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Role of Amino- and Carboxyl-Terminal Regions of $G_{\alpha z}$ in the Recognition of G_i-Coupled Receptors

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SUMMARY

Many G_i-coupled receptors are known to interact with the pertussis toxin (PTX)-insensitive G_z protein. Given that the α subunits of G_i and G_z share only 60% identity in their amino acid sequences, their receptor-interacting domains must be highly similar. By swapping the carboxyl termini of αi2 and αz with each other or with those of αt, α12, and α13, we examined the relative contributions of the carboxyl-end 36 amino acids of the α chains toward receptor recognition. Chimeric α chains lacking the site for PTX-catalyzed ADP-ribosylation were coexpressed with the type II adenylyl cyclase (AC II) and one of several G_i-coupled receptors (formyl peptide, dopamine D_2, and δ-opioid receptors) in human embryonic kidney 293 cells. The αi2/αz chimera was able to interact with both aminergic and peptidergic receptors, resulting in $\beta\gamma$-mediated stimulation of AC II in the presence of agonists and PTX. Functional and mutational analyses of αi2/αz revealed that this chimera can inhibit the endogenous ACs of 293 cells. Similarly, the αz/αi2 chimera seemed to retain the abilities to interact with receptors and inhibit cAMP accumulation. Fusion of the carboxyl-terminal 36 amino acids of αz to a backbone of αt1 produced a chimera, αt1/αz, that did not couple to any of the G_i-coupled receptors tested. Interestingly, an α13/αz chimera (with only the last five amino acids switched) displayed differential abilities to interact with receptors. Signals from aminergic, but not peptidergic, receptors were transduced by α13/αz. A similar construct, α12/αz, behaved just like α13/αz. These results indicated that "αi-like" or "αz-like" sequences at the carboxyl termini of α subunits are not always necessary or sufficient for specifying interaction with G_i-coupled receptors.

A host of clinically important drug receptors use heterotrimeric ($\alpha\beta\gamma$) G proteins belonging to the G_i subfamily for signal transduction. The G_i-coupled receptors include those that bind catecholamines, acetylcholine, serotonin, histamine, opioids, chemoattractants, chemokines, and many neuropeptides. Molecular determinants conferring selectivity at the receptor/G protein interface are beginning to be mapped out (1). Ample evidence is available in support of the notion that the carboxyl terminus of the α subunit of G_i proteins is one of the major contact sites with receptors. Attachment of an ADP-ribose moiety to the carboxyl tail of α_i by PTX prevents receptor-induced G_i activation (2). Antisera against the extreme carboxyl termini of the α_i chains can effectively disrupt receptor-induced inhibition of AC (3). Moreover, replacement of the last five amino acids of αq with the αi2 carboxyl end sequence allows the chimeric αq/αi2 protein to transduce signals from receptors that are normally coupled to G_i proteins (4). The importance of the carboxyl terminus in receptor recognition is also supported by studies with other α chains. An *unc* mutation at residue 389 uncouples αs from the β-adrenoceptor (5), whereas synthetic peptides derived from the carboxyl terminus of αt1 (rod transducin) have been shown to curtail rhodopsin/αt1 interaction (6).

Although less apparent, the amino terminus may also contribute toward the binding of αi subunits to receptors. Inferences can be drawn from the observations that the mastoparan-induced activation of G_o is abolished by point mutations (7) or tryptic digestion (8) of the amino terminus of αo. Synthetic peptides derived from the amino terminus of αt1 have been shown to inhibit effective interactions between rhodopsin and αt1 (6, 9). More recently, analysis of the crystal structures of αi1 (10) and αi1β1γ2 (11) have suggested that both amino and carboxyl termini may be involved in the coupling of the α chain to the receptor. However, because the α chain amino terminus is also involved in binding to the $\beta\gamma$ complex (11, 12), its contribution to receptor coupling may be indirect.

The cysteine residue at the carboxyl termini of αi chains, which can be ADP-ribosylated by PTX, does not seem to be required for receptor coupling. For instance, substitution of

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ABBREVIATIONS: PTX, pertussis toxin; AC, adenylyl cyclase; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; FMLP, formyl-methionyl-leucyl-phenylalanine; hCG, human choriogonadotropin; LHR, luteinizing hormone receptor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

this cysteine in αo with serine removes the PTX-induced inhibition but does not affect the transduction of hormonal signals via G_o (13). A similar mutation on αi2 produced a functional αi2 chain that acquired resistance to PTX (14). Perhaps the most convincing evidence comes from the demonstration that G_z, a PTX-insensitive G protein (15, 16), can mediate hormonal inhibition of AC by interacting with a wide variety of G_i-coupled receptors (17–22). Despite the fact that αi and αz share only ~60% identity in their amino acid sequences, their functional similarity suggests that they may share similar domains for receptor coupling. As a first step toward identifying the critical molecular determinants for receptor recognition by G_i proteins, we constructed a series of chimeric α subunits by swapping the carboxyl termini of αi2 and αz with each other or with those of other α subunits, such as αt1, α12, and α13. Their functional coupling to receptors was then assessed by measuring βγ-mediated stimulation of AC II in human embryonic kidney 293 cells transiently coexpressing the various chimeras.

Experimental Procedures

Materials. The human FMLP receptor and the murine δ-opioid receptor cDNAs (both in the pCDM8 vector) were generous gifts from Dr. F. Boulay (Laboratoire de Biochemie, Grenoble, France) and Dr. C. Evans (University of California, Los Angeles), respectively. The rat μ-opioid receptor cDNA (in the pRc/CMV vector) and the mouse κ-opioid receptor (in the pCMV6 vector) were kindly provided by Dr. L. Yu (Indiana University School of Medicine, Indianapolis, IN) and Dr. G. Bell (University of Chicago, Chicago, IL), respectively. cDNAs encoding the α subunits of G_{12} and G_{13} were supplied by Dr. M. Simon (California Institute of Technology, Pasadena). Other cDNAs were constructed or obtained as previously described (17, 23). PTX was purchased from List Biological Laboratories (Campbell, CA). hCG was generously provided by the National Pituitary Agency (Bethesda, MD). Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (CRL-1573, Rockville, MD). [^{3}H]Adenine was purchased from Amersham (Arlington Heights, IL) Plasmid purification columns were obtained from Qiagen (Hilden, Germany). Antiserum 3A-170 against the carboxyl terminal of αz was obtained from Gramsch Laboratories (Schwabhausen, Germany). Specific antisera for αz (SC-388), α12 (SC-409), and α13 (SC-410) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Construction of chimeric α subunits. The αi2/αz chimera (in pcDNA1) was made by replacing a *Bgl*II/*Not*I fragment of mouse αi2 with the corresponding sequence from rat αz, so the last 36 residues of αi2 were substituted with αz sequence. αz/αi2 was created in a converse manner. The constitutively active αi2/αz-QL and αz/αi2-QL chimeras were constructed in a similar manner by using αi2-Q205L (23) and αz-Q205L (17) as the starting materials. To construct the αt1/αz chimera, αt1 in pcDNA1 was digested with *Bgl*II and *Nde*I, and the 1.2-kb fragment was replaced with a 1.5-kb *Bgl*II/*Nde*I cognate fragment from αz. The αz/αt1 chimera was made in a similar fashion, and the αz/αt1-QL chimera was made with αz-Q205L as the source of αz sequence. Construct identity was verified by restriction mapping. The cDNAs encoding the α12/αz and α13/αz chimeras were kindly provided by Henry Bourne (University of California, San Francisco). The source and construction of other cDNAs were previously reported (17, 23).

Cell culture and transfection. Human embryonic kidney 293 cells were maintained and transfected as previously reported (24). Briefly, cells were cultured in Eagle's minimum essential medium containing 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in 5% CO_2 at 37°. Cells were seeded onto 12-well plates at ~1×10^5 cells/well. One day later, cells were transfected with medium containing the desired cDNAs along with 400 μg/ml DEAE-Dextran and 0.1 mM chloroquine for ≤2 hr at 37°. The cells were then shocked with 10% (v/v) dimethylsulfoxide in phosphate-buffered saline and returned to growth medium. The efficiency of transfections was routinely monitored through coexpression of β-galactosidase as a reporter.

cAMP accumulation. The transfected 293 cells were labeled 1 day later with [^{3}H]adenine (1 μCi/ml) in minimum essential medium containing 1% (v/v) fetal calf serum. Where indicated, 100 ng/ml PTX was added simultaneously. After 16–20 hr, the cells were assayed for cAMP levels in response to various drugs as previously described (24). cAMP accumulations were determined in the presence of 1 mM 1-methyl-3-isobutylxanthine at 37° for 30 min. Results are expressed as the ratios of [^{3}H]cAMP to total [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]cAMP pools. Absolute values for cAMP accumulation varied between experiments, but variability within a given experiment was <10% in general.

Immunodetection of chimeric α subunits. Membranes were prepared from transiently transfected 293 cells. Briefly, transfected cells were harvested in phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) containing 10 mM EDTA. Cells were resuspended in lysis buffer (50 mM Tris·HCl containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 1 mM EGTA, 5 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.4) and lysed by one cycle of freeze-thawing followed by 10 passages through a 27-guage needle. After removal of nuclei by centrifugation, membranes were collected, washed, and resuspended in lysis buffer. Protein concentrations were determined using the BioRad (Hercules, CA) Protein Assay Kit. For each sample, 75 μg of membrane proteins was separated on a μ polyacrylamide-sodium dodecyl sulfate gel and electrophoretically transferred to polyvinylidene difluoride membranes. Localization of protein markers on the polyvinylidene difluoride membrane was by Ponceau S staining. The following antisera were used: αz-specific 3A-170 and SC-388 for αt1/αz and αz/αt1, respectively; α12-specific SC-409 for α12/αz; and α13-specific SC-410 for α13/αz. Antigen/antibody complexes were visualized by enhanced chemiluminescence using the ECL kit from Amersham.

Results

Chimeras of αi2 and αz can interact with the δ-opioid receptor. The last 36 amino acids at the carboxyl termini of αi2, αz, and αt chains share substantial identity (~70%); the overall charge distribution of this region varies between α chains due to differences in three to five amino acids. A conserved *Bgl*II restriction site allows the swapping of the carboxyl terminal ends of these α subunits with each other after digestion of their cDNAs with restriction endonuclease and religation of corresponding fragments. The carboxyl terminal region of αi2 contains three charged residues (K331, K346, and D351) that are not found in the αz sequence. To test whether the αi2/αz chimera can interact with typical G_i-coupled receptors, we made use of a recombinant assay system that uses the βγ-mediated stimulation of AC II. This assay is based on the finding that in the presence of an activated αs, many receptors have the ability to stimulate AC II activity by releasing the βγ subunits from G_i and G_z proteins (20, 22, 25–27). We have thus cotransfected 293 cells with cDNAs encoding AC II, αs-Q227L (a constitutively active mutant of αs; Ref. 28), δ-opioid receptor, and one of several α subunits in their wild-type (αi2 and αz) or chimeric (αi2/αz and αz/αi2) forms. We have previously shown that under these experimental conditions, 293 cells exhibited

stimulation of cAMP production in response to activation of G_i-coupled receptors (20, 22, 26, 27), indicating the expression of a functional AC II in this cell system.

There was a ~2-fold enhancement in cAMP production on stimulation with 100 nM of the δ-selective agonist DPDPE of cells cotransfected with either wild-type or chimeric forms of the αi2 or αz subunits (Fig. 1). These results suggest that (a) the δ-opioid receptors can interact with both G_i and G_z proteins to stimulate AC II (as previously observed in Ref. 20) and (b) the chimeric constructs have retained their ability to interact with the δ-opioid receptor. The functional coupling of αi2/αz with the δ-opioid receptor was also confirmed by the effect of PTX treatment (100 ng/ml for 16–20 hr) on cAMP production. The stimulation of DPDPE-induced AC II activity in 293 cells cotransfected with αi2/αz was insensitive to PTX treatment, as was also observed for wild-type αz (Fig. 1). The partial reduction in cAMP levels observed is due to PTX inactivation of endogenous G_i-mediated AC II stimulation (Fig. 1, *vector control*). PTX treatment completely abolished the DPDPE-induced stimulation of AC II in cells cotransfected with αz/αi2. This response was similar to that of cells cotransfected with αi2 (Fig. 1), suggesting that the αz/αi2 chimera behaves more or less like an αi2. When compared with the control (vector DNA in place of the α subunit cDNA), 293 cells coexpressing αi2, αz, αi2/αz, or αz/αi2 all exhibited enhanced responses to DPDPE. The apparent enhancement of the AC II response is probably attributed to an increase in the amount of releasable βγ subunits as a result of expressing exogenous α subunits (22, 26).

Inhibition of AC by αi2/αz and αz/αi2. We have previously shown that both αi2 and αz inhibit cAMP accumulation to similar extents (17, 23). To investigate whether the chimeric αi2/αz has retained its ability to negatively regulate AC, we cotransfected the 293 cells with cDNAs encoding the rat LHR, δ-opioid receptor, and one of three α subunits (αi2, αz, or αi2/αz). Coexpression of LHR allows the targeting of transfected cells, and activation of this receptor by 5 ng/ml hCG raises the cAMP content to a level at which inhibition can be easily detected (23). In this system, 100 nM DPDPE was shown to inhibit consistently the hCG-stimulated cAMP production by 40–60% (20). In the current study, coexpression of αz, but not αi2, conferred PTX resistance to the DPDPE-induced inhibitory response (Fig. 2). In cells coexpressing the αi2/αz chimera, the DPDPE-mediated inhibition of cAMP accumulation was only slightly reduced by PTX treatment, suggesting that αi2/αz can indeed inhibit AC in an αz-like manner (Fig. 2).

Negative regulation of AC by the αi2/αz and αz/αi2 chimeras was also indirectly monitored by examining the ability of αi2/αz-QL and αz/αi2-QL to constitutively inhibit hCG-stimulated cAMP accumulation. We have previously shown that the GTPase-deficient mutants (QL mutants) of both αi2 and αz constitutively inhibit AC in 293 cells (17, 23). Using a similar paradigm, we tested whether the same point mutation in αi2/αz and αz/αi2 chimeras would result in the constitutive suppression of AC activity. Coexpression of wild-type αi2/αz or αz/αi2 with LHR did not affect the hCG-induced stimulation of AC activity because the cAMP levels were similar to that obtained when cells were cotransfected with the vector pcDNA1 instead of the α subunit constructs. However, their QL counterparts inhibited the hCG response by ~50% (Fig. 3). The magnitude of inhibition by αi2/αz-QL and αz/αi2-QL was similar to those produced by αi2-QL and αz-QL (Fig. 3). Both αi2/αz and αz/αi2 chimeras were apparently able to mediate inhibition of AC by adopting the GTP-bound active conformation.

Receptor coupling to αt1/αz and αz/αt1. Like αi and αz chains, αt1 and αt2 (rod and cone transducins, respectively) belong to the subfamily of G_i proteins. Although they are related, the transducins seem to couple exclusively to the opsin receptors and not to any of the G_i-coupled receptors. By constructing chimeras between αt1 and αz, we investigated

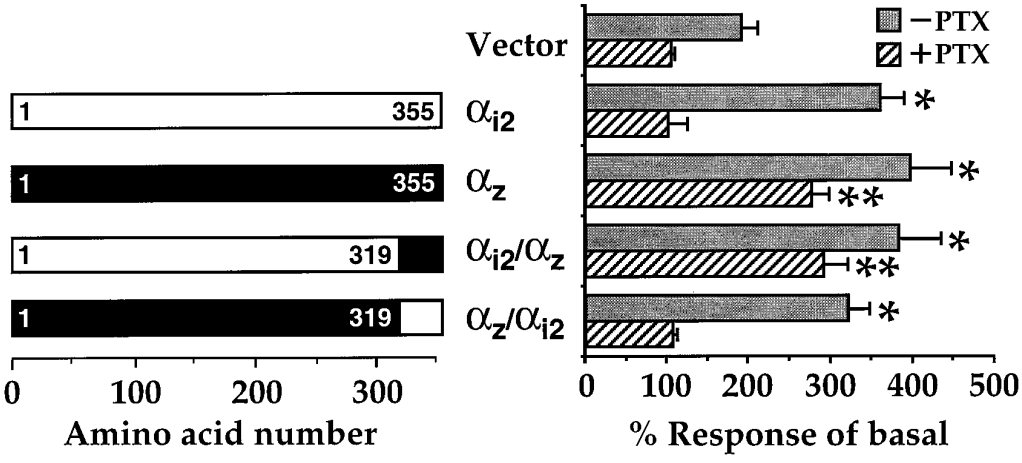

Fig. 1. Potentiation of DPDPE-induced stimulation of AC II by αi2/αz and αz/αi2 chimeras. The 293 cells were transiently cotransfected (DEAE-Dextran method), labeled with [^{3}H]adenine (1 μCi/ml), and then assayed for cAMP accumulation in the absence or presence of 100 nM DPDPE as described in the text. Cotransfections were with AC II (0.25 μg/ml), αs-Q227L (0.025 μg/ml), the δ-opioid receptor (0.25 μg/ml), and 0.15 μg/ml concentration of one of the following cDNAs: αi2, αz, αi2/αz, αz/αi2, or pcDNA1 vector (control). Cells were subjected to PTX treatment (100 ng/ml, 16 hr) where indicated. Data shown represent triplicate determinations in a single experiment; two independent experiments yielded similar results. Results are expressed as percent stimulation of cAMP formation in the presence of DPDPE compared with that measured in the absence of DPDPE. Basal values are expressed as the ratio ($\times 10^3$) of cAMP to total adenine nucleotides and ranged from 6.55 ± 0.52 to 9.24 ± 0.43. *, DPDPE-stimulated cAMP accumulation was significantly different from that observed in the vector-transfected cells (paired Bonferroni *t* test, $p < 0.05$). **, Significantly higher than basal cAMP accumulation in PTX-treated cells (paired *t* test, $p < 0.05$). *Left*, schematic of the various wild-type and chimeric α subunits used in the cotransfections.

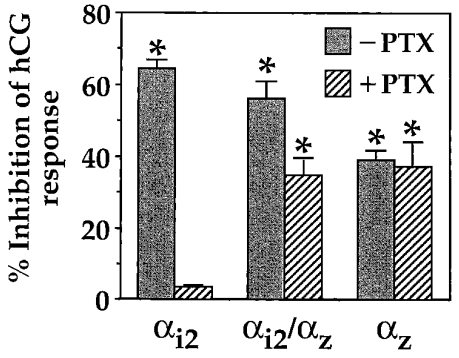

Fig. 2. PTX-insensitive inhibition of AC by the αi2/αz chimera. The 293 cells were cotransfected with the LHR cDNA (0.15 μg/ml), the δ-opioid receptor cDNA (0.25 μg/ml), and 0.15 μg/ml concentration of an α subunit (αi2, αz, or αi2/αz). Transfected cells were labeled with [^{3}H]adenine in the absence or presence of PTX (100 ng/ml) and then assayed for cAMP accumulation in response to hCG (5 ng/ml) with or without DPDPE (100 nM). Data shown represent the mean ± standard deviation of triplicate determinations of one of three experiments with similar results. Results are expressed as percent inhibition of the hCG-stimulated activity in the presence of DPDPE compared with that measured in the presence of hCG alone. hCG-stimulated cAMP accumulation ranged from 13.3 ± 0.7 to 16.1 ± 1.6. *, DPDPE significantly inhibited the hCG response (paired *t* test, $p < 0.05$).

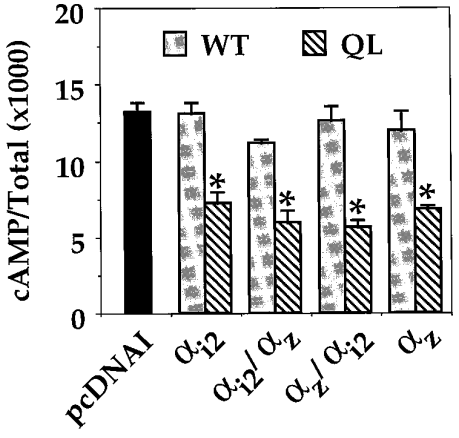

Fig. 3. Constitutive inhibition of AC by αi2/αz-QL and αz/αi2-QL. The 293 cells were cotransfected with the LHR cDNA (0.15 μg/ml) with or without 0.15 μg/ml pcDNA1 or one of the following α subunits in their wild-type (*WT*) or constitutively activated (*QL*) form: αi2, αz, αi2/αz, or αz/αi2. Transfected cells were assayed for cAMP accumulation in response to hCG (5 ng/ml). Data shown represent the mean ± standard deviation of triplicate determinations of one of three experiments with similar results. *, hCG responses were significantly inhibited in cells coexpressing the QL mutants (paired *t* test, $p < 0.05$).

whether the receptor recognition determinants of αz are indeed located on its two termini. As shown in Fig. 4, coexpression of αt1 attenuated the DPDPE-induced cAMP formation in the AC II transient transfection system. This is in agreement with the notion that αt1 is a βγ scavenger (25). Interestingly, blockade of the βγ-mediated stimulation of AC II was also observed with cells coexpressing the αt1/αz chimera (Fig. 4). Both αt1 and αt1/αz significantly inhibited the ability of DPDPE to stimulate AC II ($p < 0.05$, three experiments). By behaving like αt1, the αt1/αz chimera seemed to be unable to release βγ subunits when the δ-opioid receptor was activated. Lack of coupling to the δ-opioid receptor by αt1/αz was further demonstrated by its inability to provide PTX resistance to cells coexpressing this chimera (Fig. 4). On the contrary, the αz/αt1 chimera enhanced the DPDPE-induced stimulation of AC II. The potentiating effect of αz/αt1 suggested that this chimera can interact with the δ-opioid receptor and release βγ subunits in addition to those released from endogenous Gi proteins. Because αz/αt1 contains the PTX-catalyzed ADP-ribosylation site, the DPDPE-induced AC II response was sensitive to PTX treatment (Fig. 4). Introduction of the QL mutation into αz/αt1 produced a constitutively active chimera that can inhibit AC in a receptor-independent fashion.[2] Taken together, these results indicate that the carboxyl-end 36 amino acids of αt1 can substitute for cognate sequence on αz but that sequence integrity at the amino terminal of αz is also needed for recognition of G_i-coupled receptors.

The δ-opioid receptor does not couple to α12/αz or α13/αz. It has previously been shown that G_i-coupled receptors can activate chimeric α subunits if the last few residues of the chimeras compose a "G_i-like" sequence (4, 29). These reports tend to place more weight on the importance of the carboxyl terminus of the α chains in receptor recognition and contradict our findings with the αt1/αz chimera. We therefore examined the ability of the δ-opioid receptor to stimulate AC II via βγ subunits released from chimeric α12/αz and α13/αz subunits. Both chimeras have the last five amino acids changed to an αz sequence (29). Cells were cotransfected with cDNAs encoding AC II, α_s-Q227L, the δ-opioid receptor, and one of five α subunits (αz, α12, α13, α12/αz, and α13/αz). The transfected cells were then assayed for cAMP accumulation in the absence or presence of 100 nM DPDPE. Except for cells coexpressing αz, no enhancement of DPDPE-induced stimulation of AC II activity was seen with any of the transfected cells (Fig. 5). Moreover, only coexpression of αz provided PTX resistance to the transfected cells. Hence, the δ-opioid receptor was unable to couple to α12 or α13, and the presence of the last five amino acids of αz was insufficient to forge an interaction. Lack of coupling to α12 and α13 have already been demonstrated with the μ-opioid receptor (26).

Chimeric αi2/αz does not discriminate between various G_i-coupled receptors. Many receptors can apparently discriminate between different G proteins. For example, somatostatin receptor subtype 3 selectively couples to G_{i1} rather than G_{i2} or G_{i3} (30), whereas the complement C5a receptor prefers G_{16} to G_{11} (31). There is no hard and fast rule that if a particular G protein interacts with one receptor, it will couple to other receptors of the same class. Hence, we examined the ability of αi2/αz chimera to interact with a panel of G_i-coupled receptors by using the same transient transfection strategy. Coexpression of either αz or αi2/αz with the other two opioid receptor subtypes (μ and κ) in 293 cells conferred PTX resistance in the stimulation of AC II activity by the opioid ligands tested (Table 1). In addition, both αz and αi2/αz mediated PTX-insensitive stimulation of AC II on activation of the FMLP receptor (Table 1) (27). G_z is known to interact with receptors for aminergic neurotransmitters (17). Indeed, under similar experimental conditions, both α_2-adrenergic and dopamine D_2 receptors were able to activate the αi2/αz chimera leading to βγ-mediated stimulations of AC II (Table 1). Collectively, these results suggest that the αi2/αz does not discriminate among different G_i-coupled receptors.

[2] M. K. C. Ho, and Y. H. Wong, unpublished observations.

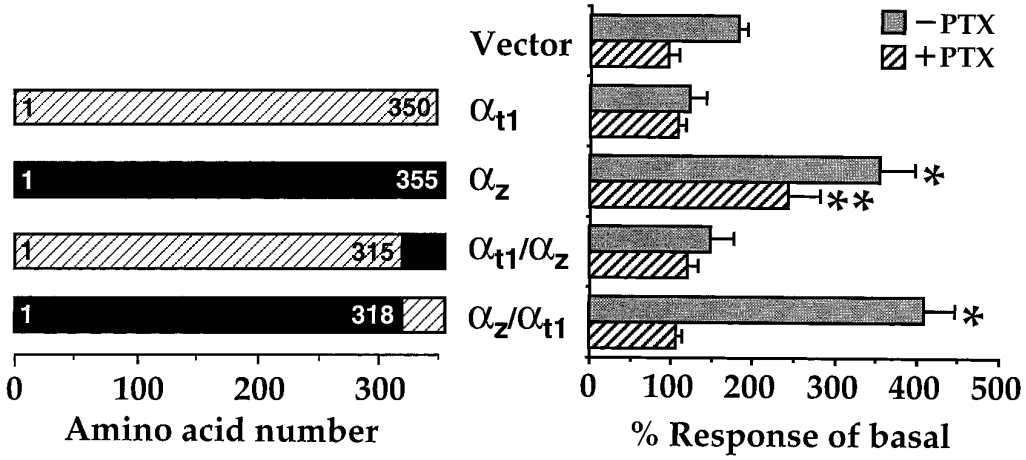

Fig. 4. Blockade of DPDPE-induced stimulation of AC II by αt1 and αt1/αz chimeras. 293 cells were cotransfected with various cDNAs as described in the legend to Fig. 1, except that the following α subunits were used: αt1, αz, αt1/αz, or αz/αt1. Transfected cells were assayed for cAMP accumulation as for Fig. 1. Data shown represent triplicate determinations in a single experiment; two independent experiments yielded similar results. Results are expressed as percent stimulation of cAMP formation in the presence of DPDPE compared with that measured in the absence of DPDPE. The basal values are expressed as the ratio ($\times 10^3$) of cAMP to total adenine nucleotides and ranged from 5.71 ± 0.31 to 8.47 ± 0.44. *, DPDPE-stimulated cAMP accumulation was significantly different from that observed in the vector control (paired Bonferroni *t* test, $p < 0.05$). **, Significantly higher than basal cAMP accumulation in PTX-treated cells (paired *t* test, $p < 0.05$). *Left*, different wild-type and chimeric α subunits.

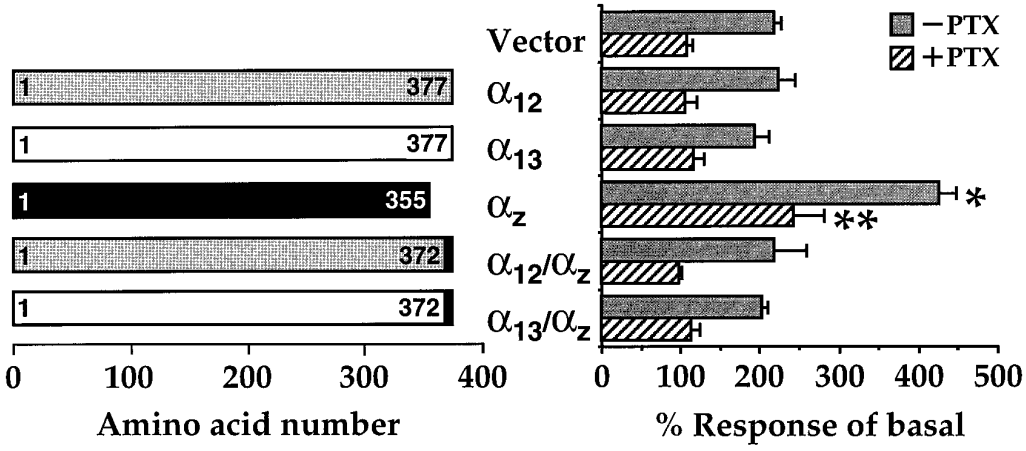

Fig. 5. Lack of δ-opioid receptor coupling to α12/αz and α13/αz chimeras. The 293 cells were cotransfected with various cDNAs as described in the legend to Fig. 1, except that the following α subunits were used: αz, α12, α13, α12/αz, or α13/αz. Transfected cells were assayed for cAMP accumulation as for Fig. 1. Data shown represent triplicate determinations in a single experiment; two independent experiments yielded similar results. Results are expressed as percent stimulation of cAMP formation in the presence of DPDPE compared with that measured in the absence of DPDPE. The basal values are expressed as the ratio ($\times 10^3$) of cAMP to total adenine nucleotides and ranged from 5.83 ± 0.72 to 8.33 ± 0.68. *, DPDPE-stimulated cAMP accumulation is significantly different from that observed in the vector control (paired Bonferroni *t* test, $p < 0.05$). **, Significantly higher than basal cAMP accumulation in PTX-treated cells (paired *t* test, $p < 0.05$). *Left*, wild-type and chimeric α subunits used in the study.

The α12/αz and α13/αz chimeras distinguish aminergic from peptidergic receptors. Although αi2/αz seemed to interact with a large variety of G_i-coupled receptors, the inability of the δ-opioid receptor to activate α12/αz and α13/αz suggested that the latter chimeras may not be as promiscuous. Activation of the Na^+/H^+ exchanger by the dopamine D_2 receptor via α13/αz has been previously demonstrated (29). Coupling of the dopamine D_2 receptor to α13/αz was therefore examined in the AC II transient transfection assays. The 293 cells cotransfected with AC II, αs-Q227L, dopamine D_2 receptor, and α13/αz were treated with PTX before cAMP assays. Activation of the dopamine D_2 receptor by 10 μM quinpirole increased cAMP accumulation by ~100% (Table 1). As a control for complete inactivation of endogenous G_i proteins by PTX, similar transfections were performed in which α13/αz was replaced by αi2. No agonist-induced stimulation of AC II was observed in control cells (Table 1), suggesting that the quinpirole response was indeed transduced by α13/αz. Replacement of the dopamine D_2 receptor with the α_2-adrenoceptor in the cotransfections produced cells in which 10 nM UK-14304, an α_2-adrenoceptor agonist, stimulated AC II in a PTX-resistant manner (Table 1). However, when we tested the μ-opioid, κ-opioid, and FMLP receptors under the same experimental conditions, none of these peptidergic receptors were able to interact with α13/αz (Table 1). The receptor-coupling profile of α12/αz was essentially identical to that of α13/αz (Table 1). Both α12/αz and α13/αz seemed to recognize aminergic but not peptidergic G_i-coupled receptor.

To confirm the expression of α12/αz and α13/αz chimeras,

TABLE 1

Receptor-mediated stimulation of AC II via PTX-insensitive wild-type and chimeric α subunits

The 293 cells were cotransfected with cDNAs encoding AC II (0.25 μg/ml), αs-Q227L (0.025 μg/ml), 0.25 μg/ml concentration of a G_i-coupled receptor, and one of several α subunits: αi2, αi2/αz, αz, α12, α13, α12/αz, or α13/αz. The following receptors were tested: α2-adrenergic, dopamine D_2, FMLP, δ-opioid, μ-opioid, and κ-opioid. Transfected cells were treated with PTX and assayed for cAMP accumulation in the absence or presence of receptor-selective agonists. Agonists used include 10 nM UK-14304 for α2-adrenoceptor, 10 μM quinpirole for dopamine D_2 receptor, 200 nM FMLP for FMLP-receptor, 100 nM DPDPE for δ-opioid receptor, 100 nM [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin for μ-opioid receptor, and 100 nM U50,488 for κ-opioid receptor. Data shown represent mean ± standard error of three or four independent experiments of triplicate determinations. Results are expressed as percent stimulation of cAMP formation in the presence of agonist compared with that measured in the absence of agonist (basal). The basal values expressed as the ratio (×10^3) of cAMP to total adenine nucleotides and ranged from 4.77 ± 0.81 to 9.46 ± 0.85.

G_α	Agonist-induced PTX-insensitive stimulation of AC II					
	α_2-Adrenoceptor	Dopamine D_2 receptor	FMLP receptor	δ-Opioid receptor	μ-Opioid receptor	κ-Opioid receptor
			% of basal			
αi2	107 ± 6	91 ± 11	92 ± 13	96 ± 8	96 ± 7	97 ± 5
αi2/αz	405 ± 47[a]	297 ± 17[a]	275 ± 31[a]	276 ± 16[a]	256 ± 15[a]	243 ± 19[a]
αz	336 ± 28[a]	307 ± 18[a]	239 ± 16[a]	235 ± 28[a]	271 ± 41[a]	213 ± 11[a]
α12	97 ± 4	107 ± 22	107 ± 21	99 ± 16	91 ± 13	104 ± 10
α12/αz	297 ± 27[a]	194 ± 27[a]	112 ± 15	107 ± 13	102 ± 12	110 ± 16
α13	116 ± 13	115 ± 12	122 ± 22	111 ± 19	98 ± 3	96 ± 7
α13/αz	267 ± 11[a]	178 ± 19[a]	115 ± 14	111 ± 8	99 ± 6	101 ± 5

[a] cAMP accumulation was significantly stimulated by agonist-induced receptor activation (paired *t* test, $p < 0.05$).

293 cells were transiently cotransfected with cDNAs encoding the AC II, αs-Q227L, and δ-opioid receptor in the absence (mock) or presence of various wild-type and chimeric constructs. Immunodetection of α12/αz and α13/αz in membranes prepared from transiently transfected 293 cells indicated that both chimeras were indeed be expressed (Fig. 6). The levels of protein expression of α12/αz and α13/αz were similar to those observed in membranes from α12- or α13-transfected cells (Fig. 6) and were comparable to the transient expressions of other exogenous α subunits in 293 cells (17, 23). Thus, the inability of α13/αz and α12/αz to interact with peptidergic receptors was not due to a lack of protein expression. It is also noteworthy that α12, but not α13, is endogenously expressed in 293 cells (Fig. 6). Expressions of αt1/αz and αz/αt1 in transiently transfected 293 cells were also confirmed by immunodetection with αz-specific antisera (Fig. 6).

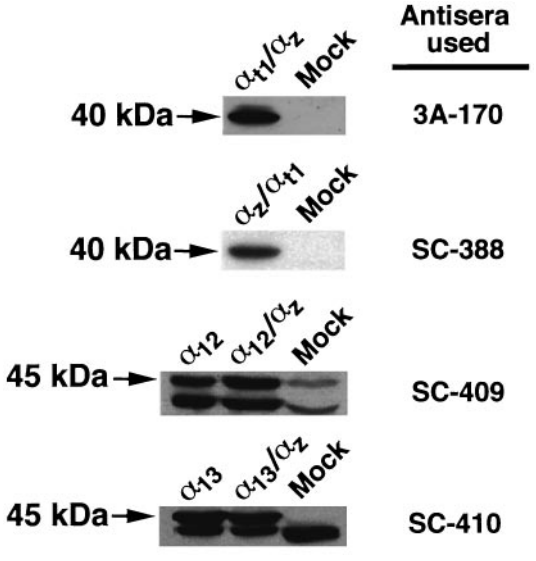

Fig. 6. Immunodetection of chimeric α subunits. The 293 cells were transiently cotransfected with cDNAs encoding the AC II, αs-Q227L, and δ-opioid receptor in the absence (*Mock*) or presence of various wild-type and chimeric constructs. Plasma membranes were prepared 48 hr after transfection. Membrane proteins (75 μg) were separated on a 12.5% polyacrylamide-sodium dodecyl sulfate gel and electrophoretically transferred to polyvinylidene difluoride membranes. Protein markers were localized by Ponceau S staining, and the chimeras were immunodetected with the specific antisera as indicated. Two independent experiments with different batches of membrane proteins yielded similar results.

Discussion

To achieve fidelity in signal integration and amplification, input from a large number of distinct receptors must be processed with precision by the G proteins. Specificity in G protein/receptor interactions has been postulated to reside at both the amino and the carboxyl termini of the G protein α subunit (1). Participation of the two termini as integral components of a putative receptor contact domain has been confirmed by studying the crystal structures of heterotrimeric G_{t1} (12) and G_{i1} proteins (11). Because numerous G_i-coupled receptors possess the ability to use G_z to mediate PTX-insensitive inhibition of AC, the receptor recognition domains of G_i and G_z must be highly similar. By comparison, the first 36 residues of αi2 and αz share ~63% identity, whereas the last 36 amino acids exhibit ~83% identity. The higher homology found between the carboxyl termini of αi2 and αz suggests that the carboxyl terminal region may be more critical for receptor recognition.

Our studies with the αi2/αz and αz/αi2 chimeras clearly demonstrate that the carboxyl termini of αi2 and αz are interchangable with respect to interaction with receptors. However, replacement of the carboxyl-terminal 36 residues of αt1 with an αz sequence (exhibiting ~75% identity) did not permit the resultant αt1/αz chimera to interact with G_i-coupled receptors. Hence, molecular determinants other than the carboxyl-terminal sequences are apparently required for the recognition of G_i-coupled receptors. Similar inferences can be drawn from studies using the αz/αt1 chimera. Because the αz/αt1 chimera was able to interact functionally with G_i-coupled receptors, the last 36 residues of αt1 must be highly homologous to those of αi2 and αz. If the carboxyl terminus constitutes the only receptor contact site on αt1, then G_{t1} should be activatable by G_i-coupled receptors, yet G_{t1} does not interact appreciably with G_i-coupled receptors (22, 26). Presumably, the amino-terminal regions of αt1 may be more important for conferring specificity in the interaction

of G_{t1} with the rhodopsin receptor, whereas the carboxyl end of αz (and $\alpha i2$) may play a more critical role in the interaction with G_i-coupled receptors.

A previous study using αq chimeras illustrated the importance of the last five residues of $\alpha i2$ and αz in the recognition of G_i-coupled receptors (4). If the first 30–40 residues of the α subunit contribute toward the formation of the receptor contact surface, then the amino termini of αq, αz, and $\alpha i2$ must bear substantial resemblance. However, sequence analysis of this region indicates ~50% identity between αq and αz (or $\alpha i2$) chains. A similar degree of identity can be found between the amino termini of $\alpha t1$ and αz, but the $\alpha t1/\alpha z$ chimera was unable to interact productively with G_i-coupled receptors. It seems that even when the carboxyl terminus contains the necessary residues, discrete sequence motifs within the amino-terminal region may dictate whether an α subunit can interact with G_i-coupled receptors. Because the extreme amino terminus of many α subunits contains sites for fatty acylation and covalent modifications and the putative receptor contact domain has substantial overlaps with the $\beta\gamma$-binding surface (11, 12), it is difficult to determine which residues are absolutely critical for receptor recognition. Furthermore, G protein α subunit/receptor interactions may involve a series of conformational changes on association with the $\beta\gamma$ complex, which may add to receptor/G protein specificity and stabilization of interactions. At least for the two splice variants of G_o, the exact composition of the $\alpha\beta\gamma$ subunits decrees which G_i-coupled receptors can be recognized by G_o (32, 33). Moreover, failure to properly bind $\beta\gamma$ subunits may result in the inability of the α subunit to interact with receptors (34).

The structural integrity of chimeras composed of $\alpha i2$ and αz sequences deserves further comment. We have shown not only that the $\alpha i2/\alpha z$ and $\alpha z/\alpha i2$ chimeras can interact with a variety of G_i-coupled receptors nut also that they can adopt an active GTP-bound conformation to inhibit AC. Additional support can be drawn from the fact that PTX abolished the interaction between receptors and $\alpha z/\alpha i2$, suggesting that the $\alpha z/\alpha i2$ chimera can maintain a structure recognizable by the toxin. The PTX sensitivity of $\alpha z/\alpha i2$ is in direct contrast with an $\alpha s/\alpha i2$ chimera that does not serve as a substrate for PTX (35). Because the amino and carboxyl termini of the α subunit lie in close proximity, replacement of the amino-terminal sequence of $\alpha i2$ with an αs sequence may disrupt the PTX recognition surface, whereas the αz sequence is sufficiently homologous to $\alpha i2$ that PTX can ADP-ribosylate the $\alpha z/\alpha i2$ chimera. By the same analogy, structural integrity of the $\alpha z/\alpha t1$ chain must be properly preserved because this chimera can interact with receptors in a PTX-sensitive manner.

Analysis of the $\alpha 12/\alpha z$ and $\alpha 13/\alpha z$ chimeras revealed an interesting profile of receptor coupling specificity. Aminergic receptors were able to interact with both $\alpha 12/\alpha z$ and $\alpha 13/\alpha z$ chimeras, albeit at a weaker efficacy than with the $\alpha i2/\alpha z$ chimera. The peptidergic receptors, on the other hand, were totally unable to interact with the $\alpha 12/\alpha z$ and $\alpha 13/\alpha z$ chimeras. It remains to be determined whether the difference in coupling to $\alpha 12/\alpha z$ and $\alpha 13/\alpha z$ chimeras is generally applicable to all G_i-coupled aminergic and peptidergic receptors. The lack of coupling to peptidergic receptors was not due to inadequate expression of $\alpha 12/\alpha z$ and $\alpha 13/\alpha z$. In addition, each of the peptidergic receptor tested was apparently expressed in sufficient quantities to interact with both αz and $\alpha i2/\alpha z$. Although we cannot exclude the possibility that coexpression with $\alpha 12/\alpha z$ or $\alpha 13/\alpha z$ may affect the expression of peptidergic receptors, this explanation is probably incorrect because the expression of FMLP receptors (as determined by radioligand binding assays) was unaffected by the type of α subunits being coexpressed (27). It is more likely that the $\alpha 12/\alpha z$ and $\alpha 13/\alpha z$ chimeras are incapable of interacting with peptidergic receptors. The precise molecular determinants that specify receptor/G protein interactions are not well understood. For a receptor to couple efficiently to a G protein, the respective contact surfaces on both components must be complementary to each other. A G_q family member, G_{16}, has recently been contrived as an universal adapter for G protein-coupled receptors (36). The α subunit of G_{16} may have the most "fitting" or "flexible" receptor contact surfaces among the G proteins. By constructing chimeras composed of $\alpha 16$ and $\alpha 11$ (which does not interact with G_i-coupled receptors) sequences, it has been shown that the specificity of G_{16} coupling to the C5a receptor is primarily found within the amino-terminal 209 residues of $\alpha 16$ (31). Whether the same regions are also necessary for $\alpha 16$ to interact with other receptor types remains to be resolved. By building on the information obtained from chimera studies and crystal structures of heterotrimeric G proteins, further investigations may provide insights on the minimum molecular determinants for α subunits to interact with receptors.

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