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Crosstalk between $G\alpha_i$- and $G\alpha_q$-coupled receptors is mediated by $G\beta\gamma$ exchange

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ABSTRACT **Activation of $G\alpha_i$-coupled receptors often causes enhancement of the inositol phosphate signal triggered by $G\alpha_q$-coupled receptors. To investigate the mechanism of this synergistic receptor crosstalk, we studied the $G\alpha_i$-coupled adenosine A_1 and α_{2C} adrenergic receptors and the $G\alpha_q$-coupled bradykinin B_2 and a UTP-preferring P2Y receptor. Stimulation of either $G\alpha_i$-coupled receptor expressed in COS cells increased the potency and the efficacy of inositol phosphate production by bradykinin or UTP. Likewise, overexpression of $G\beta_1\gamma_2$ resulted in a similar increase in potency and efficacy of bradykinin or UTP. In contrast, these stimuli did not affect the potency of direct activators of $G\alpha_q$; a truncated $G\beta_3$ mutant had no effect on the receptor-generated signals whereas signals generated at the G-protein level were still enhanced. This suggests that the $G\beta\gamma$-mediated signal enhancement occurs at the receptor level. Almost all possible combinations of $G\beta_{1-3}$ with $G\gamma_{2-7}$ were equally effective in enhancing the signals of the B_2 and a UTP-preferring P2Y receptor, indicating a very broad specificity of this synergism. The enhancement of the bradykinin signal by (*i*) $G\alpha_i$-activating receptor ligands or (*ii*) cotransfection of $G\beta\gamma$ was suppressed when the B_2 receptor was replaced by a $B_2G\beta_2$ fusion protein. $G\beta\gamma$ enhanced the B_2 receptor-stimulated activation of G-proteins as determined by GTPγS-induced decrease in high affinity agonist binding and by B_2 receptor-enhanced [^{35}S]GTPγS binding. These findings support the concept that $G\beta\gamma$ exchange between $G\alpha_i$- and $G\alpha_q$-coupled receptors mediates this type of receptor crosstalk.**

Seven transmembrane domain receptors signal via heterotrimeric G-proteins. So far, 21 different gene products encoding $G\alpha$ subunits, 5 $G\beta$, and 11 $G\gamma$ subunits have been distinguished. With these subunits, the formation of >1,000 different G-protein heterotrimers is conceivable. This panoply of combinations is theoretically sufficient to provide each individual G-protein-coupled receptor with a specific G-protein heterotrimer. For the various $G\alpha$ subunits, specific interactions with receptors have been defined by various approaches. In addition, injection of antisense oligonucleotides or the transfection of a ribozyme specific for defined $G\beta$ or $G\gamma$ differentially inhibited receptor-mediated signals, suggesting that in intact cells receptors are specifically coupled to defined $G\beta\gamma$-heterodimers (1–5). These findings argue for a receptor-specific G-protein heterotrimer interaction. In contrast, transfection experiments revealed that a single combination, $G\beta_1\gamma_2$, is equally effective in enhancing signals of several G-protein coupled receptors (6), and most biochemical assays investigating $G\beta\gamma$ interactions with $G\alpha$ and with effector molecules have shown little specificity (7–10). Thus, it is unclear whether the direct interaction of defined $G\beta\gamma$ subunits with a receptor is selective. This question is fundamental because a lack of biochemical specificity between different $G\beta\gamma$ combinations would allow different receptors to share a common pool of $G\beta\gamma$ subunits.

Under physiological conditions, such nonselective interactions might be important for the crosstalk between different receptors. It has been known for several years that stimulation of $G\alpha_i$-coupled receptors can enhance the inositol phosphate signals triggered by activation of $G\alpha_q$-coupled receptors, even though stimulation of the $G\alpha_i$-coupled receptors alone often has no effect on inositol phosphates (11–13). It is thought that $G\beta\gamma$ subunits are responsible for this effect (12–16) because relatively high concentrations of agonists are necessary. However, the mechanism of such an enhancement by $G\beta\gamma$ subunits has remained unclear. Direct stimulation of phospholipase Cβ by $G\beta\gamma$ has been reported (17), and this stimulation can occur in addition to that by $G\alpha_q$ (18). However, this stimulation is not synergistic (18–19), and this leaves the question unanswered of whether direct stimulation of phospholipase Cβ by $G\beta\gamma$ subunits is indeed the mechanism for the enhancing effects caused by $G\alpha_i$-coupled receptors.

To analyze the mechanism of this synergistic receptor crosstalk and the role of $G\beta\gamma$ in this process, we chose the $G\alpha_q$-coupled bradykinin B_2 and a UTP-preferring P2Y receptor (20–21), and the $G\alpha_i$-coupled adenosine A_1 and the α_{2C} adrenergic receptors. These receptors also are expressed together in native cells and are known to enhance each other's signaling (15, 22). We report that the receptor crosstalk can occur at the level of the receptors themselves and propose that exchange of $G\beta\gamma$ subunits by the $G\alpha_i$- and the $G\alpha_q$-coupled receptors mediates this type of receptor crosstalk.

MATERIALS AND METHODS

Construction of Expression Vectors. All of the cDNAs used in these studies were ligated into an expression vector that is driven by the cytomegalovirus promoter. Identity of the subcloned cDNAs coding for bovine $G\gamma_{1,2,3,5,7}$, for mouse $G\gamma_4$, and for human $G\beta_{1-3}$ was determined by DNA sequencing. The cDNA coding for a B_2 receptor-Gβ2 fusion protein was constructed by overlap extension using PCR as described (23). The following primers were used for the PCRs: sense-B_2, 5′-TCCCTCTAGAAGCTTATGTTCTCTCCCTGGAG-3′; antisense-B_2, 5′-TGCTCCAGCTCACTCATCTGTCTGCTCCCTGCCCA-3′; sense-Gβ_2, 5′-TGGGCAGGGAGCAGACAGATGAGTGAGCTGGAGCA-3′; antisense-Gβ_2, 5′-ATCCGAGCTCGAATTCTTAGTTCCAGATCTTGA-3′. The resulting PCR-product was ligated into the *Hin*dIII/*Eco*RI sites of the pcDNA3 vector (Invitrogen). An N-terminally truncated mutant of the Gβ_3 subunit (amino acids 45–340) was created by PCR. Identity of the constructs was confirmed by DNA sequencing.

Cell Transfection and Determination of Inositol Phosphate Levels. COS-1 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% (vol/vol) fetal calf

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Abbreviation: PLC, phospholipase C.

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serum and were kept in a 92.5% air/7.5% CO_2 atmosphere. Cells were transfected by using Lipofectamine (GIBCO/BRL), or the DEAE-dextran method with 5 μg of DNA/ml of the DEAE-dextran solution (6). The transfection efficiency was usually 20–30% as determined by staining of the cells with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) after transfection with a plasmid for β-galactosidase (6). For determination of inositol phosphate levels, cells were seeded on 12- or 6-well plates coated with 0.1% (wt/vol) gelatine in PBS and were transfected with 5 μg of DNA/ml of transfection mixture. In cotransfection experiments, the total amount of DNA was held constant with plasmid pcDNA3 (Invitrogen). Forty-eight hours after transfection, cells were assayed for inositol phosphate levels or for binding of [2,3-prolyl-3,4-^{3}H]bradykinin (specific activity 98 Ci/mmol) (Amersham) or were scraped for membrane preparation as described (24, 25).

Measurement of Changes in the Intracellular Free Ca^{2+} Concentration. Intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, of HEK-293 cells transfected with cDNAs coding for the B_2 or the $B_2G\beta_2$ receptor was determined on detached cells by fura-2/AM as described (24). Fluorescence at 510 nm was recorded. Excitation wavelength alternated between 340 and 380 nm in intervals of 40 ms. Changes in $[Ca^{2+}]_i$ are given as the ratio between 340 and 380 nm.

Immunoblotting. Proteins of transfected COS-1 cells were resolved by SDS/PAGE on 10% acrylamide gels. Proteins were transferred to polyvinylidenedifluoride membranes by using semidry blotting. Proteins (Gβ subunits, B_2 receptor) were detected on immunoblots as described (24) by anti-Gβ common (Transduction Research, Framingham, MA) and anti-B_2 receptor antibodies (24), respectively.

RESULTS

Enhancement of Gα_q-Coupled Receptor Signals by Stimulation of Gα_i-Coupled Receptors. To analyze the mode of receptor crosstalk between Gα_i and Gα_q-coupled receptors, we chose the Gα_q-coupled bradykinin B_2 and a UTP-preferring P2Y receptor, and the Gα_i-coupled adenosine A_1 and α_{2C} adrenergic receptors. Expression of α_{2C}-adrenergic or A_1 adenosine receptors in COS cells and stimulation with 1 μM medetomidine (α_{2C}-adrenergic receptor) or cyclohexyladenosine (A_1 adenosine receptor) did not per se result in the generation of inositol phosphates. However, their stimulation increased the potency and the efficacy of UTP and bradykinin. Fig. 1 shows this for the α_{2C}-adrenergic receptor; its stimulation increased the potency of UTP from 3.5 ± 0.4 μM to 1.5 ± 0.4 μM (Fig. 1*A*) and that of bradykinin from 2.0 ± 0.2 nM to 0.9 ± 0.1 nM (Fig. 1*B*) (n = 3). In addition, the efficacy of both hormones was increased by the costimulation of the α_{2C}-adrenergic receptor to 158 ± 7% and 137 ± 6%, respectively. Very similar results were obtained for the coexpression and costimulation of the A_1 adenosine receptor (data not shown).

Effects of G$\beta\gamma$ on Receptor- and G$_\alpha$-Triggered Stimulation of PLC Activity. Do G$\beta\gamma$ subunits released from Gα_i mediate the enhancement of Gα_q-stimulated phospholipase C (PLC) activity? We expressed G$\beta\gamma$ subunits and compared their effects with those of Gα_i-coupled receptor stimulation. Expression of G$\beta\gamma$ subunits also caused an increase in the potency and efficacy of UTP and bradykinin (Fig. 1 *A* and *B*), and this enhancement was virtually indistinguishable from that caused by stimulation of the α_2-adrenergic receptor. The EC_{50} values were increased from 3.5 ± 0.4 μM to 1.5 ± 0.5 μM for UTP and from 2.0 ± 0.2 nM to 0.9 ± 0.2 nM for bradykinin. These results are indeed compatible with the view that stimulation of Gα_i-coupled receptors enhances PLC activation by causing an increase in free G$\beta\gamma$ subunits at the cell membrane.

Two different mechanisms are conceivable for how G$\beta\gamma$ subunits could enhance a receptor-stimulated signal: (*i*) Free G$\beta\gamma$ subunits may somehow activate PLC together with Gα_q,

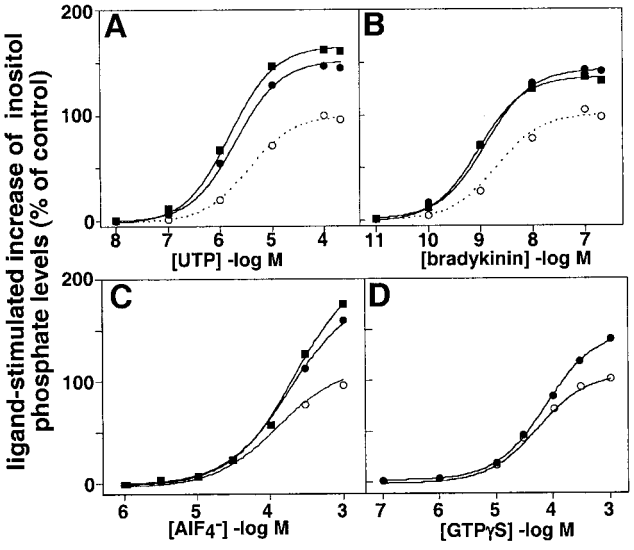

FIG. 1. G$\beta\gamma$-dependence of the inositol phosphate responses to UTP (*A*), bradykinin (*B*), AlF_4^- (*C*), and GTPγS (*D*) in mock-transfected COS cells (○) or in COS cells transfected with plasmids encoding for G$\beta_1\gamma_2$ (●), or for the α_{2C}-adrenergic receptor (■). Cells expressing α_{2C}-receptors were costimulated with 1 μM medetomidine. For GTPγS stimulation (*D*), cells were permeabilized with 10 μM digitonin in a nominally Ca^{2+}-free buffer containing 138 mM KCl instead of NaCl. The ligand-stimulated increase of the inositol phosphate levels is given as percent of the maximum increase in mock-transfected control cells (100%), which was 2,430 ± 180, 2,100 ± 130, 650 ± 70, and 380 ± 40 cpm for AlF_4^-, GTPγS, UTP, and bradykinin, respectively. Expression of α_{2C}-adrenergic receptors and stimulation by medetomidine did not significantly alter basal inositol phosphate levels. Basal inositol phosphate levels determined in the absence (320 ± 25 cpm) or presence (540 ± 30 cpm) of G$\beta_1\gamma_2$ were subtracted from the respective ligand-stimulated values to determine the pure "ligand-stimulated increase." Data are from a representative experiment that was reproduced three times with similar results.

or (*ii*) expression of G$\beta\gamma$ subunits may specifically increase receptor-mediated signals. To discriminate between both mechanisms, we stimulated Gα proteins directly with AlF_4^- or GTPγS (Fig. 1 *C* and *D*). G$\beta\gamma$ subunits increased the maximum AlF_4^- and GTPγS-stimulated signals (Fig. 1 *C* and *D*). However, the potencies of AlF_4^- and GTPγS were not enhanced by G$\beta\gamma$ but were actually slightly decreased from 110 ± 20 μM to 190 ± 20 μM and from 50 ± 5 μM to 72 ± 15 μM (n = 3), respectively. Concomitant Gα_i-coupled α_{2C} receptor stimulation had similar effects on the AlF_4^--stimulated signal (Fig. 1*C*).

Different Effects of a Gβ_3 Mutant on AlF_4^- Versus Receptor-Stimulated Signals. To further discriminate between the two different modes of G$\beta\gamma$-mediated signal enhancement, we created an N-terminally truncated Gβ_3 mutant (amino acids 45–340) with reduced affinity for the Gγ subunit. The G$\beta\gamma$-receptor interaction depends on the Gγ subunit (26). Therefore, a Gβ mutant with reduced affinity for the Gγ subunit should have a reduced capacity to interact with receptors. The Gβ_3 mutant expressed in COS cells exhibited a slightly decreased apparent molecular weight compared with the wild-type protein in immunoblot (Fig. 2*A*). The mutant was still capable of significantly increasing basal inositol phosphate levels (Fig. 2*B*) and of enhancing an AlF_4^--stimulated signal (Fig. 2*C*). The stimulatory effects mediated by the truncated Gβ_3 depended on the cotransfection of Gγ_7, suggesting that the truncated Gβ_3 still interacted weakly with Gγ subunits (Fig. 2 *B* and *C*). However, expression of the truncated Gβ_3 mutant did not significantly enhance the UTP- or bradykinin-stimulated signals (Fig. 2 *D* and *E*). Thus, the mode of G$\beta\gamma$-mediated signal enhancement at the receptor level seems

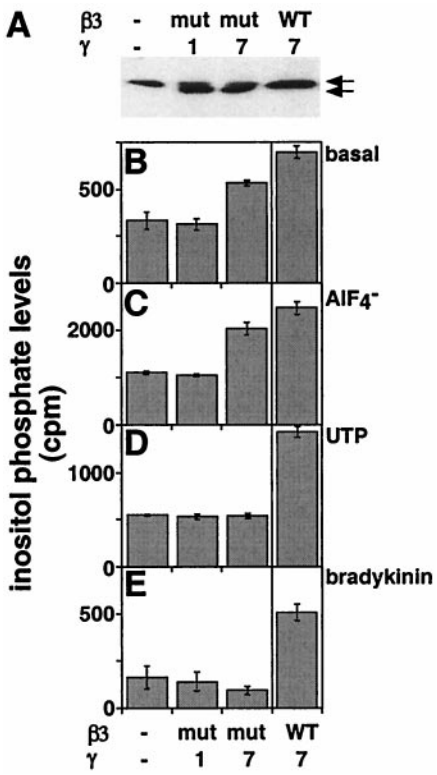

FIG. 2. Effect of a $G\beta_3$ mutant (amino acids 45–340) on basal, AlF_4^--, UTP-, and bradykinin-stimulated inositol phosphate levels. (*A*) Immunoblot of COS cells transfected with the $G\beta_3$ mutant ($\beta_3$mut) or with wild-type $G\beta_3$ (WT). $G\gamma_1$ and $G\gamma_7$ were cotransfected as indicated. As a control, mock-transfected COS cells were used (-). (*B–E*) Inositol phosphate levels of COS cells expressing the $G\beta_3$ mutant ($\beta_3$mut) together with $G\gamma_{1,7}$ or wild-type $G\beta_3$ (WT) with $G\gamma_7$. In *B*, basal inositol phosphate levels were determined. The increase in inositol phosphate levels over the respective basal levels after stimulation by AlF_4^- (300 μM $AlCl_3$/10 mM NaF), by UTP (100 μM), and by bradykinin (100 nM) are given in *C*, *D*, and *E*, respectively. Data are from a representative experiment (triplicates ± SD) that was reproduced three times with similar results.

to be different from the stimulatory effect of $G\beta\gamma$ at the G-protein level.

Enhancement of B_2 and UTP-Preferring P2Y Receptor Signals by Defined $G\beta\gamma$ Subunits. The above-described findings raise the possibility that the synergism might be exerted at the receptor level: for example, by exchange of $G\beta\gamma$ subunits between $G\alpha_i$ and $G\alpha_q$-coupled receptors. A prerequisite for such a mechanism is the ability of a given receptor to interact equally well with a broad spectrum of $G\beta\gamma$ combinations because, initially, $G\alpha_q$- and $G\alpha_i$-coupled receptors might well be coupled to different $G\beta\gamma$ dimers. We therefore determined the interaction of defined $G\beta\gamma$ subunits with the B_2 and UTP-preferring P2Y receptor. For this analysis, we chose $G\beta_{1-3}$ and $G\gamma_{1-7}$ because the specific $G_{\beta\gamma}$-receptor complexes delineated from antisense studies (1–5) are all composed of these subunits. These $G\beta\gamma$ combinations were expressed in COS cells. Equal protein expression of the various $G\beta\gamma$ combinations was verified by immunoblotting with anti-$G\beta_{common}$ antibodies (Fig. 3*A*). Compared with mock-transfected cells, the $G\beta$ signal of overexpressing cells was increased ≈2-fold. Considering a transfection efficiency of 20–30% (as determined by staining of cells transfected with a β-galactosidase expression plasmid) (data not shown), a 2-fold increase in intensity suggests a 4- to 6-fold overexpression of $G\beta$ in the transfected cells. All combinations of $G\beta_{1-3}$ with $G\gamma_{2-7}$ were equally effective in stimulating basal PLC activity (Fig. 3*B*). The effect of $G\beta_1\gamma_1$ (=transducin $G\beta\gamma$) was only

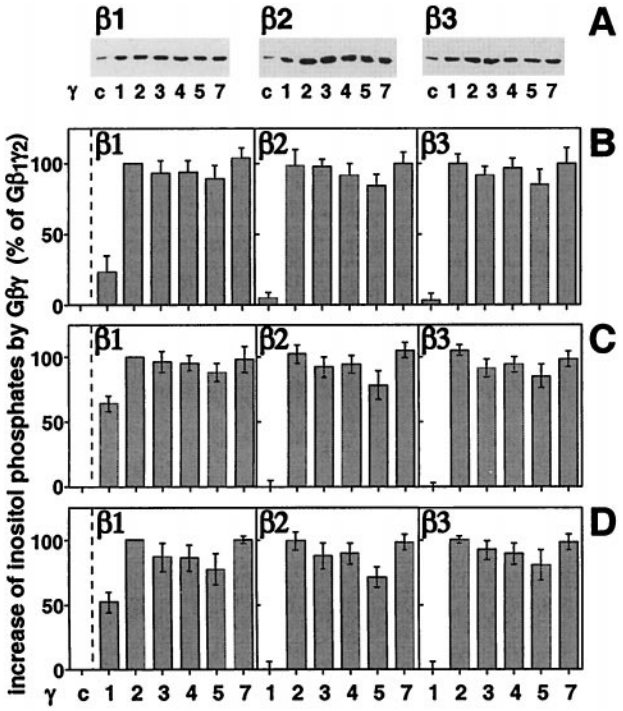

FIG. 3. $G\beta\gamma$-mediated enhancement of basal, bradykinin-, and UTP-stimulated inositol phosphate levels. (*A*) Immunoblot of COS cells transfected with combinations of cDNAs coding for $G\beta_{1-3}$ and $G\gamma_{1-7}$. Note that $G\gamma_6$ is lacking because it is identical with $G\gamma_2$. $G\beta$ subunit expression was detected by a monoclonal anti-$G\beta_{common}$ antibody. (*B*) Increase of basal inositol phosphate levels of COS cells after coexpression of $G\beta_{1-3}$ and $G\gamma_{1-7}$. The values represent the increase of basal inositol phosphate levels after $G\beta\gamma$ expression compared with mock-transfected control cells (0%) and are given as percent of maximum (100%) determined with $G\beta_1\gamma_2$. Expression of $G\beta_1\gamma_2$ increased basal inositol phosphate levels (240–380 cpm) 1.7- to 1.9-fold. (*C* and *D*) $G\beta\gamma$-mediated enhancement of the bradykinin- [100 nM (*C*)] and UTP- [100 μM (*D*)] stimulated signals. The $G\beta\gamma$-mediated signal enhancement over the signal of the mock-transfected control cells (0%) is given as percent of maximum stimulation (100%) after $G\beta_1\gamma_2$ expression. In mock-transfected control cells, the UTP- and bradykinin-stimulated increase over basal was 580–880 and 210–430 cpm, respectively. In $G\beta_1\gamma_2$-expressing cells, the UTP and the bradykinin-stimulated increase over basal was 1,370–1,850 cpm and 540–1,300 cpm, respectively. Data presented are the means (±SEM) of 3–7 different experiments.

≈25% of maximum (Fig. 3*B*), and $G\beta_2\gamma_1$ and $G\beta_3\gamma_1$ did not significantly increase basal inositol phosphate levels. These findings confirm earlier data (27–28) that $G\beta_2$ and $G\beta_3$ associate weakly with $G\gamma_1$.

Subsequently, the effects of $G\beta\gamma$ expression on the two different $G\alpha_q$-coupled receptors were determined. Combinations of $G\beta_{1-3}/G\gamma_{2,3,4,7}$ enhanced the bradykinin and UTP-stimulated increase in inositol phosphate levels equally well (Fig. 3 *C* and *D*). Complexes with $G\gamma_5$ also enhanced the hormone-stimulated signals, though to a slightly lesser extent. Taken together, the interaction of the various $G\beta\gamma$ subunits with the signaling cascades of the B_2 and the UTP-preferring P2Y receptor is characterized by a remarkable lack of coupling specificity, which allows combinations formed of $G\beta_{1-3}$ and $G\gamma_{2-7}$ to enhance efficiently the bradykinin- or UTP-mediated signals. This lack of specificity should enable these receptors to accept different $G\beta\gamma$ dimers released from various differentially coupled receptors.

Signaling of a B_2 Receptor-$G\beta_2$ Fusion Protein. To test the hypothesis that the enhancement of $G\alpha_q$-coupled receptor signaling by $G\alpha_i$-coupled receptors reflects exchange of $G\beta\gamma$ subunits between two different receptors, we constructed a

fusion protein between the B_2 receptor and $G\beta_2$. Transient transfection of COS cells resulted in the expression of the fusion protein as determined by immunoblot stained with anti-$G\beta$ antibodies, which showed the appearance of an ≈95-kDa protein in addition to the endogenous $G\beta$ subunits of ≈35 kDa (Fig. 4*A*). The apparent molecular weight of ≈95 kDa corresponds to the expected size of B_2 receptor plus $G\beta$ subunit. When the immunoblot was stained with anti-B_2 receptor antibodies (24), again a protein of ≈95 kDa was detected in $B_2G\beta_2$-expressing cells whereas a protein of ≈60 kDa was apparent in wild-type B_2 receptor-expressing cells (Fig. 4*B*). These findings confirm that the expressed protein consists of a $G\beta_2$ subunit fused to the B_2 receptor.

The affinity of the fusion protein for the agonist bradykinin as determined by radioligand binding was 0.4 ± 0.2 nM (data not shown); this value was similar to the affinity of the wild-type receptor (0.8 ± 0.3 nM). To determine whether the fused $G\beta_2$ subunit altered the mode of receptor activation, we determined the B_2-receptor-mediated rise in $[Ca^{2+}]_i$. The bradykinin-stimulated rise in $[Ca^{2+}]_i$ in cells expressing the fusion protein was virtually identical to cells expressing the wild-type receptor, suggesting that the fused $G\beta$ subunit did not alter the kinetics of agonist-stimulated receptor activation (Fig. 4 *C* and *D*). Also, the EC_{50} value for the bradykinin-induced rise in inositol phosphate levels of the B_2 receptor-fusion protein was similar to the wild-type B_2 receptor (Fig. 4*E*). Thus, B_2 receptors with C-terminally fused $G\beta$ subunit interact efficiently with $G\alpha$ proteins of COS cells.

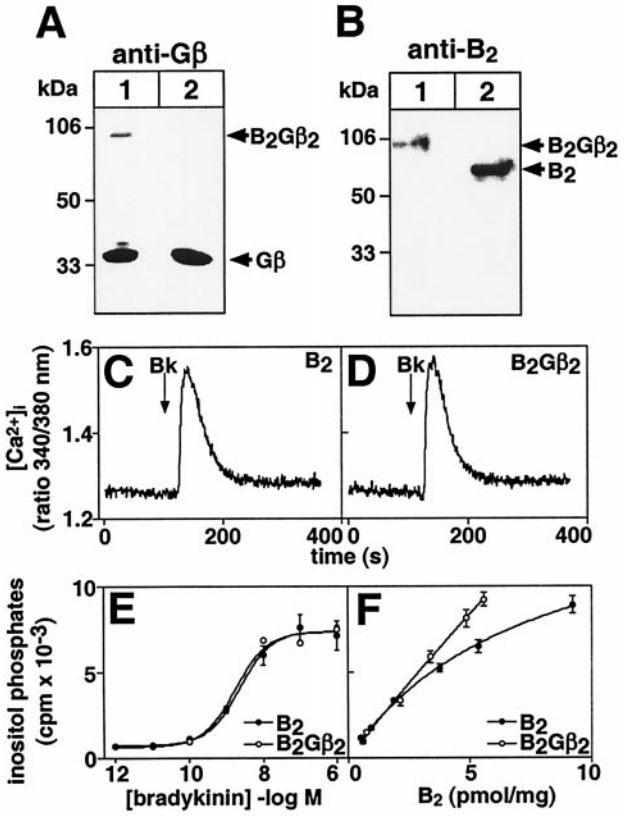

FIG. 4. Characterization of a fusion protein between the B_2 receptor and the $G\beta_2$ subunit. (*A* and *B*) Expression of the $B_2G\beta_2$-fusion protein in COS cells (lane 1). Cells transfected with the wild-type B_2 receptor served as a control (lane 2). The immunoblots were analyzed with anti-$G\beta_{common}$ (*A*) or anti-B_2 receptor (*B*) antibodies. (*C* and *D*) Bradykinin-stimulated rise in $[Ca^{2+}]_i$ in fura-2-loaded HEK-293 cells transfected with the wild-type B_2 receptor (*C*) or with the $B_2G\beta_2$ fusion protein (*D*). At the time point indicated by an arrow, 100 nM bradykinin (Bk) was added. (*E*) Bradykinin-induced rise in inositol phosphate levels in COS cells transfected with the wild-type B_2 receptor or with the $B_2G\beta_2$-fusion protein. Cells expressed 6.1 pmol/mg protein of wild-type and 3.9 pmol/mg protein of $B_2G\beta_2$ receptor. Data are from a representative experiment (triplicates ± SD) that was reproduced three times with similar results. (*F*) Inositol phosphate levels of COS cells expressing different levels of wild-type B_2 receptors or of $B_2G\beta_2$ fusion protein after stimulation by 100 nM bradykinin. The amount of expressed receptors is given as pmol/mg protein. Data are from a representative experiment (triplicates ± SD) that was reproduced three times with similar results.

Does the fused $G\beta_2$ subunit enhance the $B_2G\beta_2$ receptor signal? To address this question, we compared the maximum bradykinin-stimulated signals of the wild-type and the $B_2G\beta_2$ receptor at different receptor expression levels. With expression levels exceeding 2 pmol/mg protein, the signal of the $B_2G\beta_2$ receptor at a given receptor number was significantly increased compared with the wild-type receptor (Fig. 4*F*). This finding suggests that the C-terminally fused $G\beta_2$ subunit is functionally active and enhances the agonist-stimulated signal of the $B_2G\beta_2$ receptor similarly as seen with separately expressed $G\beta(\gamma)$ subunits and the wild-type B_2 receptor.

Inhibition of $G\beta\gamma$ Exchange in the $B_2G\beta_2$ Receptor Fusion Protein. We next asked whether the fused $G\beta_2$ subunit could suppress the enhancement of the B_2 receptor signal by $G\beta\gamma$ subunits and determined the effect of $G\beta_1\gamma_2$ coexpression on the bradykinin signal. Cotransfection with $G\beta_1\gamma_2$ increased the inositol phosphate production triggered by the wild-type B_2 receptor by up to 60% (Fig. 5*A*). In contrast, the signal of the $B_2G\beta_2$ receptor was not significantly enhanced by cotransfection of $G\beta_1\gamma_2$ (Fig. 5*A*). Thus, we conclude that, under the conditions applied (*i*) the overexpressed $G\beta\gamma$ did not enhance B_2 receptor-mediated signaling by direct synergistic activation of PLC, (*ii*) $G\beta\gamma$ needs access to the B_2 receptor, and (*iii*) this access can be blocked by fusion of $G\beta_2$ to the receptor's C terminus. A small potentiating effect of expressed $G\beta_1\gamma_2$ could be observed when the fusion protein was expressed at lower levels (<1.5 pmol/mg protein), suggesting true competition between "free $G\beta\gamma$" and the $G\beta$ subunit fused to the B_2 receptor (data not shown).

Costimulation of the $B_2G\beta_2$ Fusion Protein and $G\alpha_i$-Coupled Receptors. Finally, we triggered the release of $G\beta\gamma$ subunits by stimulation of the two different $G\alpha_i$-coupled

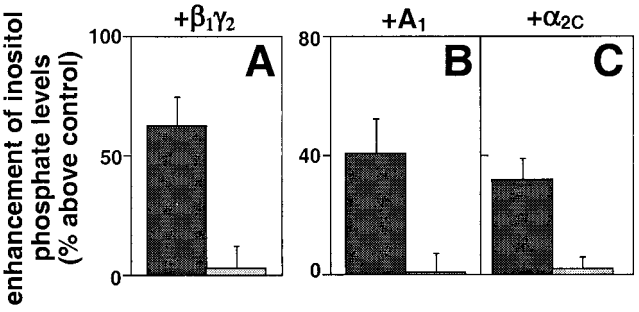

FIG. 5. (*A*) Effect of $G\beta\gamma$ subunit expression on signaling by wild-type B_2 receptors and the $B_2G\beta_2$ fusion protein. The $G\beta_1\gamma_2$-mediated enhancement of the bradykinin-stimulated signal (100 nM) was determined on COS cells expressing the B_2 receptor (■) or the $B_2G\beta_2$ fusion protein (▨) together with $G\beta_1\gamma_2$. The enhancement is given as percent above control cells (0%): i.e., cells transfected with wild-type B_2 receptor or with $B_2G\beta_2$ fusion protein alone. (*B* and *C*) Suppression of crosstalk with $G\alpha_i$-coupled receptors in a $B_2G\beta_2$ fusion protein. COS cells expressing the B_2 receptor (■) or the $B_2G\beta_2$ fusion protein (▨) together with the A_1 adenosine (*B*) or the α_{2C}-adrenergic (*C*) receptor were stimulated with 100 nM bradykinin plus 1 μM N^6-cyclohexyladenosine in the presence of 0.5 units/ml adenosine deaminase (*B*) or plus 1 μM medetomidine (*C*). Expression levels of the transfected B_2 receptors were 3.3 ± 0.4 pmol/mg protein. Expression levels of the transfected A_1 and α_{2C} receptor were 2.1 ± 0.4 and 1.8 ± 0.3 pmol/mg protein, respectively. Data are given as percent above control (0%): i.e., stimulation with bradykinin alone. All data are the means (±SEM) of three different experiments.

receptors used above, i.e., the A_1 adenosine and the α_{2C} adrenergic receptor, and determined again the effects on the signals generated by wild-type B_2 and $B_2G\beta_2$ receptors. By using wild-type B_2 receptors, the costimulation of either A_1 adenosine receptors (Fig. 5*B*) or of α_{2C}-adrenergic receptors (Fig. 5*C*) enhanced the maximal signal of the B_2 receptor. However, stimulation of either of the two $G\alpha_i$-coupled receptors did not alter the inositol phosphate signal of the $B_2G\beta_2$-fusion (Fig. 5 *B* and *C*). Thus, the $G\beta_2$ subunit fused to the C terminus of the B_2 receptor was capable of suppressing the A_1- or α_{2C}-receptor-mediated enhancement of the B_2 receptor's signal.

Effect of $G\beta\gamma$ on B_2 Receptor-Stimulated G-Protein Activation. The experiments suggest that $G\beta\gamma$ enhances a receptor-stimulated signal by direct interaction with the receptors. Two mechanisms are conceivable: (*i*) $G\beta\gamma$ may increase the number of functional holotrimeric G-proteins that can be activated by a given receptor, or (*ii*) the $G\beta\gamma$-receptor interaction may enhance the receptor-stimulated $G\alpha$ activation: i.e., guanine nucleotide exchange. Expression of $G\beta_1\gamma_7$ did not significantly increase the number of high affinity binding sites for [^{3}H]bradykinin (Fig. 6*A*; $t = 0$). Because the number of high affinity binding sites reflects the interaction of functional holotrimeric G-proteins with an activated receptor, we conclude that an increase in the number of functional $G\alpha\beta\gamma$ heterotrimers was not responsible for the $G\beta\gamma$-mediated signal enhancement at the receptor level. By contrast, $G\beta1\gamma7$ expression accelerated the GTPγS-induced decrease in the number of high affinity [^{3}H]bradykinin binding sites (Fig. 6*A*), and $G\beta\gamma$ subunits purified from bovine brain enhanced the B_2 receptor-stimulated GTPγS binding (Fig. 6*B*). Thus, $G\beta\gamma$ subunits accelerated the agonist-stimulated guanine nucleotide exchange: i.e., activation of the $G\alpha$ subunit.

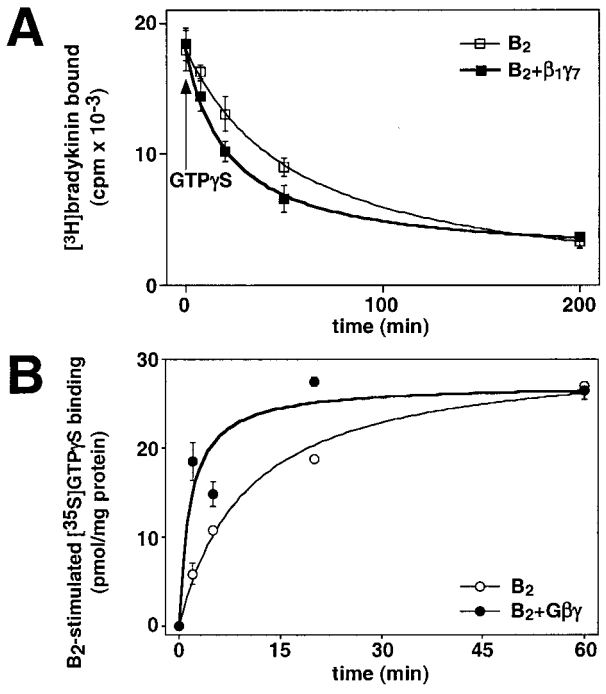

FIG. 6. (*A*) Effect of $G\beta\gamma$ subunit expression on binding of [^{3}H]bradykinin. Membranes of COS cells transfected with B_2 receptor alone or with B_2 receptor together with $G\beta_1\gamma_7$ were incubated for 1 h at 4°C with 5 nM [^{3}H]bradykinin. At $t = 0$, GTPγS (1 μM) was added, and the cells were placed at 30°C. Specific high affinity binding of [^{3}H]bradykinin was determined at the time points indicated. Data are from a representative experiment (triplicates ± SD) that was reproduced three times with similar results. (*B*) B_2 receptor-stimulated GTPγS binding. Membranes of B_2 receptor expressing HEK-293 cells (5 μg protein/50 μl) were incubated in the absence (B_2) or presence (B_2+$G\beta\gamma$) of 50 nM $G\beta\gamma$ purified from bovine brain, and bradykinin-stimulated [^{35}S]GTPγS binding was determined at the time points indicated.

DISCUSSION

Signal adaptation to new inputs is the basis for efficient signaling networks. Mechanisms to sense and to adapt to stimulatory or inhibitory inputs are generally summarized under the term "crosstalk." An increasing number of different crosstalk mechanisms is emerging between G-protein-coupled receptors involving all levels of the signaling cascade(s). A crosstalk mechanism on the initial step of signal generation, the receptor-G-protein interface has so far not been demonstrated. In fact, antisense experiments demonstrating preferential coupling of specific receptors to specific G-protein heterotrimers in intact cells (1–4) might appear to preclude such a cross-talk at the receptor/G-protein level.

Stimulation of two $G\alpha_i$-coupled receptors, the α_{2C}-adrenergic and the A_1 adenosine receptor, enhanced the potency and efficacy of the signal generated by two $G\alpha_q$-coupled receptors, a UTP-preferring P2Y and the B_2 receptor. This signal enhancement was very similar to that caused by overexpression of $G\beta\gamma$ and is most likely caused by release of $G\beta\gamma$ from $G\alpha_i$. It seems to be exerted proximal to $G\alpha_q$, i.e., at the receptor level, because (*i*) an N-terminally truncated $G\beta_3$ mutant was still capable of increasing basal and AlF_4^--stimulated signals but had no effect on the receptor-mediated signals, and (*ii*) a $G\beta_2$ subunit fused to the B_2 receptor substituted for the signal enhancement caused by stimulation of $G\alpha_i$-coupled receptors or by $G\beta\gamma$. Thus, access of $G\beta\gamma$ to the receptors appears to be essential for this mechanism, and this access is (partially) precluded by the fused $G\beta_2$-moiety. The $G\alpha_i$-coupled receptors appear to provide the $G\alpha_q$-coupled receptors with additional $G\beta\gamma$ subunits (Fig. 7). $G\beta\gamma$ subunits seem to promote $G\alpha$ activation by accelerating the rate of receptor-stimulated GTP-binding (Fig. 7). A similar effect of $G\beta\gamma$ subunits has been observed *in vitro* for the rhodopsin-stimulated $G\alpha_t$ activation (29). Because $G\beta\gamma$ subunits suppress the GDP/GTP exchange in the absence of an activated receptor by direct $G\alpha$ interaction (30), the $G\beta\gamma$-receptor interaction seems to be a prerequisite for the $G\beta\gamma$-mediated enhancement of $G\alpha$ activation. Although $G\beta\gamma$ subunits may enhance a receptor-stimulated signal at several steps of the signaling cascade (cf. introduction), a major component may be contributed by the newly discovered mode of receptor crosstalk. This crosstalk relies on $G\beta\gamma$ exchange between $G\alpha_i$-coupled and $G\alpha_q$-coupled receptors, thereby accelerating receptor-stimulated GTP-binding of $G\alpha_q$ (Fig. 7).

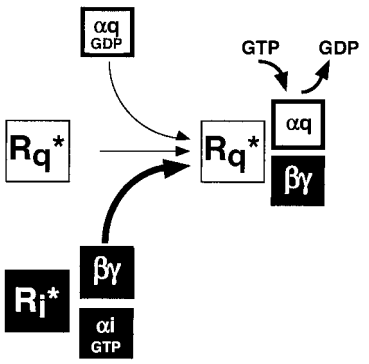

FIG. 7. Model of $G\beta\gamma$-mediated crosstalk between $G\alpha_i$ and $G\alpha_q$-coupled receptors. $G\beta\gamma$ transfer from an activated $G\alpha_i$ coupled receptor (R_i*) to an activated $G\alpha_q$-coupled receptor (R_q*) enhances the receptor-stimulated GDP/GTP exchange of $G\alpha_q$.

Although the mode of receptor crosstalk between $G\alpha_i$ and $G\alpha_q$-coupled receptors was analyzed in COS cells, the observed mechanism seems to be common to a variety of different $G\alpha_i$- and $G\alpha_q$-coupled receptors in recombinant and primary cell systems (15, 16, 31). It did not depend on the overexpression of $G\beta\gamma$ subunits because the endogenous levels of $G\beta\gamma$ subunits in COS cells were sufficient to promote receptor crosstalk. In intact nonstimulated cells, different receptors appear to be preferentially coupled to different heterotrimeric G-proteins (1–5). Therefore, efficient $G\beta\gamma$-mediated crosstalk must rely on a lack of biochemical coupling specificity between receptors and different $G\beta\gamma$ dimers. Indeed, combinations of $G\beta_{1-3}$ with $G\gamma_{2-7}$ were all about equally efficient in enhancing the UTP- and bradykinin-stimulated signals. Complexes with $G\gamma_5$ were only slightly less efficient (70–90% of maximum), an effect that is most likely attributable to a less efficient interaction with phospholipase C (U.Q., unpublished data) and not to a less efficient interaction with the receptors. One might argue that overexpression of $G\beta\gamma$ subunits may cover small differences in potency between several combinations, but the low efficiency of $G\beta_1\gamma_1$ is in good agreement with earlier results in which $G\beta_1\gamma_1$ was 10-fold less potent than $\beta_1\gamma_2$ in stimulating PLCβ_3 activity (9), the main component of $G\beta\gamma$-stimulated PLC activity in COS cells (32). Thus, the receptor-$G\beta\gamma$ interaction lacks coupling specificity and thus fulfills the proposed prerequisite for efficient $G\beta\gamma$-based crosstalk.

$G\beta\gamma$ subunits do not seem to exchange per se; they must be mobilized by receptor activation. Receptor signaling via $G\alpha_i$ and $G\alpha_q$ often leads to opposing effects in the same cell: e.g., stimulation of A_1 adenosine receptors induces vasoconstrictor responses in pulmonary vascular beads whereas B_2 receptors mediate vasodilation (33); stimulation of α_{2C}-adrenergic or A_1 adenosine receptors suppresses norepinephrine release from sympathetic nerve endings, which is stimulated by activation of B_2 receptors (22, 34). Therefore, the concomitant release of $G\beta\gamma$ subunits after $G\alpha_i$-receptor stimulation may dampen the generated signal by lowering the threshold for the antagonizing $G\alpha_q$-coupled receptor via $G\beta\gamma$ transfer. Thus, the $G\beta\gamma$-mediated mode of receptor crosstalk may act as a feedback loop to balance inhibitory and stimulatory inputs.

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