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The β6/α5 regions of $G\alpha_{i2}$ and $G\alpha_{oA}$ increase the promiscuity of $G\alpha_{16}$ but are insufficient for pertussis toxin-catalyzed ADP-ribosylation

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Abstract

Replacement of β6/α5 region at the C-terminus on $G\alpha_{16}$ with $G\alpha_z$-specific residues has been shown to broaden the promiscuity of $G\alpha_{16}$. Here, we substituted the last 44 residues of $G\alpha_{16}$ with the corresponding region from either $G\alpha_{i2}$ or $G\alpha_{oA}$ (16i44 and 16o44). 16i44 and 16o44 chimeras were more effective than $G\alpha_{16}$ at coupling to G_i-linked δ-opioid, μ-opioid, and *Xenopus* melatonin MT_{1c} receptors when coexpressed in green monkey fibroblast (COS-7) cells. 16i44, but not 16o44, also enhanced the formyl peptide-induced stimulation of phospholipase C activity. Both chimeras were resistant to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation, despite the fact that pertussis toxin partially inhibited the chimera-mediated stimulation of phospholipase Cβ. The use of $G\alpha_{t1}$ as a Gβγ scavenger revealed that the pertussis toxin-sensitivity can be attributed to endogenous Gβγ subunits released from $G_{i/o}$. Although incorporation of a $G\alpha_i$-like β6/α5 region into the C-terminus of $G\alpha_{16}$ increases its promiscuity, this region is not sufficient to support recognition by pertussis toxin.

Keywords: ADP-ribosylation; G protein; Receptor; Pertussis toxin; Phospholipase C

1. Introduction

Heterotrimeric (αβγ) G proteins are grouped into four subfamilies according to their coupling specificity in the signal transduction pathway (Simon et al., 1991). Members belonging to the $G\alpha_q$, $G\alpha_i$, $G\alpha_s$ and $G\alpha_{12}$ subfamilies have different and yet overlapping specificities for G-protein-coupled receptors. The more promiscuous G proteins, such as the human $G\alpha_{16}$ and its mouse homologue $G\alpha_{15}$, belong to the $G\alpha_q$ subfamily (Amatruda et al., 1991). A promiscuous G protein is highly desirable for linking orphan G-protein-coupled receptors to intracellular signaling and both $G\alpha_{15}$ and $G\alpha_{16}$ have been employed for deorphanizing G-protein-coupled receptors as well as to study their signaling (Kostenis, 2001). Despite its ability to link a variety of $G\alpha_q$-, $G\alpha_i$- and $G\alpha_s$-coupled receptors to the activation of phospholipase Cβ (Offermanns and Simon, 1995; Lee et al., 1998), $G\alpha_{16}$ remains unable to recognize a subset of G-protein-coupled receptors. These include the chemokine CCR2a receptor (Kuang et al., 1996), the α_{1A}- and α_{1C}-aderenoceptors (Wu et al., 1992), the *Xenopus* melatonin MT_{1c} receptor (Lai et al., 2002), the SLC-1 receptor (Saito et al., 1999), and the thyrotropin-releasing hormone receptor (Quick et al., 1996).

There is now a substantial body of literature on the molecular basis of receptor/G protein interactions. A series of elegant studies by several groups has identified five regions on the Gα subunit that participate in receptor recognition (reviewed in Bourne, 1997). They include the α2 helix, the β6–α5 loop, the α5 helix, and the extreme N- and C-termini of the Gα. Among these regions, the C-terminus appears to play a determining role in receptor recognition. By replacing C-terminal residues in $G\alpha_q$ (Conklin et al., 1993) and $G\alpha_s$ (Liu et al., 1995; Conklin et al., 1996), Conklin and coworkers have demonstrated that the receptor coupling specificity of the Gα can be modified radically. $G\alpha_q$ chimeras harboring the last five residues of $G\alpha_{i2}$ or $G\alpha_{oA}$ (termed qi5 and qo5, respectively) exhibit improved capability in recognizing G-protein-coupled receptors (Conklin et al., 1993; Joshi et al., 1999). Manipulation of the amino acid sequence at the N-terminus of the Gα subunit can also lead to altered recognition towards G-protein-coupled receptors (Kostenis et al., 1997; Ho and Wong, 2000). Capitalizing on these reports, we have recently

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broadened the promiscuity of $G\alpha_{16}$ by incorporating $G\alpha_z$-specific sequences at the carboxyl terminus of $G\alpha_{16}$ (Mody et al., 2000). The two $G\alpha_{16/z}$ chimeras, termed 16z25 and 16z44, can efficiently mediate agonist-induced stimulation of phospholipase Cβ for a large selection of G_i-linked receptors, including the melatonin MT_{1c} receptor and chemokine CCR2a receptor that are incapable of interacting with $G\alpha_{16}$ (Mody et al., 2000; Liu et al., 2003). These $G\alpha_{16/z}$ chimeras have proven useful in delineating the signal transduction mechanisms of taste receptors (Nelson et al., 2002) and may become invaluable tools in deorphanizing G-protein-coupled receptors.

In constructing the $G\alpha_{16/z}$ chimeras, $G\alpha_z$ was selected as the donor because of its resistance to ADP-ribosylation by pertussis toxin as well as its ability to interact with practically all G_i-linked receptors (Ho and Wong, 2001). The former characteristic allows the effects of the chimeras to be distinguished from those of the pertussis toxin-sensitive $G_{i/o}$ proteins, while the latter feature provides for versatility. However, a recent study suggests that not all G_i-linked receptors possess the ability to associate with G_z (Francken et al., 2000). The human 5-HT_{5A} receptor recognizes G_i and G_o, but not G_z, in a baculovirus expression system. Thus, it is conceivable that the 16z25 and 16z44 chimeras may not be able to recognize all G_i-linked receptors. In the 16z44 chimera, the α4−β6−α5 regions are composed of 44 $G\alpha_z$-specific residues that share approximately 60% identity among members of the $G\alpha_i$ subfamily. The central question to be examined in this study is whether substitution of the same region of $G\alpha_{16}$ with $G\alpha_i$- or $G\alpha_o$-specific sequences would increase the promiscuity of the resultant chimeras. We have constructed $G\alpha_{16/i}$ and $G\alpha_{16/o}$ chimeras, termed 16i44 and 16o44, by substituting the last 44 residues of $G\alpha_{16}$ with cognate regions from either $G\alpha_{i2}$ or $G\alpha_{oA}$. These chimeras were then transiently transfected into COS-7 cells along with different G-protein-coupled receptors to assess their ability to stimulate phospholipase Cβ activity. Since the extreme C-terminus of $G\alpha_{i/o}$ contains the site for pertussis toxin-catalyzed ADP-ribosylation, the resultant chimeras might serve as substrates for the toxin. In view of the fact that the qi5 and qo5 chimeras are resistant to pertussis toxin-catalyzed ADP-ribosylation (Joshi et al., 1998), we have also assessed the pertussis toxin sensitivity of 16i44 and 16o44. Our results suggest that both 16i44 and 16o44 exhibit enhanced promiscuity toward G_i-coupled receptors, but they do not serve as targets for pertussis toxin-catalyzed ADP-ribosylation.

2. Materials and methods

2.1. Materials

cDNAs of rat μ-opioid and mouse δ-opioid receptors were generous gifts from L. Yu (University of Cincinnati College of Medicine), D. Evans (UCLA) and G. Bell (University of Chicago, IL, USA), respectively. The human formyl peptide receptor in the pCDM8 vector was kindly provided by Dr. F. Boulay (Laboratories de Biochemie, Grenoble, France). *Xenopus* melatonin MT_{1c} receptor was a kind gift from Dr. Steven Reppert (Massachusetts General Hospital, Boston). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, USA). *myo*-[^{3}H]Inositol was purchased from NEN (Boston, MA, USA). $G\alpha_{16}$-specific antiserum against the epitope CLTEDEKAAARVDQE (residues 13–27 of $G\alpha_{16}$) was custom made by Gramsch Laboratories (Schwabhausen, Germany). Cell culture reagents were from Life Technologies (Grand Island, NY, USA) and other chemicals were from Sigma (St. Louis, MO).

2.2. Construction of chimeras

Using polymerase chain reaction (PCR) methods, Gα chimeras were constructed from cDNAs encoding the human $G\alpha_{16}$, mouse $G\alpha_{i2}$, or rat $G\alpha_{oA}$. The cDNAs were all previously subcloned into pcDNA1 at either *Xba*I (for $G\alpha_{16}$) or *Eco*RI (for both $G\alpha_{i2}$ and $G\alpha_{oA}$). The C-terminal 44 residues of $G\alpha_{16}$ were replaced by the corresponding amino acids of $G\alpha_{i2}$ to produce the 16i44 chimera. The 16o44 chimera was constructed from $G\alpha_{16}$ and $G\alpha_{oA}$ in a similar manner. Primers were designed to produce two half-products which overlap with each other. A 5′ fragment was made with the reverse chimeric primer and T7 primer, and the 3′ fragment was generated with the forward chimeric primer and the SP6 primer. The two half-fragments were then annealed together to create a full-length product using only T7 and SP6 primers. The 30i16i44 chimera was similarly constructed using 16i44 as one of the template. Primers used in the construction of chimeras are listed below with their corresponding nucleotide sequences for $G\alpha_{i2}$ and $G\alpha_{oA}$ underlined. 16i44/AS, GTC TTT GCG CTT ATT GCT GCC CTC GGG GCC; 16i44/S, GGC CCC GAG GGC AGC AAT AAG CGC AAA GAC; 16o44/AS, GGG TGA GCG GTT TTT GCT GCC CTC GGG GCC; 16o44/S, GGC CCC GAG GGC AGC AAA AAC CGC TCA CCC; 30i16i44/S, GAC GGC GAG AAG GCG CGC GGG GAG CTG AAG; 30i16i44/AS, CTT CAG CTC CCC GCG CGC CTT CTC GCC GTC. Reactions were performed under the following amplification conditions: 94 °C for 60s, 56 °C for 60s and 72 °C for 90s for 35 cycles with Robocycler40 from Stratagene (La Jolla, CA). 16i44 and 16o44 were checked with restriction mapping and then subcloned into pcDNA1 using *Hin*dIII and *Eco*RV, 30i16i44 was subcloned into *Hin*dIII sites. The constructs were subsequently sequenced by means of dideoxynucleotide sequencing.

2.3. Transfection of green monkey fibroblast (COS-7) cells

Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (v/v), 50 U/ml penicillin and 50 μg/ml streptomycin were used for COS-7 cells cultivation at 37 °C incubator with 5% CO_2. One day before transfection,

cells were seeded into 12-well plates at 1.5×10^5 cells/well. Same transfection method was used as described before (Wong, 1994). Qiagen column chromatography purified cDNA samples (250 ng/ml each) were incubated in growth medium mixture with 250 μg/ml DEAE-dextran and 100 μM chloroquine for 3 h. After incubation, cells were shocked for 1 min at room temperature in phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide (v/v) which was then rinsed with PBS alone once and replaced with growth media for 24 h. A cotransfection with β-galactosidase indicated 50–60% transfection efficiency after staining.

2.4. Inositol phosphate accumulation assay

Each well of 12-well plates containing transfected COS-7 cells were incubated with 750 μl of inositol-free DMEM with 5% fetal calf serum and 2.5 μCi/ml *myo*-[^{3}H]inositol for 18–24 h with or without 100 ng/ml pertussis toxin. Labeling medium was aspirated and replaced with 1 ml of inositol phosphate assay medium alone (DMEM buffered with 20 mM HEPES, 20 mM LiCl, pH 7.5). After 10-min incubation, the medium was replaced with 1 ml assay medium containing the appropriate agonists and the cells were incubated for 1 h at 37 °C. Reactions were stopped using 750 μl of 20 mM formic acid and kept at 4 °C for at least 30 min. Sequential ion-exchange chromatography separated [^{3}H]inositol phosphates from other labeled inositol species as described previously (Tsu et al., 1995). Results are expressed as the average ratio of [^{3}H]inositol phosphates to total [^{3}H]inositol species. Variations of triplicates within a given experiments were generally less than 10%.

2.5. Flurometric measurement of Ca^{2+} mobilization

COS-7 cells were seeded into black-walled, clear-bottomed 96-well tissue culture plates at a density of 2×10^4/well. Transfection cocktails were prepared in antibiotics-free OptiMEM with Lipofectamine 2000 (Invitrogen) as described in the manufacturer's manual, and added directly onto the cells in a final volume of 100 μl. Transfected cells were labeled 24 h later with 2 μM Fluo-4 (Molecular Probes) in 200 μl of assay buffer (Hanks' balanced salt solution buffered with 20 mM of HEPES, pH 7.5, with 2.5 mM of freshly made probenecid) for 60 min at 37°C. Agonists were prepared in assay buffer five times the final working concentration and distributed in clear V-bottomed 96-well plates. Changes in fluorescence were detected in the Fluorometric imaging Plate Reader (FLIPR®I) with an excitation wavelength of 488 nm. Background fluorescence was adjusted to the range of 8000–12,000 units by altering the laser power and exposure time (typically 0.4 W and 0.4 s, respectively). Fifty microliters of each agonist solution was added to the corresponding wells and the fluorescent emission (between 510 and 560 nm) was monitored for 3 min. Results were expressed as changes in fluorescent intensity units (FIU). Concentration–response curves were generated by determination of the maximal change in FIU of each data set. Dose-dependent responses were analyzed using GraphPad Prism 3.

2.6. Membrane protein preparation and immunodetection

Transfected COS-7 cells were washed with Ca^{2+}/Mg^{2+}-free PBS and harvested using 5 ml of Ca^{2+}/Mg^{2+}-free PBS containing 10 mM EDTA. Cells were spun down briefly at $200 \times g$ for 5 min at 4 °C and then resuspended in hypotonic lysis buffer (50 mM Tris–HCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine–HCl and 1 mM dithiothreitol, pH 7.4). After one cycle of freeze-thawing followed by 10 passages through a 27-gauge needle, nuclei were spun down and membranes were collected by spinning the supernatant for 15 min at $15{,}000 \times g$. Membrane pellets were resuspended in lysis buffer and protein concentrations were determined by Bio-Rad Protein Assay Kit. Fifty micrograms of each protein sample was resolved on 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes through electroblotting. Chimeras were detected by a custom-made antiserum against $G\alpha_{16}$ N-terminus (residues 13–27) and visualized using the Amersham Biosciences ECL™ Western kit. The 30i16i44 chimera was detected by an antiserum that recognizes the C-terminus of transducin, $G\alpha_{i1}$ and $G\alpha_{i2}$ (NEI-801 by NEN Life Science Products).

2.7. In vitro translation

In vitro translation was carried out to produce $G\alpha_{i2}$, $G\alpha_{oA}$, 16i44 and 16o44 using the Promega Transcend™ tRNA system according to the manufacturer's protocol at 30 °C for 90 min. Aliquots (2–3 μl) of the translation mixtures were analyzed on a 12.5% SDS-PAGE and detected using streptavidin peroxidase conjugate (Calbiochem) and visualized on film.

2.8. [^{32}P]ADP-ribosylation

Pertussis toxin-catalyzed ADP-ribosylation was carried out as described previously (Wong et al., 1988). Briefly, 50 μg/ml of pertussis toxin was preactivated at 30 °C for 1 h in 50 mM dithiothreitol (DTT). The reaction mixture contained 100 mM Tris-HCl (pH 7.5) with 10 mM ATP, 0.04 M thymidine, 80 mM arginine, 0.8 M bovine serum albumin, 0.4 M K_3PO_4 (pH 7.5), 20 mM ADP-ribose, 40 mM $MgCl_2$, 100 mM EDTA, 20 mM GTP, 50 μg membrane proteins. The labeling reaction was initiated with the addition of 25 μM [α-^{32}P]NAD together with 5 μg of preactivated pertussis toxin and incubated at 30 °C for 30 min, then it was stopped with a NAD-wash containing 50 mM Tris–HCl (pH 7.5), 5 μM NAD and 1 mM EDTA. Protein samples of $G\alpha_{16}$ and chimeras were then resolved on 12.5% SDS-PAGE and exposed on film for visualization.

3. Results

3.1. Design of $G\alpha_{16/i/o}$ chimeras

A unique structural feature of $G\alpha_{16}$ (and $G\alpha_{15}$) is an insertion of 11 residues (amino acids 325–336; based on the alignment of all mammalian Gα subunits using the Clustal X program) between the α4 helix and the β6 strand. In a previous study (Mody et al., 2000), we replaced half of the α4/β6 insertion by $G\alpha_z$ residues without shortening the insertion. The resultant chimera, termed 16z44, has the last 44 amino acids composed of $G\alpha_z$-specific residues and exhibits enhanced promiscuity towards G_i-coupled receptors. We adopted the same approach to tag on 44 residues from either $G\alpha_{i2}$ or $G\alpha_{oA}$ to the $G\alpha_{16}$ backbone and constructed the 16i44 and 16o44 chimeras, respectively. As compared to 16z44, 16i44 and 16o44 have, respectively, 10 and 16 nonidentical residues (Fig. 1). Based on the crystal structures of $G\alpha_{i1}$ (Wall et al., 1995), the N- and C-termini are predicted to be in close proximity and may both participate in receptor recognition and docking with pertussis toxin. To assess whether the N-terminus of $G\alpha_{i2}$ is required for efficient coupling to G_i-linked receptors and ADP-ribosylation, we replaced the entire putative region of αN helix of 16i44 with that of the first 30 residues of $G\alpha_{i2}$. The resultant 30i16i44 chimera is shorter than $G\alpha_{16}$ by nine residues (Fig. 1).

3.2. Coupling of 16i44 and 16o44 to G_i-linked receptors

We expressed 16i44 and 16o44 transiently in COS-7 cells to determine if the chimeras can be functionally expressed and whether they are able to stimulate inositol phosphate production following activation by G_i-linked receptors. COS-7 cells were contransfected with cDNAs encoding $G\alpha_{16}$, 16z44, 16i44 or 16o44 together with one of the following receptors (at 0.25 μg/ml per construct): μ-opioid receptor, δ-opioid receptor, melatonin MT_{1c} receptor, and formyl peptide receptor. Transfected cells were assayed for inositol phosphate formation with or without the addition of appropriate agonists. In COS-7 cells coexpressing the μ-opioid receptor with one of the chimeras, 300 nM of the μ-opioid receptor-selective agonist, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-Enkephalin acetate salt (DAMGO), significantly stimulated inositol phosphate production by three- to four-fold above the corresponding basal values (Fig. 2). In agreement with our previous report (Mody et al., 2000), DAMGO produced a small but insignificant stimulation of phospholipase Cβ activity in cells coexpressing $G\alpha_{16}$. When COS-7 cells were cotransfected with the melatonin MT_{1c} receptor and one of the chimeras, 1 μM 2-iodomelatonin produced significant stimulations of approximately twofold increase of inositol phosphate levels (Fig. 2) but not when coexpressed with $G\alpha_{16}$. Similarly, agonist activation of the δ-opioid receptor resulted in significant stimulations of around threefold inositol phosphates increases in cells coexpressing 16z44, 16i44 or 16o44 (Fig. 2). Since the δ-opioid receptor is able to interact with $G\alpha_{16}$ more efficiently than μ-opioid receptor (Lee et al., 1998), a 2.4-fold increase inositol phosphate response was observed in cells coexpressing $G\alpha_{16}$. It should be noted that although the fold stimulations observed between $G\alpha_{16}$ and the chimeras were similar, the actual magnitudes of basal as well as agonist-induced responses were much higher with the chimeras. Another receptor tested was the formyl peptide receptor, where its coexpression with 16z44, 16i44 or 16o44 resulted in agonist-induced inositol phosphate productions that ranged from 2.7- to 6.6-fold increases (Fig. 2). A sixfold increase of inositol phosphate stimulation was obtained in formyl-Met-Leu-Phe (fMLP)-treated COS-7 cells coexpressing the formyl peptide receptor and $G\alpha_{16}$, the strongest $G\alpha_{16}$-mediated response among the different G_i-linked

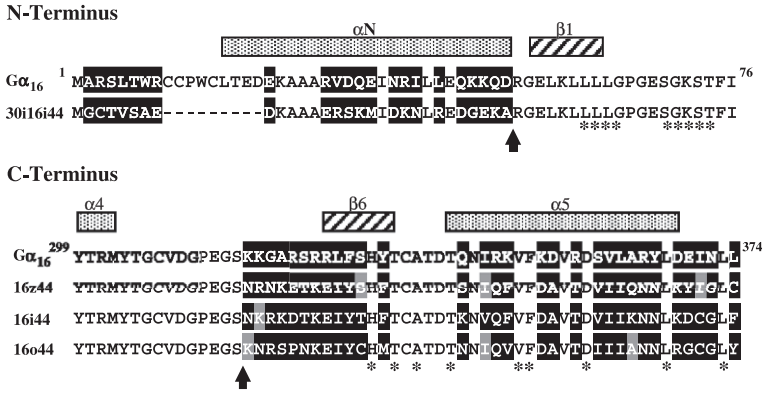

Fig. 1. Alignment of amino acid sequences of $G\alpha_{16}$ and the chimeras. Sequences were aligned using the Clustal X program and gaps represented by dashed lines were introduced for better alignment of the sequences. Arrows underneath the sequences mark the junction points between $G\alpha_{16}$ and $G\alpha_z$, $G\alpha_{i2}$ or $G\alpha_{oA}$ sequences. Shaded areas in black represent nonidentical sequences of the chimeras to $G\alpha_{16}$, while strictly conserved amino acids among G protein families are marked with asterisks. Shaded areas in gray represent identical amino acids between $G\alpha_{16}$ and one of the three members of the $G\alpha_i$ subfamily. Putative secondary structures are based on the $G\alpha_{t1}$ crystal structures and are represented as shaded horizontal bars above the sequences. Numbers shown are the relative positions of the amino acids of $G\alpha_{16}$.

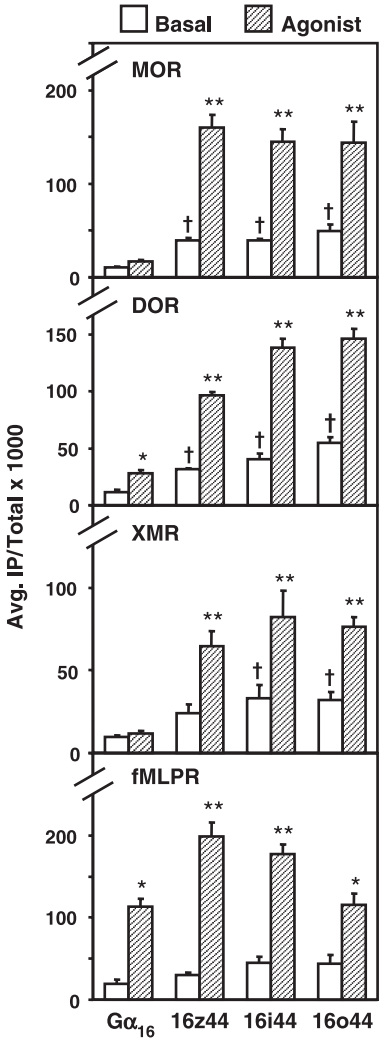

Fig. 2. Coupling of μ-opioid receptor (MOR), δ-opioid receptor (DOR), melatonin MT_{1c} receptor (XMR) and formyl peptide receptor (fMLPR) to $Gα_{16}$, 16z44, 16i44 and 16o44 chimeras. COS-7 cells were cotransfected with cDNAs (0.25 μg/ml) encoding each receptor with one of the G proteins. Transfected cells were labeled with *myo*-[^{3}H]inositol and assayed for inositol phosphate (IP) formation in the absence (basal) or presence of the appropriate ligands: 300 nM DAMGO, 300 nM DPDPE, 1 μM 2-iodomelatonin and 200 nM fMLP. *Agonist-induced inositol phosphates response was significantly higher than the corresponding basal value; **agonist-induced inositol phosphates response was significantly higher than the corresponding agonist-induced response of $Gα_{16}$ expressing cells; †basal inositol phosphates response by chimeras was significantly higher than basal inositol phosphates formation by $Gα_{16}$; Bonferroni *t* test, $P<0.05$.

receptors tested. These results are in general agreement with our previous findings where 16z44 exhibited enhanced capability to interact with a panel of G_i-coupled receptors (Mody et al., 2000; Liu et al., 2003). With the exception of 16o44-mediated formyl peptide receptor responses, inositol phosphate stimulation produced by the chimeras were significantly higher than that produced by $Gα_{16}$. We have also tested the 16i44 and 16o44 chimeras against several G_s-linked receptors. Both chimeras exhibited efficient coupling to the dopamine D1, dopamine D5 and vasopressin V2 receptors (data not shown). Overall, 16i44 and 16o44 were more efficient than $Gα_{16}$ in coupling to G_i-linked receptors, while they were coupled to G_s-linked receptors with similar proficiency as $Gα_{16}$.

Next we examined if the more robust responses associated with the chimeras were due to higher expression levels of the chimeras as compared to $Gα_{16}$. We used Western blotting to check the level of protein expression in COS-7 cells transfected with $Gα_{16}$, 16i44, 16o44 or 16z44. An antiserum that recognizes the N-terminus of $Gα_{16}$ (residues 13–27) was used for the immunodetection of the chimeras. As shown in Fig. 3A, all three chimeras were expressed at levels similar to, or slightly less than, that of $Gα_{16}$. These results suggest that the improved agonist-induced responses mediated by the 16i44 and 16o44 chimeras were not due to exceptionally high levels of protein expression relative to $Gα_{16}$.

Our initial assessment of the 16i44 and 16o44 chimeras suggested that they are as effective as the previously characterized 16z44 chimera in interacting with G-protein-coupled receptors that are incapable of recognizing $Gα_{16}$. To further compare 16i44 and 16o44 against 16z44, we examined the efficiency of coupling between these three chimeras and two selected G_i-linked receptors. The μ-opioid receptor and melatonin MT_{1c} receptor were chosen because of their weak coupling to $Gα_{16}$. Each receptor was coexpressed with either $Gα_{16}$ or one of the three chimeras in COS-7 cells and assayed for inositol phosphate accumulation in response to increasing concentrations of the corresponding agonist. Activation of the μ-opioid receptor by DAMGO did not stimulate PLCβ activity in cells coexpressing the receptor and $Gα_{16}$ (Fig. 4). In contrast, DAMGO dose-dependently stimulated the formation of inositol phosphates with EC_{50} values of 1.5, 1.3 and 1.1 nM in the presence of 16z44,

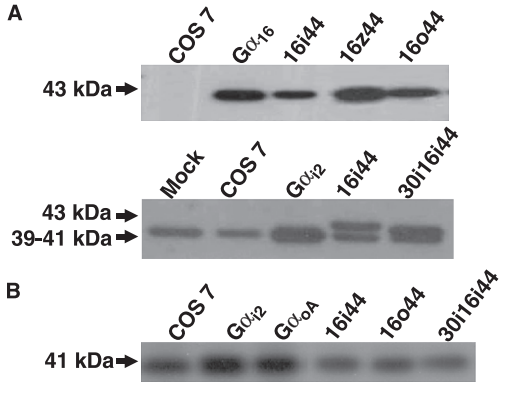

Fig. 3. Immunodetection and [^{32}P]ADP-ribosylation of chimeric Gα subunits. COS-7 cells were transfected with cDNAs encoding either wild-type or chimeric Gα subunits as indicated. (A) Membrane proteins (30 μg) prepared from untransfected COS-7 cells, mock transfected, or the transfectants were probed with anti-$Gα_{16}$ (upper panel) or anti-$Gα_{i2}$ (lower panel) antisera. (B) Selected membrane preparations were subjected to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation and the labeled substrates were resolved on a 12.5% SDS-PAGE gel and visualized by autoradiography.

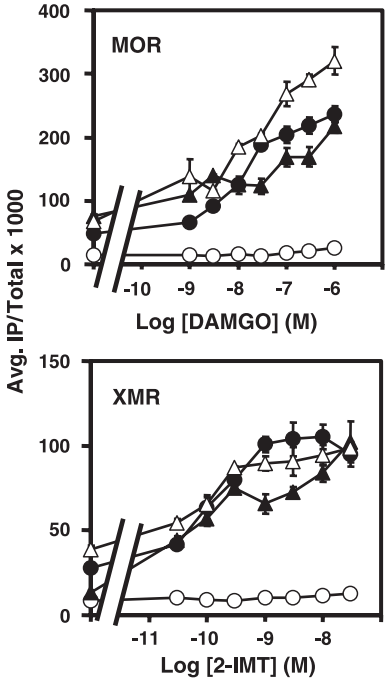

Fig. 4. Dose-dependent agonist stimulation of phospholipase Cβ by the μ-opioid receptor (MOR) and melatonin MT_{1c} receptor (XMR) via $G\alpha_{16}$, 16z44, 16i44 or 16o44. COS-7 cells were transiently transfected with 0.25 μg/ml of cDNAs encoding one of the two selected receptors and a Gα subunit: $G\alpha_{16}$ (○), 16z44 (●), 16o44 (△), or 16i44 (▲). Transfected cells were assayed for inositol phosphate (IP) formation in the absence or presence of increasing concentrations of DAMGO (1 nM to 1 μM) or 2-iodomelatonin (30 pM to 100 nM). Data shown are means ± S.D. of triplicate determinations in a single experiment; two separate experiments yielded similar results.

16i44 and 16o44, respectively. Likewise, melatonin MT_{1c} receptor coupled weakly to $G\alpha_{16}$ (Fig. 4) but it efficiently stimulated the phospholipase Cβ activity in the presence of 16z44, 16i44 and 16o44 with EC_{50} values of 100, 50 and 90 pM, respectively. Thus, both 16i44 and 16o44 appeared to be as efficient as 16z44 in mediating agonist-induced stimulation of phospholipase Cβ. Similar findings were observed with 16i44 and 16o44 in agonist-induced Ca^{2+} mobilization assays using the FLIPR®I. DAMGO dose-dependently elevated intracellular Ca^{2+} signals with EC_{50} values of 117, 2.3, 1.7 and 1.7 nM in the presence of $G\alpha_{16}$, 16z44, 16i44 and 16o44, respectively (Fig. 5). Likewise, the melatonin MT_{1c} receptor had little or no coupling with $G\alpha_{16}$ but efficient Ca^{2+} responses were observed with 16z44, 16i44 and 16o44 with EC_{50} values of 0.9, 0.4 and 0.4 nM, respectively (Fig. 5).

3.3. Coupling of 30i16i44 to G_i-linked receptors

There is as yet no consensus on the requirement of the N-terminus of the Gα subunit for receptor recognition. Although the N-terminus of $G\alpha_{16}$ does not appear to be essential for receptor recognition (Lee et al., 1995), the N-termini of $G\alpha_z$ (Ho and Wong, 2000) and $G\alpha_q$ (Kostenis et al., 1997) are critical for their association with receptors. To assess whether the N-terminus of $G\alpha_{i2}$ facilitates interactions with receptors, we constructed the 30i16i44 chimera where the putative αN helix of 16i44 was replaced by that of $G\alpha_{i2}$. COS-7 cells were cotransfected with cDNAs encoding one of three G-protein-coupled receptors (μ-opioid, δ-opioid or fMLPR; at 0.25 μg/ml per construct) and 30i16i44, and then assayed for inositol phosphate formation in the absence or presence of appropriate agonists. Expression of 30i16i44 was confirmed by immunodetection with an anti-$G\alpha_{i2}$ antiserum (Fig. 3A). Since the αN helix of $G\alpha_{i2}$ is shorter than that of $G\alpha_{16}$ by nine amino acids (Fig. 1), the deduced molecular weight of 30i16i44 is slightly smaller than that of 16i44. Accordingly, 16i44 was detected as a distinct immunoreactive band whereas the immunoreactivity of 30i16i44 was in between 16i44 and the endogenous $G\alpha_{i2}$ (Fig. 3A). The immunoreactivity of 30i16i44-expressing cells was comparable to that of cells transfected with 16i44 or $G\alpha_{i2}$, and was higher than that of the control cells (untransfected or mock transfected cells; Fig. 3A). In COS-7 cells coexpressing the μ-opioid receptor with 30i16i44, basal inositol phosphate production was significantly higher than that of the $G\alpha_{16}$-expressing cells (Fig. 6). In contrast to 16i44, the 30i16i44 chimera did not significantly enhance μ-opioid

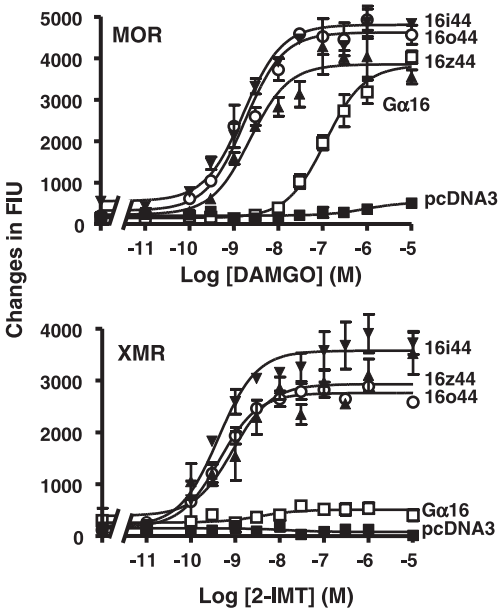

Fig. 5. Dose-dependent agonist stimulation of Ca^{2+} mobilization by the μ-opioid receptor (MOR) and melatonin MT_{1c} receptor (XMR) via $G\alpha_{16}$, 16z44, 16i44 or 16o44. COS-7 cells were transiently transfected with the indicated cDNAs (0.20 μg/ml) using Lipofactamine™ 2000. pcDNA 3 (■) or $G\alpha_{16}$ (□) or 16z44 (▲) or 16i44 (▼) or 16o44 (○). Transfected cells were assayed in a FLIPR®I and calcium mobilizations were recorded as changes in fluorescence intensity unit (FIU) unit in the absence or presence of increasing concentrations of DAMGO or 2-iodomelatonin (1 pM to 10 μM). Data shown are means ± S.D. of triplicate determinations in a single experiment; two separate experiments yielded similar results.

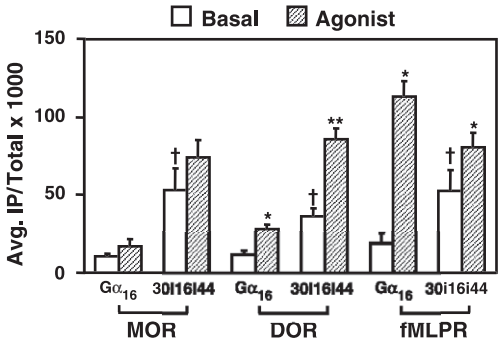

Fig. 6. Coupling of μ-opioid receptor (MOR), δ-opioid receptor (DOR) and formyl peptide receptor (fMLPR) to 30i16i44. COS-7 cells were transfected with cDNAs (0.25 μg/ml) encoding the appropriate receptors and 30i16i44 which were further assayed for inositol phosphate (IP) accumulation in the absence (basal) or presence of the appropriate ligands: 300 nM DMAGO, 300 nM DPDPE and 200 nM fMLP. *Agonist-induced inositol phosphate response was significantly higher than the corresponding basal value; **agonist-induced inositol phosphate response was significantly higher than the corresponding basal value as well as the agonist-induced response by $G\alpha_{16}$; †basal inositol phosphate response by chimeras was significantly higher than basal inositol phosphate formation by $G\alpha_{16}$; Bonferroni *t* test, $P<0.05$.

receptor mediated stimulation of inositol phosphate production. When coexpressed with 30i16i44, activation of the δ-opioid receptor by DPDPE significantly stimulated the production of inositol phosphates to a level higher than that observed in cells coexpressing $G\alpha_{16}$ (Fig. 6) but lower than those obtained with 16i44 (Fig. 2). Although 30i16i44 generated significant production of inositol phosphates upon agonist-induced activation of the formyl peptide receptor, the response was no better than that of $G\alpha_{16}$ (Fig. 6). In cells expressing either the δ-opioid receptor or the formyl peptide receptor, the presence of 30i16i44 appeared to elevate basal inositol phosphate levels. Overall, 30i16i44 was able to link G_i-coupled receptors to phospholipase Cβ activation, albeit less efficient than $G\alpha_{16}$ or 16i44.

3.4. 16i44 and 16o44 chimeras were resistant to pertussis toxin-catalyzed ADP-ribosylation

Although the 16i44 and 16o44 chimeras possess the C-terminal cysteine residue that acts as the site for pertussis toxin-catalyzed ADP-ribosylation, they do not necessarily serve as substrates for the toxin. Previous studies with $G\alpha_{s/i}$ chimeras (Osawa et al., 1990) as well as the qi5 and qo5 chimeras (Joshi et al., 1998) indicate that the C-terminus ADP-ribose acceptor site alone is not sufficient for pertussis toxin recognition. We therefore investigated the effects of pertussis toxin treatment on the signaling properties of 16i44 and 16o44. COS-7 cells coexpressing $G\alpha_{16}$, 16z44, 16i44 or 16o44 together with a G_i-linked receptor were treated with or without pertussis toxin (100 ng/ml, 16 h) prior to assaying for agonist-induced inositol phosphate production. As shown in Fig. 7, pertussis toxin produced small but insignificant inhibitions in most of the combinations of receptors and G proteins tested. In cells coexpressing the μ-opioid receptor and either $G\alpha_{16}$ or 16i44, treatment with pertussis toxin significantly suppressed the agonist-induced phospholipase Cβ responses (Fig. 7). Pertussis toxin also inhibited agonist-induced phospholipase Cβ activity in cells coexpressing the δ-opioid receptor and $G\alpha_{16}$, 16i44 or 30i16i44 (Fig. 7). In contrast, only the 16i44-mediated phospholipase Cβ response was attenuated by pertussis toxin in cells coexpressing the formyl peptide receptor (Fig. 7). Although the 16i44-mediated activation of phospholipase Cβ was inhibited by pertussis toxin for all three receptors tested, the inhibition was incomplete. Given that these receptors could not utilize endogenous G proteins to activate phospholipase Cβ (Mody et al., 2000), total blockade should be observed if 16i44 served as a substrate of pertussis toxin. This raised the possibility that the chimeras could not be ADP-ribosylated by pertussis toxin.

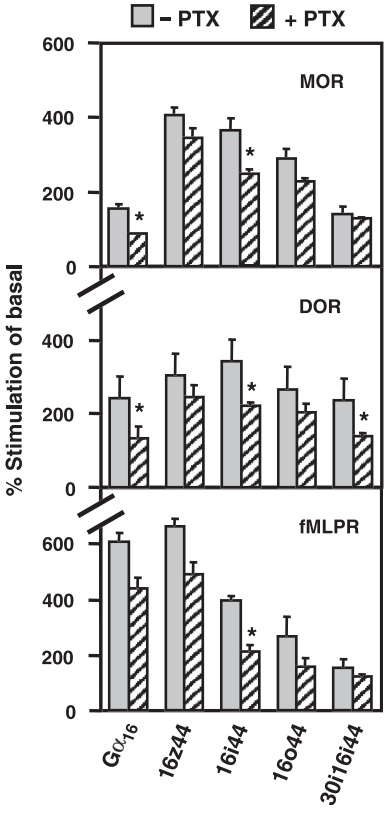

Fig. 7. Effects of pertussis toxin on chimera-mediated activations of phospholipase Cβ. COS-7 cells were transfected with μ-opioid receptor (MOR), δ-opioid receptor (DOR), or formyl peptide receptor (fMLPR) and $G\alpha_{16}$ or one of the indicated chimeras (0.25 μg/ml of cDNA). Transfectants were treated with or without pertussis toxin (PTX, 100 ng/ml, 16 h) as indicated. Inositol phosphate (IP) production was assayed with or without 300 nM DAMGO, 300 nM DPDPE or 200 nM fMLP. Results were expressed as percentage stimulation of basal inositol phosphate formation. *Agonist-induced responses were significantly inhibited by pertussis toxin; Bonferroni *t* test, $P<0.05$.

To verify whether the chimeras were indeed substrates of pertussis toxin, plasma membrane preparations containing $G\alpha_{16}$, 16i44, 16o44 and 30i16i44 (as confirmed by immunodection) were subjected to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. Since the $G\alpha_{16}$-based constructs have a deduced molecular weight of approximately 43 kDa, they could be distinguished from the endogenous $G\alpha_{i/o}$ (39/41 kDa) proteins. As shown in Fig. 3B, only endogenous pertussis toxin substrates were [^{32}P]ADP-ribosylated. The extent of [^{32}P] labeled substrate was highest in cells overexpressing $G\alpha_{i2}$, whereas the intensities of [^{32}P]-labeled substrates in 16i44-, 16o44- and 30i16i44-expressing cells were similar to the control. These results indicated that 16i44, 16o44 and 30i16i44 could not be efficiently labeled by pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. We have also performed pertussis toxin-catalyzed [^{32}P]ADP-ribosylation on in vitro translated protein products. $G\alpha_{16}$, 16z44, 16i44, 16o44 and 30i16i44 were prepared by using the Promega Transcend™ tRNA system, and their expressions were verified on a 12.5% SDS-PAGE using the streptavidin peroxidase conjugate. None of the translated protein products served as substrates for pertussis toxin-catalyzed [^{32}P]ADP-ribosylation (data not shown). These studies suggested that 16i44, 16o44 and 30i16i44 were either resistant to pertussis toxin-catalyzed ADP-ribosylation or served as poor substrates of the toxin.

3.5. Involvement of Gβγ in chimera-mediated stimulation of phospholipase Cβ

If the $G\alpha_{16/i}$ and $G\alpha_{16/o}$ chimeras could not be ADP-ribosylated by pertussis toxin, then how could the toxin inhibit agonist-induced phospholipase Cβ responses as observed in Fig. 7? A plausible explanation lies with the Gβγ subunits. We have previously demonstrated that G_{16}-dependent stimulation of phospholipase Cβ by the δ-opioid receptor is partially mediated via the Gβγ subunits released upon the activation of G_i proteins (Chan et al., 2000). This component of the agonist-induced activation of phospholipase Cβ is thus sensitive to pertussis toxin treatment (Fig. 7). To test the involvement of Gβγ subunits in the 16i44-, 16o44- and 30i16i44-dependent stimulation of phospholipase Cβ, we used the Gα subunit of transducin ($G\alpha_{t1}$) as a Gβγ scavenger to block the agonist-induced response. In line with our previous observations (Chan et al., 2000), the presence of $G\alpha_{t1}$ significantly inhibited the ability of DPDPE to stimulate phospholipase Cβ in cells coexpressing the δ-opioid receptor and $G\alpha_{16}$ (Fig. 8). Similar results were obtained with the 16z44-, 16i44- and 16o44-mediated activation of phospholipase Cβ by DPDPE (Fig. 8). In contrast, the DPDPE-induced stimulation of phospholipase Cβ in cells coexpressing the δ-opioid receptor and 30i16i44 was unaffected by the presence of $G\alpha_{t1}$. Although the effect of $G\alpha_{t1}$ varied between different $G\alpha_{16}$ chimeras, it indicated the involvement of Gβγ subunits in phospholipase Cβ activation.

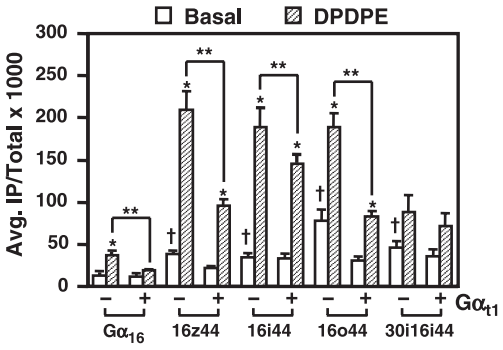

Fig. 8. DPDPE-induced stimulation of phospholipase Cβ by δ-opioid receptor via $G\alpha_{16}$, 16z44, 16i44, 16o44 and 30i16i44 can be partially inhibited by a Gβγ-scavenger, $G\alpha_{t1}$. COS-7 cells were cotransfected with the δ-opioid receptor and $G\alpha_{16}$ or one of the chimeras with or without $G\alpha_{t1}$ (all at 0.25 μg/ml of cDNA). Cells were then assayed for inositol phosphate production in the absence or presence of 300 nM DPDPE. *DPDPE significantly induced inositol phosphate (IP) accumulation over basal values; **$G\alpha_{t1}$ significantly reduced the DPDPE-induced response; †basal inositol phosphate response by chimeras was significantly higher than basal inositol phosphate formation by $G\alpha_{16}$; Bonferroni *t* test, $P<0.05$.

Similar results were obtained with the μ-opioid receptor (unpublished data).

4. Discussion

The importance of the two extreme termini of the Gα subunit in receptor recognition is well supported by a host of experimental data amassed from biochemical, immunological, pharmacological, and crystallographic studies (Bourne, 1997). In particular, regions close to the extreme C-terminal end of the Gα subunit appear to define the fidelity of its association with receptors (Conklin et al., 1993, 1996). We have previously shown that the promiscuity of $G\alpha_{16}$ can be enhanced by replacing its C-terminal β6/α5 region with $G\alpha_z$-specific sequences (Mody et al., 2000; Liu et al., 2003). The present study further demonstrates that such enhancement was not unique to $G\alpha_z$, because cognate regions from $G\alpha_{i2}$ and $G\alpha_{oA}$ could also confer increased promiscuity to $G\alpha_{16}$. $G\alpha_{16}$ chimeras harboring the last 44 residues of $G\alpha_z$, $G\alpha_{i2}$ or $G\alpha_{oA}$ were more efficient than parental $G\alpha_{16}$ in linking receptor activation to the stimulation of phospholipase Cβ. The N-terminal αN helix of $G\alpha_{i2}$, on the other hand, did not enhance the promiscuity of $G\alpha_{16}$.

The α5 helix is a known receptor contact site where $G\alpha_z$, $G\alpha_{i2}$ and $G\alpha_{oA}$ show 64% sequence identity (Fig. 1). The α5 helix of $G\alpha_{16}$ shows at most 40% sequence identity to members of the G_i family. The ability of 16i44 and 16o44 to enhance the recognition of G_i-coupled receptors in much the same way as the previously characterized 16z44 is therefore not surprising. Like 16z44, 16i44 and 16o44 efficiently linked G_i-coupled receptors to the stimulation of PLCβ (as well as calcium mobilization) and were also capable of coupling to G_s-linked receptors, confirming the importance of the swapped regions (β6–α5 loop and α5 helix) of $G\alpha_{16}$

in determining the specificity of receptor coupling. The C-terminal regions of $G\alpha_z$, $G\alpha_{i2}$ and $G\alpha_{oA}$ appear to be interchangeable with respect to their receptor coupling, as indicated by this as well as previous studies (Tsu et al., 1997). The presence of a more "G_i/G_o-like" tail enhanced the coupling behavior of $G\alpha_{16}$, an already promiscuous G protein, by allowing it to efficiently interact with G_i-linked receptors that are otherwise poorly recognized by $G\alpha_{16}$ (e.g. μ-opioid and melatonin MT_{1c} receptor). Alignment of the C-terminal sequences of the different Gα subunits reveal that only 9 out of the last 44 residues are conserved. Among members of the $G\alpha_i$ subfamily, sequence identity in this region increases to 25 residues. Members within the $G\alpha_s$ and $G\alpha_{12}$ subfamilies also exhibit high degree of homology in this region, suggesting that the β6−α5 loop and the α5 helix might indeed determine the specificity of receptor interaction. If so, $G\alpha_{16}$ could be engineered to better recognize G_s- and G_{12}-coupled receptors and thus facilitate attempts to deorphanize G-protein-coupled receptors.

Structurally, the close proximity of the two termini suggests that the αN helix might also play a role in receptor recognition. The N-termini of $G\alpha_q$ and $G\alpha_z$ have indeed been shown to participate in receptor recognition (Kostenis et al., 1997; Ho and Wong, 2000). However, incorporation of the αN helix into the 16i44 backbone failed to offer enhanced promiscuity to the resultant 30i16i44 chimera, with diminished ability to interact with the δ-opioid receptor. The involvement of the αN helix in receptor recognition remains controversial and difficult to elucidate, especially since this domain is subjected to fatty acylation and serve as a contact site for the Gβγ subunits (Wall et al., 1995).

One of the interesting observations pertaining to the present study is the ineffectiveness of pertussis toxin to completely suppress the 16i44- and 16o44-mediated responses. The incorporation of the C-terminal cysteine at the −4 position was supposed to render both 16i44 and 16o44 chimeras sensitive to pertussis toxin-catalyzed ADP-ribosylation. Since the G_i-coupled receptors are incapable of stimulating phopholipase Cβ in COS-7 cells except when coexpressed together with $G\alpha_{16}$ or the chimeras, agonist-induced inositol phosphate formation should be completely abolished by pertussis toxin if the 16i44 and 16o44 were inactivated by the toxin. Yet, pertussis toxin only partially suppressed the 16i44-mediated responses, with negligible inhibitory effects on the 16o44-mediated responses. Differences in the pertussis toxin-sensitivities of 16i44 and 16o44 might be explained in terms of the intrinsic properties of G_i and G_o as substrates for pertussis toxin-catalysed ADP-ribosylations. Purified G_o is less sensitive to ADP-ribosylation by pertussis toxin than G_i (Neer et al., 1984). Both 16i44 and 16o44 failed to be [^{32}P]ADP-ribosylated by pertussis toxin (Fig. 3B) despite the presence of the three penultimate amino acid residues, Cys-Gly-Leu, in these chimeras. It should be noted that other factors may also affect the petussis toxin-catalyzed ADP-ribosylation reaction. For instance, the association of Gβγ dimers to the Gα subunits can increase the V_{max} of this reaction (Tsai et al., 1984; Graf et al., 1992). Because of its close proximity to the C-terminus, the primary structure of the N-terminus of Gα might also be important for recognition by pertussis toxin (Osawa et al., 1990). $G\alpha_i$ chimeras containing the N-terminus of $G\alpha_s$ (Freissmuth and Gilman, 1989; Hingorani and Ho, 1988) or $G\alpha_q$ (Joshi et al., 1998) are resistant to pertussis toxin inactivation, whereas those containing the N-terminus of $G\alpha_z$ (Ho and Wong, 2000) are sensitive to pertussis toxin treatment. These studies indicate that the C-terminal ADP-ribose acceptor site alone is not sufficient for pertussis toxin recognition and the N-terminus of the Gα subunit might play a role in providing additional contact sites for pertussis toxin. However, the presence of the αN helix of $G\alpha_{i2}$ at the N-terminus of 30i16i44 did not create an efficient substrate for pertussis toxin. The failure of 30i16i44 to serve as a substrate for pertussis toxin suggests that other structural motifs might be required for docking to pertussis toxin, or that the $G\alpha_{16}$ backbone failed to orientate the two termini into a conformation recognizable by pertussis toxin.

It may be argued that if the chimeras could not be [^{32}P]ADP-ribosylated by pertussis toxin, then agonist-induced phospholipase Cβ activities should not be affected by the pertussis toxin treatment. Yet, significant inhibitions were observed for $G\alpha_{16}$- and 16i44-mediated inositol phosphate productions in pertussis toxin-treated cells (Fig. 7). The observed inhibitions by pertussis toxin can be attributed to the involvement of Gβγ in the activation of phospholipase Cβ. Many G_i-linked receptors are incapable of activating phospholipase Cβ through the $G_{i/o}$ proteins but can do so when a G_q-mediated pathway is simultaneously activated (Selbie and Hill, 1998). This augmentation is believed to be mediated by Gβγ subunits released upon dissociation from the Gα subunit. When phospholipase Cβ is activated by a member from the $G\alpha_q$ family (such as $G\alpha_{16}$), it becomes responsive to stimulation by the Gβγ subunits released by endogenous $G_{i/o}$ (Chan et al., 2000). The use of $G\alpha_{t1}$ as a Gβγ scavenger in the present study confirms the participation of Gβγ subunits in agonist-induced stimulation of phospholipase Cβ. Agonist-induced activation of phospholipase Cβ was impaired when the Gβγ-scavenger $G\alpha_{t1}$ was cotransfected with chimeric Gα subunits. Suppressions exerted by $G\alpha_{t1}$ were most obvious in cells expressing 16z44, 16i44 and 16o44, but was not significant in those expressing 30i16i44. The suppressive effect of $G\alpha_{t1}$ is unlikely to be due to competition of expression because it has been demonstrated that coexpression of $G\alpha_{t1}$ does not affect the expression of $G\alpha_{16}$ (Chan et al., 2000). The extent to which this reflects the participation of Gβγ released from G_i is unclear because Gβγ from the chimeras might also contribute in the activation process. This might explain why the inhibitory profiles of pertussis toxin and $G\alpha_{t1}$ are not identical.

Taken together, the present study demonstrates that the β6−α5 loop and α5 helix at the C-terminus, but appar-

ently not the αN helix at the N-terminus, contribute towards defining the specificity of receptor recognition. Further studies are needed to better define the relative contributions of individual residues in forming the receptor contact surface. The inability of pertussis toxin to ADP-ribosylate the $G\alpha_{16/i/o}$ chimeras is consistent with similar studies on $G\alpha_{s/i}$ (Osawa et al., 1990) and $G\alpha_{q/i}$ (Joshi et al., 1998) chimeras. Structural motifs in the Gα subunit other than the two termini must participate in the binding of pertussis toxin. Given that the pertussis toxin binding regions on $G\alpha_{i/o}$ may overlap with those required for interaction with receptors, it is reasonable to assume that the receptor contact sites involve more than just the C-terminus of the Gα subunit. Upon receptor activation, the chimeras appear to both directly and indirectly stimulate phospholipase Cβ. One of the mechanisms might involve the Gβγ complex. Presumably, the chimera-mediated stimulation of phospholipase Cβ is augmented by endogenous pertussis toxin-sensitive $G_{i/o}$ proteins that release Gβγ subunits to further activate phospholipase Cβ. Irrespective of the mechanism of action, $G\alpha_{16}$ chimeras such as the 16z44 are invaluable tools for delineating G-protein-coupled receptors signaling (Nelson et al., 2002) and for drug discovery (Liu et al., 2003). The 16i44 and 16o44 chimeras may well expand this collection of molecular tools.

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