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Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor

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The chemokine receptor, CCR-5, a G protein-coupled receptor (GPCR) which mediates chemotactic responses of certain leukocytes, has been shown to serve as the primary co-receptor for macrophage-tropic human immunodeficiency virus type 1 (HIV-1). Here we describe functional coupling of CCR-5 to inhibition of forskolin-stimulated cAMP formation via a pertussis toxin-sensitive G_i protein mechanism in transfected HEK 293 cells. In response to chemokines, CCR-5 was desensitized, phosphorylated and sequestered like a prototypic GPCR only following overexpression of G protein-coupled receptor kinases (GRKs) and β-arrestins in HEK 293 cells. The lack of CCR-5 desensitization in HEK 293 cells in the absence of GRK overexpression suggests that differences in cellular complements of GRK and/or β-arrestin proteins could represent an important mechanism determining cellular responsiveness. When tested, the activity of CCR-5 as an HIV-1 co-receptor was dependent neither upon its ability to signal nor its ability to be desensitized and internalized following agonist stimulation. Thus, while chemokine-promoted cellular signaling, phosphorylation and internalization of CCR-5 may play an important role in regulation of chemotactic responses in leukocytes, these functions are dissociable from its HIV-1 co-receptor function.

Keywords: chemokine receptors/desensitization/HIV-1 co-receptor/internalization/phosphorylation

Introduction

Chemokine receptors were recently shown to play an important role in human immunodeficiency virus type 1 (HIV-1) infection by serving as essential cofactors for HIV-1 entry (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). Chemokines are 70–90 amino acid major inflammatory peptides that have been implicated in migration and activation of leukocytes (Murphy, 1994). They can be subdivided into CXC and CC subfamilies according to the position of conserved cysteine residues, which are either separated by one amino acid (X) or are adjacent to one another (Murphy, 1994). CXC chemokines predominantly activate neutrophils and appear to be important in acute inflammatory responses, whereas CC chemokines generally target myeloid and lymphoid cells as well as basophils and eosinophils and are thought to be involved in chronic and allergic inflammation (Murphy, 1994). Chemokines bind to a family of G protein-coupled receptors (GPCRs) that are differentially expressed in blood cells (Power and Wells, 1996). CXC chemokines bind CXC-specific receptor subtypes (CXCR-1, CXCR-2, CXCR-3 and CXCR-4) and CC chemokines recognize a second subgroup of chemokine receptors (CCR-1, CCR-2a, CCR-2b, CCR-3, CCR-4 and CCR-5), each of which shows distinct but overlapping ligand binding specificity (D'Souza and Harden, 1996; Power and Wells, 1996). In contrast, Duffy antigen, the malaria trypanosome receptor, recognizes both CXC and CC chemokines.

The primary cell surface receptor for HIV-1 infection is the CD4 glycoprotein. However, expression of human CD4 in animal cell lines is not sufficient to render these susceptible to HIV-1 infection, thus suggesting that a co-receptor might be required (Maddon *et al.*, 1986; Clapham *et al.*, 1991). Subsequent analysis of several primary isolates of HIV-1 showed that these could replicate in primary T cells and macrophages but could not infect the $CD4^+$ T cell lines utilized to grow laboratory-adapted isolates of HIV-1. Conversely, these laboratory-adapted or T cell (T)-tropic HIV-1 isolates proved unable to infect $CD4^+$ macrophages that are permissive for primary or macrophage (M)-tropic HIV-1 isolates (Gartner *et al.*, 1986). These different cell tropisms, which were subsequently mapped to the HIV-1 envelope protein (O'Brien *et al.*, 1990; Hwang *et al.*, 1991), suggested that two distinct co-receptors for T- and M-tropic HIV-1 might exist. Identification of human CXCR-4 and CCR-5 as, respectively, the co-receptor for T-tropic and M-tropic HIV-1 isolates (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996) has fully validated this hypothesis. Of interest, it has also been demonstrated that the endogenous ligands for CXCR-4 [stromal cell-derived factor-1 (SDF-1)] and CCR-5 [macrophage inflammatory protein-1α (MIP-1α), MIP-1β and regulated upon activation normal T expressed and secreted (RANTES)] can prevent infection by either T-tropic or M-tropic HIV-1 respectively (Cocchi *et al.*, 1995; Bleul *et al.*, 1996; Oberlin *et al.*, 1996; Raport *et al.*, 1996; Samson *et al.*, 1996).

Although identification of CCR-5 as a cofactor for HIV-1 infection represents a breakthrough, little is known about signal transduction and regulation of this chemokine receptor. In general, agonist binding to GPCRs activates a signaling cascade mediated by intracellular second messengers, which is counteracted by intrinsic cellular mechanisms which rapidly attenuate receptor signaling. The mechanisms underlying GPCR desensitization have been particularly well studied using the β_2 adrenergic

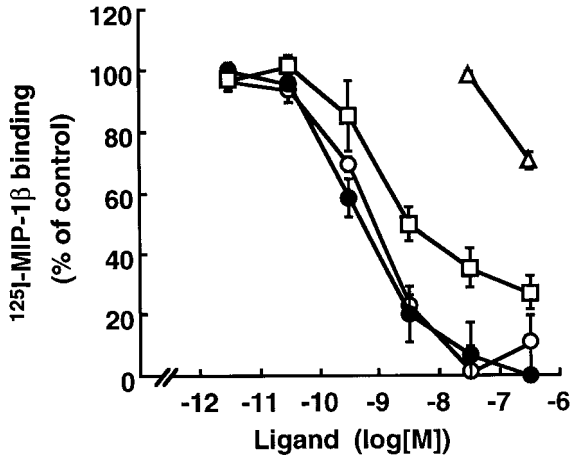

Fig. 1. Competition for specific [^{125}I]MIP-1β binding to membranes of HEK 293 cells transiently expressing 12CA5 epitope-tagged CCR-5. Experimental details are described under Materials and methods. The unlabeled ligands added in the binding assays are MIP-1α (○), MIP-1β (●), RANTES (□) and MCP-1 (△). The values are the mean ± SE of three separate experiments performed in duplicate.

receptor (β_2AR). The process involves phosphorylation by second messenger-dependent protein kinases and receptor-specific G protein-coupled receptor kinases (GRKs), which facilitate binding of arresting proteins (β-arrestins) to the receptor, resulting in further uncoupling of receptor–G protein interactions (Premont *et al.*, 1995; Ferguson *et al.*, 1996a,b). In addition, β-arrestins participate in receptor sequestration/internalization, the process responsible for re-establishment of normal responsiveness, by serving as GPCR adaptor proteins (Ferguson *et al.*, 1996c; Goodman *et al.*, 1996; Zhang *et al.*, 1996). High levels of expression of GRKs and β-arrestins have been observed in blood leukocytes (Chuang *et al.*, 1992; Parruti *et al.*, 1993), suggesting a conserved role for these proteins in regulating CCR-5 functions. Therefore, we examined whether the same mechanisms regulating activity of prototypic GPCRs (functional coupling, phosphorylation, desensitization and internalization) applied to CCR-5. In addition, we tested whether these processes might influence the ability of CCR-5 to serve as a co-receptor for HIV-1 infection.

Results

Expression and functional coupling of CCR-5 in HEK 293 cells

To examine the functional coupling of CCR-5, human embryonic kidney (HEK) 293 cells were transiently transfected with an expression vector, pCMV5/CCR-5, directing expression of a 12CA5 N-terminal epitope-tagged human CCR-5 (Bieniasz *et al.*, 1997). Expression of the receptor was detected by both flow cytometry, using an anti-12CA5 antibody, and by radioligand binding assay, using [^{125}I]MIP-1β as a radiolabeled ligand. To examine the potency of chemokine peptides in inhibiting radioligand binding, we examined competition for binding of [^{125}I]MIP1-β in the presence of MIP-1α, MIP1-β, RANTES and MCP-1 (Figure 1). MIP-1α, MIP1-β and RANTES competed specific binding of [^{125}I]MIP1-β with IC_{50} values (the half-maximal inhibitory concentrations) of 0.80 ± 0.09, 0.63 ± 0.46 and 8.5 ± 9.1 nM respectively, whereas MCP-1 showed lower binding affinity. The IC_{50} values and the order of affinity were consistent with those reported previously using cloned CCR-5 (Raport *et al.*, 1996; Samson *et al.*, 1996), indicating that the epitope-tagged receptor was not impaired in its ability to bind ligand when compared with the native receptor.

CCR-5 has been reported in a permanently transfected 3T3 cell line to elicit increases in intracellular Ca^{2+} concentrations in response to stimulation with MIP1-α, MIP1-β and RANTES (Deng *et al.*, 1996; Farzan *et al.,* 1997). However, in HEK 293 cells transiently transfected with CCR-5 no increases in inositol phosphates were observed in response to agonist stimulation (data not shown). Instead, chemokines exhibited the capacity to inhibit forskolin-induced cAMP accumulation in a dose-dependent manner (Figure 2A), whereas in cells not transfected with CCR-5, chemokines did not reduce forskolin-stimulated cAMP formation (data not shown). In these experiments CCR-5 was co-transfected with adenylyl cyclase type V (ACV), which enhanced the signal specifically in transfected cells, but was not required to observe CCR-5-mediated inhibition of cAMP accumulation. All subsequent functional experiments were performed in the presence of ACV co-expression. Forskolin-stimulated cAMP formation was reduced in a dose-dependent manner by MIP-1α, MIP1-β and RANTES, but not MCP-1 (Figure 2A). MIP-1β and RANTES were more potent than MIP-1α. EC_{50} values (the effective concentration for half-maximal response) of MIP-1α, MIP1-β and RANTES were 3.6 ± 2.1, 0.42 ± 0.11 and 0.31 ± 0.07 nm respectively. MIP-1α and RANTES have lower and higher potency respectively compared with binding affinity. A similar discrepancy in the rank order of affinity of these peptides between binding and signaling potency has also been observed with interactions of these chemokines with CCR-1 and CCR-5 (Neote *et al.*, 1993; Raport *et al.*, 1996). The results of these studies and others might suggest that MIP-1α may act as a partial CCR-5 agonist (Neote *et al.*, 1993; Raport *et al.*, 1996) MIP-1β-mediated inhibition of forskolin-induced cAMP formation was pertussis toxin (PTX) sensitive (Figure 2B), indicating that CCR-5 couples to G_i-mediated inhibition of the cAMP signaling cascade (Ui, 1984; Gilman, 1987) in addition to G_q-coupled increases in intracellular Ca^{2+} concentrations, as is often observed for a G_i/G_q-coupled receptor (Deng *et al.*, 1996; Farzan *et al.*, 1997).

Desensitization of CCR-5

Chemokine receptors are known to be desensitized rapidly *in vivo* (Murphy, 1994). However, except for MCP-1 (Franci *et al.*, 1996), the mechanism(s) by which this is achieved has not been clearly delineated. The ability of CCR-5 to couple effectively to G_i-mediated inhibition of forskolin-induced cAMP production in HEK 293 cells provided an assay with which to begin to examine CCR-5 desensitization in cell culture. To test CCR-5 desensitization, receptor-expressing cells were preincubated with or without MIP-1β (30 min), washed with an acid solution to remove ligand and then tested for dose-dependent inhibition of forskolin-stimulated cAMP formation by MIP-1β. In HEK 293 cells transfected with CCR-5 alone no desensitization of the dose–response relationship with MIP-1β was observed (Figure 3A). However, when the

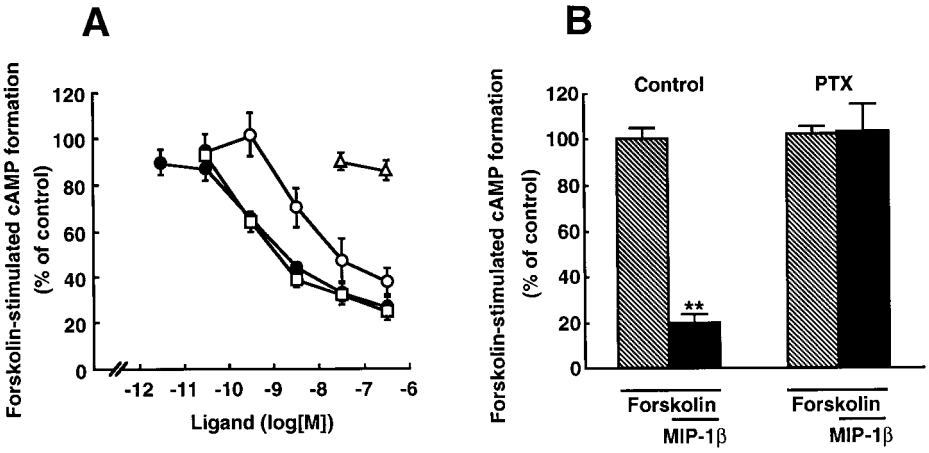

Fig. 2. Functional coupling of CCR-5. (**A**) Effect of chemokine peptides on forskolin-stimulated cAMP formation in HEK 293 cells transiently expressing CCR-5 and ACV. HEK 293 cells expressing CCR-5 and ACV were incubated with the indicated concentrations of MIP-1α (○), MIP-1β (●), RANTES (□) and MCP-1 (△) in the presence of 1 μM forskolin for 30 min and then assessed for cAMP formation. The values are the mean ± SE of three separate experiments performed in triplicate. (**B**) Effect of PTX on agonist-induced inhibition of forskolin-stimulated cAMP formation in HEK 293 cells transiently expressing CCR-5 and ACV. Transfected cells were pretreated with or without 100 ng/ml PTX for 20 h, incubated with 1 μM forskolin in the presence or absence of 100 nM MIP-1β for 30 min and then assessed for cAMP formation. Data presented were taken from a representative of two separate experiments and the values are the mean ± SD of triplicate determinations. $^{**}P < 0.01$ compared with incubation with forskolin alone.

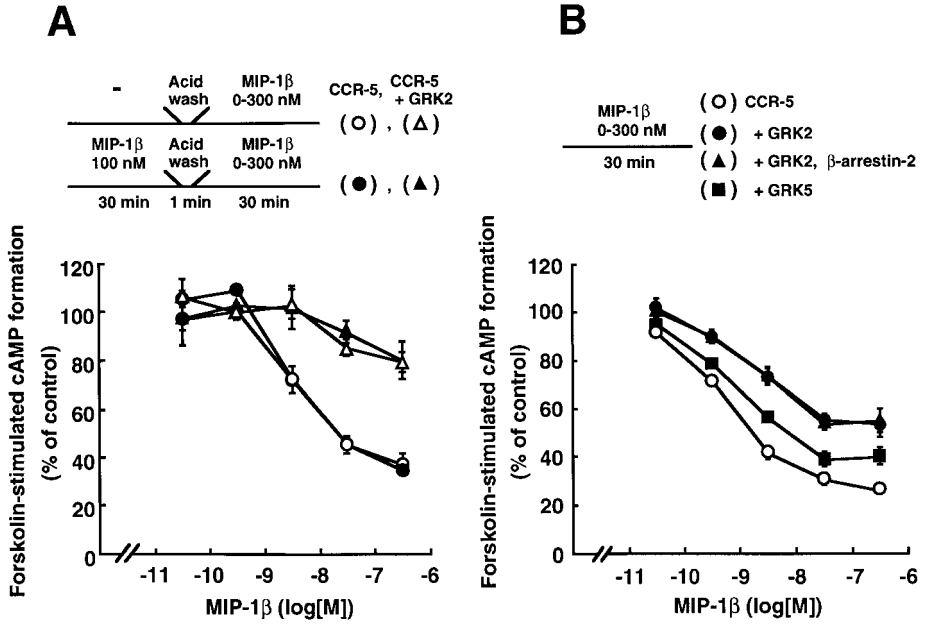

Fig. 3. Desensitization of CCR-5. (**A**) HEK 293 cells expressing CCR-5 and ACV, together with (△, ▲) or without (○, ●) GRK2 were pretreated (▲, ●) or not (△, ○) with 100 nM MIP-1β for 30 min at 37°C. After rinsing once with 50 mM glycine and 150 mM NaCl, pH 3.0, for 1 min, the cells were incubated with the indicated concentrations of MIP-1β in the presence of 1 μM forskolin for 30 min and then cAMP formation determined. Data presented were taken from a representative of two separate experiments and the values are the mean ± SD of triplicate determinations. (**B**) HEK 293 cells expressing CCR-5 and ACV (○), together with GRK2 (●), GRK2 plus β-arrestin-2 (▲) or GRK5 (■) were incubated with the indicated concentrations of MIP-1β in the presence of 1 μM forskolin for 30 min. cAMP formation was then determined. The values are the mean ± SE of three to four separate experiments performed in triplicate.

cells were transfected with CCR-5 together with a GRK2 expression plasmid (similar expression levels of CCR-5 were confirmed by flow cytometry) MIP-1β-stimulated inhibition of cAMP formation was virtually abolished, suggesting that GRK2 phosphorylation potently regulates functional coupling of CCR-5. Using this protocol, CCR-5 responsiveness was markedly attenuated even in the absence of pre-exposure to ligand, likely as a consequence of rapid GRK-mediated receptor phosphorylation ($t_{1/2}$ = 15 s; Roth *et al.*, 1991) during the 30 min time period over which the cells were exposed to the high affinity peptide ligand (Figure 3A). The effect of GRK overexpression on CCR-5 desensitization was not limited to GRK2 (Figure 3B). Like GRK2, in the absence of pre-exposure to ligand, overexpression of GRK5 desensitized MIP-1β-mediated inhibition of forskolin-induced cAMP accumulation, albeit less effectively (Figure 3B). Co-expression of β-arrestin-2 with GRK2 did not further enhance GRK2-mediated impairment of the CCR-5-mediated inhibitory response to cAMP formation (Figure 3B).

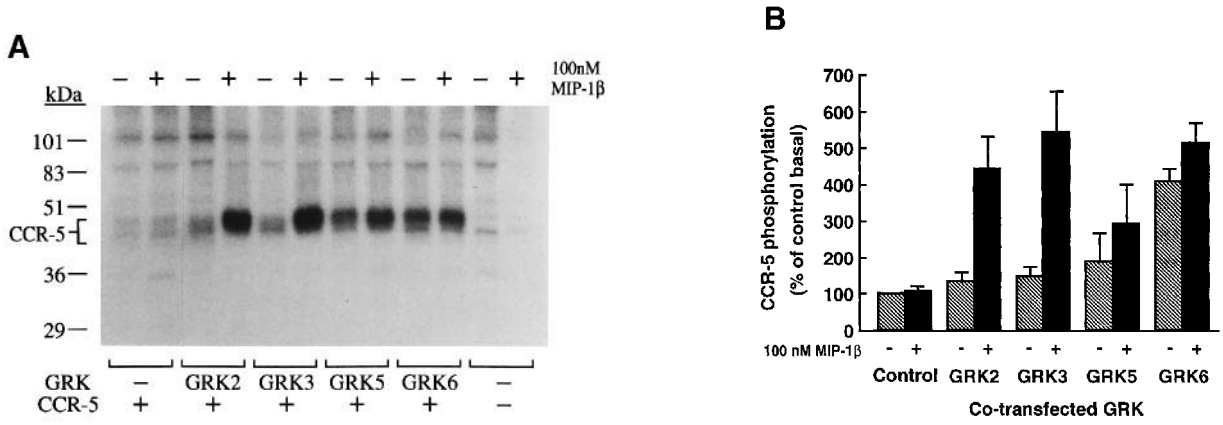

Fig. 4. Phosphorylation of CCR-5. (**A**) Effect of overexpression of GRKs on CCR-5 phosphorylation. HEK 293 cells transiently expressing CCR-5 together with the indicated GRKs were metabolically labeled with [^{32}P]orthophosphate and stimulated or not with 100 nM MIP-1β for 10 min at 37°C. Immunoprecipitated receptors were subjected to 10% SDS–PAGE. Mock-transfected cells were treated identically. The autoradiograph shown is a representative of three separate experiments. (**B**) The radioactivity migrating at the position of CCR-5 was quantitated using a PhosphorImager. Receptor phosphorylation was expressed as percentage radioactivity compared with unstimulated control cells. The values are the mean ± SE of three separate experiments.

The difference in the extent of GRK2-mediated desensitization observed in Figure 3A versus B likely represents interexperimental variability in GRK expression levels achieved following transient transfection of HEK 293 cells. However, the profile of desensitization in the absence of a desensitizing pre-stimulus is similar to that described for the β_1-adrenergic and D1 dopamine receptors following GRK overexpression (Freedman *et al.*, 1995; Tiberi *et al.*, 1996).

Phosphorylation of CCR-5 by GRKs

GRKs mediate GPCR desensitization by rapidly phosphorylating receptors in response to agonist activation (15–30 s; Roth *et al.*, 1991). Therefore, to address whether CCR-5 desensitization following GRK overexpression was a consequence of increased receptor phosphorylation, we examined whole-cell phosphorylation of CCR-5 following a 10 min exposure to 100 nM MIP-1β. CCR-5 does not contain any consensus sites for phosphorylation by either protein kinase A or protein kinase C (Samson *et al.*, 1996), consequently any receptor phosphorylation observed in the absence of co-transfected GRKs is presumably mediated by endogenously expressed GRKs. In HEK 293 cells expressing CCR-5 alone no agonist-dependent receptor phosphorylation was detected (Figure 4A). However, overexpression of either GRK2 or GRK3 resulted in a dramatic increase in CCR-5 phosphorylation (Figure 4A), 4.4- and 5.4-fold over basal respectively (Figure 4B). In contrast, overexpressed GRK5 increased basal phosphorylation but did not enhance agonist-dependent CCR-5 phosphorylation as extensively (2.9-fold over basal non-transfected with GRKs) as compared with GRK2 and GRK3. These results mirror the agonist-mediated desensitization pattern presented in Figure 3B. GRK6 overexpression primarily resulted in increased agonist-independent CCR-5 phosphorylation.

Agonist-promoted internalization of CCR-5

Recent data from our laboratory have demonstrated that GRK phosphorylation facilitates internalization of β_2AR, by promoting binding of β-arrestin to the receptor, which then acts as a GPCR endocytic adaptor protein (Ferguson *et al.*, 1996c; Zhang *et al.*, 1996). However, β-arrestin-dependent endocytosis of other GPCRs has not been reported. Therefore, we tested whether CCR-5 was internalized in response to MIP-1β stimulation and whether internalization was β-arrestin dependent, as for β_2AR (Figure 5A). CCR-5 internalization following a 30 min exposure to 100 nM MIP-1β was measured by flow cytometry following labeling of cell surface receptors with anti-12CA5 antibody and internalization was quantitated as loss of cell surface immunofluorescence (Barak *et al.*, 1994). When expressed alone in HEK 293 cells CCR-5 was internalized poorly (17% loss of cell surface receptors) and overexpression of GRK2, β-arrestin-1 and β-arrestin-2 individually did little to enhance internalization of the receptor. However, when GRK2 was overexpressed with either β-arrestin-1 or β-arrestin-2 a synergistic increase in CCR-5 internalization was observed (34 and 44% respectively). In contrast, when the receptor was expressed with the β-arrestin mutants β-arrestin-1-V53D and β-arrestin-2-V54D its internalization was inhibited (11%) or unchanged respectively. These mutant β-arrestins when expressed at high levels compete with endogenous β-arrestins for the receptor but are unable to mediate receptor sequestration and thus function as dominant negative mutants.

We have reported that β-arrestin targets GPCRs for dynamin-dependent, clathrin-coated vesicle-mediated endocytosis and that this process can be blocked by a dynamin I mutant, dynamin I-K44A (Zhang *et al.*, 1996). Therefore, since CCR-5 internalization was responsive to GRK phosphorylation and β-arrestin binding, we tested whether dynamin I-K44A overexpression might impair CCR-5 internalization. Dynamin I-K44A when expressed alone modestly impaired CCR-5 internalization, but when expressed with both GRK2 and β-arrestin-1 or β-arrestin-2 abolished GRK- and β-arrestin-mediated sequestration of the receptor (Figure 5A).

Cell type specificity for G protein-coupled receptor internalization has also been observed, i.e. β_2AR is sequestered well in HEK 293 cells but poorly in COS-7 cells (Zhang *et al.*, 1996). Consequently, we tested the ability of CCR-5 to be internalized in COS-7 cells. In the absence

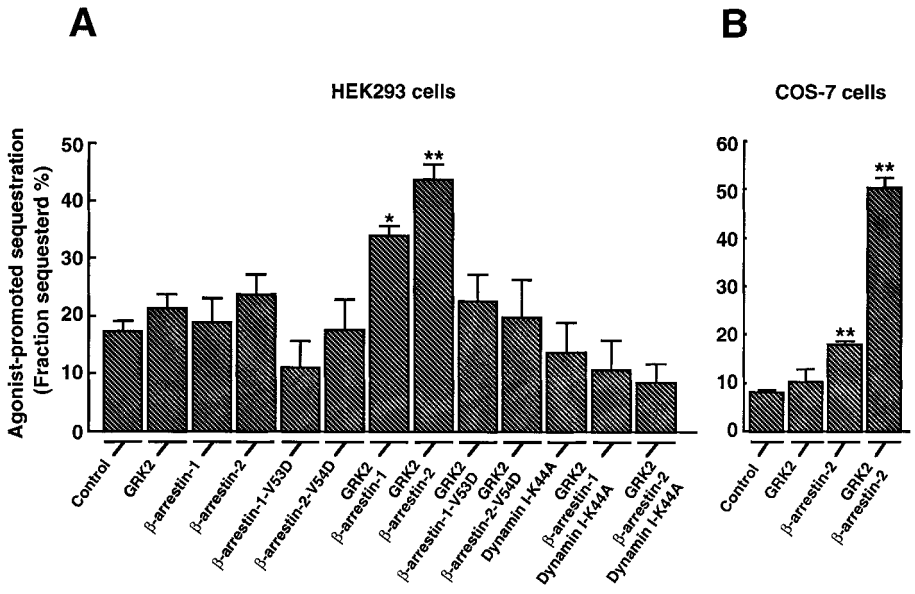

Fig. 5. Agonist-promoted sequestration of CCR-5. Effect of GRK2, β-arrestin-1, β-arrestin-2, β-arrestin-1-V53D, β-arrestin-2-V54D and dynamin I-K44A overexpression on agonist-promoted sequestration of CCR-5. HEK 293 (**A**) or COS-7 cells (**B**) co-expressing 12CA5 epitope-tagged CCR-5 and the indicated proteins were incubated with or without 100 nM MIP-1β for 30 min, following which sequestration of CCR-5 was assessed by flow cytometry using monoclonal anti-12CA5 antibody. The values are the mean ± SE of three to seven separate experiments. $*P < 0.05$ and $**P < 0.01$ compared with control.

of co-transfected GRK2 and β-arrestins, internalization of the receptor in COS-7 cells was low when compared with HEK 293 cells (8 versus 17%; Figure 5B). However, while GRK overexpression had little effect on CCR-5 internalization in COS-7 cells, overexpression of β-arrestin-2 significantly increased the proportion of internalized receptor (18%). Interestingly, co-expression of GRK2 and β-arrestin-2 resulted in a synergistic increase in the extent of CCR-5 internalization in COS-7 cells (Figure 5B) and was identical to that observed in HEK 293 cells. These results are consistent with data obtained for β_2AR, which has been demonstrated to be sequestered rather than down-regulated following short-term agonist stimulation (Barak *et al.*, 1994).

Expression of GRK and β-arrestin in human T cell lines

To examine whether the effect of overexpression of GRK2 and β-arrestins on CCR-5 desensitization and agonist-promoted internalization in transfected in HEK 293 cells might be physiologically relevant to native blood cells, we examined and compared endogenous expression levels of these proteins in both HEK 293 and a human T cell line, PM1. The PM1 cell line is a $CD4^+$, $CCR\text{-}5^+$ clone derived from the neoplastic T cell line HUT78 and was chosen because it is known to be susceptible to a wide range of HIV-1 isolates, including M-tropic isolates (Lusso *et al.*, 1995). Immunoblots for GRK2 and GRK3 expression levels in PM1 cells and HEK 293 cells expressing CCR-5 with and without GRK2, β-arrestin-2 or both GRK2 and β-arrestin-2 revealed that GRK2 expression in PM1 cells is substantially greater than that found endogenously in HEK 293 cells. In fact, the endogenous expression level of GRK2 in PM1 cells was equivalent to that observed following GRK2 overexpression in HEK 293 cells (Figure 6A). The expression level of β-arrestin in PM1 cells was ~1.6-fold higher than that of the

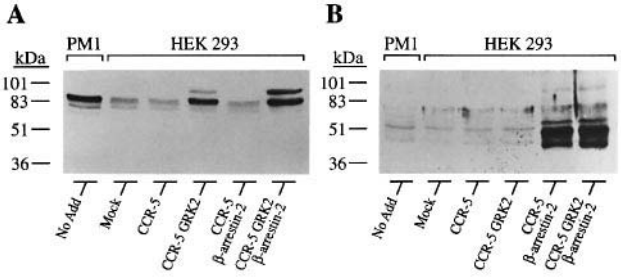

Fig. 6. Expression of GRK and β-arrestin in PM1 cells and HEK 293 cells. Expression levels of GRK (**A**) and β-arrestin (**B**) were assessed by Western blot analysis of 100 and 50 μg total protein respectively, prepared from PM1 cells and HEK 293 cells transfected with plasmids encoding the indicated proteins, using antibody cross-reactive for both GRK2 and 3 (A) or both β-arrestin-1 and -2 (B).

untransfected HEK 293 cells. These results indicate that overexpression of GRK2 and β-arrestins in HEK 293 cells likely provides a good experimental model system which recapitulates to some extent the endogenous cellular milieu in which the CCR-5 receptor is normally active.

Receptor signaling mechanisms and co-receptor function for HIV-1 entry

Although CCR-5 was shown to interact with a complex of CD4 and gp120 (Trkola *et al.*, 1996; Wu *et al.*, 1996), it was not known whether the co-receptor function of CCR-5 in HIV-1 infection might be related to normal functioning of the receptor, i.e. signaling and internalization. To begin to address these questions we constructed a CCR-5 mutant, Y297A (Figure 7A), which, based upon the study of β_2AR and other receptors, might be impaired in its ability to signal and perhaps even its ability to be internalized (Barak *et al.*, 1994; Slice *et al.*, 1994; Hunyady *et al.*, 1995; Ferguson *et al.*, 1995). The Y297A mutant is similar to the Y326A mutant described previously for β_2AR, which is impaired in its ability to signal, serve as

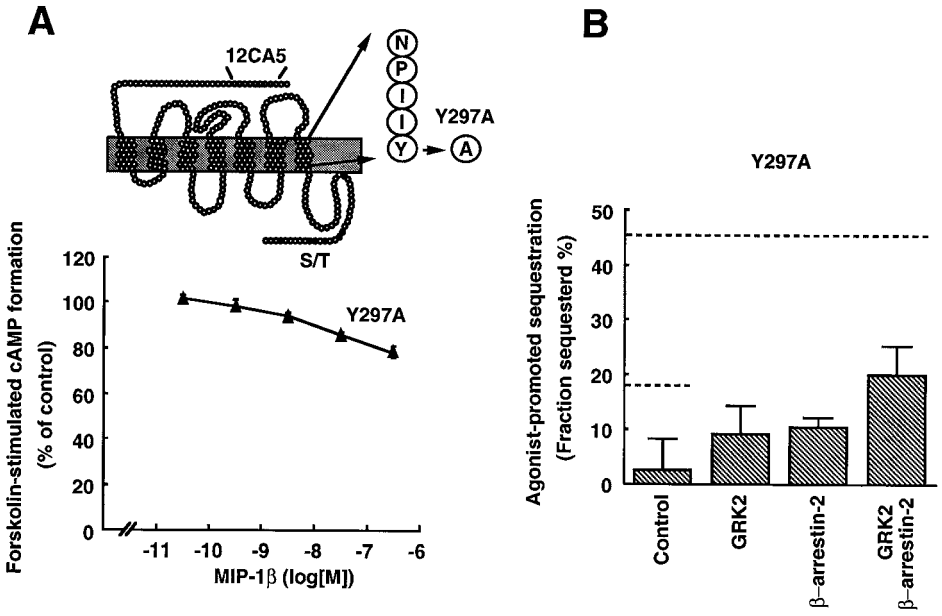

Fig. 7. Construction and characterization of a CCR-5 mutant. (**A**) Illustration of the Y297A mutant (inset) and coupling to inhibition of forskolin-stimulated cAMP formation in receptor-expressing cells. HEK 293 cells expressing Y297A (▲) mutant receptors together with ACV were incubated with the indicated concentrations of MIP-1β in the presence of 1 μM forskolin for 30 min and then monitored for cAMP formation. The values are the mean ± SE of three separate experiments performed in triplicate. (**B**) Sequestration of Y297 mutant receptor. HEK 293 cells co-expressing 12CA5 epitope-tagged Y297 mutant receptor and the indicated proteins were incubated with or without 100 nM MIP-1β for 30 min, following which sequestration of Y297 mutant receptor was assessed by flow cytometry using monoclonal anti-12CA5 antibody. The values are the mean ± SE of four separate experiments. The dashed lines indicate the level of sequestration of wild-type CCR-5 with (upper line) or without (lower line) co-expression of GRK2 and β-arrestin-2 (see Figure 5).

a substrate for GRK-mediated phosphorylation and be sequestered. When evaluated, the Y297A mutant was found to be functionally impaired in its ability to inhibit forskolin-stimulated cAMP formation (Figure 7A). Agonist-promoted internalization was also impaired for the Y297A mutant compared with wild-type CCR-5, not only when cells were transfected with CCR-5 alone, but also when co-transfected with GRK2, β-arrestin-2 or both GRK2 and β-arrestin-2 (Figure 7B).

CCR-5 has been demonstrated to serve as the co-receptor for M-tropic HIV infection. Therefore, it was of particular interest to examine whether CCR-5 coupling, desensitization and/or internalization might regulate its function as a co-receptor for HIV-1 entry. We addressed whether altered CCR-5 signaling, phosphorylation and internalization affected CCR-5 co-receptor function in HIV-1 infection in two ways. First, we examined whether the uncoupled Y297A mutant supported HIV-1 infection. Second, we examined whether the effect of overexpressed GPCR regulatory proteins, to increase CCR-5 phosphorylation and/or either increase or decrease CCR-5 internalization, might affect the ability of CCR-5 to function as an HIV-1 co-receptor. In these experiments HEK 293 cells were transfected with an HIV-1 proviral expression vector encoding the M-tropic BaL proviral DNA (Hwang *et al.*, 1991) and used as HIV-1-producing cells. HIV-1 indicator cells were generated by transfection of HEK 293 cells with an expression vector encoding each of the receptors and regulatory proteins together with both a human CD4 expression vector and an indicator construct containing the HIV-1 LTR linked to a secreted alkaline phosphatase (SEAP) indicator gene (Berger *et al.*, 1988), as previously described (Bieniasz *et al.*, 1997). The results of these experiments are illustrated in Figure 8. Transfection of increasing amounts of both wild-type and Y297A receptors resulted in progressively more HIV-1 infection (Figure 8A). In addition, co-transfection of wild-type CCR-5 with GRK2, β-arrestin-1, β-arrestin-1-V53D, GRK2 and β-arrestin, dynamin I or dynamin I-K44A did not result in any significant change in HIV-1 infection compared with cells transfected with receptor alone (Figure 8B). These results indicate that functional coupling, phosphorylation and internalization do not contribute significantly to the co-receptor function of CCR-5 for HIV-1 entry.

Discussion

The present experiments provide evidence that the functional activity of CCR-5, the co-receptor for M-tropic HIV-1 and a member of the chemokine responsive subset of the GPCR superfamily, is regulated by the same mechanisms delineated for prototypic GPCRs. In response to chemokine stimulation, CCR-5 can couple through G_i to inhibition of intracellular cAMP formation in HEK 293 cells and, in the presence of appropriate regulatory proteins (GRKs and β-arrestins), the signaling function of this receptor is desensitized. In addition, agonist-stmulated CCR-5 internalization, like β_2AR, is β-arrestin dependent. Moreover, through the use of mutant receptors and over-expression of GPCR regulatory proteins, we demonstrate that the signaling function of CCR-5, its phosphorylation and desensitization as well as its internalization in response to ligand binding are all events that can be dissociated from the ability of CCR-5 to support HIV-1 infection in host cells.

Expression of CCR-5 in HEK 293 cells demonstrates

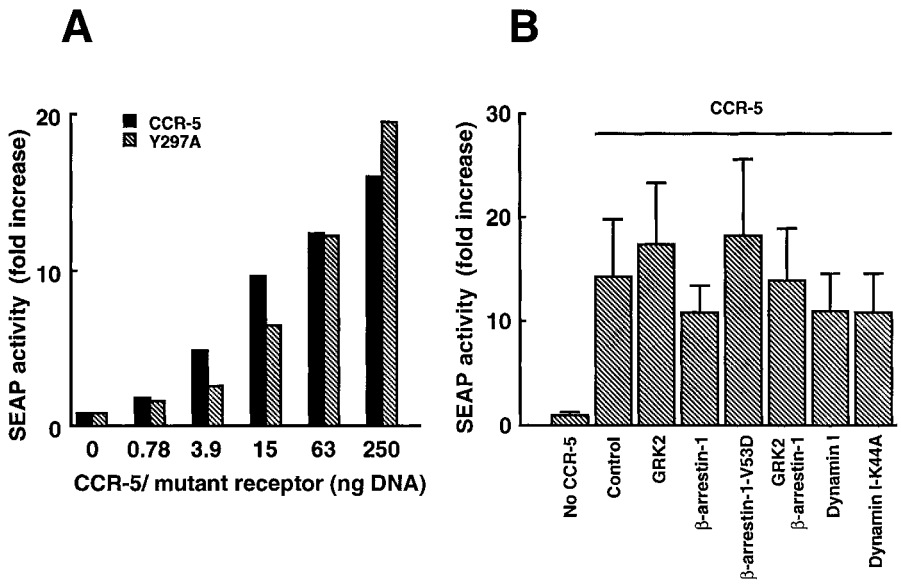

Fig. 8. Co-receptor function of CCR-5 and Y297A mutant receptor for entry of HIV-1. (**A**) Indicator cells were generated by transfection of HEK 293 cells with the indicated concentrations of plasmids encoding either wild-type CCR-5 or Y297A mutant receptor, together with plasmids encoding CD4 and the pCMV5/HIV/SEAP indicator construct. Co-receptor function for HIV-1 entry was assessed by measuring SEAP activity after co-cultivation with HIV-1 BaL-producing cells. Co-receptor function was expressed as fold increase in SEAP activity compared with the activity when co-cultivated with non-virus-producer cells. Data are representative of three separate experiments. (**B**) HEK 293 cells transfected with plasmids encoding CCR-5 (50 ng), the indicated protein (1 μg each), CD4 and pCMV5/HIV/SEAP were used as indicator cells. Co-receptor function was determined by measuring SEAP activity after co-cultivation with HIV-1 BaL-producing cells. The values are the mean ± SD of four separate experiments.

that in response to the chemokines MIP-1α, MIP-1β and RANTES, CCR-5 couples efficiently to inhibition of intracellular cAMP levels in these cells. CCR-5 signaling, which was monitored in the presence of co-transfected ACV in order to enhance the agonist-generated signal in transfected cells, was presumably through the G protein subtype G_i, as the response was sensitive to PTX (Ui, 1984; Gilman, 1987). In contrast to the recent study of Deng *et al.* (1996), reporting chemokine-stimulated Ca^{2+} mobilization in CCR-5-expressing cells, but similar to the findings of Raport *et al.* (1996), we were unable to detect phosphatidylinositol hydrolysis in response to chemokines in HEK 293 cells transfected with CCR-5 (data not shown). These apparent dissimilarities might reflect both differences in the cellular complements of G proteins and the sensitivities of the assays used to demonstrate G_q coupling, Ca^{2+} mobilization and inositol phosphate formation. Nonetheless, the biochemical complements of signaling molecules in individual cell lines might determine the selectivity of the signal transduction pathway (Raport *et al.*, 1996), the heterologous HEK 293 cell expression system has been used to examine the signaling function of many GPCRs and now provides an excellent G_i-coupled reporter system in which to study not only the signaling function of CCR-5, but its potential mechanisms of regulation.

Chemokine receptors cause chemotactic and pro-adhesive effects in leukocytes in response to locally produced chemoattractants and not only activation but desensitization of chemokine receptor responsiveness is thought to play a critical role in maintaining the ability of leukocytes to sense a gradient of chemoattractant (Murphy, 1994). For instance, a short exposure (30 min) of monocytes to MIP-1β results in a decreased chemotactic response when cells are re-exposed to either MIP-1α or MIP-1β (Wang *et al.,* 1993). Interestingly, in transfected HEK 293 cells expressing CCR-5 and ACV alone exposure to chemokine failed to produce desensitization of the cAMP inhibitory response. However, when the cells were transfected to overexpress GRK2, MIP-1β-induced inhibition of cAMP accumulation was profoundly attenuated even without prior incubation with MIP-1β. This observation correlated well with agonist-induced CCR-5 phosphorylation, which was also only apparent following overexpression of GRKs.

Interestingly, during the preparation of this manuscript it was reported that another chemokine receptor (MCP-1R) was specifically phosphorylated by GRK2 in microinjected *Xenopus* oocytes (Franci *et al.*, 1996). These results suggest that GRK-mediated phosphorylation may represent a common mechanism by which chemokine receptor desensitization is achieved. However, unlike MCP-1R, CCR-5 could be phosphorylated by GRK2, -3, -5 and -6. While all four GRKs tested proved to phosphorylate CCR-5, selectivity of the GRKs was also evident. Whereas GRK2 and GRK3 overexpression led to increases in phosphorylation primarily in the presence of agonist, GRK5 and GRK6 phosphorylated CCR-5 even in the absence of agonist stimulation. Such a level of selectivity and agonist independence of GRK phosphorylation has been previously observed with other GPCRs (Ménard *et al.*, 1996; Oppermann *et al.*, 1996; Tiberi *et al.*, 1996). Nonetheless, CCR-5 seems to differ from other prototypes of the GPCR family, such as the β_1-adrenergic, β_2-adrenergic, dopamine D1A and angiotensin II type 1A receptors, which can all be desensitized and phosphorylated in an agonist-dependent fashion by the endogenous complement of GRKs in HEK 293 cells (Ferguson *et al.*, 1995; Freedman *et al.*, 1995; Oppermann *et al.*, 1996; Tiberi *et al.*, 1996).

Previous studies have shown that peripheral blood

leukocytes contain high levels of mRNA for GRKs (Chuang *et al.*, 1992). Interestingly, when the levels of expression of GRK2 obtained by overexpression in HEK 293 cells were compared with a T cell clone naturally expressing CCR-5 (PM1 cells), the apparent levels of GRK2 protein expressed were very similar if not higher in PM1 cells. These findings suggested that the conditions established by overexpression of GRKs in HEK 293 cells recapitulate closely those of cells naturally expressing CCR-5 and that CCR-5 likely requires higher levels of GRK to be effectively desensitized. This observation raises the possibility that variation in the complement of regulatory proteins such as GRKs may be a physiologically important mechanism determining the specificity of regulation of certain GPCRs. In addition, these results suggest that GPCRs may have evolved to match the environment in which they are naturally expressed, such that regulation of their activity might vary in different cell types or tissues. Further studies will be required to ascertain whether GRK-mediated phosphorylation may contribute to CCR-5 desensitization in its natural environment. In addition, the inability of overexpressed β-arrestin to enhance GRK-mediated CCR-5 desensitization might suggest that β-arrestins play a more important role in internalization than desensitization of CCR-5.

Studies with β_2AR have demonstrated that GRK-mediated phosphorylation promotes binding of β-arrestins, which not only can serve to further uncouple the receptor, but also act as intracellular trafficking molecules specifically targeting GPCRs to clathrin-coated vesicles for endocytosis and eventual dephosphorylation, recycling to the plasma membrane and resensitization (Ferguson *et al.*, 1996c; Goodman *et al.*, 1996; Zhang *et al.*, 1996; Krueger *et al.*, 1997). Recently it was speculated that these same mechanisms might be involved in chemokine receptor internalization (Franci *et al.*, 1996). When tested, CCR-5 was internalized poorly when expressed in either HEK 293 or COS-7 cells, with or without GRK2, β-arrestin-1 or β-arrestin-2 individually. However, overexpression of GRK2 with either β-arrestin-1 or β-arrestin-2 resulted in a dramatic increase in chemokine-stimulated CCR-5 sequestration. While it is possible that loss of cell surface CCR-5 immunofluorescence does not discriminate between receptor sequestration and down-regulation, the similarity between the mechanisms utilized for both CCR-5 and β_2AR internalization suggests that the phenomenon being measured here was sequestration. The present results with CCR-5 demonstrate the first example of a synergistic relationship between GRK-mediated phosphorylation and β-arrestin binding for sequestration of a wild-type receptor. This synergism is mechanistically identical to GRK- and arrestin-mediated desensitization of rhodopsin and β_2AR observed previously in protein reconstitution experiments (Attramadal *et al.*, 1992; Lohse *et al.*, 1992) and recapitulates data obtained with the Y326A β_2AR mutant (Ferguson *et al.*, 1996c; Menard *et al.*, 1997). The inability of CCR-5 to be internalized in HEK 293 cells parallels the inability of β_2ARs to be sequestered in COS-7 cells which express lower levels of β-arrestins (Zhang *et al.*, 1996; Menard *et al.*, 1997). Perhaps the lower sensitivity to endogenous β-arrestin expression levels exhibited by CCR-5 is related to a relatively short intracellular third loop when compared with other GPCRs that have been demonstrated to effectively interact with β-arrestins *in vitro* (Gurevich *et al.*, 1995). While PM1 cells do not express dramatically higher levels of β-arrestin protein when compared with HEK 293 cells, Parruti *et al.* (1993) reported that β-arrestin mRNA was very abundant in peripheral blood leukocytes. This observation suggests that higher β-arrestin expression levels might be expected in other blood cell types.

The recent identification of CCR-5 as a cofactor for M-tropic HIV-1 and the ability of chemokines to prevent HIV-1 infection has heightened interest in whether normal functioning of the receptor might contribute to the infection process. Our ability to access functional coupling, desensitization, phosphorylation and sequestration of CCR-5 in HEK 293 cells presented a unique opportunity to examine whether signaling, functional regulation and, most importantly, intracellular trafficking of CCR-5 were involved in its function as a cofactor. The results of these studies indicate that neither functional coupling of the receptor nor phosphorylation, desensitization and/or internalization are required for function as a cofactor for HIV-1 infection. Rather, it appears that CCR-5 serves as an anchoring protein for HIV-1 infection. This idea is supported not only by studies of chimeric receptors derived from CCR-5 and CCR-2b, where it was recently shown that one receptor lacking co-receptor function still stimulated PI hydrolysis and another receptor subserved cofactor function but failed to couple to PI hydrolysis (Atchison *et al.*, 1996), but also by studies using functionally uncoupled CCR-5 point mutants (Farzan *et al.*, 1997). In recent studies, using chimeric mouse and human CCR-5, we and others have reported that interaction between the HIV-1 envelope protein gp120 and CCR-5 is complex and that not only are multiple regions of CCR-5 involved in gp120 binding, but different HIV-1 isolates recognize distinct regions of the receptor (Atchison *et al.*, 1996; Rucker *et al.*, 1996; Bieniasz *et al.*, 1997).

It has been proposed that down-modulation of co-receptors via clathrin-coated pits might be involved in the process of HIV-1 entry (Lapham *et al.*, 1996). However, in the present study we found that HIV-1 infection was neither increased by overexpression of proteins enhancing CCR-5 internalization (GRK and β-arrestins) nor diminished by overexpression of proteins expected to block CCR-5 internalization via clathrin-coated pits (β-arrestin-1-V53D and dynamin I-K44A) in cells co-expressing CCR-5. While our results do not rule out the possibility that receptor desensitization and internalization processes might contribute to chemokine-dependent inhibition of HIV-1 entry, PTX treatment of PM1 cells failed to block RANTES-mediated inhibition of HIV-1 infection (Cocchi *et al.*, 1996; Oravetz *et al.*, 1996).

In conclusion, this study provides evidence that CCR-5 desensitization and internalization involve GRK- and β-arrestin-dependent mechanisms and, along with the recent results of Franci *et al.* (1996), suggest that these mechanisms may be applicable generally to other chemokine receptors, such as the other HIV cofactor fusin, CXCR-4. However, these mechanisms, as well as functional coupling of the receptor subtypes, do not appear to be necessary for function of CCR-5 as a cofactor for M-tropic HIV-1. The similarity of functional regulation of CCR-5 to that of β_2AR indicates that mechanisms of

desensitization and resensitization play an important role in leukocyte chemoattractant responsiveness. Finally, this study provides direct experimental evidence that the relative ability of a particular GPCR to be both desensitized and sequestered is intimately related to endogenous expression levels of GRKs and β-arrestins in the cell in which it is expressed.

Materials and methods

Materials

Materials were obtained from the following sources: minimal essential medium, phosphate-free Dulbecco's modified Eagle's medium (DMEM), RPM-1 medium, phosphate-buffered saline (PBS), fetal bovine serum, normal goat serum and gentamicin from Life Technologies; HEK 293 cells and COS-7 cells were from the American Type Culture Collection; PM1 cells (Cocchi *et al.*, 1995; Lusso *et al.*, 1995) were obtained from the AIDS Research and Reference Reagent Program; MIP-1α, MIP-1β, RANTES and MCP-1 from R & D Systems; [^{125}I]MIP-1β, [2,8-^{3}H]adenine, [8-^{14}C]cAMP and [^{32}P]orthophosphate from DuPont-New England Nuclear; anti-12CA5 monoclonal antibody, forskolin and protease-free bovine serum albumin (BSA) from Boehringer Mannheim; 3-isobutyl-1-methylxanthin (IBMX), Nonidet P-40, protease inhibitors and Fc-specific fluorescein-labeled goat anti-mouse antibody from Sigma Chemicals; PTX from Calbiochem; protein A–Sepharose 4 fast flow from Pharmacia Biotech Inc; ECL Western blotting analysis system from Amersham Corp. All other chemicals and reagents were purchased from Fisher or VWR.

Cell culture and transfection

HEK 293 cells were grown in minimal essential medium with Earle's salts, and supplemented with heat-inactivated fetal bovine serum (10% v/v) and gentamicin (100 μg/ml). The cells were seeded at a density of 2×10^6 cells/100 mm dish and transiently transfected using a modified calcium phosphate method (Cullen, 1987). COS-7 cells were grown in DMEM supplemented with heat-inactivated fetal bovine serum (10% v/v) and gentamicin (100 μg/ml). Transient transfection of COS-7 cells was achieved in the same manner as for HEK 293 cells. Expression plasmids for 12CA5 epitope-tagged human CCR-5 (pCMV5/CCR-5) (Bieniasz *et al.*, 1997), canine ACV (pcDNAI/ACV) (Ishikawa *et al.*, 1992), bovine GRK2 (pcDNAI/GRK2), bovine GRK3 (pcDNAI/GRK3) (Freedman *et al.*, 1995), bovine GRK5 (pcDNAI/GRK5) (Premont *et al.*, 1994), human GRK6 (pCMV5/GRK6) (Ménard *et al.*, 1996), rat β-arrestin-1 (pCMV5/β-arrestin-1), rat β-arrestin-1-V53D (pCMV5/β-arrestin-1-V53D), rat β-arrestin-2 (pCMV5/β-arrestin-2), rat β-arrestin-2-V54D (pCMV5/β-arrestin-2-V54D) (Ferguson *et al.*, 1996c), rat dynamin I (pCB1/dynamin I), rat dynamin I-K44A (pCB1/dynamin I-K44A) (Zhang *et al.*, 1996) and human CD4 (pCMV5/CD4) (Bieniasz *et al.*, 1997) were as previously described. Cells were transfected with 2.5–5.0 μg/dish CCR-5 cDNA, 2.5 μg/dish ACV cDNA and 5.0 μg/dish other plasmid DNAs unless otherwise specified. PM1 cells were grown in RPM-1 medium supplemented with heat-inactivated fetal bovine serum (10% v/v) and gentamicin (100 μg/ml).

Ligand binding of CCR-5

For determination of ligand binding selectivities of 12CA5 epitope-tagged human CCR-5 expressed in HEK 293 cells, isolation of crude membranes and subsequent ligand binding assays were performed essentially as described previously (Shigemoto *et al.*, 1990; Raport *et al.*, 1996). Competition binding experiments for CCR-5 receptor were carried out using [^{125}I]MIP-1β. Cell membranes (13–30 μg/ml) were incubated with 100 pM [^{125}I]MIP-1β for 90 min in 0.25 ml binding solution containing 50 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% BSA. Bound ligand was separated on glass fiber filters (Whatman GF/C) pre-soaked in 0.3% polyethyleneimine and 0.2% BSA by vacuum filtration, washed four times with 4 ml cold wash buffer (10 mM HEPES, pH 7.4, 0.5 mM NaCl and 0.5% BSA) and quantitated with a γ counter. All experiments were carried out at least three times in duplicate. Specific binding was calculated by subtracting non-specific binding, determined in the presence of 300 nM unlabeled MIP-1β, from total binding. Specific binding activity amounted to 80% of total binding activity.

cAMP assay

HEK 293 cells transfected with CCR-5 cDNA and ACV cDNA (Ishikawa *et al.*, 1992), with or without GRK and β-arrestin cDNAs, were labeled overnight with 1 μCi/ml [^{3}H]adenine in minimal essential medium supplemented with 5% fetal bovine serum. The cells were washed with fresh medium containing 10 mM HEPES, pH 7.4 and 0.2% BSA, treated with 1 mM IBMX for 15 min and then stimulated with 1 μM forskolin, alone or with chemokines. After incubation for 30 min at 37°C, the medium was aspirated and the reaction was terminated with a stop solution containing 2.5% percholic acid, 0.1 mM cAMP and 4 nCi/ml [^{14}C]cAMP. The cAMP levels were quantitated as previously described (Salomon, 1991). For analysis of the effect of pretreatment with MIP-1β on forskolin-stimulated cAMP formation, transfected HEK 293 cells were pretreated with or without 100 nM MIP-1β for 30 min at 37°C. After rinsing once with 50 mM glycine and 150 mM NaCl, pH 3.0, for 1 min, the cells were incubated with the indicated concentrations of MIP-1β in the presence of 1 μM forskolin for 30 min and then assessed for cAMP formation.

Whole-cell phosphorylation assay

HEK 293 cells seeded in 6-well dishes were washed twice with phosphate-free DMEM without serum (37°C) and then labeled for 60 min at 37°C with 0.75 ml/well [^{32}P]orthophosphate (100 μCi/ml) in the same medium. Duplicate pairs of matching wells containing labeled cells were then treated with an additional 0.75 ml serum- and phosphate-free DMEM containing 200 nM MIP-1β (100 nM final concentration) and incubated at 37°C for 10 min. Cells were washed twice with ice-cold Dulbecco's PBS and scraped into lysis buffer (0.4 ml/well) (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.5 mM phenylmethysulfonyl fluoride, 10 μg/ml benzamidine and 1 μg/ml pepstatin A, pH 7.4), following which duplicate wells were combined. The cells were solubilized for 1 h at 4°C on an inversion wheel. Insoluble material was removed by centrifugation at 436 000 *g* for 15 min. After removal of aliquots for protein determinations, equal volumes of cell lysates were precleared for 1 h at 4°C with 100 μl 20% protein A–Sepharose suspension in lysis buffer containing 2% BSA. Epitope-tagged CCR-5 was immunoprecipitated by incubating lysates with 15 μg anti-12CA5 antibody and 100 μl 20% protein A–Sepharose suspension for 2 h at 4°C. After three washes with ice-cold lysis buffer, immune complexes were eluted from protein A–Sepharose beads by incubation with SDS sample buffer for 10 min at 65°C. Samples were loaded onto 10% SDS–polyacrylamide gels with equivalent amounts of receptors in each lane. The amount of receptor in each sample was assessed by flow cytometry (Barak *et al.*, 1994) and was multiplied by the protein content of each immunoprecipitation tube. The receptor content of each sample was normalized to the sample with the least receptor content by dilution with sample buffer. Samples were then subjected to SDS–PAGE followed by autoradiography. The extent of receptor phosphorylation was quantitated using a Molecular Dynamics phosphorimaging system and ImageQuant software.

Sequestration

Receptor sequestration was assessed by flow cytometry as described previously (Barak *et al.*, 1994). In brief, sequestration was defined as the fraction of total cell surface receptors which, after exposure to agonist, were removed from the plasma membrane and thus were not accessible to antibodies from the outside of the cells. The cells were exposed to 100 nM MIP-1β for 30 min at 37°C before antibody staining.

Western blotting

HEK 293 cells grown in 6-well dishes were washed twice with ice-cold Dulbecco's PBS and scraped into lysis buffer (0.4 ml/well), followed by solubilization for 1 h at 4°C on an inversion wheel. PM1 cells grown in a 75 cm^2 flask were harvested by centrifugation, washed and solubilized in the same manner. Insoluble material was removed by centrifugation at 436 000 *g* for 15 min. Equivalent protein amounts from the different total protein preparations (50–100 μg) were subjected to SDS–PAGE followed by electroblotting with a Millipore Milliblot semi-dry electroblotting system onto nitrocellulose membranes. The membranes were blocked with PBS containing 1% low fat skimmed milk, 5% normal goat serum and 0.05% Tween 20 at 4°C overnight. Blots were incubated with 1:2500 diluted antiserum cross-reactive for both GRK2 and -3 (Arriza *et al.*, 1992) or for both β-arrestin-1 and -2 (Attramadal *et al.*, 1992) in PBS containing 1% low fat skimmed milk and 0.05% Tween 20 for 1 h at room temperature. After incubation with the antiserum, the membranes were washed three times for 15 min in PBS containing 1%

low fat skimmed milk and 0.05% Tween 20 and then incubated for 1 h at room temperature with 1:2000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG supplied with the ECL Western blotting analysis system in PBS containing 1% low fat skimmed milk and 0.05% Tween 20. The membranes were washed three times in PBS containing 1% low fat skimmed milk and 0.05% Tween 20 and then exposed using the ECL Western blotting analysis system.

Mutant receptor

A point mutation (Tyr297→Ala) in CCR-5 was generated by site-directed mutagenesis using PCR to mutate codon 297 TAT (Tyr) to GCG (Ala) (Y297A mutant). The integrity of the nucleotide sequence was confirmed by dideoxy DNA sequencing analysis.

Cell fusion assay for co-receptor function

Transient virus indicator cells were generated by co-transfection of HEK 293 cells in 35 mm dishes with expression vectors encoding CD4 (pCMV5/CD4, 400 ng), CCR-5 or mutant receptors (0–250 ng) and an indicator construct (pCMV5/HIV/SEAP, 400 ng) containing the HIV-1 LTR linked to the secreted alkaline phosphatase (SEAP) indicator gene (Bieniasz *et al.*, 1997). For analyses of the effect of overexpression of GRK2, β-arrestin-1, β-arrestin-1-V53D, dynamin I and dynamin I-K44A on cofactor function of CCR-5, 1 μg expression plasmid encoding these proteins was transfected with 50 ng pCMV5/CCR-5. Simultaneously, virus-producing cells were generated by transfection of HEK 293 cells with 2 μg HIV-1 proviral construct pBaL (Hwang *et al.*, 1991). At 48 h after transfection producer and indicator cells were harvested by trypsinization and equal numbers of cells (~5×10^4) were co-cultivated for 48 h. Culture supernatants were then harvested and SEAP activity was determined as described previously (Berger *et al.*, 1988).

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