Arrestin Regulates MAPK Activation and Prevents NADPH Oxidase-dependent Death of Cells Expressing CXCR2*

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Activation of CXCR2 IL-8 receptor leads to activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and rapid receptor endocytosis. Co-immunoprecipitation and co-localization experiments showed that arrestin and CXCR2 form complexes with components of the ERK1/2 cascade following ligand stimulation. However, in contrast to the activation of the β2-adrenergic receptor, arrestin was not necessary for ERK1/2 phosphorylation or receptor endocytosis. In contrast, β-arrestin 1/2 double knockout cells showed greatly enhanced phosphorylation of ERK1/2, as well as phosphorylation of the stress kinases p38 and c-Jun N-terminal protein kinase. The stimulation of stress kinases in arrestin double knockout cells could be attenuated in the presence of diphenylene iodonium (DPI), an inhibitor of the NADPH oxidase, suggesting that reactive oxidant species (ROS) participated in mitogen-activated protein kinase (MAPK) activation. ROS could indeed be detected in IL-8-stimulated β-arrestin 1/2 knockout cells, and cytoplasmic Rac was translocated to the membrane fraction, which is a prerequisite for oxidation. The oxidative burst induced cell death within 6 h of IL-8 stimulation of these cells, which could be prevented in the presence of DPI. These results indicate a novel function for arrestin, which is protection from an excessive oxidative burst, resulting from the sustained stimulation of G-protein-coupled receptors that cause Rac translocation.

Seven transmembrane receptors are usually referred to as G-protein-coupled receptors (GPCRs)1 because of their signaling through G-proteins, although their function is not limited to that pathway (1). In the G-protein pathway GPCR activation by agonist leads to replacement of GDP on the G subunit of the βγ dimer. Both subunits are capable to activate numerous signaling cascades including phospholipase C and adenyl cyclase. The activated receptors are quickly phosphorylated by GPCR kinases, which increase the affinity of the receptors for β-arrestin. β-Arrestin binding to the GPCR leads to the termination of the interaction of the receptor with its respective G-protein (2) thereby arresting G-protein-mediated signaling. However, GPCRs coupled to arrestins can signal in a G-protein-independent fashion leading to the activation of extracellular signal-regulated kinase (ERK1/2) (3, 4), Src (5), and JNK (6). These kinase cascades assemble as complexes with β-arrestin and GPCRs.

The ubiquitously expressed ERK1/2, members of the mitogen-activated protein kinase (MAPK) family, are important cellular regulators (7). They can be activated by a variety of extracellular signals including growth factors, phospholipids, cytokines, hormones, and neurotransmitters, mediating a wide spectrum of cellular functions, including cell cycle regulation, cellular proliferation, differentiation, movement, and angiogenesis (8). ERK1/2 activation occurs through dual phosphorylation on a Thr-Xaa-Tyr motif within the activation loop by upstream kinases (9). The ERK1/2 signaling pathway consists of a three kinase module comprised of a MAPK (ERK1/2), a MAPK activator (MAPK/ERK kinase, MEK), and a MEK activator (MEK kinase, MEKK), which are sequentially phosphorylated. Although ERK activation by GPCRs is often a Ras-dependent event, additional pathways can be involved. These vary for different receptors and occasionally cell type, and may be initiated by PKA, PKC, through protein-tyrosine kinases (e.g. EGF receptor, Src, FAK), or direct interaction with the β-arrestin scaffold (10). Similar MAPK cascades consist of the stress-activated p38 and JNK (11).

In addition to its complex formation with signaling cascades arrestin also associates with clathrin and the clathrin adaptor AP-2, which target the GPCR into coated pits and leads to endocytosis of the receptor (12). In the case of the β2-adrenergic receptor β-arrestin is absolutely necessary for receptor internalization (13), and internalization of the angiotensin II type 1A receptor is largely inhibited in the absence of arrestin (13). However, the role of β-arrestin in receptor internalization is not universal; for instance arrestin is not required in endocytosis of the N-formyl peptide receptor (14).

CXCR2 receptor, which is activated by IL-8 and gro-α, is a GPCR primarily coupled to G1 (15), and is expressed by neutrophils, monocytes, microvascular endothelial cells, some fibroblasts, and cancer cells. It is rapidly internalized following receptor activation (16) in a process that involves dynamin (17) and AP-2 (18), and therefore appears to involve clathrin-coated pits. However, a truncated receptor, which is not phosphorylated, and hence should have a low affinity for arrestin is normally internalized (18), which raises the question whether arrestin plays a role during endocytosis of CXCR2. Here we
show that CXCR2 forms complexes with arrestin, but that these complexes are not necessary for receptor internalization or ERK1/2 phosphorylation. In contrast, MAPK activation was enhanced in β-arrestin 1/2 double knockout cells to the point, where cells responded with cell death rather than cell proliferation.

CXCR2 is one of a number of G-coupledGPCRs on neutrophils, which include CXCR1, the formyl peptide receptor, and C5a receptor. These last three receptors are all important for neutrophil chemotaxis in vitro and in vivo. The physiological role of CXCR2 on these cells is, however not clear. Neutrophils clearly express functional CXCR2 receptors, which respond to stimulation with calcium mobilization, actin polymerization, and moderate enzyme release (19), but show little effect on activation pathways important for the immune surveillance functions of the neutrophil. Stimulation of neutrophilic CXCR2 neither supports chemotaxis (20), nor does it cause a respiratory burst (21). There is no good explanation for this behavior other that the rate of phosphorylation, which can be detected within seconds of stimulation, and consequent uncoupling from the G-protein may supercede the rate of assembly of the NADPH oxidase complexes.

While the NADPH oxidase system is best described in neutrophils (22), adherent cell types including endothelial and epithelial cells, smooth muscle cells and fibroblasts can produce intracellular oxidants following receptor activation (23–25). Although some of the components of these NADPH oxidase systems differ from those of the neutrophilic complexes (26), activation of all these systems depends on translocation of Rac from the cytoplasm to the cell membrane (27), and activation of a flavin-dependent membrane-bound cytochrome, which is inhibited by diphenylethion (DPI) (28).

Stimulation of various cell types expressing CXCR2 (neutrophils and microvascular endothelial cells, which express CXCR2 constitutively, NIH3T3 cells and HEK293 cells transfect with CXCR2) failed to respond with a respiratory burst (21). However, in β-arrestin 1/2 double knockout fibroblasts stimulation with IL-8 caused a pronounced intracellular respiratory burst, which was responsible for later cell death. It therefore appears that arrestin is instrumental in suppressing or terminating activation of the respiratory burst in cells expressing CXCR2.

**EXPERIMENTAL PROCEDURES**

Materials—Raf-1 kinase inhibitor and DPI were purchased from Calbiochem (San Diego, CA). Anti-c-Raf, anti-MEK, anti-GFP, anti-β-arrestin 2, and anti-Rac1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antiphospho-ERK1/2, anti-ERK1/2, antiphospho-p38, anti-p38, antiphospho-JNK, and anti-Myc antibodies were from Cell Signaling (Beverly, MA); anti-FLAG antibody was obtained from Sigma. Protein A beads were from Amersham Biosciences (Arlington Heights, IL). γ-[32P]ATP was from PerkinElmer Life Sciences (Boston, MA). IL-8 and SDF-1 were expressed and purified from E. coli (Novagen/EMD Biosciences, La Jolla, CA). Following purification, the clarified cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, blocked with 3% dry milk in TBS-Tween, and exposed to specific primary antibodies as described for each experiment. Antibody binding was detected using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies and enhanced chemiluminescence (ECL, Amersham Biosciences). Phosphoblots were re-probed with a second antibody, e.g. anti-ERK1/2 antibody to assure equal loading.

Subcellular Fractionation—For the isolation of membrane fractions, cells were stimulated for the indicated times and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μM sodium vanadate, and 10 μM NaF) and clarified by centrifugation. The supernatants were incubated with primary antibodies for 2 h at 4 °C, followed by capture of the immunocomplexes with protein A beads for 1 h at 4 °C. The immunoprecipitates were washed twice with lysis buffer and once with phosphate-buffered saline to remove nonspecifically bound proteins. The bound proteins were analyzed by immunoblotting.

For immunoblotting, the clarified cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, blocked with 3% dry milk in TBS-Tween, and exposed to specific primary antibodies as described for each experiment. Antibody binding was detected using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies and enhanced chemiluminescence (ECL, Amersham Biosciences). Phosphoblots were re-probed with a second antibody, e.g. anti-ERK1/2 antibody to assure equal loading.

To isolate nuclear fractions, cells were vortexed in buffer A (10 mM HEPES, pH 8.0, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM DTT, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin) containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM sodium vanadate, and 10 μM NaF. The pellets were washed three times with buffer A (containing 1 mM Na₃VO₄, 1 mM DTT, 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin), incubated on ice for 15 min following by another 2 min centrifugation at 4 °C. The supernatants (nuclear fraction) were transferred to clean tubes containing 5% Laemmli sample buffer, boiled, and separated on SDS polyacrylamide gels.

Raf Kinase Assay—Washed c-Raf immunoprecipitates were subjected to 40 μl of kinase buffer (30 mM HEPES, pH 7.4, 10 mM MnCl₂, 5 mM MgCl₂, 1 mM DTT, 2 mM Na₃VO₄, 25 mM β-glycerophosphate, 50 μM ATP) containing 15 μCi of γ-[32P]ATP and 0.4 μg of purified kinase inactive MEK1 (Upstate Biotechnologies), and then incubated at 30 °C for 30 min. Kinase reactions were stopped by adding Laemmli sample buffer and boiling, followed by separation on SDS polyacrylamide gels, gel transfer, and autoradiography. To assess equal loading, membranes were probed with anti-c-Raf antibody and stained with the ECL kit.

Fluorescence Microscopy—For fluorescence microscopy, HEK 293 cells grown on collagen-coated glass coverslips were transiently co-transfected with different combination of plasmids as described in the figure legends. After stimulation with 100 nM IL-8 or SDF-1 for the times indicated at 37 °C, cells were fixed for 25 min with 4% paraformaldehyde in phosphate-buffered saline and mounted with AntiFade (Molecular Probes, Eugene, OR). Z-stack images were taken on a Leica DM ER microscope (Leitz, Bannockburn, IL) connected with an ORCA camera (Hamamatsu Photonics, Hamamatsu City, Japan) and deconvoluted using Openlab 3.1.5 software (Improvision, Boston, MA).

To detect apoptosis an annexin V-fluorescein isothiocyanate/pro-
RESULTS

IL-8-mediated Translocation of Arrestin and ERK1/2 Cascade Components in Cells Expressing CXCR2—Since arrestin translocation plays an important role in MAPK activation and endocytosis of a number of GPCRs (34), we asked whether activation of CXCR2 caused translocation of arrestin and the components of the MAPK cascade to the plasma membrane. As expected, arrestin, and the kinases c-Raf and MEK, were translocated to the membrane fraction of CXCR2-expressing cells following stimulation with IL-8 (Fig. 1A). This translocation of arrestin was seen in HEK293 cells stably expressing CXCR2-GFP fusion protein (Fig. 1A) as well as in RBL2H3, rat basophilic leukemia cells (Fig. 1B), transfected with wild-type CXCR2. For comparison the same experiment was repeated in CXCR4-expressing cells, which showed only a rather subtle translocation of the same components to the plasma membrane (Fig. 1C).

Activation of MAPK—Arrestin translocation has been shown to play an important role in MAPK activation of many GPCRs, e.g. the β2-adrenergic receptor (35), the AT1a-angiotensin receptor (36), and the protease-activated receptor 2 (PAR-2) thrombin receptor (37). Therefore MAPK phosphorylation was assessed in CXCR2-expressing cells stimulated with IL-8. IL-8 was found to induce a robust, but transient ERK1/2 activation in all cell types tested (HEK293, RBL2H3, and SYF cells) (Fig. 2A), which was maximal around 2 min of stimulation. This activation did not depend on Src activity as it did not appear decreased in SYF cells, which are deficient of Src, Yes, and Fyn. Although activation of CXCR2 can cause transactivation of the EGF receptor (38), this pathway played only a minor role in signaling to ERK1/2, and inhibition of the EGF receptor with AG1478 showed no effect on ERK1/2 phosphorylation (results not shown). In contrast, the ERK1/2 response was almost entirely inhibited by a Raf-1 kinase inhibitor (Fig. 2B). Kinase-deficient Raf (Raf K375M) similarly attenuated ERK1/2 phosphorylation (Fig. 2B). Involvement of c-Raf could further be shown directly in Raf activity assay, in which MEK1 is phosphorylated in the presence of activated c-Raf (Fig. 2C). Since c-Raf has been shown to be part of multicomponent complexes formed between the MAPK cascade, arrestin, and some GPCRs, we next asked whether this was the case for CXCR2.

Coimmunoprecipitation and Colocalization of CXCR2, Arrestin, and Components of the MAPK Cascade—Since activation of the Raf/MAPK cascade has been shown to be mediated through complex formation with arrestin, e.g. in the β2-adrenergic receptor system (1), we determined whether IL-8 would induce similar complexes. For this purpose, HEK293 cells expressing CXCR2-GFP were transiently transfected with β-arrestin 2-FLAG, Myc-c-Raf, and ERK2-RFP expression plasmids. After stimulation with IL-8, the amount of ERK2 and c-Raf in complex with β-arrestin 2 and CXCR2 increased considerably as shown in Fig. 3, A and B, and a high level of phosphorylation of ERK2 was detected in the complexes. This could be shown by immunoprecipitation with either an antibody, which recognizes the FLAG tag fused to arrestin (Fig. 3A) or an antibody that recognizes GFP, which is fused to CXCR2 (Fig. 3B). Similarly, components of the ERK cascade could be coimmunoprecipitated with GFP- or FLAG-arrestin following IL-8 stimulation of the untagged CXCR2, indicating that it was specific for the receptor and not a function of the GFP (Fig. 3C). These results provide strong evidence that CXCR2 stimulation causes recruitment of complexes consisting of β-arrestin, c-Raf, and ERK2, in which arrestin appears to function as a scaffold for signal transduction. As shown in Fig. 3D, the absence of ERK2 had no effect on the association of c-Raf with β-arrestin 2, whereas c-Raf expression significantly enhanced the precipitation of phospho-ERK2 with β-arrestin 2. Raf kinase inhibitor or dominant-negative c-Raf mutant both attenuated the complex formation (Fig. 3D). These results indicated that ERK2 activation and association with β-arrestin 2 depended on c-Raf, and that the interaction of c-Raf with β-arrestin 2 facilitated ERK2 activation, suggesting an integral role of c-Raf in these complexes, which was compatible with the fact that Raf-1 inhibitor almost totally blocked IL-8 induced ERK1/2 activation (see Fig. 3E).
FIG. 2. ERK1/2 phosphorylation in cells transfected with CXCR2 and stimulated with 100 nM IL-8 is largely dependent on c-Raf activation. Whole cell lysates of cells stimulated with IL-8 for the indicated times were analyzed by Western blotting with antiphospho-ERK1/2 antibody. Subsequently the blots were stripped and reblotted with anti-ERK1/2 antibody. A, time course of ERK1/2 phosphorylation in HEK293, SYF (Src/Yes/Fyn-deficient) and RBL2H3 cells transfected with CXCR2/CXCR2-GFP. B, effect of Raf-1 inhibitor and dominant-negative c-Raf mutant (K375M) on ERK1/2 phosphorylation in IL-8-stimulated HEK293 cells expressing CXCR2-GFP as indicated. C, Raf activity assay: cell lysates of CXCR2-GFP expressing HEK293 cells + or − IL-8 were used in a MEK1 phosphorylation assay as described under “Experimental Procedures,” and separated on SDS polyacrylamide gels. Phospho-MEK was detected by autoradiography, the c-Raf loading control by Western blotting. In each case one experiment representative of three is shown.

2B). Complex formation could also be visualized by fluorescence microscopy of cells transfected with CXCR2-GFP, β-arrestin 2, and ERK2-RFP or with CXCR2, β-arrestin 2-GFP, and ERK2-RFP. In unstimulated cells CXCR2-GFP was located in the plasma membrane, GFP-fused arrestin homogenously in the cytoplasm and to a lesser extent in the nucleus (results not shown). Following stimulation with IL-8, co-localization between CXCR2 and ERK2 or between β-arrestin 2 and ERK2 could be detected in intracellular vesicles (Fig. 3E). These findings together with previous reports showing that GPCR-arrestin complexes are essential for MAPK activation (10), suggested that arrestin played an integral role in CXCR2-mediated MAPK activation. One known function of β-arrestin in complex with seven transmembrane receptors and components of the MAPK cascade, is to retain these complexes in the cytoplasm rather than to allow translocation of MAPK to the nucleus, where it induces transcriptional activation (36). However, in CXCR2-expressing cells a major fraction of ERK1/2 phosphorylation following stimulation with IL-8 was detected in the nuclear fraction (Fig. 3F). So the function of CXCR2-mediated ERK1/2 nuclear translocation remains to be investigated.

MAPK Activation in β-Arrestin 1/2 Knockout Cells—The finding of CXCR2/β-arrestin/MAPK complexes together with previous reports showing that GPCR-β-arrestin complexes are essential for MAPK activation suggested that β-arrestin play an essential role in CXCR2-mediated MAPK activation. To prove this point ERK1/2 phosphorylation was determined in MEFs expressing CXCR2 and stimulated with IL-8. The stress kinases p38 and JNK that are only poorly activated by IL-8 stimulation of CXCR2-transfected cells, were highly phosphorylated in the arrestin knockout cells (Fig. 4, B and C). Although there appeared a slight delay in receptor endocytosis compared with arrestin-containing cells, receptor endocytosis in β-arrestin knockout cells was complete by 5–10 min.

Effect of Dominant-Negative β-Arrestin on CXCR2 Functions—Similar results concerning β-arrestin function were obtained with a second approach using a dominant-negative arrestin mutant (arrestin319–418). In the presence of dominant-negative β-arrestin, IL-8-induced ERK1/2 phosphorylation was prolonged (Fig. 5A). In contrast, SDF-1-mediated ERK1/2 activation in CXCR4-transfected cells was attenuated in the presence of dominant-negative β-arrestin (Fig. 5B). Similarly, expression of dominant-negative β-arrestin in HEK293 cells showed no effect on IL-8-mediated CXCR2 endocytosis, but prevented SDF-1-induced internalization of CXCR4 (Fig. 5C).
IL-8 Causes Cell Death in β-Arrestin 1/2 Knockout Cells—
The activation of MAP kinases was so excessive in β-arrestin 1/2 knockout cells that it appeared likely that it might lead to cell death (41, 42) rather than cell activation. Indeed stimulation of these cells with IL-8 caused apoptotic appearance with cells rounding up and de-adhering over a period of several hours (Fig. 6A). This was confirmed by annexin V staining. At 3 h 11% of the cells stained positive for annexin V, an early indicator of apoptosis, whereas only 2.5% of all cells took up propidium iodide, an indicator of late apoptosis or necrosis. By 5 h 60.9% of the annexin V-positive cells were also stained by propidium iodide (results not shown).

Because of the prominent translocation of Rac to the membrane fraction in these cells, we hypothesized that oxidative stress due to activation of the NADPH oxidase system was the pathway that mediated this behavior. When an inhibitor of the oxidative burst, DPI, was added during the incubation with IL-8, cell rounding could be prevented (Fig. 6A). IL-8-mediated cell death was not observed in MEFs, in which one of the two β-arrestins had been knocked out (Fig. 6A), indicating that either of the β-arrestins could protect from cell death.

Furthermore, DPI attenuated phosphorylation of p38 (Fig. 6B) suggesting that activation of this stress kinase was at least partially mediated by ROS. DPI showed little effect on ERK1/2 and JNK phosphorylation (results not shown), suggesting these kinases were activated directly and not secondary to oxidant production.

Detection of ROS in IL-8-stimulated β-Arrestin 1/2 Knockout Cells:

Fig. 3. Co-immunoprecipitation of CXCR2, arrestin and components of the MAPK cascade. HEK293 cells were transiently transfected with CXCR2-GFP, FLAG-arrestin 2, Myc-c-Raf, and ERK2-RFP. The cell lysates were immunoprecipitated with anti-FLAG to precipitate arrestin (A) or with anti-GFP to precipitate CXCR2-GFP (B). Washed precipitates were Western blotted with anti-FLAG/arrestin, anti-Myc-c-Raf, antiphospho-ERK1/2, or anti-ERK1/2 antibodies as indicated. C, HEK293 cells transfected with untagged CXCR2 and either GFP- or FLAG-arrestin 2 in the absence or presence of IL-8 were immunoprecipitated with anti-GFP or anti-FLAG and Western-blotted with antiphospho-ERK1/2 or anti-ERK1/2 antibody as indicated. D, role of c-Raf in IL-8-mediated β-arrestin/ERK complexes. Top panel, effect of increasing concentrations of Myc-c-Raf on ERK1/2 phosphorylation in IL-8-stimulated HEK293 cells. Cells were transfected with DNA constructs as indicated in the figure, immunoprecipitated with anti-FLAG/arrestin, and blots were developed with anti-Myc-c-Raf, antiphospho-ERK1/2 or anti-ERK1/2 antibody. Bottom panel, CXCR2-expressing cells were transfected with FLAG-β-arrestin 2, Myc-c-Raf, ERK2-RFP, and K375M c-Raf as indicated and stimulated with IL-8 for 2 min. The effect of Raf-kinase inhibitor was also tested. Samples were immunoprecipitated with anti-FLAG/arrestin, and blots were developed with antiphospho-ERK1/2 or anti-ERK1/2. E, co-localization between CXCR2, β-arrestin, and ERK. HEK293 cells were transiently transfected with CXCR2-GFP, β-arrestin 2, and ERK2-RFP (top panel) or with wt-CXCR2, GFP-β-arrestin 2, and ERK2-RFP (bottom panel), and stimulated with 100 nM IL-8 for 2 min. Images were taken on a fluorescent microscope (×100 objective) and deconvoluted. Arrows indicate some of the areas of co-localization between CXCR2 and ERK2 or between β-arrestin and ERK2. F, nuclear translocation of phospho-ERK1/2 in HEK293 cells expressing CXCR2. After stimulation with IL-8 for 2 to 10 min, cell lysates were separated into nuclear and cytoplasmic fractions and Western-blotted with antiphospho-ERK. Representative Western blots are shown from at least three independent observations for each protein.
To verify an oxidative burst, two methods were used. First, the formation of the blue NBT-diformazan from NBT was followed microscopically. This method is used as a clinical test for NADPH oxidase function in neutrophils, but it is usually not sensitive enough to detect ROS formation in non-phagocytic cells. However, in \( \beta \)-arrestin 1/2−/− cells IL-8 caused intensive blue staining in the presence of NBT (Fig. 6C). This was not observed in any other cell type stimulated with IL-8, including neutrophils, which normally form NBT-diformazan, when stimulated with \( N \)-formyl peptide or C5a. The formation of NBT-diformazan in IL-8 stimulated \( \beta \)-arrestin-deficient cells could be prevented in the presence of DPI (Fig. 6C). Again, either \( \beta \)-arrestin 1 or \( \beta \)-arrestin 2 could prevent oxidant formation as shown in Fig. 6C. In contrast, stimulation with SDF-1 failed to induce NBT-diformazan formation (Fig. 6C) and did not result in cell death (results not shown).

Second, the formation of red fluorescent resorufin from amplex red was detected in the membrane fraction of MEF \( \beta \)-arrestin 1/2 knockout cells (43). Even cell membrane fractions of unstimulated \( \beta \)-arrestin 1/2 knockout cells showed a considerable portion of Rac in the membrane fraction of these cells even in the absence of stimulation.

**DISCUSSION**

We show here that CXCR2 forms complexes with \( \beta \)-arrestin and components of the MAPK cascade. However, in contrast to the situation for the \( \beta \)-adrenergic receptor or the AT1a-receptors (10, 36), these complexes appear to attenuate MAPK activation rather than promote it. \( \beta \)-arrestin-deficient cells showed a markedly increased activation of the various MAP kinases. In
Role of Arrestin in CXCR2 Signaling

A plasmid encoding β-arrestin (5 μg of DNA) and CXCR2-GFP (1 μg of DNA) was transfected into HEK293 cells as indicated. Cells were then treated with 100 nM IL-8 for the indicated times. Cell lysates were prepared, and ERK1/2 phosphorylation was detected by Western blotting (one experiment representative of four). B, HEK293 cells expressing CXCR4-GFP were transfected with β-arrestin as indicated and stimulated with 50 nM SDF-1 for 5 min. ERK1/2 phosphorylation was detected as described above. C, HEK293 cells were transiently transfected with CXCR2-GFP or CXCR4-GFP and dominant-negative β-arrestin. Following stimulation with 100 nM IL-8 or 50 nM SDF-1 for 15 min GFP fluorescent images were obtained with a confocal microscope (×63 objective).

CXR2-GFP and CXCR4-GFP in HEK293 cells

|          | no stim. | 15 min + IL-8 | 15 min + IL-8 |
|----------|----------|---------------|---------------|
| CXCR2-GFP|          |               |               |
| CXCR4-GFP|          |               |               |
| dom. neg. arrestin (μg) | - | 1 | 5 |

MAPK, and cytochrome c leakage from mitochondria (47), it did not investigate the mechanism, which causes cell death. Our results clearly indicate that β-arrestin-deficient cells undergo oxidative stress when stimulated with IL-8, and that ROS are responsible for the cell death. This was first suggested by the protective effect of DPI, which inhibits NADPH oxidase, and then confirmed by the ability to detect ROS in IL-8-stimulated β-arrestin −/− MEFS. Furthermore, IL-8 caused translocation of Rac to the membrane fraction, which is an essential component of the NADPH oxidase complex (22, 48). Although some oxidant formation was already observed in unstimulated β-arrestin knockout cells, in particular if membrane fractions were used, in which cellular antioxidant enzymes would have been removed, this amount appeared below the threshold dose that caused cell death.

Activation of CXCR2 caused translocation of Raf and Rac to the membrane fraction. In the presence of β-arrestin this led to a transient activation of ERK1/2 and Rac (39). When β-arrestin was absent, the activation was sustained and ultimately caused cell death. Although we did not investigate CXCR4-mediated response in much detail, it appears that CXCR4 behaves like the β2-adrenergic receptor, where β-arrestin is necessary for receptor endocytosis and enhances MAPK activation. SDF-1, which did not cause ROS production, failed to recruit Rac. It is worth noting that only GPCRs, which were endocytosed in an arrestin-independent fashion, like CXCR2, but not like CXCR4, were capable of mediating cell death of arrestin deficient cells (47). Since Rac plays an essential role in endocytosis (49) as well as in the activation of the oxidative burst, these two functions appear intimately linked. One may speculate that GPCRs that cause apoptosis in β-arrestin-deficient cells are generally able to recruit Rac.

Furthermore, these results are compatible with a recent report in which it was shown that β-arrestin 1 is anti-apoptotic following stimulation of the insulin-like growth factor 1 receptor in a phosphatidylinositol kinase (PI 3-kinase)-mediated mechanism (50). Because PI 3-kinase is necessary for Rac activation (51), it appears that certain receptor kinases may activate the same β-arrestin-dependent pathway as the Rac-activating GPCRs.

It has been shown that endocytosis of CXCR2 is dependent on dynamin (52). At the time of the publication of this report, dynamin was primarily seen as a component of clathrin-mediated endocytosis. While dynamin certainly plays a role in clathrin-mediated endocytosis, this is not its only function. Both caveolar uptake and type 1 phagocytosis, which is mediated by Rac, are dynamin-dependent (53, 54). While we cannot exclude that CXCR2 is endocytosed in an β-arrestin/clathrin-dependent fashion in normal cells, there exists an alternate internalization pathway in β-arrestin-deficient cells, which presumably is dynamin/Rac dependent (54). In phagocytic cells this pathway is referred to as phagocytosis type 1 (55).

CXCR2, C5a receptor and formyl peptide receptors, are all expressed in neutrophils and can all activate Rac. This causes a respiratory burst for the formyl peptide receptor and C5a-receptors, but not following stimulation of CXCR2 (21). If the oxidative burst, the consequence of Rac translocation, is terminated or inhibited in the presence of β-arrestin, the rate of GPCR phosphorylation and of β-arrestin recruitment, will determine the extent of the oxidative burst. Phosphorylation of CXCR2 is extremely rapid, - it appears complete within 15 s (30), and maximal complex formation with arrestin was detected by 2 min, which is undoubtedly an overestimate of the time required due to the poor time resolution of preparing cell lysates form adherent cells. It therefore appears likely that the rate of complex formation with arrestin supercedes the rate of assembly of the NADPH
oxidase in the case of CXCR2. Such a system allows the fine tuning of activation of the NADPH oxidase, which is essential for the protection from bacterial infection, but at the same time deleterious to oxidant producing cells.

Since all cells express β-arrestin, it will have to be determined whether there are situations in which β-arrestin concentrations are too low to limit the NADPH oxidase activation following stimulation with GPCR ligands. It is intriguing in this respect that cellular arrestin levels can be considerably decreased due to ubiquitin-mediated protein degradation in containing cells. Such an apoptosis inhibitory function for Hsp70 has been described (62). It will have to be shown, whether the binding of Hsp70 depends on the presence of β-arrestin.

Although many pathways lead from stimulation of a seven transmembrane receptor to MAPK activation, the majority of the ERK1/2 phosphorylation following IL-8 stimulation of CXCR2 depended on activation of a Ras/Raf pathway independent of the cell type tested. It was associated with translocation of Raf to the membrane fraction, a prerequisite for Raf activation (63). Although IL-8 can transactivate the EGF receptor (38, 64), and this transactivation is important for CXCR2-dependent cell migration of endothelial cells, this pathway made only a minor contribution to the overall ERK1/2 phosphorylation in the cell types tested (HEK293 and SYF) used here. Decreases in ERK1/2 phosphorylation were barely perceptible in AG1478-treated cells. Similarly, inhibition of Src with PP1, or using the Src family-deficient SYF cells, had no effect on ERK1/2 phosphorylation.

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REFERENCES

1. Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Nat. Rev. Mol. Cell. Biol. 3, 639–650
2. Budd, D. C., Rae, A., and Tobin, A. B. (1999) J. Biol. Chem. 274, 12355–12360
3. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) J. Cell Biol. 148, 1267–1292
4. Lee, J., Cacalano, T., Toy, K., Moore, M. W., and Wood, W. I. (1995) J. Immunol. 155, 2159–2164
5. Ahn, S., Maudsley, S., Luttrelly, L. M., Lefkowitz, R. J., and Daaka, Y. (1999) J. Biol. Chem. 274, 1185–1188
6. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) Science 290, 1515–1518
7. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) Physiol. Rev. 79, 143–180
8. Nishida, E., and Gotob, Y. (1993) Trends Biochem. Sci. 18, 128–131
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