Chimeric $G\alpha_q$ mutants harboring the last five carboxy-terminal residues of $G\alpha_{i2}$ or $G\alpha_o$ are resistant to pertussis toxin-catalyzed ADP-ribosylation

Sushma A. Joshi[1], Kenneth P.K. Fan[2], Vanessa W.S. Ho[3], Yung H. Wong*

Department of Biology and the Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

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Abstract Three widely-used $G\alpha_q$ chimeras harboring the last five residues of $G\alpha_i$, $G\alpha_o$ and $G\alpha_z$ (qi5, qo5 and qz5) were examined for their ability to serve as substrates for pertussis toxin (PTX)-catalyzed ADP-ribosylation. In COS-7 cells coexpressing one of the three opioid receptors (μ, δ, and κ) and a $G\alpha_q$ chimera, agonist-induced stimulation of phosphoinositide-specific phospholipase C (PI-PLC) was largely insensitive to PTX treatment. Only the qi5-mediated stimulation of PI-PLC by κ-opioids was partially inhibited by PTX. In βγ-release assays, PTX treatment did not affect the ability of opioid receptors to activate these chimeras. [^{32}P]ADP-ribosylation labeled $G\alpha_{i/o}$ but not qi5 or qo5, although the expression of these chimeras was confirmed by immunodetection. Thus, $G\alpha_q$ chimeras with a $G\alpha_{i/o}$-like tail are insensitive to PTX treatment.

Key words: Chimera; G protein; Opioid receptor; Pertussis toxin; Signal transduction

1. Introduction

Heterotrimeric (αβγ) G proteins transduce extracellular signals detected by specific cell-surface receptors into intracellular messages. A large variety of effector molecules are regulated by 20 distinct G proteins which are classified into four subfamilies (G_i, G_s, G_q and G_{12}) based on the homologies of their α subunits [1]. Many receptors for neurotransmitters, hormones, neuropeptides, and photons utilize members of the G_i subfamily for signal transduction. Seven of the eight members in the G_i subfamily (except G_z) are substrates of pertussis toxin (PTX).

PTX is derived from *Bordetella pertussis*. It is a heterohexameric protein divided into A (Active) and B (Binding) subunits. The A subunit possesses ADP-ribosyltransferase activity and the B subunit, which is a pentamer, confers binding specificity to the enzyme. The heterohexamer catalyzes the ADP-ribosylation of Gα subunits at a cysteine residue four amino acids from the carboxy-terminus, using NAD as the donor [2]. This covalent modification uncouples the G proteins from the activated receptors. The specificity of PTX-catalyzed ADP-ribosylation has proven extremely useful in delineating signal transduction pathways in which the role of a G protein is suspected [3], but this reaction depends on a number of factors. The V_{max}, but not the K_m of this reaction is dependent on the association of βγ dimers to the Gα subunits [4,5]. Mutational analysis at the C-terminus of $G\alpha_o$ points to the importance of the three penultimate amino acid residues, Cys-Gly-Leu, in PTX-catalyzed ADP-ribosylation [6]. However, studies with $G\alpha_{s/i}$ chimeras indicate that the C-terminus ADP acceptor site alone is not sufficient for PTX recognition [7,8]. The primary structure of the N-terminus of Gα, which is in close proximity to the C-terminus [9], is also important in PTX recognition [7].

To date, although the mechanism by which PTX recognizes Gα is not fully understood, PTX treatment has become a widely used experimental strategy to characterize Gα subunits. PTX-sensitive and -insensitive Gα subunits are often differentiated solely on the basis of the presence of a cysteine residue near the C-terminus [6]. Recent attempts to determine the specificity and fidelity of receptor-G protein coupling have relied heavily on the construction of mutant as well as chimeric G proteins. Because of their wide spectrum of receptor linkage, members of the G_i subfamily are often selected as donors in the design of chimeric G proteins. A series of chimeras constructed between $G\alpha_q$ and $G\alpha_i$, $G\alpha_o$, or $G\alpha_z$ [10] have proven to be extremely useful in delineating the specificity of receptor-G protein coupling [11]. Some of these $G\alpha_q$ chimeras contain the PTX-catalyzed ADP-ribosylation site. Due to their widespread use in different receptor systems [12–14], it has become important to confirm if these $G\alpha_q$ chimeras are indeed PTX-sensitive. In the present study, we provide biochemical evidence that two such chimeras, qi5 and qo5, are resistant to modification by PTX. Our results show that Gα chimeras containing a $G\alpha_{i/o}$ like C-terminus cannot be assumed to be PTX-sensitive.

2. Materials and methods

2.1. Materials

The cDNAs encoding the rat μ-opioid, mouse δ- and κ-opioid receptors were generous gifts from L. Yu (University of Cincinnati College of Medicine), C. Evans (UCLA), and G. Bell (University of Chicago, IL, USA), respectively. The $G\alpha_q$ chimeras (qi5, qo5, and qz5) were kindly donated by H.R. Bourne and B. Conklin (UCSF). Type II adenylyl cyclase (AC II) cDNA was obtained from R. Reed (Johns Hopkins University, Baltimore, MD, USA). PTX was pur-

*Corresponding author. Fax: (852) 2358 1559.
E-mail: boyung@ust.hk

[1]Center for Biotechnology, Northwestern University, 1801 Maple Avenue, Evanston, IL 60201, USA.

[2]Downing College, University of Cambridge, Cambridge, UK.

[3]New Hall, University of Cambridge, Cambridge, UK.

Abbreviations: AC II, type II adenylyl cyclase; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; IP, inositol phosphates; PTX, pertussis toxin; PI-PLC, phosphoinositide-specific phospholipase C

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chased from List Biological Laboratories (Campbell, CA, USA). The $G\alpha_q$-specific antiserum E-17 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [^{3}H]Adenine and [^{3}H]*myo*-inositol were purchased from Amersham International (Buckinghamshire, UK) and DuPont-NEN (Boston, MA, USA), respectively. Cell culture reagents were from Life Technologies (Grand Island, NY, USA) and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and cDNA transfections

COS-7 cells (ATCC CRL-1651) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 50 units/ml penicillin and 50 μg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded in 12-well plates at 1.2×10^5 cells per well 24 h before transfection. Cells were transfected with various cDNAs by the DEAE-dextran method as previously described [15]. Briefly, Qiagen purified cDNAs were added to growth media containing 100 nM chloroquine and 250 μg/ml DEAE-dextran. The cells were exposed to the transfection cocktail for 3.5 h then shocked with 10% DMSO (v/v) in phosphate buffered saline (PBS) for 1 min. The cells were washed with PBS before returning to growth media for 24 h. HEK 293 cells (ATCC CRL-1573) were cultured in Eagle's minimal essential medium (MEM) with 10% FCS. 2×10^5 cells were transfected for 1.5 h with 400 μg/ml of DEAE-dextran. Other conditions and procedures were the same as with COS-7 cells.

2.3. Inositol phosphates (IP) and cAMP assays

Transfected cells were labeled with [^{3}H]*myo*-inositol (2.5 μCi/ml) or [^{3}H]adenine (1 μCi/ml) in growth media, with or without PTX (100 ng/ml) for 18–24 h. Labeled cells were assayed for inositol phosphates (IP) or cAMP accumulation as described previously [15,16]. Absolute measurements of IP or cAMP accumulation varied between experiments, but variability within a given experiment was generally $<10\%$.

2.4. Membrane protein preparation and immunodetection of chimeric $G\alpha_q$ subunits

COS-7 cells were grown on 150-mm dishes to 70–80% confluence. Transfection was performed as in 12-well plates with proper adjustments to the volumes and amounts of the reagents used. Transfected cells were incubated with growth media at normal growth conditions for 48 h for the expression of exogenous proteins. Membranes were prepared from the transfected cells by one cycle of freeze-thawing followed by 10 passages through a 27-gauge needle [17]. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. For each sample, 75 μg of membrane proteins were separated on a 12.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Protein markers on the membrane were localized by Ponceau S staining. Immunodetection of $G\alpha_q$ chimeras by the $G\alpha_q$-specific antiserum E-17 (Santa Cruz Biotechnology) was visualized by chemiluminescence using the ECL kit from Amersham.

2.5. [^{32}P]ADP-ribosylation

Pertussis toxin-catalyzed ADP-ribosylation of membrane-bound proteins was performed as described previously [18]. PTX (50 μg/ml) was preactivated at 25°C for 1 h in 50 mM DTT. ADP-ribosylation was carried out in 0.1 ml (final volume) of 100 mM Tris-HCl (pH 7.5) containing 10 mM thymidine, 1 mM EDTA, 1 mM L-α-dimyristol phosphatidylcholine, 2.5 mM $MgCl_2$, 1 mM ATP, 50–75 μg protein of membranes, 25 μM [α-^{32}P]NAD^+, and 5 μg of preactivated pertussis toxin (90 min, 25°C). The PTX substrates were identified by precipitating the proteins immediately after ADP-ribosylation by chloroform/methanol and subjected to 12.5% SDS-polyacrylamide gel electrophoresis and autoradiography. The gel was exposed to Fuji RX film at −70°C for 1–2 days.

3. Results and discussion

Chimeric $G\alpha_q$ subunits containing the last few residues of $G\alpha_i$ or $G\alpha_o$ [10] can link G_i-coupled receptors to the stimulation of PI-PLC, and thus provide alternative means to study ligand-receptor interactions [12] as well as determining the fidelity of receptor-G protein coupling [11,13,14]. We have previously demonstrated that the three opioid receptors (μ, δ and κ) exhibit differential abilities to stimulate $G\alpha_q$ chimeras (qi5, qo5 and qz5; with the last five residues altered) in heterologous expression systems [14]. Of the three $G\alpha_q$ chimeras, both qi5 and qo5 contain the PTX-catalyzed ADP-ribosylation site. Despite their widespread use, it has not been unequivocally established that qi5- and qo5-mediated responses can be abolished by PTX. If so, PTX-sensitivity might serve as a distinguishing feature between wild-type $G\alpha_{q/11}$ and these two $G\alpha_q$ chimeras. A previous study with dopamine D_2 receptor and qi4 (a chimera which is functionally similar to qi5) indicated that qi4-mediated stimulation of PI-PLC is sensitive to PTX whereas the qz5 response is insensitive [10]. We investigated whether the interplay between the opioid receptors and $G\alpha_q$ chimeras are similarly affected by PTX. COS-7 cells were cotransfected with cDNAs encoding the opioid receptors and the $G\alpha_q$ chimeras. Transfected cells were treated with or without PTX (100 ng/ml) and subsequently assayed for IP production in response to opioid agonists. Except for μ-receptor and qo5, each pair of opioid receptor and $G\alpha_q$ chimera allowed opioid agonists to stimulate PI-PLC in transfected COS-7 cells (Fig. 1). This is in accordance with our previous study [14]. The agonist-induced stimulation of PI-

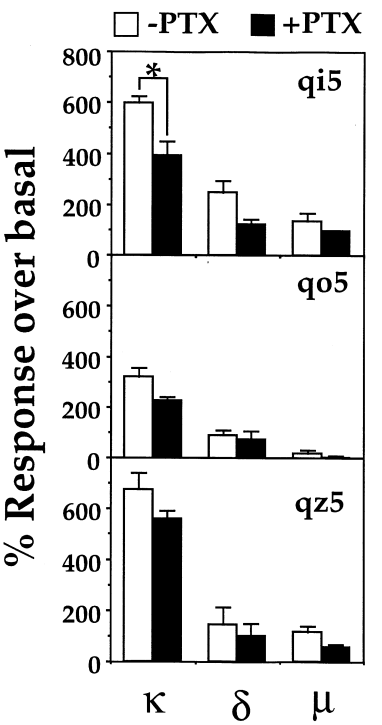

Fig. 1. Effect of PTX treatment on IP formation in cells coexpressing the opioid receptors and $G\alpha_q$ chimeras. COS-7 cells were transiently cotransfected with cDNAs (0.25 μg/ml), encoding an opioid receptor (κ, δ, or μ) and one of the three $G\alpha_q$ chimeras (qi5, qo5, or qz5) as indicated. Transfected cells were labeled with [^{3}H]inositol in the absence or presence of PTX (100 ng/ml) and subsequently assayed for responses to the corresponding opioid agonist (U-50488H, DPDPE, or DAGO) at 300 nM. KOR, κ-opioid receptor; DOR, δ-opioid receptor; MOR, μ-opioid receptor. Results are expressed as percent stimulation of IP production over basal response which ranged from 10.5 ± 1.4 to 13.2 ± 4.6. Data shown are mean ± S.D. of triplicate determinations from a typical experiment; two additional experiments gave similar results. *, PTX significantly reduced the ability of U-50488 to stimulate the formation of IP with the qi5 chimera; paired Bonferroni *t*-test, $P<0.05$.

PLC was only slightly reduced by PTX treatment in cells coexpressing every combination of $G\alpha_q$ chimeras and the μ- or δ-opioid receptor (Fig. 1). PTX partially inhibited (~30%) the PI-PLC response in cells coexpressing the κ-opioid receptor and qi5, but not when qi5 was replaced by either qo5 or qz5 (Fig. 1). Under similar experimental conditions, opioid-induced inhibition of adenylyl cyclase is completely abolished by PTX (data not shown but see [16,19,20]).

To confirm that the functions of qi5 and qo5 are insensitive to PTX treatment, we assessed the ability of these chimeras to release βγ subunits upon activation by agonist-bound opioid receptors. In the presence of activated $G\alpha_s$, G_i-coupled receptors are known to stimulate type II adenylyl cyclase (AC II) through the βγ subunits released from the PTX-sensitive $G\alpha_i$ [16,21]. Previous studies have demonstrated that the opioid receptors can stimulate AC II in transiently transfected HEK 293 cells via the endogenous G_i/G_o proteins [16,19,20]. To test if opioid receptors can stimulate AC II through $G\alpha_q$ chimeras, we cotransfected HEK 293 cells with cDNAs encoding AC II, $G\alpha_s$RC (a mutationally activated form of $G\alpha_s$), and the κ-opioid receptor in the absence and presence of $G\alpha_q$ chimeras. We switched to HEK 293 cells because this system has a better signal-to-noise ratio for cAMP assays. In control cells transfected with pcDNA1 instead of a $G\alpha_q$ chimera, U-50 488-stimulated cAMP accumulation was completely abolished by PTX treatment (Fig. 2). However, coexpression of qi5, qo5, or qz5 with the κ-opioid receptor rendered the agonist-induced cAMP accumulation insensitive to PTX (Fig. 2). Similar results were seen with the δ-opioid receptor (data not shown). The inability of PTX to prevent βγ release in these studies suggests that, like qz5, both qi5 and qo5 are insensitive to PTX treatment.

In order to verify the results obtained from the functional studies, we performed immunodetection of the $G\alpha_q$ chimeras and PTX-catalyzed [^{32}P]ADP-ribosylation on membranes prepared from transfected cells. COS-7 cells were cotransfected with cDNAs encoding the δ-opioid receptors and the $G\alpha_q$ chimeras, and the transfected cells were harvested two days later for the preparation of plasma membranes. Using a $G\alpha_q$-

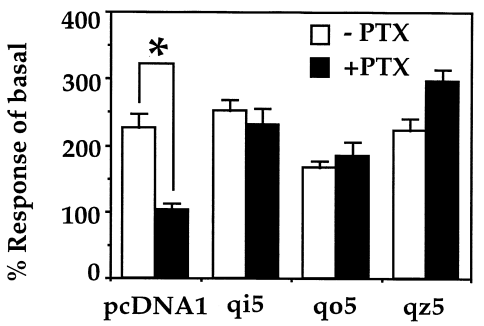

Fig. 2. U-50 488-induced stimulation of AC II by the $G\alpha_q$ chimeras. HEK 293 cells were cotransfected with AC II (0.25 μg/ml), κ-opioid receptor (0.25 μg/ml), α_sRC (0.025 μg/ml), and 0.25 μg/ml of the pcDNAI vector as a control or one of the $G\alpha_q$ chimeras (qi5, qo5, or qz5). Cells were labeled with [^{3}H]adenine, with or without PTX (100 ng/ml) and assayed for cAMP accumulation in response to 300 nM U-50 488. Results are expressed as percent stimulation of cAMP formation in the presence of U-50 488, compared with that measured in the absence of the agonist. The basal value varied between 1.3 ± 0.1 to 3.5 ± 0.1. Data represent triplicate determinations in a single experiment; two independent experiments gave similar results. *, PTX significantly reduced the ability of U-50 488 to activate AC II; paired Bonferroni *t*-test, $P < 0.05$.

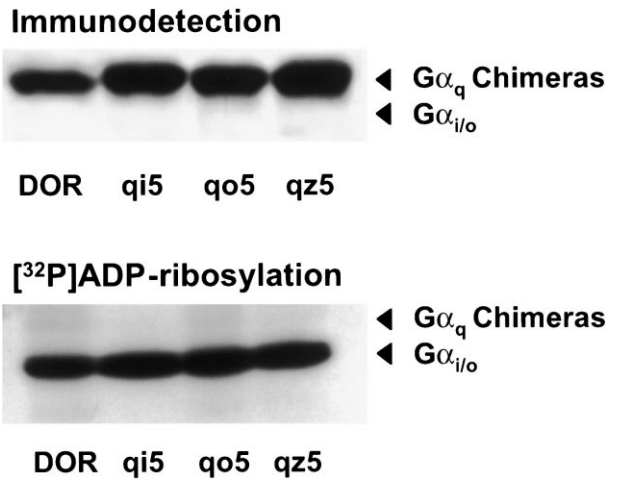

Fig. 3. [^{32}P]ADP-ribosylation and immunodetection. COS-7 cells were cotransfected with cDNA encoding the δ-opioid receptor without (DOR) or with one of the $G\alpha_q$ chimeras qi5, qo5 or qz5. Membranes were prepared from the transfected cells as described in Section 2. The G proteins in membranes from these COS-7 cells were separated by 12.5% SDS-polyacrylamide gel electrophoresis and detected by immunoblotting with a $G\alpha_q$-specific antiserum (upper panel). The membranes were also subjected to [^{32}P]ADP-ribosylation with PTX. [^{32}P]ADP-ribosylated substrates were resolved on a 12.5% SDS-polyacrylamide gel and visualized by autoradiography (lower panel). Arrowheads indicate the relative positions of $G\alpha_q$ chimeras and $G\alpha_{i/o}$ proteins.

specific antiserum, we confirmed that qi5, qo5 and qz5 were expressed to similar levels beyond endogenous $G\alpha_q$ in the transfected cells (Fig. 3). The same membranes were then subjected to PTX-catalyzed [^{32}P]ADP-ribosylation. Since the $G\alpha_q$ chimeras have a deduced molecular weight of 43 kDa, they should be easily differentiated from the endogenous PTX substrates, $G\alpha_{i/o}$ (39–41 kDa). As shown in Fig. 3, only $G\alpha_{i/o}$ subunits were [^{32}P]ADP-ribosylated in membranes prepared from control and $G\alpha_q$ chimera-expressing cells. Similar results were obtained with the κ-opioid receptor and, collectively, they indicate that qi5 and qo5 are poor substrates for PTX-catalyzed ADP-ribosylation.

Since qz5 lacks the PTX target residue, it is not surprising that the toxin fails to block agonist-induced activation of PI-PLC in cells coexpressing qz5. However, the almost complete lack of PTX-sensitivity of qi5- and qo5-mediated PI-PLC stimulation is enticing. The cysteine and glycine residues at −4 and −3 positions of the carboxy-terminal are important for ADP-ribosylation by PTX [6]. As the qi5 and qo5 chimeras contain these residues, they should be ADP-ribosylated, whereas the absence of cysteine makes the qz5 chimera PTX-insensitive. Although it has previously been demonstrated that a $G\alpha_q$ chimera, the qi4, is sensitive to PTX [10], inhibition of the qi4-mediated response by the toxin was incomplete. In fact, the coupling of dopamine D_2 receptor to qi4 is only partially (~50%) inhibited by PTX [10]. Thus our results are in line with the previous findings. Moreover, studies with $G\alpha_{s/i}$ chimeras indicate that the C-terminus ADP acceptor site alone is not sufficient for PTX recognition [8,9]. Given that the PTX binding regions on $G\alpha_{i/o}$ may be similar, if not identical, to those required for interaction with receptors, it is reasonable to assume that the PTX contact sites involve more than just the last five residues of the Gα subunit. The N-terminus of the Gα subunit is known to interact with recep-

tors and βγ subunits [22]. It should also be noted that the κ-opioid induced stimulation of PI-PLC mediated via qi5 was partially sensitive to PTX. Slight differences in the PTX-sensitivities of qo5 and qi5 might be explained in terms of the intrinsic properties of G_i and G_o as substrates for PTX-catalyzed ADP-ribosylations. Purified G_i is more sensitive to ADP-ribosylation by PTX than G_o [23]. Taken together, the present study demonstrated that $G\alpha_q$ chimeras containing $G\alpha_{i/o}$-like C-terminus are poor substrates of PTX. The PTX-insensitivity of qi5 and qo5 can be used to distinguish their responses from endogenous G_i proteins, and thus further broadens their usage in characterizing G_i-coupled receptors.

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