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Cutting Edge: Differential Regulation of Chemoattractant Receptor-Induced Degranulation and Chemokine Production by Receptor Phosphorylation

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Abstract

Phosphorylation of G protein-coupled receptors and the subsequent recruitment of β-arrestin play an important role in desensitization of receptor-mediated responses, including degranulation in leukocytes. In this study, we report that receptor phosphorylation also provides a stimulatory signal for CCR ligand 2 (CCL2) production. C3a stimulated degranulation in a basophilic leukemia RBL-2H3 cell expressing wildtype C3aR or a phosphorylation-deficient mutant (ΔST-C3aR). In contrast, C3a caused CCL2 production only in C3aR but not eΔST-C3aR cells. Furthermore, overexpression of G protein-coupled receptor kinase 2 resulted in enhancement of both ligand-induced receptor phosphorylation and CCL2 production but inhibition of degranulation. Agonist activation of C3aR, but not ΔST-C3aR, led to the translocation of green fluorescent protein tagged β-arrestin 2 from the cytoplasm to the plasma membrane. These data demonstrate that receptor phosphorylation, which provides a turn off signal for degranulation, is essential for CCL2 production.

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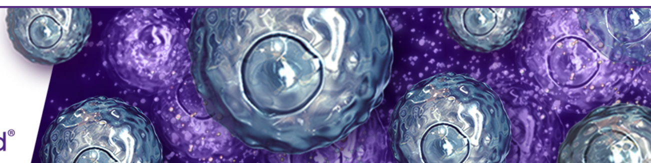

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CUTTING EDGE

Cutting Edge: Differential Regulation of Chemoattractant Receptor-Induced Degranulation and Chemokine Production by Receptor Phosphorylation[1]

Jasimuddin Ahamed,* Bodduluri Haribabu,† and Hydar Ali[2]*

Phosphorylation of G protein-coupled receptors and the subsequent recruitment of β-arrestin play an important role in desensitization of receptor-mediated responses, including degranulation in leukocytes. In this study, we report that receptor phosphorylation also provides a stimulatory signal for CCR ligand 2 (CCL2) production. C3a stimulated degranulation in a basophilic leukemia RBL-2H3 cell expressing wild-type C3aR or a phosphorylation-deficient mutant (ΔST-C3aR). In contrast, C3a caused CCL2 production only in C3aR but not ΔST-C3aR cells. Furthermore, overexpression of G protein-coupled receptor kinase 2 resulted in enhancement of both ligand-induced receptor phosphorylation and CCL2 production but inhibition of degranulation. Agonist activation of C3aR, but not ΔST-C3aR, led to the translocation of green fluorescent protein tagged β-arrestin 2 from the cytoplasm to the plasma membrane. These data demonstrate that receptor phosphorylation, which provides a turn off signal for degranulation, is essential for CCL2 production. ***The Journal of Immunology,*** **2001, 167: 3559–3563.**

Chemoattractant receptors on leukocytes mediate host defense against bacterial infection and are also involved in allergic diseases (1–3). Bacterial-derived formylated peptides (fMLP), and the complement components C3a and C5a stimulate exocytotic release of granules in basophils and eosinophils (4–6). In addition, fMLP, C3a, and C5a up-regulate the chemokine CCR ligand 2 (CCL2,[3] formerly, monocyte chemoattractant protein 1 (MCP-1)) mRNA and induce protein release (7, 8). The biological effects of chemoattractants are mediated by cell surface G protein-coupled receptors via the activation of divergent signaling pathways (8–10). For example, fMLP and C5a-induced degranulation is mediated via the activation of protein kinase C and mobilization of Ca^{2+} but not extracellular signal-regulated kinases (ERK) (11, 12). In contrast, chemoattractant-induced CCL2 production depends on ERK phosphorylation but appears to be mediated independently of protein kinase C activation (8).

Receptor phosphorylation by G protein-coupled receptor kinase (GRK) and the subsequent recruitment of β-arrestin are essential for uncoupling of receptors from G protein. In COS cells, coexpression of C3a receptors with GRK is associated with an enhancement of ligand-induced C3aR phosphorylation and inhibition of phosphoinositide hydrolysis (13). Furthermore, phosphorylation-deficient chemoattractant receptors expressed in rat basophilic leukemia RBL-2H3 cells couple more efficiently to G proteins and stimulate a more sustained Ca^{2+} mobilization and enhanced degranulation when compared with wild-type receptors (14, 15). Recent studies revealed that receptor phosphorylation by GRK and β-arrestin recruitment, which provide an inhibitory signal for G protein activation, mediate ERK activation by a number of G protein-coupled receptors (16–18). Based on these findings, we hypothesized that receptor phosphorylation could provide a stimulatory signal for chemokine production. To test this hypothesis, we used the C3aR as a model for chemoattractant receptors and generated transient transfectants expressing wild-type or phosphorylation-deficient receptors in RBL-2H3 cells. The results presented herein demonstrate that chemoattractant receptor-induced CCL2 production requires receptor phosphorylation. Furthermore, this Gi-independent signal interacts with Gi-dependent ERK phosphorylation and Ca^{2+} mobilization to induce CCL2 production.

Materials and Methods

Materials

[^{32}P]Orthophosphate was purchased from NEN Life Sciences Products (Boston, MA). fMLP, PP2, fluphenazine, and U0126 were purchased from Calbiochem (La Jolla, CA). Recombinant C5a was purchased from Sigma (St. Louis, MO). Purified C3a was obtained from Advanced Research Technologies (San Diego, CA). BAPTA-AM, indo-1 AM, and pluoronic F127 were purchased from Molecular Probes (Eugene, OR). Rabbit anit-ERK1 and anti-phospho-ERK Abs were obtained from New England Biolabs (Beverly, MA). 12CA5 Ab and PE -labeled secondary were obtained from Roche Molecular Biochemicals (Indianapolis, IN) and Southern Biotechnology Associates (Birmingham, AL), respectively. Pertussis toxin (PTX) and all tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). The ECL Western blotting analysis kit was purchased from Amersham (Arlington Heights, IL). The CCL2 sandwich ELISA kit was purchased from BioSource International (Camarillo, CA).

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[3] Abbreviations used in this paper: CCL2, CCR ligand 2 (formerly known as MCP-1), GFP, green fluorescent protein; βarr2, β-arrestin 2; GRK, G protein-coupled receptor kinase; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin.

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Cell culture, transfection, receptor phosphorylation, ERK activation, and β-hexosaminidase release assay

RBL-2H3 cells were maintained as monolayer cultures in DMEM supplemented with 15% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (11). The phosphorylation-deficient mutant of C3aR (ΔST-C3aR) was constructed by PCR. The 5′ oligonucleotide corresponding to the hemagglutinin epitope tag of the C3aR was used with a 3′ oligonucleotide complementary to the C3aR tail replacing all serine and threonine residues with alanine. The resulting PCR product was cloned into the mammalian expression vector pcDNA3 and the entire receptor cDNA was sequenced. Transient transfection in RBL-2H3 cells was performed by electroporation (11). For each transfection, 20 μg of total cDNA was used, with a ratio of 1:1 for C3aR:GRK2 and 1:4 for C3aR:ΔST-C3aR and β-arrestin 2 (βarr2)-green fluorescent protein (GFP). Empty pcDNA3 vector was used for mock transfection (15). Cells were cultured in complete growth medium for 16–18 h after transfection and used for experiments. Cell surface receptor expression was determined by incubating receptor or mock-transfected cells with 12CA5 Ab, followed by PE -labeled secondary Ab, and analyzed on a FACStar^{PLUS} flow cytometer (BD Biosciences, Mountain View, CA). Receptor phosphorylation, ERK1/ERK2 activation, and β-hexosaminidase release assay were performed as described by us previously (1, 11, 19).

Assay of CCL2 production by ELISA

For measurement of CCL2, RBL-2H3 cells (0.4×10^6/well) were cultured in complete growth medium overnight. Cells were stimulated with C3a and supernatants were collected 6 h later, centrifuged, and stored frozen at −80°C until analysis. CCL2 production was quantified by a sandwich ELISA kit as described in the manufacturer's protocols. The reaction was read at 450 nm in an ELISA plate reader.

Confocal microscopy

Cells were observed using a laser scanning confocal microscope (Olympus Fluoview; Olympus, Melville, NY) with a ×60 lens. Cells expressing hemagglutinin-tagged receptors and βarr2-GFP were plated on 35-mm glass bottom dishes (Mat Tek, Ashland, MA). The cells were stimulated with 100 nM C3a for 1 min at 37°C. The reaction was stopped by adding three volumes of cold PBS, and the cells were then washed and fixed with 2% paraformaldehyde solution for 30 min at room temperature. To visualize cell surface receptor expression, cells were incubated with 12CA5 Ab followed by Texas Red-conjugated secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The GFP was excited using a 488-nm argon/krypton laser and Texas Red was excited at 543 nm and detected at 515- to 540- and 570-nm band-pass filters, respectively.

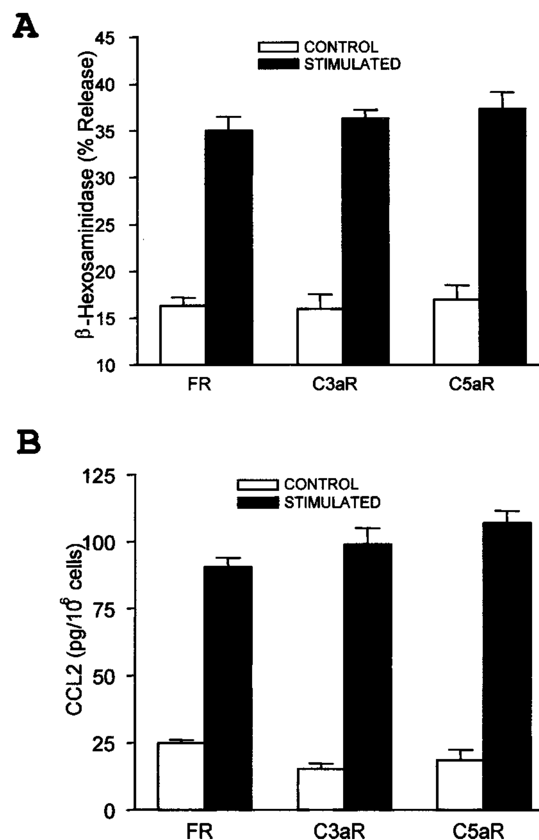

FIGURE 1. Chemoattractant receptor-induced degranulation and CCL2 production. RBL-2H3 cells transiently expressing receptors for fMLP (FR), C3a (C3aR), and C5a (C5aR) were stimulated with fMLP (1 μM), C3a (100 nM), and C5a (100 nM), respectively. β-Hexosaminidase release (*A*) and CCL2 production (*B*) were determined as described in *Materials and Methods*. The data are the mean ± SEM of three experiments performed in triplicate.

Results and Discussion

Chemoattractants stimulate degranulation and chemokine production in leukocytes (8, 10, 20–22). Recently, C3a has also been shown to stimulate chemokine production in epithelial cells (23). As shown in Fig. 1, fMLP, C3a and C5a stimulated degranulation and chemokine CCL2 production in RBL-2H3 cells transiently expressing their appropriate receptors. Receptor phosphorylation is an important mechanism for the desensitization of G protein-coupled receptor-mediated responses, including degranulation in leukocytes (14, 15, 17, 19). To determine the role of receptor phosphorylation on chemoattractant receptor-induced CCL2 production, wild-type and a phosphorylation-deficient C3aR (C3aR and ΔST-C3aR, Fig. 2*A*) were generated. Flow cytometric analysis with hemagglutinin epitope tag-specific Ab (12CA5) was used to determine the cell surface expression of C3aR and ΔST-C3aR (Fig. 2*B*). As shown in Fig. 2*C*, C3a caused phosphorylation of C3aR but not ΔST-C3aR. The effect of C3a on CCL2 production was then determined. C3a stimulated CCL2 production only in cells expressing C3aR but not ΔST-C3aR (Fig. 2*D*). The possibility that ΔST-C3aR is defective in coupling to G protein is unlikely as C3a stimulated a sustained Ca^{2+} mobilization (data not shown) and caused enhanced degranulation (Fig. 2*E*) in cells expressing this mutated receptor. These findings suggest that receptor phosphorylation, which provides a turn off signal for degranulation, is required for chemokine production.

Agonist-induced receptor phosphorylation is mediated by a family of protein kinases collectively known as GRK. Four members of this family, GRK2, GRK3, GRK5, and GRK6, are widely expressed in various cell types including leukocytes (24, 25). However, RBL-2H3 cells predominantly express GRK2 (26). Overexpression of GRK2 in COS cells, which do not endogenously express this protein, leads to enhancement of C3a-induced receptor phosphorylation and inhibition of phosphoinositide hydrolysis (13). To further test the role of receptor phosphorylation on C3a-induced responses in RBL-2H3 cells, we generated transient transfectants coexpressing C3aR and GRK2. FACS analysis revealed that expression of GRK2 had no effect on the cell surface expression of C3aR (Fig. 3*A*). GRK2 overexpression resulted in an ~2-fold increase in C3a-induced receptor phosphorylation (*inset,* Fig. 3*A*). This increase in receptor phosphorylation was associated with ~2-fold enhancement of C3a-induced CCL2 production (Fig. 3*B*) but a significant inhibition of degranulation (Fig. 3*C*). These findings support the notion that receptor phosphorylation, which turns off degranulation, is absolutely essential for chemokine production.

We have previously shown that fMLP, C3a, and C5a stimulated CCL2 production in a human mast cell line, HMC-1 (8). We suggested that chemoattractant receptor-induced ERK phosphorylation interacts synergistically with Ca^{2+}/calcineurin-dependent activation of NFAT to induce chemokine gene expression (8).

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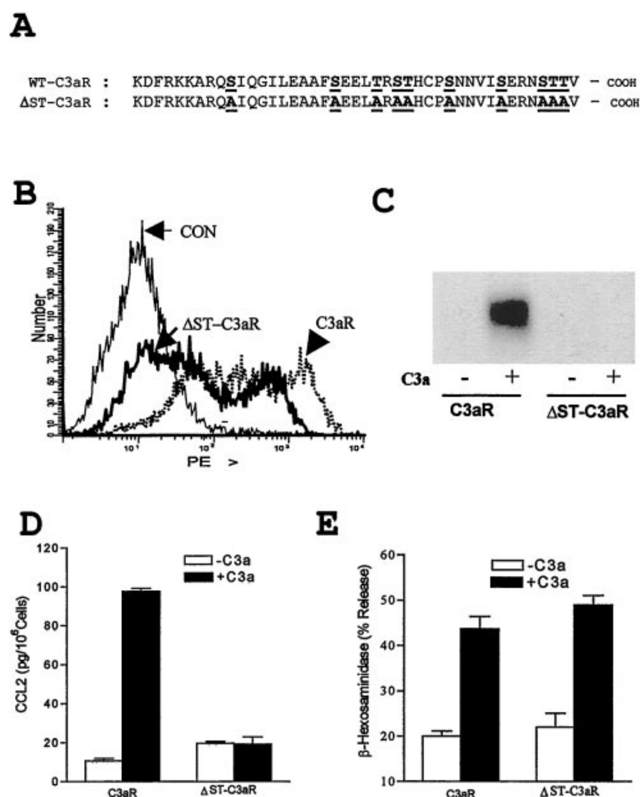

FIGURE 2. Receptor phosphorylation differentially regulates degranulation and CCL2 production. *A,* Amino acid sequences of the carboxyl-terminal tails of wild-type (WT-C3aR) and phosphorylation-deficient (ΔST-C3aR) C3aRs. Potential phosphorylation sites and their mutations are depicted by bold and underlining. Transient transfectants were generated in RBL-2H3 cells expressing C3aR or ΔST-C3aR. *B,* Cell surface receptor expression was determined by FACS analysis. Mock-transfected cells were used as a control (CON). *C,* ^{32}P-labeled cells were incubated in the absence (−) or presence (+) of C3a (100 nM) for 5 min and receptor phosphorylation was determined. *D,* Cells were cultured in growth medium and stimulated with C3a (100 nM) for 6 h. The supernatants were removed and assayed for CCL2 production by ELISA. *E,* Cells were stimulated with C3a (100 nM) for 10 min. The supernatants were removed and assayed for degranulation by measuring the release of β-hexosaminidase. The data shown are one of three similar experiments.

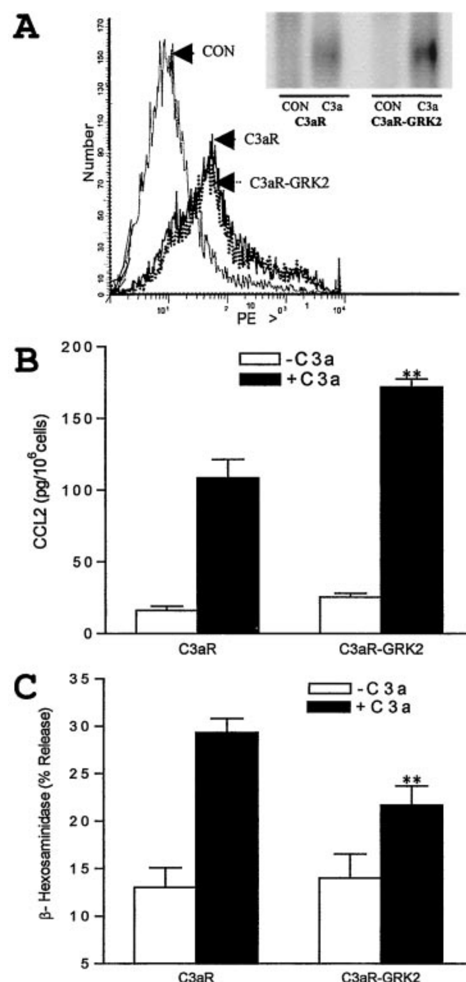

FIGURE 3. GRK2 overexpression enhances C3a-induced CCL2 production but inhibits degranulation. Transient transfectants were generated in RBL-2H3 cells expressing C3aR (C3aR) or C3aR plus GRK2 (C3aR-GRK2). *A,* Receptor expression was determined by FACS analysis. Mock-transfected cells were used as control (CON). The *inset* in *A* shows ligand-induced C3aR phosphorylation. C3a-induced CCL2 production (*B*) and β-hexosaminidase release (*C*) were also determined. **, Statistical significance based on Student's *t* test with $p \leq 0.05$.

Receptor phosphorylation by GRK and subsequent β-arrestin-mediated internalization via clathrin-coated pits is an important mechanism for activation of ERK by a number of G protein-coupled receptors (27, 28). This raises the possibility that chemoattractant receptor-induced chemokine production could involve receptor phosphorylation and β-arrestin-dependent ERK activation. To test this possibility, transient transfectants were generated in RBL-2H3 cells coexpressing C3aR or ΔST-C3aR and βarr2-GFP conjugate. As shown in Fig. 4*A*, C3a caused a rapid translocation of βarr2-GFP from the cytosol to the membrane in C3aR cells. In contrast, C3a did not induce this response in ΔST-C3aR cells. PTX, which had no effect on ligand-induced receptor phosphorylation (Fig. 4*B*) or βarr2-GFP translocation (data not shown), caused substantial inhibition of C3a-induced CCL2 production (Fig. 4*C*) and ERK phosphorylation (Fig. 4*D*). Furthermore, C3a stimulated equivalent ERK phosphorylation in both C3aR and ΔST-C3aR cells (Fig. 4, *D* and *E*). These findings suggest that although receptor phosphorylation is required for β-arrestin recruitment, it is not involved in C3a-induced ERK phosphorylation. Interestingly, DeFea et al. (29) recently showed that wild-type and phosphorylation-deficient Gαq-coupled protease-activated receptors cause ERK phosphorylation via different mechanisms with distinct roles for Src activation. However, we found that the Src inhibitor PP2 had no effect on ligand-induced ERK phosphorylation in C3aR or ΔST-C3aR cells (Fig. 4, *D* and *E*). This indicates that unlike the situation with a protease receptor, C3a-stimulated ERK phosphorylation in C3aR and ΔST-C3aR cells is mediated via a shared mechanism that does not involve receptor phosphorylation or Src activation. These data also indicate that C3a-induced receptor phosphorylation and ERK activation are mediated via independent pathways and that these pathways interact synergistically to induce CCL2 production.

Although β-arrrestin does not appear to be involved in C3a-induced ERK activation in RBL-2H3 cells, it is quite possible that this adapter molecule interacts with other signaling pathways to induce CCL2 production. Consistent with this notion, McDonald et al. (30) recently demonstrated that ligand-stimulated βarr2 recruitment leads to assembly of a signaling complex resulting in the activation of c-Jun amino-terminal kinase 3. Barlic et al. (31) showed that β-arrestin associates with two Src family tyrosine kinases, Hck and c-Fgr, in IL-8-stimulated neutrophils and RBL-2H3 cells expressing the chemokine receptor CXCR1. The demonstration in the present study that a Src inhibitor, PP2, which had

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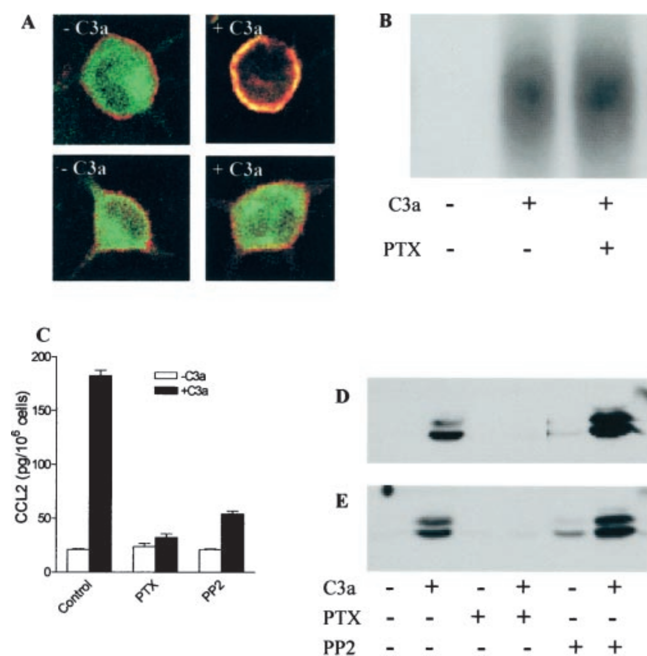

FIGURE 4. Roles of Gi-dependent and Gi-independent signaling pathways on C3a-induced CCL2 production. *A,* RBL-2H3 cells coexpressing C3aR and βarr2-GFP (*upper panels*) or ΔST-C3aR and βarr2-GFP (*lower panels*) were stimulated with C3a (100 nM) for 1 min and translocation of βarr2-GFP was determined by confocal microscopy. *B,* Cells were incubated in the absence or presence of PTX (100 ng/ml, overnight) and C3a (100 nM, 5 min) and receptor phosphorylation was determined. *C,* Cells expressing C3aR were preincubated with PTX or PP2 (10 μM, 60 min), stimulated with C3a (100 nM, 6 h), and CCL2 production was determined by ELISA. C3aR (*D*) and ΔST-C3aR (*E*) cells were preincubated with the indicated inhibitors, stimulated with C3a (100 nM) for 5 min, and ERK phosphorylation was determined by Western blotting using phospho-ERK-specific Ab. The data shown are one of three similar experiments.

no effect on C3a-induced ERK phosphorylation, caused substantial inhibition of CCL2 production, (Fig. 4, *C* and *D*) raises the possibility that a Src family member might provide a link between receptor phosphorylation and chemokine production. It will be important in future studies to identify the Src family tyrosine kinase or other proteins that interact with the phosphorylated receptor and possibly β-arrestin to induce chemokine production.

In summary, using RBL-2H3 cells as a model, we have shown that receptor phosphorylation inhibits chemoattractant-induced degranulation. Moreover, we have demonstrated that receptor phosphorylation sets into motion a stimulatory pathway for the transcriptional regulation (8) leading to chemokine production. Thus, the present study reveals the novel finding that GRK performs a dual role in chemoattractant receptor-induced biological responses: it switches off degranulation and at the same time turns on chemokine production.

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