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Chemokine Receptor CCR5 Functionally Couples to Inhibitory G Proteins and Undergoes Desensitization

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Abstract Chemokine receptor CCR5 is not only essential for chemotaxis of leukocytes but also has been shown to be a key coreceptor for HIV-1 infection. In the present study, hemagglutinin epitope-tagged human CCR5 receptor was stably expressed in Chinese hamster ovary cells or transiently expressed in NG108–15 cells to investigate CCR5-mediated signaling events. The surface expression of CCR5 was confirmed by flow cytometry analysis. The CCR5 agonist RANTES stimulated [^{35}S]GTPγS binding to the cell membranes and induced inhibition on adenylyl cyclase activity in cells expressing CCR5. The effects of RANTES were CCR5 dependent and could be blocked by pertussis toxin. Furthermore, overexpression of Giα2 strongly increased both RANTES-dependent G-protein activation and inhibition on adenylyl cyclase in cells cotransfected with CCR5. These data demonstrated directly that activation of CCR5 stimulated membrane-associated inhibitory G proteins and indicated that CCR5 could functionally couple to G-protein subtype Giα2. The abilities of CCR5 to activate G protein and to inhibit cellular cAMP accumulation were significantly diminished after a brief prechallenge with RANTES, showing rapid desensitization of the receptor-mediated responsiveness. Prolonged exposure of the cells to RANTES caused significant reduction of surface CCR5 as measured by flow cytometry, indicative of agonist-dependent receptor internalization. Our data thus demonstrated that CCR5 functionally couples to membrane-associated inhibitory G proteins and undergoes agonist-dependent desensitization and internalization. J. Cell. Biochem. 71:36–45, 1998.

Key words: chemokine receptor CCR5; G-protein activation; receptor desensitization; internalization

Chemokine receptors are a group of cell-surface receptors that mediate activation and chemotaxis of different leukocytes. Through interacting with chemokines, chemokine receptors play important roles in acute and chronic inflammatory processes. Chemokines are divided into CXC and CC (α and β) chemokine subfamilies according to the relative position of the first two conserved cysteines. Chemokine receptors have been reported to facilitate the entry of human immunodeficiency virus type 1 stains (HIV-1) into cells and amplification cycles [Clapham and Weiss, 1997]. Studies have shown that the β-chemokine receptor CCR5 serves as an entry cofactor for macrophage tropic stains of HIV in primary CD4$^+$ T cells and macrophages, whereas T-cell tropic HIV can use the α-chemokine receptor CXCR4 to infect CD4$^+$ T cells [Clapham and Weiss, 1997; Moore, 1997; Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996]. Other chemokine receptors such as CCR2B and CCR3 can also serve as entry cofactors for HIV-1 under certain conditions [Connor et al., 1997; He et al., 1997; Choe et al., 1996; Doranz et al., 1996]. The essential role of CCR5 is also indicated by resistance to HIV-1 infection of individuals with the defective CCR5 alleles [Rana et al., 1997; Liu et al., 1996].

The chemokine receptors possess the seven-transmembrane domain structure and thus belong to the G-protein-coupled receptor (GPCR) superfamily [Power and Wells, 1996]. Activa-

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tion of chemokine receptors induces chemotaxis, elevation of intracellular calcium, and activation of phospholipase C. These signal responses can be blocked by pertussis toxin (PTX), suggesting that PTX-sensitive Gi/Go proteins are required for chemokine-mediated signal transduction processes [Combadiere et al., 1996; Kuang et al., 1996; Alkhatib et al., 1997]. It has been demonstrated that chemokine receptors can couple to various G proteins including $G\alpha_i$, $G\alpha_q$, and $G\alpha_{16}$ [al-Aoukaty et al., 1996; Arai and Charo, 1996; Murphy, 1996]. However, CCR5-mediated G-protein activation has not been demonstrated directly.

With exposure to an agonist, GPCRs undergo acute desensitization, (rapid receptor uncoupling from G proteins) and internalization (receptor endocytosis from the cell surface) [Freedman and Lefkowitz, 1996]. Recent reports have shown that CC chemokines, RANTES, MIP-1α, and MIP-1β, inhibit HIV infection [Alkhatib et al., 1996; Cocchi et al., 1995, 1996; Deng et al., 1996; Dragic et al., 1996; Paxton et al., 1996] by interacting with CCR5 [Alkhatib et al., 1996; Cocchi et al., 1996; Deng et al., 1996; Dragic et al., 1996], probably through induction of chemokine receptor internalization. Agonist treatment has been reported to cause desensitization, phosphorylation, and sequestion of CCR5 after overexpression of GPCR kinases (GRKs) and β-arrestins in human embryonic kidney (HEK) 293 cells [Aramori et al., 1997].

HIV-1 infects the central nervous system and causes dementia associated with AIDS [Price et al., 1988]. CCR5, CXCR4, and CCR3 have been shown to express functionally not only in leukocytes but also in microglia cells and neurons in the central nervous system and efficiently promote HIV infection there [He et al., 1997; Hesselgesser et al., 1997]. In the present study, we used Chinese hamster ovary (CHO) cells stably expressing CCR5 and neuroblastoma × glioma hybrid (NG108–15) cells as a transient transfection system to study CCR5-mediated cellular signaling events in responses to RANTES. Our results demonstrate that CCR5 functionally couples to the inhibitory G proteins and undergoes desensitization and internalization in these cells in an agonist-dependent manner.

MATERIALS AND METHODS

Reagents

Recombinant human RANTES, a specific agonist of CCR5, was purchased from R&D Systems (Minneapolis, MN). Mouse monoclonal antibody 12CA5 against the influenza hemagglutinin (HA) epitope (YPYDVPDYA) was obtained from Boehringer Mannheim (Mannheim, Germany). Mouse monoclonal antibody against Giα2 was obtained from NeoMarkers (Fremont, CA). [^{35}S]GTPγS (1,250 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA), and [^{3}H]cAMP was obtained from Amersham (Arlington Heights, IL). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

Cloning

The full-length cDNA encoding CCR5 was cloned by polymerase chain reaction from Human Leukocyte Marathon-Ready cDNA (Clon-Tech, Palo Alto, CA) by using specific primers designed based on the published sequence [Raport et al., 1996; Samson et al., 1996], and the 5′ primer contained sequence encoding the HA epitope. The amplified human CCR5 cDNA fragment encoding the HA epitope tag was then subcloned into the expression vector pcDNA3 (Invitrogen, La Jolla, CA) to generate pcDNA-CCR5 plasmid, which will express CCR5 with an HA tag at the N-terminus. The DNA sequence was verified, and the absence of any Taq polymerase error was confirmed by sequencing.

Transfection and Receptor Expression

NG108–15 cells (American Type Culture Collection, Rockville, MD) were plated in 60-mm tissue culture dishes at 6×10^5 cells/dish 20 h before transfection in Dulbecco's Minimum Eagle's Medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Transfection was performed using 5 μg pcDNA-CCR5/6×10^5 cells and the calcium phosphate-DNA coprecipitation method, as described by Sambrook et al. [1987]. The transiently transfected NG108–15 cells (NG-CCR5) were harvested and used 48 h after transfection.

CHO cells were transfected with pcDNA-CCR5 by using LipofectAMINE (Life Technologies, Bethesda, MD) and following the manufacturer's instructions. The transfectants were placed and maintained in medium containing 1 mg/ml Geneticin (Gibco-BRL) 48 h after transfection. Geneticin-resistant clones were selected, and the expression of CCR5 was evalu-

ated by using 12CA5 and fluorescence-activated cell-sorting (FACS) analysis. The cells were maintained in medium containing 250 μg/ml Geneticin after clonal selection. A clonal CHO cell line stably expressing the HA-tagged CCR5 (CHO-CCR5) was used in the present study.

GTPγS Binding Assay

The assay was carried out as described elsewhere [Tian et al., 1993; Cheng et al., 1997]. Cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM EGTA at 4°C, and the lysate was centrifuged at 30,000*g* for 10 min. The membrane pellet (containing 15 μg protein) was resuspended and incubated at 30°C for 1 h in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 40 μM GDP, and 0.5 nM [^{35}S]GTPγS (1,200 Ci/mmol) in the presence or absence of RANTES in a total volume of 100 μl. The reaction was terminated by adding 4 ml phosphate buffered saline (PBS) and then immediately filtered through GF/C filters under vacuum. The filters were washed and counted by liquid scintillation spectrophotometry. Data were obtained by means of duplicate samples. Basal binding was determined in the absence of RANTES, and nonspecific binding was obtained in the presence of 10 μM GTPγS. The percentage of stimulated [^{35}S]GTPγS was calculated as $100 \times (cpm_{sample} - cpm_{nonspecific})/(cpm_{basal} - cpm_{nonspecific})$. Cells were cultured in serum-free medium for 12 h before agonist treatment to reduce basal GTPγS binding in the experiments without Giα2 cotransfection.

Cyclic AMP Assay

Cells (serum-starved for 12 h in the experiments without Giα2 cotransfection) were challenged with RANTES in the presence of 10 μM forskolin and 500 μM 1-methyl-3-isobutylxanthine (IBMX) at 37°C for 10 min. The reactions were terminated with 1 N perchloric acid and neutralized with 2 M K_2CO_3. The cAMP level of each sample was determined by using radioimmunoassay, as described previously [Cai et al., 1996; Ma et al., 1997]. Data were averages of duplicate samples and are presented as a percentage of control (in the presence of forskolin alone).

Flow Cytometry

Live cells were labeled with monoclonal antibody (mAb) 12CA5 in PBS containing 2% bovine serum albumin at 4°C for 1 h. After washing twice with cold PBS, the cells were treated with fluorescein isothiocyanate (FITC)-conjugated, affinity-purified goat anti-mouse IgG (Tago, Burlingame, CA) in PBS. The cells were washed twice in PBS, resuspended in the same buffer, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Live cells were gated by light scatter, and 3,000–5,000 cells were acquired for each point. Basal cell fluorescence intensity was determined with cells not transfected with CCR5 or with cells incubated only with the FITC-labeled goat anti-mouse antibody. Mean fluorescence of all live cells were used for calculation [Schmid et al., 1988; Trapaidze et al., 1996].

Internalization

CHO and NG108–15NG cells expressing CCR5 were incubated with different concentrations of RANTES for the time indicated at 37°C in DMEM. At the end of incubation, the medium was removed and cells were washed twice with ice-cold PBS. Cells were then exposed to mAb 12CA5 and FITC-conjugated affinity-purified goat anti-mouse IgG (Tago) for flow cytometry, as described elsewhere [Trapaidze et al., 1996].

Western Blotting

Cells were lysed in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2% sodium dodecylsulfate (SDS), and 1% 2-mercaptoethanol. Aliquots (15 μg protein) of the whole cell extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellose membranes. Immunoblotting was performed by using the ECL kit (Amersham) according to the manufacturer's protocols.

RESULTS

Surface Expression of CCR5

Plasmid pcDNA-CCR5 carrying the complete coding sequence of the human CCR5 was constructed and used to transfect NG108–15 cells transiently. The expressed CCR5 was tagged at the N-terminus with the influenza HA epitope to facilitate analysis. Expression of the tagged CCR5 was examined by using flow cytometry after staining with 12CA5 and FITC-conjugated secondary antibody 48 h after transiently transfecting NG108–15 cells with pcDNA-CCR5. Surface expression of CCR5 was de-

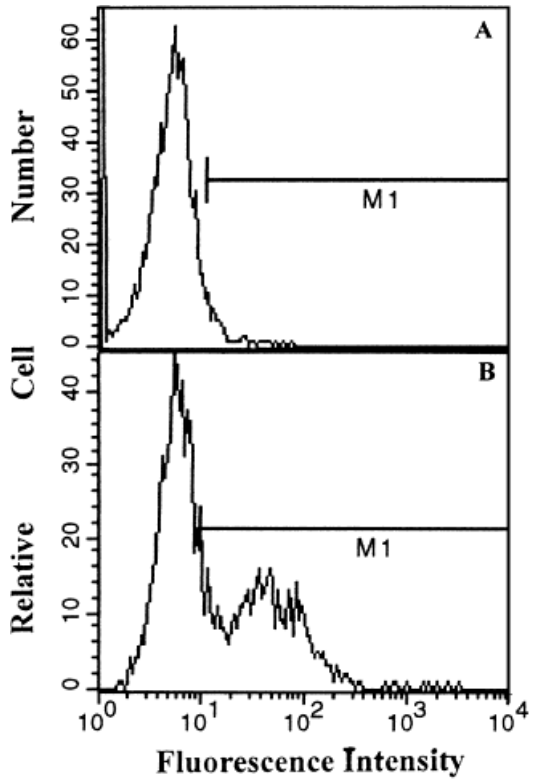

Fig. 1. Surface expression of CCR5. Flow cytometry analysis of NG-CCR5 cells were carried out after incubation with FITC-labeled goat anti-mouse antibody following incubation with mAb 12CA5 (**B**) or with FITC-labeled goat anti-mouse antibody alone (**A**). Untransfected NG108-15 cells showed negative staining under the same conditions (data not shown).

tected (Fig. 1), indicating that the exogenous CCR5 was expressed on cell surface in a grossly correct conformation. Typically, approximately 30% transiently transfected NG108–15 cells expressed the receptor, whereas the remainder was not fluorescently positive. Immunofluorescence staining of the clonal cell line CHO-CCR5 also showed the surface expression of CCR5 (data not shown).

Activation of PTX-Sensitive G Proteins

The [^{35}S]GTPγS binding assay has been widely applied to determine the agonist-dependent activation of PTX-sensitive G proteins mediated by GPCRs, including opioid, muscarinic, fromyl peptide, and α_2-adrenergic receptors [Traynor and Nahouaki, 1995]. Therefore, we examined the ability of RANTES, an agonist of CCR5, to activate membrane-associated G proteins by using this assay. RANTES stimulated [^{35}S]GTPγS binding significantly, to levels of 160% and 190% of the unstimulated controls, in NG-CCR5 and CHO-CCR5 cells, respectively (Fig. 2A), whereas no significant RANTES-dependent [^{35}S]GTPγS binding was observed in either NG108–15 or CHO cells, which do not express the chemokine receptors. These results show that the increase in [^{35}S]GTPγS binding was agonist and receptor dependent and thus

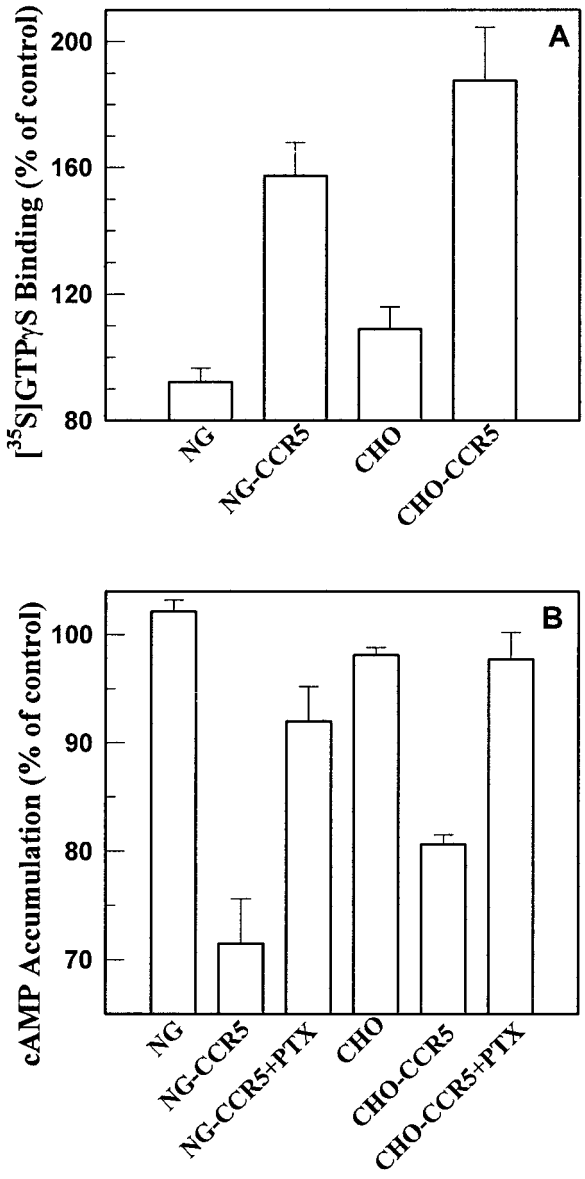

Fig. 2. CCR5-mediated activation of inhibitory G protein. [^{35}S]GTPγS binding to the cell membranes (**A**) and forskolin-stimulated cAMP accumulation (**B**) were determined in NG108–15 (NG), NG-CCR5, CHO, and CHO-CCR5 cells after stimulation with 10^{-10} M RANTES and with or without PTX pretreatment (100 ng/ml) for 24 h. The data are represented as a percentage of the control (in the absence of RANTES). The figure is representative of three independent experiments. Each value is the average of duplicate samples and represents means ± SD.

indicate that interaction of RANTES with CCR5 activates membrane-associated G proteins.

Activation of the inhibitory G proteins inhibits adenylyl cyclase activity and causes decrease of cellular cAMP level. Treatment of CHO-CCR5 and NG-CCR5 cells with RANTES resulted in a significant inhibition of foskolin-stimulated accumulation of cellular cAMP, and the RANTES-induced inhibition was abolished by treatment with PTX (Fig. 2B). These data clearly indicate that CCR5 functionally couples to the inhibitory G protein.

Functional Coupling to Giα2

It has been shown that Giα2 is one of the major subtypes of inhibitory G proteins present in NG108–15 cells [Roerig et al., 1992]. To verify that CCR5 functionally couples to the inhibitory G proteins, Giα2 was coexpressed with CCR5 in NG108–15 cells. The results showed that overexpression of Giα2 strongly increased both RANTES-promoted [^{35}S]GTPγS binding (Fig. 3A,G) and RANTES-induced inhibition of adenylyl cyclase in cells cotransfected with CCR5 (Fig. 3C), indicating that CCR5 could functionally couple to Giα2 in NG108–15 cells. Furthermore, the effects of RANTES on G-protein activation and cyclase activity in these cells were concentration dependent (Fig. 3B,D) and could be blocked by PTX completely (Fig. 3E,F). The extents of stimulation on CCR-mediated response following transfection of Giα2 are parallel with levels of Giα2 expression (Fig. 3G).

Desensitization of CCR5-Mediated Functional Responsiveness

Agonist treatment rapidly reduces responsiveness (desensitization) of G-protein-coupled receptors, which is a common feature of many GPCRs [Freedman and Lefkowitz, 1996]. Aramori et al. [1997] reported that CCR5 undergoes desensitization only after overexpression of GRKs and β-arrestins in HEK 293 cells. In the present study, the responsiveness of CCR5 to RANTES was determined after a 10-min pretreatment with different concentrations of the same agonist in NG108–15 cells cotransfected with CCR5 and Giα2. As shown in Figure 4A,B, the abilities for CCR5 to stimulate G protein activation and to inhibit cellular cAMP production were significantly diminished after prechallenge with RANTES at all concentrations tested ($P < 0.05$). Responsiveness of CCR5 decreased by more than 80% after prechallenging the cells with 0.1 nM RANTES for 10 min as observed in both assays (Fig. 4A,B). Western blot analysis showed much higher expression of GRK2 and β-arrestin in NG108 cells than in HEK 293 cells (Fig. 4C). Taken together, these results demonstrate that CCR5, like other GPCRs, undergo acute desensitization in NG108–15 cells in the absence of enormous exendogenous GRKs and arrestins in NG108–15 cells.

Agonist-Induced Internalization of CCR5

Internalization of receptors causes a reduction of surface receptors and has been implicated as one mechanism of downregulation of the GPCR-mediated cellular responses [Freedman and Lefkowitz, 1996]. The HA epitope and its mAb 12CA5 have been widely used in the studies of numerous receptors and other proteins. Activation, desensitization, and internalization of G-protein-coupled receptors such as beta2-adrenergic receptor [Barak et al., 1994], opioid receptors [Pei et al., 1995; Zhao et al., 1997], and chemokine receptors [Rucker et al., 1996; Aramori et al., 1997; Bieniasz et al., 1997] have been investigated by using the N-terminal HA-tagged receptors. Studies have also demonstrated that the HA epitope tag at the N-terminus of the receptor does not affect ligand binding and agonist-dependent signal transduction of CCR5 [Aramori et al., 1997; Bieniasz et al., 1997]. By using FACS analysis, we observed the characteristics of CCR5 internalization. Exposure of CHO-CCR5 cells to RANTES caused a left shift of the fluorescence peak value (data not shown). The mean fluorescence intensity of CHO-CCR5 cells was reduced more than 40% after a 30-min incubation with 10 nM RANTES, indicating a significant reduction in the number of CCR5 on the cell surface. Further kinetic study in CHO-CCR5 cells showed that the agonist-induced receptor internalization occurred in a time-dependent manner, with nearly 60% of CCR5 internalized 2 h after the challenge with RANTES (Fig. 5A). RENTES-induced CCR5 internalization in NG-CCR5 cells was quantitatively comparable to that in CHO-CCR5 cells (Fig. 5A). The concentration dependence of RANTES in the induction of CCR5 internalization was also observed in CHO-CCR5 (Fig. 5B) and NG-CCR5 cells (data not shown) by using FACS analysis. Taken together, our data demonstrate that CCR5 undergoes agonist-induced internalization.

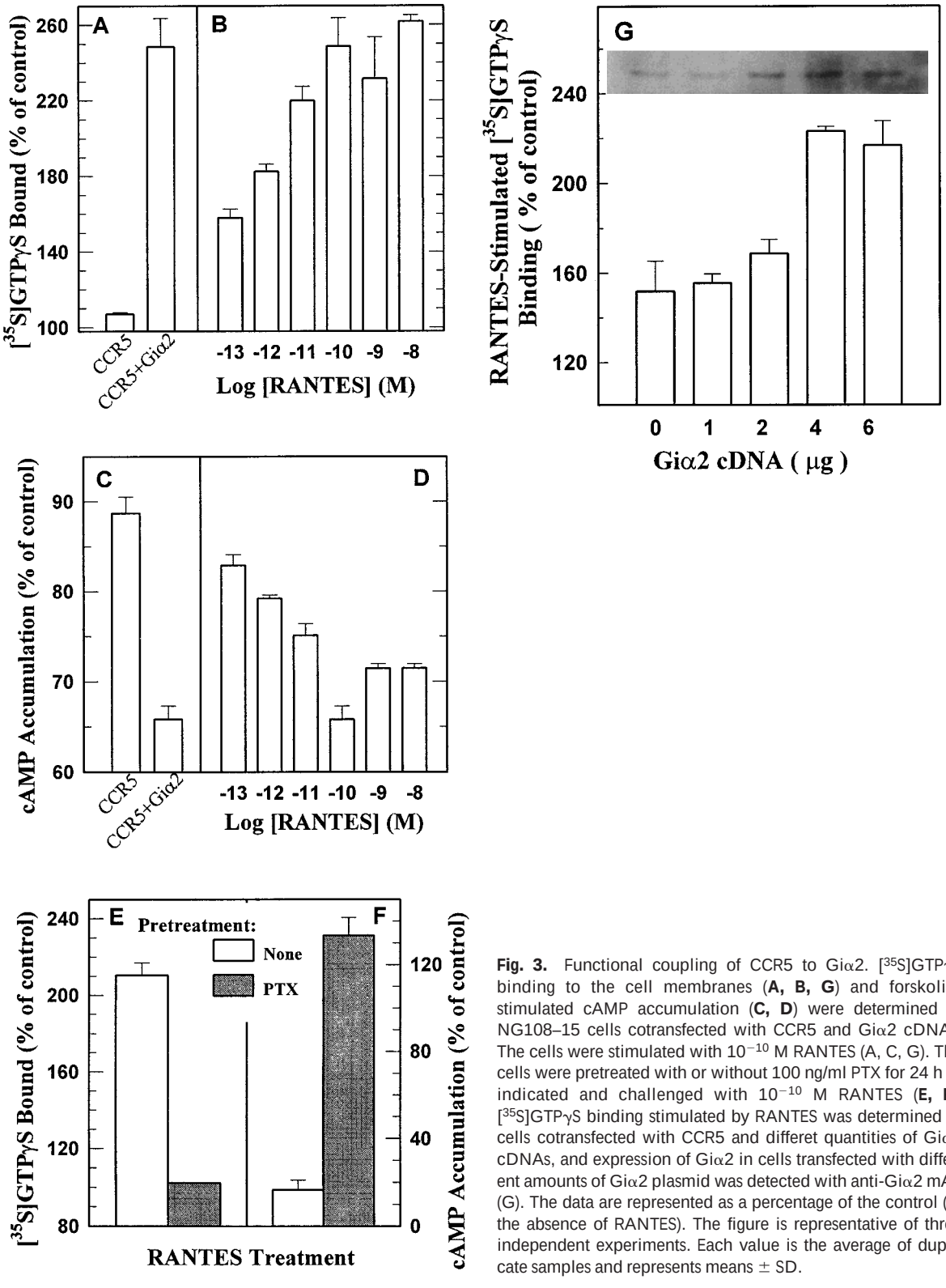

Fig. 3. Functional coupling of CCR5 to Giα2. [^{35}S]GTPγS binding to the cell membranes (**A, B, G**) and forskolin-stimulated cAMP accumulation (**C, D**) were determined in NG108–15 cells cotransfected with CCR5 and Giα2 cDNAs. The cells were stimulated with 10^{-10} M RANTES (A, C, G). The cells were pretreated with or without 100 ng/ml PTX for 24 h as indicated and challenged with 10^{-10} M RANTES (**E, F**). [^{35}S]GTPγS binding stimulated by RANTES was determined in cells cotransfected with CCR5 and differet quantities of Giα2 cDNAs, and expression of Giα2 in cells transfected with different amounts of Giα2 plasmid was detected with anti-Giα2 mAb (G). The data are represented as a percentage of the control (in the absence of RANTES). The figure is representative of three independent experiments. Each value is the average of duplicate samples and represents means ± SD.

DISCUSSION

The recent establishment of chemokine receptors as cofactors for HIV infection has contributed significantly to understanding HIV transmission and the AIDS epidemic. It also signifies the importance of understanding the basic characteristics of chemokine receptor activation and the subsequent events. Functional coupling of CCR5 to inhibition of forskolin-stimulated cAMP formation through a PTX-sensitive (G protein) mechanism has been observed in HEK 293 cells [Aramori et al., 1997; Gosling et al., 1997]. However, activation of the inhibitory G proteins by an agonist of CCR5 has not been shown. In the present study, we have demonstrated directly that agonist stimulation of CCR5 results in activation of membrane-associated inhibitory G proteins and that CCR5 can functionally couple to Giα2 and undergo acute desensitization in NG108–15 cells.

CCR5, like other GPCRs, underwent rapid desensitization with exposure to agonist, which may represent a common feature of chemokine receptors. Although the molecular mechanisms of CCR5 desensitization is not known, receptor phosphorylation and the subsequent uncoupling of receptor from G proteins are likely involved, as has been demonstrated in many other GPCRs [Freedman and Lefkowitz, 1996]. On the intracellular domains, especially at the carboxyl terminal tail of CCR5, there are rich serine and threonine residues, and some of the residues are potential phosphorylation sites for GRKs and are conserved in all β-chemokine receptors [Raport et al., 1996; Samson et al., 1996]. Agonist stimulation has been shown to induce strongly the phosphorylation of CCR2B (probably at its carboxyl terminus), and the receptor phosphorylation leads to desensitization and internalization of CCR2B [Franci et al., 1996]. Thus, receptor phosphorylation is likely involved in the desensitization of CCR5. Aramori et al. [1997] reported that CCR5 is desensitized, phosphorylated, and sequestered but only after overexpression of GRKs and β-ar-

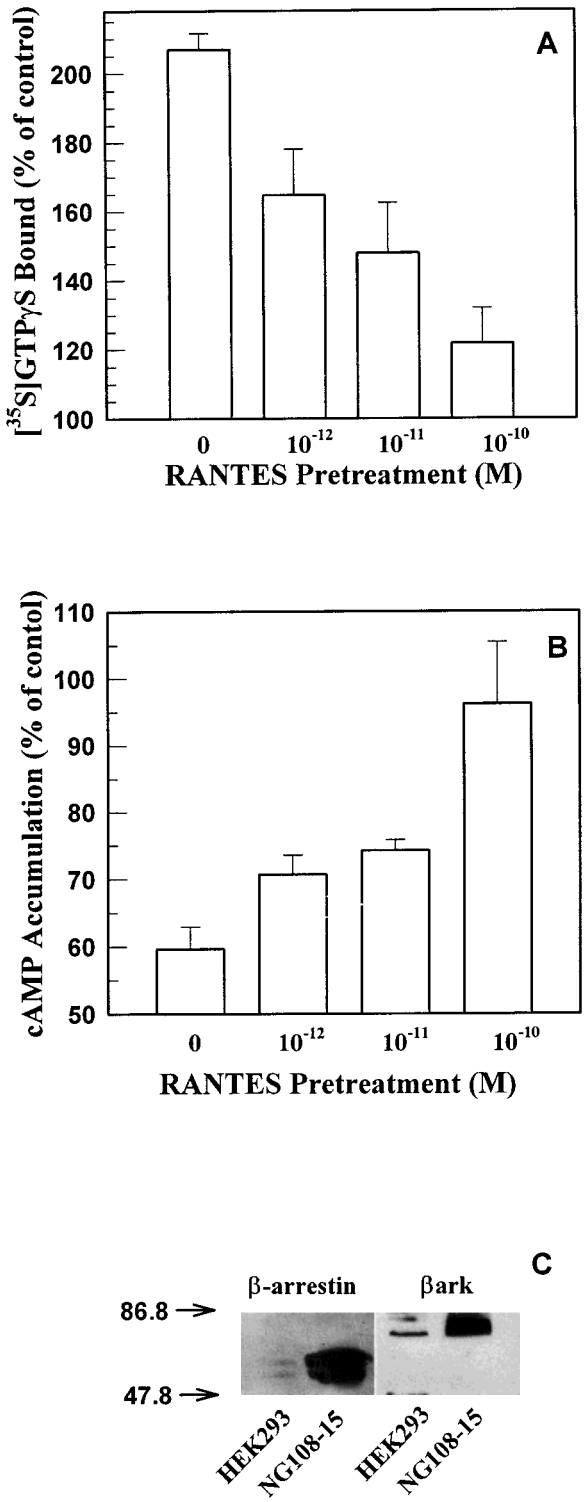

Fig. 4. Desensitization of CCR5. [^{35}S]GTPγS binding to the cell membranes (**A**) and forskolin-stimulated cAMP accumulation (**B**) were determined in NG-CCR5 cells. The cells were pretreated with 0, 10^{-12}, 10^{-11}, or 10^{-10} M RANTES at 37°C for 10 min. After washing twice with PBS, the cells were challenged again with 10^{-11} M RANTES at 37°C for 10 min. The data are represented as percentages of control (in the absence of RANTES). The figure is representative of three independent experiments. Each value is the average of duplicate samples and represents means ± SD. (**C**): Expression of endogenous GRKs and β-arrestins in NG108–15 and HEK 293 cells were analyzed by Western blot analysis by using equal amounts of cytosolic protein (15 μg) and antibodies to GRK2 and β-arrestin.

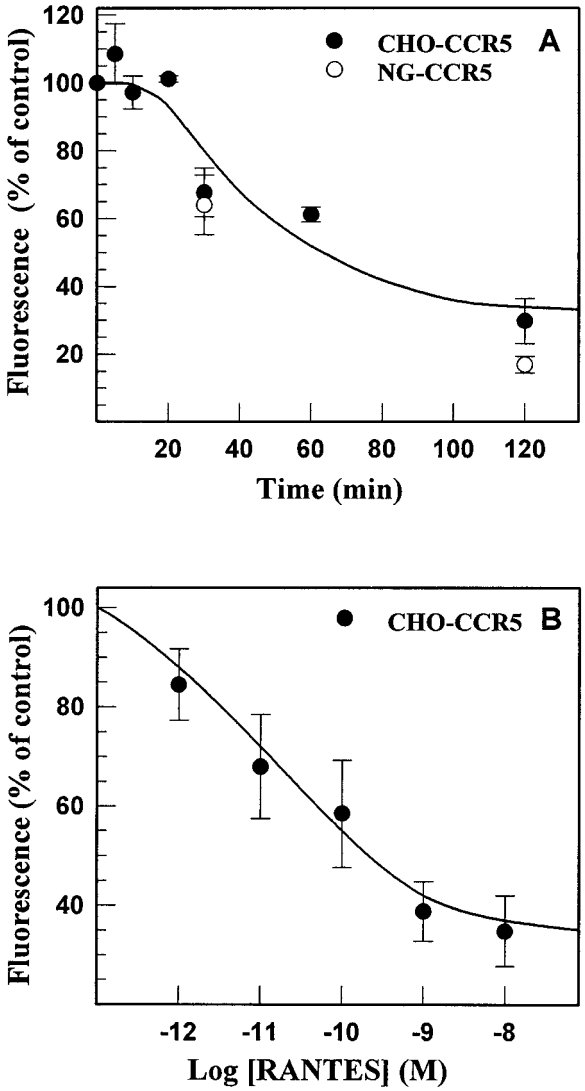

Fig. 5. Kinetics and concentration-dependence of CCR5 internalization. NG-CCR5 and CHO-CCR5 cells were treated with 10^{-8} M RANTES at 37°C for the times indicated (**A**) or with different concentrations of RANTES at 37°C for 30 min (**B**), and surface receptors were analyzed by flow cytometry by using mAb 12CA5. Receptor internalization is indicated by a reduction in the fluorescence in the cells measured. Untransfected CHO cells showed negative staining under the same conditions, and mean fluorescence did not change significantly after the RANTES treatment (data not shown). Results were presented as means ± SD. The autofluorescence of the cells is subtracted from the mean fluorescence, and the value obtained at time 0 (A) or absence of RANTES (B) is taken as 100%.

restins in HEK 293 cells. In the present study, we were able to observe desensitization of CCR-mediated responsiveness at both levels of activation of inhibitory G protein and inhibition of cyclase and internalization of CCR5 without introduction of exogenous GRKs or β-arrestins in NG-CCR5 cells. This may reflect the difference of concentrations of GRKs and β-arrestins in NG108–15 cells and HEK 293 cells. Western blot analysis showed that the level of endogenous β-arrestins and GRKs in NG108–15 cells were several fold higher than that in HEK 293 cells (Fig. 4C). Furthermore, high levels of β-arrestin mRNA and protein were found in peripheral blood leukocytes, and its expression is regulated by cellular cAMP [Parruti et al., 1993]. Hence, the level of β-arrestins may regulate, through chemokine receptors, the responsiveness of leukocyte to chemokines.

Receptor desensitization and internalization are two major means for cells to reduce quickly the receptor-mediated responsiveness to overwhelming stimulation to keep their normal functions and to avoid potential damages. Although the latest development has demonstrated that facilitation of HIV entry, signaling, and internalization are independent functions of chemokine receptors [Atchison et al., 1996; Aramori et al., 1997; Farzan et al., 1997; Gosling et al., 1997], reducing surface receptor density by promoting internalization of the HIV coreceptors appears to be a promising strategy to prevent HIV infection [Amara et al., 1997; Moore, 1997]. It has been reported that direct application of the chemokines RANTES, MIP-1α, and MIP-1β inhibit HIV entry to cells [Cocchi et al., 1995, 1996]. Studies on the mechanisms of CCR5-mediated signaling process and resultant desensitization and internalization of chemokine receptors thus would have therapeutic potential in the struggle against AIDS and other viral diseases.

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