THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 5, Issue of January 29, pp. 2824–2828, 1999

Printed in U.S.A.

$G\alpha_i$ Is Not Required for Chemotaxis Mediated by G_i-coupled Receptors*

(Received for publication, November 9, 1998)

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Pertussis toxin inhibits chemotaxis of neutrophils by preventing chemoattractant receptors from activating trimeric G proteins in the G_i subfamily. In HEK293 cells expressing recombinant receptors, directional migration toward appropriate agonist ligands requires release of free G protein $\beta\gamma$ subunits and can be triggered by agonists for receptors coupled to G_i but not by agonists for receptors coupled to two other G proteins, G_s and G_q. Because activation of any G protein presumably releases free $G\beta\gamma$, we tested the hypothesis that chemotaxis also requires activated α subunits ($G\alpha_i$) of G_i proteins. HEK293 cells were stably cotransfected with the G_i-coupled receptor for interleukin-8, CXCR1, and with a chimeric $G\alpha$, $G\alpha_{qz5}$, which resembles $G\alpha_i$ in susceptibility to activation by G_i-coupled receptors but cannot regulate the $G\alpha_i$ effector, adenylyl cyclase. These cells, unlike cells expressing CXCR1 alone, migrated toward interleukin-8 even after treatment with pertussis toxin, which prevents activation of endogenous $G\alpha_i$ but not that of $G\alpha_{qz5}$. We infer that chemotaxis does not require activation of $G\alpha_i$. Because chemotaxis is mediated by $G\beta\gamma$ subunits released when G_i-coupled receptors activate $G\alpha_{qz5}$, but not when G_q- or G_s-coupled receptors activate their respective G proteins, we propose that G_i-coupled receptors transmit a necessary chemotactic signal that is independent of $G\alpha_i$.

As it migrates to a site of infection or tissue injury, an inflammatory cell must detect a chemokine gradient and organize its cytoskeleton to move in the right direction (1–4). The signaling pathways responsible for this complex cellular response are poorly understood. Pertussis toxin, which specifically prevents receptor-dependent activation of G_i proteins, blocks chemotactic migration of neutrophils; we therefore infer that G_i proteins play essential roles in mediating the chemotactic signal. Activation of G proteins by serpentine receptors releases two potential stimulators of downstream signals, an α subunit ($G\alpha$), bound to GTP, and a free $G\beta\gamma$ subunit (5). For example, the α_i subunits of G_i proteins directly mediate inhibition of adenylyl cyclase, while the $\beta\gamma$ subunits of these proteins mediate opening of K^+ channels and stimulation of phospholipase Cβ (6).

To identify the G protein subunit that mediates chemotaxis, we have begun to study chemotaxis in a cell line, HEK293,[1] which is amenable to stable transfection with normal and mutant receptors and other signaling proteins. Endogenous $G\alpha$ subunits of HEK293 cells include α_s, α_q, α_{i1}, α_{i2}, and α_{i3}, but not α_o or α_z (7, 8). In this model we found that chemotaxis requires receptors that activate G_i and that release of free $\beta\gamma$ is essential (9). Receptors that activate two other G proteins, G_s and G_q, could not mediate chemotaxis in HEK293 cells. Abundant evidence indicates that activation of these two G proteins, like that of G_i, involves dissociation of $G\beta\gamma$ from GTP-bound $G\alpha$. Accordingly, it is reasonable to ask why the $G\beta\gamma$ released from α_s·GTP or α_q·GTP could not mimic the chemotactic effect of $G\beta\gamma$ released from α_i·GTP.

One answer is that $G\alpha_i$ itself makes the difference, by activating an essential downstream signal distinct from those triggered by $G\beta\gamma$. A second possibility is that G_i proteins are simply more abundant than G_s or G_q, and accordingly release more $G\beta\gamma$ upon activation. A third possibility is that inhibitory signals generated by the α subunits of G_s or G_q block the chemotactic response to free $\beta\gamma$. To test these possibilities, which are not mutually exclusive, we assessed chemotaxis of HEK293 cells expressing different combinations of receptors and $G\alpha$ proteins. Our results show that chemotaxis requires a receptor that can activate G_i but does not require $G\alpha_i$ itself. We propose that chemotaxis requires not only $G\beta\gamma$, but also a signaling function of G_i-coupled receptors that is distinct from activation of $G\alpha_i$.

EXPERIMENTAL PROCEDURES

Materials—Recombinant IL-8, forskolin, and rat collagen type 1 were obtained as described (9). The modified Boyden chamber was purchased from Neuroprobe, and the polycarbonate filters were procured from Poretics. Pertussis toxin was obtained from List Biologicals.

Plasmid Constructs—Wild type $G\alpha_q$ and $G\alpha_q$-Q205L, tagged with the EE epitope, were as described (10). Mutagenesis was verified by DNA sequencing. EE-tagged $G\alpha_{qz5}$ in pcDNA1 was obtained from Bruce Conklin, Gladstone Institute, San Francisco General Hospital (11). The interleukin 8 receptor type A (hereafter termed CXCR1) subcloned into pcDNA3 was obtained from Israel Charo, Gladstone Institute, San Francisco General Hospital.

Cell Culture and Transfection—HEK293 cell lines stably expressing CXCR1 and the m3-muscarinic acetylcholine receptor (m3AChR) were generated as described (9) and maintained in G418 (800 μg/ml). For double stable transfectants, a vector containing a hygromycin resistance cassette was cotransfected with the various $G\alpha$ constructs in pcDNA1. Clones stably expressing both CXCR1 and the respective $G\alpha$ constructs were selected and maintained in both G418 and hygromycin (200 μg/ml). Cell lines were propagated as described (9).

Assays—Assays of chemotaxis, cAMP accumulation, and inositol phosphate accumulation were performed as described (9).

Immunoblots—Subconfluent cells (5×10^6) were lysed in RIPA

* This work was supported in part by postdoctoral fellowships from the Howard Hughes Medical Institute and the Robert Wood Johnson Foundation (to E. R. N.) and National Institutes of Health Grants GM-27800 and CA-54427 (to H. R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HEK, human embryonal kidney; IL-8, interleukin 8; CXCR1, interleukin 8 receptor type A; m3AChR, m3-muscarinic acetylcholine receptor; GRK, G protein receptor kinase.

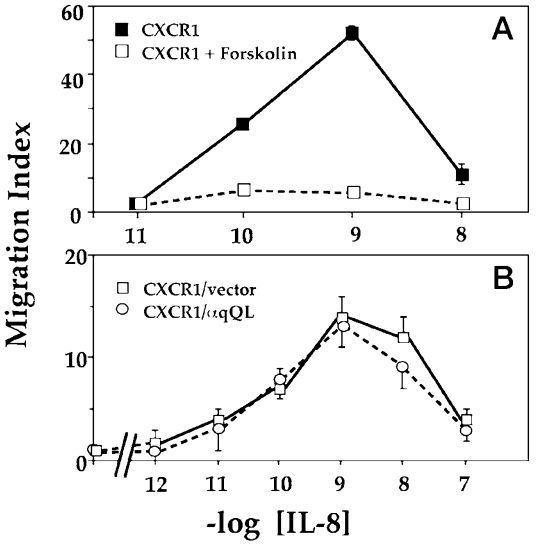

FIG. 1. **Effect of concurrent activation of $G\alpha_s$ and $G\alpha_q$ on chemotaxis toward IL-8.** Migration assays were performed in a 48-well Boyden chamber, as described (9), on cells stably expressing: CXCR1 alone (*filled* and *open squares*; *panel A*) or CXCR1 plus vector (*open squares*; *panel B*) or plus $G\alpha_q$-Q205L (*open circles*; *panel B*). Forskolin (*open squares*, *panel A*) was present at 200 μM. Values represent the mean ± S.E. of six determinations. Similar results were obtained in three or more independent experiments.

buffer containing 5% Nonidet P-40, 2.5% deoxycholate, 250 mM Tris (7.4), 750 mM NaCl, and 12.5 mM $MgCl_2$ for 30 min at 4 °C. Lysates were diluted as noted in RIPA buffer, separated on a 12% SDS gel, and transferred to polyvinylidene difluoride membranes. Membranes were probed with EE monoclonal antibody as described (10). ECL (NEN Life Science Products) was used to visualize immunoreactive bands.

RESULTS

Abundant evidence indicates that receptor activation of all trimeric G proteins causes dissociation of $G\beta\gamma$ from $G\alpha$·GTP. If so, why does receptor activation of G_i elicit chemotaxis, but release of $G\beta\gamma$ from activated G_q or G_s does not (9, 12)? One trivial explanation is that the second messengers synthesized in response to activation of G_s and G_q actually inhibit the chemotactic response that would otherwise be elicited by release of $G\beta\gamma$. Fig. 1 shows that this explanation could account for the failure of activated G_s, but not that of activated G_q, to mediate chemotaxis. Forskolin, which reproduces the stimulation of cAMP accumulation that would result from activation of $G\alpha_s$, completely inhibited the chemotactic response to IL-8 in HEK293 cells expressing the recombinant IL-8 receptor, CXCR1 (Fig. 1*A*); this result is in accord with previous observations (13–15) that cAMP inhibits the chemotactic response of neutrophils and other cells. To test whether activated $G\alpha_q$ can inhibit chemotaxis, we cotransfected cells expressing recombinant CXCR1 with a cDNA (10) encoding mutationally activated $G\alpha_q$ ($G\alpha_q$-Q205L). Expression of $G\alpha_q$-Q205L increased basal phosphoinositide accumulation more than 30-fold (result not shown), but had no effect whatever on chemotaxis toward IL-8 (Fig. 1*B*).

If activated $G\alpha_q$ cannot inhibit chemotaxis, we must ask why the release of $G\beta\gamma$ from receptor-activated G_q does not mediate chemotaxis. The simplest explanation would be that chemotaxis requires $G\alpha_i$·GTP, as well as $G\beta\gamma$. Accordingly, we asked whether CXCR1 can elicit chemotaxis when it activates a G protein containing a chimeric $G\alpha$, $G\alpha_{qz5}$ (11), which cannot regulate activity of a direct effector of $G\alpha_i$, adenylyl cyclase. $G\alpha_{qz5}$ is identical to $G\alpha_q$ except that its C-terminal five amino acids are replaced by the corresponding sequence of $G\alpha_z$, a $G\alpha$ that responds to stimulation by G_i-coupled receptors but is not

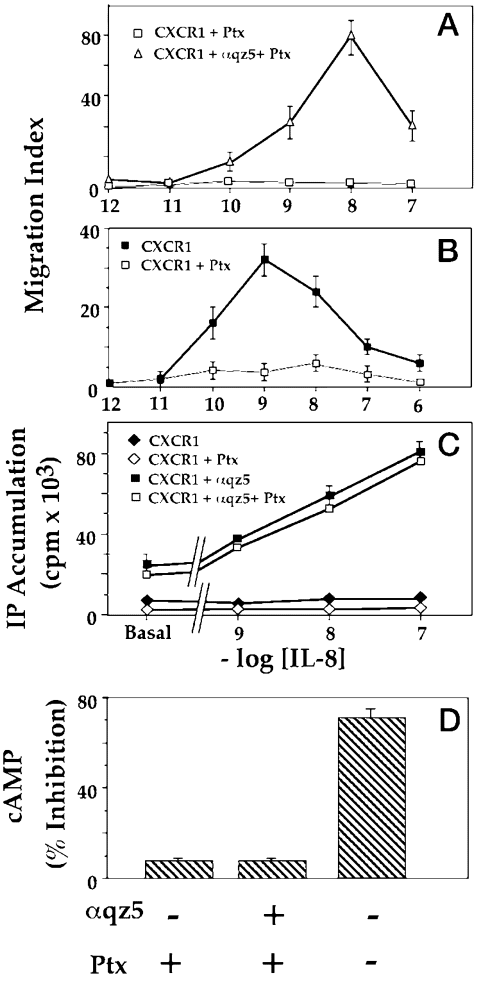

FIG. 2. **Effect of overexpressed $G\alpha_{qz5}$ on chemotactic and second messenger responses to IL-8.** *A* and *B*. Migration assays were performed as described (9) on cells expressing either CXCR1 with vector (*squares*, *panels A* and *B*) or CXCR1 with $G\alpha_{qz5}$ (*open triangles*, *panel A*). Cells were incubated without (*filled squares*, *panel B*) or with (*other symbols*, *panels A* and *B*) 500 μg/ml pertussis toxin for 4 h at 37 °C. The cells were subsequently washed and assayed as described. *C*, accumulation of of inositol phosphates in response to the indicated concentrations of IL-8 was measured. CXCR1 alone, *diamonds*; CXCR1 plus $G\alpha_{qz5}$, *squares*. Cells were treated with (*open symbols*) or without (*filled symbols*) pertussis toxin, as described for *panels A* and *B*. *D*, IL-8 (10 nM) inhibition of cAMP accumulation stimulated by forskolin (200 μM) in CXCR1-expressing cells. Cells expressed CXCR1, with or without $G\alpha_{qz5}$ and were treated with or without pertussis toxin, as indicated in the figure. Values represent percent inhibition by IL-8 of the forskolin-stimulated cAMP response. Forskolin alone increased cAMP more than 100-fold. Values represent the mean ± S.E. of six determinations for *panels A* and *B* and three determinations for *panels C* and *D*. Similar results were obtained in three or more independent experiments.

inhibited by treatment with pertussis toxin (16). Expression of recombinant $G\alpha_z$ with CXCR1 conferred on the cells the ability to migrate toward IL-8 even after treatment with pertussis toxin (result not shown); this result did not speak to the question of whether α_i is required for chemotaxis, however, because $G\alpha_z$ can mimic the inhibitory effect of $G\alpha_i$ on adenylyl cyclase (16).

We have shown that ligand-bound G_i-coupled receptors can use $G\alpha_{qz5}$ to activate the phosphoinositide pathway usually regulated by $G\alpha_q$ (11). Cells that co-expressed $G\alpha_{qz5}$ and CXCR1 migrated toward IL-8 (Fig. 2*A*). Chemotaxis was mediated by a G protein containing $G\alpha_{qz5}$, rather than by endogenous G_i, as shown by the inability of pertussis toxin to prevent chemotaxis of $G\alpha_{qz5}$-expressing cells; the toxin completely blocked chemotaxis toward IL-8 in control cells expressing

CXCR1 alone (Fig. 2*B*).

This result strongly suggests that chemotaxis in HEK293 cells does not require activated $G\alpha_i$, although it does require activation of a G_i-coupled receptor. Controls indicated that IL-8 did indeed activate $G\alpha_{qz5}$, but not $G\alpha_i$. In $G\alpha_{qz5}$-expressing cells, the chemokine stimulated accumulation of phosphoinositides, even after treatment with pertussis toxin (Fig. 2*C*). In contrast, the chemokine inhibited cAMP accumulation in the same cells only if they had not been treated with pertussis toxin (Fig. 2*D*), *i.e.* only when endogenous G_i was accessible to activation by CXCR1.

We considered a potential quantitative explanation for the previously reported (9) failure of a G_q-coupled receptor, the m3AChR, to mediate chemotaxis in HEK293 cells. Although the cellular content of $G\alpha_q$ in HEK293 cells is unknown, it is probably lower than that of the exogenous $G\alpha_{qz5}$ stably expressed in these cells. Thus, it is possible that activation of G_q does release $G\beta\gamma$, but that the amount of membrane-bound $G\alpha_q{\cdot}\beta\gamma$ available for receptor activation in cells transfected only with the m3AChR cDNA, unlike the presumably larger amount of $G\alpha_{qz5}{\cdot}\beta\gamma$ in transfected cells, cannot release sufficient amounts of $G\beta\gamma$ in response to receptor stimulation.

To test this possibility, we increased the amount of available $G\alpha_q$ by stably transfecting a cDNA encoding recombinant $G\alpha_q$ into HEK293 cells already expressing the m3AChR. Carbachol, the m3AChR ligand, failed to elicit chemotaxis even in the doubly transfected cells (Fig. 3*A*). The negative inference, that a G_q-coupled receptor cannot elicit chemotaxis, was supported by control observations (Fig. 3, *B* and *C*) indicating that recombinant $G\alpha_q$ was indeed overexpressed and responsive to receptor stimulation in these cells. Thus, expression of exogenous $G\alpha_q$ allowed greater agonist-stimulated accumulation of phosphoinositides than that observed either in cells expressing the m3AChR alone or in pertussis-toxin treated cells expressing $G\alpha_{qz5}$ and CXCR1 (Fig. 3*B*). Moreover, recombinant $G\alpha_q$ and $G\alpha_{qz5}$ were expressed to nearly identical extents in the two types of cell (Fig. 3*C*), as indicated by immunoblots with monoclonal antibodies against epitopes inserted into both $G\alpha$ proteins.

The failure of CXCR1 alone to mediate activation of phosphoinositide accumulation by IL-8 (Fig. 2*C*) is consistent with results of a previous study (17), in which recombinant CXCR1 was found to activate some but not all members of the α_q family, *i.e.* IL-8 stimulated phospholipase C in CXCR1-expressing COS-7 cells if they coexpressed α_{14}, α_{15}, or α_{16}, but did not do so in cells expressing CXCR1 alone or in combination with α_q or α_{11}. In the same study (17), CXCR1 mediated G_i- and $G\beta\gamma$-dependent activation of phospholipase C, but only in the presence of the $\beta 2$ isoform of the phospholipase. In view of this latter result, we suspect that HEK293 cells lack the $\beta 2$ isoform of the enzyme, because CXCR1 alone does not stimulate phosphoinositide accumulation in these cells (Fig. 2*C*).

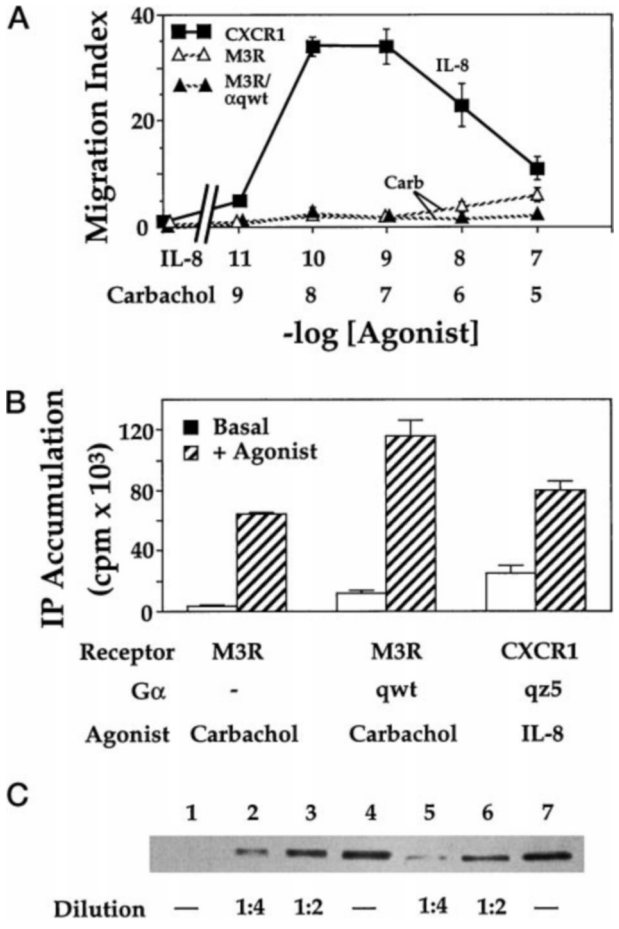

FIG. 3. **Chemotaxis and signaling in cells overexpressing the m3AChR ± wild type $G\alpha_q$.** *A*, migration assays were performed on cells expressing CXCR1 alone (*filled squares*), the muscarinic acetylcholine receptor, type 3 (m3AChR, abbreviated *MR3*) alone (*open triangles*), or the m3AChR plus wild type $G\alpha_q$ (*filled triangles*). Concentrations of IL-8 (*squares*) or carbachol (*triangles*) are indicated on the *abscissa*. *B*, inositol phosphate accumulation on cells expressing (as indicated) the m3AChR alone, the m3AChR with wild type $G\alpha_q$ (*qwt*), or CXCR1 with $G\alpha_{qz5}$ (*qz5*) and treated with or without carbachol or IL-8, as indicated. *C*, immunoblots, using a monoclonal antibody against the EE epitope) of lysates from cells expressing m3AChR alone (*lane 1*), m3AChR and epitope-labeled wild type $G\alpha_q$ (*lanes 2–4*), or CXCR1 and epitope-labeled $G\alpha_{qz5}$ (*lanes 5–7*). Lysates were undiluted (—) or diluted 1:2 or 1:4, as indicated.

DISCUSSION

Our experiments with HEK293 cells pose an intriguing twofold paradox. First, as reported earlier (9), liberation of $G\beta\gamma$ from $G\alpha\beta\gamma$ is required for chemotaxis of these cells; nonetheless, even though activation of any trimeric G protein releases $G\beta\gamma$, receptors that activate G proteins other than G_i do not mediate chemotaxis. Second, even though receptors coupled to G_i are required to mediate chemotaxis of these cells, signaling by $G\alpha_i$ itself is not required, at least in HEK293 cells. Here we discuss four speculative ways to resolve these paradoxes: G_i-coupled receptors may activate a specific subset of $G\beta\gamma$ isoforms, may generate a $G\alpha_i$-independent signal in addition to $G\beta\gamma$, may be susceptible to novel regulatory controls, or may promote co-localization in the plasma membrane of appropriate effectors with the $G\beta\gamma$ liberated by receptor activation. These explanations are not mutually exclusive.

By choosing among polypeptides encoded by five $G\beta$ and 11 $G\gamma$ genes, mammalian cells could express many different $G\beta\gamma$ isoforms (6). Does a specific $G\beta\gamma$ isoform mediate chemotaxis? If so, the responsible $G\beta\gamma$ dimer must possess specificity not only for a subset of G protein coupled receptors but also for the specific downstream effector(s) of chemotaxis. In both respects the evidence from other G protein-mediated signaling pathways is inconclusive. Receptors can select among $G\beta$ and $G\gamma$ isoforms *in vitro* (6) and in regulating neuronal Ca^{2+} channels of intact cells (18, 19). Shared specificity for one $G\beta\gamma$ isoform has not been reported, however, for any *group* of G protein-coupled receptors, including those that couple to G_i. With respect to effectors, circumstantial evidence implicates $G\gamma_2$ as an essential component of G_i-mediated stimulation of phospholipase Cβ in differentiated HL60 cells (20), and γ_5 and γ_{12} are reported to colocalize in cultured cells with vinculin and F actin, respectively (21). Nonetheless, experiments in several laboratories have failed to show significant specificity of any $G\beta\gamma$ dimer, except for the relative weakness of those containing

$G\gamma_1$ for stimulating any effector (6, 22, 23).

Does chemotaxis require a receptor to generate a third kind of signal, independent of $G\alpha_i$ and in addition to the signal(s) relayed by free $G\beta\gamma$? G protein receptor kinases (GRKs) and arrestins, two potential candidates for generators of such a signal, are involved in agonist-dependent desensitization and endocytosis of receptors. In a $G\beta\gamma$-dependent fashion, GRKs bind to and are activated by agonist-stimulated receptors; activated GRKs phosphorylate residues on the cytoplasmic face of receptors (24) and could, hypothetically, phosphorylate downstream effectors. Receptor phosphorylation by GRKs markedly enhances agonist-dependent association of receptors with arrestins, which act negatively, by competing with G proteins, to damp receptor signaling (25). Arrestins also mediate association of receptors with other proteins, including components of the endocytotic machinery of clathrin-coated pits (26). This more positive role of arrestins could serve as an analog for association with and activation of a hypothetical downstream effector of chemotaxis. No member of either the GRK or the arrestin families, however, has yet been reported to interact specifically with chemotactic or G_i-coupled receptors.

One piece of evidence indirectly suggests that chemotactic receptors may generate a signal separate from those mediated by $G\alpha_i$ and $G\beta\gamma$; a C-terminal truncation of CXCR1 markedly inhibited the chemotactic response but did not alter the receptor's ability to trigger agonist-dependent inhibition of adenylyl cyclase or stimulation of the mitogen-activated protein kinase pathway (9), responses mediated by $G\alpha_i$·GTP and $G\beta\gamma$, respectively (5, 27). C-terminal tails of receptors are implicated as sites that contribute to binding of GRKs and arrestins, and might very well bind to other target molecules as well (25).

A third possibility is that, rather than generating a signal distinct from those mediated by $G\alpha_i$ and $G\beta\gamma$, chemotactic G_i-coupled receptors are susceptible to a kind of regulatory control that does affect other receptors. For example, we could imagine that signaling by these receptors is enhanced or attenuated by a molecule that accumulates asymmetrically at the front or the back, respectively, of a cell migrating up a gradient of chemoattractant.

A fourth speculation, perhaps the most interesting, could resolve the paradox created by dependence of chemotaxis on G_i-coupled receptors and $G\beta\gamma$, but not $G\alpha_i$. In this scenario, the G_i-coupled receptor promotes liberation of $G\beta\gamma$ in microdomains of the cell that contain critical downstream effectors of chemotaxis; the receptor could do so by associating with a scaffolding protein that sequesters effectors of chemotaxis in a signaling complex. The chemotrophic pheromone response of *Saccharomyces cerevisiae* furnishes a relevant precedent: $G\beta\gamma$ mediates this response by promoting formation of a signaling complex assembled by a scaffolding protein, STE5p (28). Other data raise the possibility that G protein-coupled receptors participate in signaling complexes containing both G protein subunits and effectors. For example, experiments in a reconstituted system using pure receptor, effector, and G protein suggest that all three components participate in a membrane-bound functional complex: phospholipase Cβ, the effector of $G\alpha_q$·GTP, accelerates receptor-stimulated exchange of GTP for GDP bound to $G\alpha_q$ (29). Similarly, a recently identified scaffold protein, inaD, assembles downstream proteins involved in G protein-dependent phototransduction (including a phospholipase C) at specific subcellular locations in the retina of fruit flies (30). Note that formation of the postulated chemotaxis signaling complex might depend on both the activated receptor and $G\beta\gamma$. In this regard, protein domains located in the third intracellular loops (ic3 domains) of m2- and m3-muscarinic receptors associate with free $G\beta\gamma$ (but not with $G\beta\gamma$ complexed to $G\alpha$·GDP) and $G\beta\gamma$ and the ic3 domain appear to form a ternary complex with a receptor kinase, GRK2 (31). The unusually large ic3 domains of muscarinic receptors may be analogs of the hypothetical scaffolding proteins that associate with G_i-coupled receptors.

This fourth proposal for resolving the paradox raises an interesting question. Do all G_i-coupled receptors share the proposed ability of chemotactic receptors to organize signaling complexes that are required for chemotaxis? We and others have tested several G_i-coupled receptors, not previously identified as "professional" chemotactic receptors, for ability to mediate directional migration of cultured cells toward the appropriate ligand; the D2 dopamine receptor and the μ- and δ-opioid receptors do mediate chemotaxis, albeit not as efficiently as CXCR1 (9, 12). It is not clear whether this result can be generalized to include all, or even most, G_i-coupled receptors.

The paradox we have described is paralleled, nonetheless, by similar paradoxes in two other responses to agonists for G_i-coupled receptors: stimulation of the mitogen-activated protein kinase pathway and opening of K^+ channels. Even though $G\beta\gamma$ (but not $G\alpha_i$·GTP) mediates both responses, neither response is elicited by the $G\beta\gamma$ liberated by activating receptors that activate G proteins other than G_i (32).[2] Possible resolutions of these paradoxes include those we have outlined for resolving the paradox in chemotactic signaling.

Finally, our findings in HEK293 cells provide a starting point for dissecting the molecular basis of chemotactic signaling in neutrophils and other professionally chemotactic cells. G_i-coupled receptors and G protein subunits can serve as probes for identifying the critical but so far elusive effectors that harness the actin cytoskeleton to effect directional migration.

Acknowledgments—We thank Israel Charo, Mark von Zastrow, and members of the Bourne laboratory for useful advice and review of the manuscript.

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