translocation supports the view that MCP-1 triggers PIP\(_3\) formation via the type Ib PI 3-kinase \(\gamma\), which is activated by the \(\beta\gamma\) subunits of heterotrimeric G proteins (43).

Down-regulation of GPCR activity is caused by desensitization and receptor internalization. Both processes are intimately associated with receptor phosphorylation. Receptor internalization is mediated by G protein-coupled receptor kinases (GRK), whereas receptor desensitization is caused by second messenger-regulated protein kinases and GRK (24, 44, 45). Fig. 3E shows that eotaxin does not induce CCR2 phosphorylation and implies that the inhibitory effect of eotaxin on MCP-1-stimulated responses is not caused by receptor phosphorylation. However, rapid CCR2 phosphorylation is observed after stimulation with MCP-1. Because the requirement of MAPK activation for agonist-induced desensitization of the \(\mu\)-opioid receptor was previously shown (46, 47), we tested the effect of MEK1/2 inhibition on receptor phosphorylation. Pretreatment with the MEK inhibitor PD 98059 does not prevent MCP-1-induced phosphorylation (Fig. 3E) and receptor internalization (not shown), suggesting that MCP-1-induced CCR2 desensitization (6) does not rely on signaling pathways that require MEK/ERK activation. In fact, inhibition of MEK/ERK activation with PD98059 or U0126 does not prevent desensitization of CCR2 when cells are sequentially treated with MCP-1, and calcium mobilization is determined (not shown). Taken together, the observations shown in Fig. 3 reveal that eotaxin and MCP-1 activate distinct intracellular signal transduction pathways via CCR2.

MCP-1 treatment desensitizes CCR2 for the ability to activate the MEK/ERK pathway in response to a second stimulation with MCP-1 or eotaxin (not shown). Because eotaxin and MCP-1 use different pathways, we tested the effect of eotaxin on MCP-1-stimulated ERK activation. Fig. 4B shows that addition of MCP-1 alone or in combination with eotaxin resulted in similar degrees of ERK activation. Pretreatment of CCR2\(^+\) cells with increasing concentrations of eotaxin had minor effects on subsequent ERK activation by MCP-1 (Fig. 4A). Similarly, extending the interval between the addition of eotaxin and that of MCP-1 up to 5 min did not influence the response to the latter. However, after stimulation with eotaxin, the receptor becomes refractory to further stimulation by the same chemokine. The apparent marginal activation of ERK observed in cells restimulated with eotaxin after 1 min (Fig. 4B) could reflect the residual activity of CCR2 induced by the first addition of the chemokine. These results corroborate our observation that MCP-1 and eotaxin activate ERK through distinct pathways. Moreover, in contrast to actin polymerization and calcium mobilization, ERK activation by MCP-1 is not antagonized by eotaxin-activated pathways, suggesting that CCR2 activates different pools of ERK depending on the activating chemokine.

**Discussion**

Previously eotaxin was characterized as a natural antagonist of CCR2 that does not trigger functional responses via CCR2 (6) or as a partial agonist that at elevated concentrations can induce moderate chemotactic responses, but is unable to stimulate calcium mobilization (14). Several models for GPCR ligand interaction and signal transduction have been proposed (48, 49). Our findings show that eotaxin activates the MAPK cascade via CCR2 and that stimulation of the pathway contributes to the antagonistic action of the chemokine on CCR2 (Fig. 5). The action of eotaxin on CCR2 is complex and is not explained by competitive binding, where mutually exclusive binding of the two ligands to the same receptor site is expected (50, 51). For receptor activation by a partial agonist,
it is anticipated that combined stimulation with increasing concentrations of eotaxin should reduce the maximum response elicited by an agonist (MCP-1) to the level seen with the partial agonist alone. Our observation that eotaxin does not affect the MCP-1-stimulated activation of the MAPK cascade does not favor the classification of eotaxin as a partial agonist. The different potencies by which eotaxin and MCP-1 activate G proteins and stimulate different signal transduction pathways and pools of ERK (Fig. 5) support the view that CCR2 assumes ligand-specific, receptor-active states (48).

Receptor desensitization is closely correlated with its phosphorylation. However, in contrast to most functional responses elicited by chemokine receptor activation, internalization and phosphorylation are not inhibited by pertussis toxin (4, 23). It is assumed that GRK sense the active state of the receptor and independently of G protein activation phosphorylate the receptor, which leads to arrestin binding and internalization. The fact that eotaxin does not cause receptor phosphorylation (Fig. 3E) and internalization (6) (data not shown) provides further evidence that the chemokine induces a CCR2 conformation distinct from the conformation induced by other chemokines triggering functional responses such as MCP-1.

The observations reported in this study are consistent with a model in which eotaxin can arrest CCR2 at the membrane by a mechanism that differs from previously characterized modes of receptor desensitization (52, 53). Furthermore, MCP-1-mediated activation of ERK is not affected by pretreatment of CCR2 with eotaxin, indicating that eotaxin does not cause complete receptor desensitization. It is noteworthy that the responsiveness of CCR2 to stimulation with eotaxin ceases after the first contact with the chemokine (Fig. 4B), suggesting that ligand-specific receptor desensitization occurs nevertheless.

Several GPCR-dependent pathways leading to the activation of MAPK have been described. Most include the activation of tyrosine kinases that trigger via GRB2/SOS Ras activation. An alternative mechanism involves Rap1-GAP, which triggers Raf kinase directly (54–57). Activation of PI 3-kinase by βγ subunits, leading to GRB2/Sos stimulation, has also been reported (58). The majority of chemokines trigger a fast and transient activation of ERK that is insensitive or only moderately sensitive to wortmannin (4, 59). Our finding that eotaxin-mediated activation of ERK is fully abrogated in the presence of the PI 3-kinase inhibitor wortmannin hints at a signal transduction mechanism that is not common to other chemokines. It is conceivable that the eotaxin-induced receptor state promotes an unusual coupling mechanism that does not lead to extensive G protein activation, but requires association of the protein with the receptor (Fig. 5). The observation that pertussis toxin treatment dissociates the G protein from CCR2 supports this view. It is also plausible that eotaxin induces a state of the receptor that catalyzes the guanine nucleotide exchange of the prebound G protein, but does not trigger release of the activated G protein from CCR2. By contrast, the full CCR2 agonist MCP-1 stimulates guanine nucleotide exchange and release of the G protein from the receptor, allowing the catalysis of several cycles of G protein activation. Our finding that eotaxin and MCP-1 trigger the activation of different isoforms of type I PI 3-kinase further supports the conclusion the ligand-induced active states of CCR2 couple to distinct signal transduction pathways. Binding of MCP-1 to CCR2 causes the release of βγ from heterotrimeric G proteins, leading to the activation of PI 3-kinase γ (60, 61) and of phospholipase Cβ and calcium mobilization. Eotaxin, which appears not to trigger guanine nucleotide exchange of the G protein, moderately stimulates a p85/p110 PI 3-kinase. Additional experimental work is required to characterize this signal transduction mechanism. It can also be envisaged that additional pathways may be differentially activated by eotaxin and MCP-1.

Eotaxin-stimulated cells did not show membrane ruffles despite the evident recruitment of GFP-PH-Akt to the plasma membrane. The observation is consistent with previous reports showing that at moderate concentrations eotaxin does not induce chemotaxis via CCR2 (6, 14) and that this chemokine does not stimulate actin polymerization (Fig. 2). Activation of PI 3-kinase by MCP-1 was anticipated based on the recent observation that the lipid kinase activity is instrumental for efficient orientation of migrating cells along a chemotactic gradient (62, 63). Accordingly, MCP-1-stimulated cells showed actin polymerization and membrane ruffles to which GFP-PH-Akt is recruited. However, activation of PI 3-kinase appears unnecessary for the MCP-1/CCR2-dependent ERK activation pathway, whereas PI3P production in eotaxin-stimulated cell is critical for ERK activation.

Eotaxin and MCP-1 are coexpressed in pathologic conditions, namely allergic reactions (6). MCP-1 is a strong stimulus for lysosomal enzyme release from monocytes (64) and histamine secretion from basophils (65), whereas eotaxin attracts CCR3-positive cells without stimulating release through CCR2. Our present observations suggest that in vivo eotaxin could assume a role as a modulator under such conditions. Although eotaxin efficiently recruits leukocytes to sites of injury, at the same time it tempers the inflammatory response by attenuating secretion of proinflammatory mediators that are secreted in response to CCR2 stimulation. Because eotaxin does not trigger receptor down-regulation, it is conceivable that the cells quickly regain CCR2 functional responsiveness once eotaxin is eliminated from their environment.

Acknowledgments

We thank Drs. Jens Geginat and Gioacchino Natoli for valuable discussions, and Prof. Ian Clark-Lewis for providing the chemokines.

References


