FEBS Letters 522 (2002) 130–134 FEBS 26222

Role of intracellular loops of cannabinoid CB_1 receptor in functional interaction with $G_{\alpha16}$

Begonia Y. Ho[1], Lori Current, James G. Drewett*

Department of Pharmacology, Physiology and Therapeutics, University of North Dakota, Grand Forks, ND 58202, USA

Received 28 March 2002; revised 30 May 2002; accepted 30 May 2002

First published online 7 June 2002

Edited by Jacques Hanoune

Abstract The cannabinoid CB_1 but not the CB_2 receptor was demonstrated to couple via $G_{\alpha16}$ to activate phospholipase C after co-expression in COS7 cells. Chimeric CB_1/CB_2 receptors were used as a model to study receptor–$G_{\alpha16}$ interaction. Sequences of the second and third intracellular loops and the carboxy-terminus were substituted from the CB_1 into the CB_2 receptor. Only the triple mutant with all three regions replaced activated phospholipase C to a similar extent as the CB_1 receptor, suggesting that all three intracellular regions are required for interacting with $G_{\alpha16}$. Several sub-domains within the third intracellular loop were identified for receptor–$G_{\alpha16}$ interaction. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: $G_{\alpha16}$; Cannabinoid CB_1 receptor; Cannabinoid CB_2 receptor; Receptor–G_α coupling; G protein-coupled receptor; Mutagenesis

1. Introduction

The G protein-coupled receptor represents one of the largest protein families discovered in nature with over 1000 members. They far outnumber the G_α subunits identified so far. Therefore, each G_α subunit is able to couple to multiple receptors. Depending on their predominant signaling pathway, most G protein-coupled receptors are referred to as $G_{\alpha s}$-, $G_{\alpha i/o}$- or $G_{\alpha q}$-coupled. Among all the known G_α subunits, the human $G_{\alpha16}$ and its mouse homolog $G_{\alpha15}$ are unique because they can couple a variety of $G_{\alpha s}$-, $G_{\alpha i/o}$- and $G_{\alpha q}$-linked receptors to phospholipase C activation [1–5]. Because of this promiscuous nature, $G_{\alpha16}$ has been referred to as a universal G protein adapter with great implication for agonist screening strategies, especially in the case of orphan receptors [1].

Although $G_{\alpha15}$ and $G_{\alpha16}$ exhibit promiscuity towards G protein-coupled receptors, they cannot be considered as universal adapters. We recently reported that the cannabinoid CB_1, but not the CB_2 receptor, coupled to phospholipase C activation to increase inositol phosphate formation after its co-expression with $G_{\alpha16}$ in monkey kidney epithelial COS7 and human embryonic kidney HEK293 cells [6]. In addition to the CB_2 receptor, several G protein-coupled receptors also did not interact with $G_{\alpha16}$, including the CCR_{2a} chemokine receptor [3], the α_{1A}-adrenoceptor [7] and the MT_{1C} melatonin receptor [5].

The molecular determinant for receptor–$G_{\alpha16}$ interaction is still largely unknown. $G_{\alpha16}$ belongs to the $G_{\alpha q}$ family ($G_{\alpha11}$, $G_{\alpha q}$, $G_{\alpha14}$, $G_{\alpha15}$ and $G_{\alpha16}$) that couples to phospholipase C activation [8]. Different sequences within the third intracellular (i3) loop of the α_{1B}-adrenoceptor have been suggested to be involved in coupling to $G_{\alpha16}$ and $G_{\alpha q/11}$ [9]. The present study took advantage of the fact that the CB_1 but not the CB_2 receptor couples to $G_{\alpha16}$. Because of the high sequence homology between the two receptors and the ability of both to interact with similar ligands, they can be used as a model system for studying receptor–$G_{\alpha16}$ interaction. Using a gain-of-function approach, the i2 loop, i3 loop and carboxy-terminus of the CB_2 receptor were systematically replaced with sequences of the CB_1 receptor. We successfully created chimeric receptors that showed enhanced ability to activate phospholipase C. The present study describes the construction of a series of CB_1/CB_2 chimeras and the characterization of their ability to couple to $G_{\alpha16}$.

2. Materials and methods

2.1. Materials

The cDNA clones of the human cannabinoid CB_1 receptor, human cannabinoid CB_2 receptor and human $G_{\alpha16}$ were gifts from Dr. M. Parmentier (Université Libre de Bruxelles, Brussels, Belgium), Dr. S. Munro (Medical Research Council, London, UK) and Dr. M. Simon (California Institute of Technology, Pasadena, CA, USA), respectively. WIN55,212-2 was purchased from Research Biochemicals (Natick, MA, USA). T7 Sequenase v2.0 sequencing kit, *myo*-[2-^{3}H]inositol (115 Ci/mmol) and [^{125}I]cAMP (2000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). [^{3}H]CP55,940 (180 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Thermostable DNA polymerases and restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Cell culture reagents were purchased from Gibco BRL (Grand Island, NY, USA) or Biowhittaker (Walkersville, MD, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Fisher Scientific (Hanover Park, IL, USA) or as indicated.

*Corresponding author. Present address: College of Pharmacy, University of Cincinnati, 3223 Eden Ave, P.O. Box 670004, Cincinnati, OH 45267-0004, USA. Fax: (1)-513-558 0978.
E-mail addresses: ho.begoniay@kendle.com (B.Y. Ho), lori@current.nu (L. Current), drewetjg@ucmail.uc.edu (J.G. Drewett).

[1] Present address: Department of Clinical Development, Kendle International, 1200 Carew Tower, 441 Vine St, Cincinnati, OH 45202, USA.

Abbreviations: CP55,940, (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; WIN55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolol[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate

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PII: S0014-5793(02)02917-4

2.2. Construction of wild type and chimeric receptors

All cDNA clones used for transient transfection were inserted in eukaryotic expression plasmids 3′ to the cytomegalovirus promoter. The cDNA clones of the human cannabinoid CB_1 and CB_2 receptors were inserted into pRcCMV (Invitrogen, San Diego, CA, USA) and human $G_{\alpha16}$ was obtained as a gift in the vector pCIS.

Chimeras that contain substitutions of the entire cytoplasmic domain were generated by polymerase chain reaction (PCR)-based mutagenesis [10] with the high fidelity thermostable Vent or Vent (exo-) DNA polymerase using either the cannabinoid CB_1 or CB_2 receptor cDNA as the template. The chimeric CB_1/CB_2 PCR fragments generated using these two DNA polymerases were mostly blunt-ended and were subcloned into *Sca*I-digested pBluescript KS (Stratagene, La Jolla, CA, USA). Positive clones were transferred into the pRcCMV-based plasmids containing the cannabinoid receptor cDNA using appropriate restriction sites. Chimeras with smaller substitutions were constructed by oligonucleotide-directed mutagenesis using a Muta-gene M13 in vitro mutagenesis kit (Bio-Rad, Richmond, CA, USA). Uracil-containing single-stranded template was generated with the helper phage R408 (Promega, Madison, WI, USA) or RZ1032 (Quantum Biotechnologies, Laval, QC, Canada). All mutants were analyzed by restriction analysis and double-stranded dideoxy sequencing.

2.3. Cell culture and transfection of COS7 cells

Monkey kidney COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) in a humidified environment containing 5% CO_2 and 95% air at 37°C. COS7 cells were transfected according to the method described by Offermanns and Simon [2] and Ho et al. [6] with modifications. Cells were transfected with the wild type or chimeric cannabinoid receptor (1.25 μg) and $G_{\alpha16}$ (1.25 μg) using lipofectamine or lipofectamine 2000 (Gibco) in serum-free OPTI-MEM (Gibco). The total amount of DNA and lipofectamine used was maintained constant among different transfection experiments with the vector pRcCMV.

2.4. Determination of inositol phosphate level

The formation of inositol phosphates was measured as described [2,6]. Briefly, 20–24 h post-transfection, cells were labeled for 24–28 h with 1 μCi/ml *myo*-[2-^{3}H]inositol in inositol-free DMEM (Gibco) and 20% dialyzed fetal calf serum (Gibco). Cells were treated with WIN55,212-2 prepared in inositol-free DMEM containing 10 mM LiCl and 1 mg/ml fatty acid-free bovine serum albumin for 20 min at 37°C. Total inositol phosphate accumulation (inositol monophosphate+inositol bisphosphate+inositol 1,4,5-trisphosphate) was expressed as percent of basal level determined in vehicle-treated cells in the absence of the agonist.

2.5. Determination of cAMP level

Adenylyl cyclase activity was assayed by cAMP formation accumulated in whole cells [11]. cAMP levels were measured by radioimmunoassay using anti-cAMP antibodies (gift from Dr. A.F. Parlow of the National Hormone and Pituitary Program, Torrance, CA, USA, lot number CV-27) according to the methods of Richman et al. [12].

2.6. Radioligand binding assay

Because of the large number of chimeric receptors, the B_{max} and K_d values were calculated from competition instead of saturation binding assays according to the method described by DeBlasi et al. [13]. The preparation of membranes and binding assays were performed as reported previously [6]. Various concentrations of CP55,940 were used to compete with 0.5 nM [^{3}H]CP55,940 (1 h at 30°C). Non-specific binding was determined in the presence of 10 μM (−)-Δ^9-tetrahydrocannabinol.

2.7. Data analysis

Data shown in the figures and table are expressed as mean ± S.E.M. GraphPad Prism 3.02 (San Diego, CA, USA) was used to perform two-way analysis of variance (ANOVA) to test for any concentration-dependent effect and for the difference between the wild type and mutant receptors. The Bonferroni post-hoc test was used to test for differences between the wild type and mutant receptor at each concentration of agonist. GraphPad Prism 3.02 was also used to calculate IC_{50} values of binding assays. The B_{max} and K_d values were calculated using the following equations: $K_d = IC_{50} - L$ and $B_{max} = (B_o \times IC_{50})/L$, where L is the concentration of the radioligand and B_o is the specifically bound radioligand determined in the absence of the competitor [13].

Table 1
Binding parameters and inositol phosphate (IP) response of wild type (WT) and chimeric cannabinoid receptors

	B_{max} (pmol/mg protein)	K_d (nM)	IP response (%)
Wild type			
WT CB_1	0.34 ± 0.13 (5)	0.58 ± 0.11 (6)	161.45 ± 3.89 (32)
WT CB_2	0.40 ± 0.17 (5)	0.67 ± 0.15 (5)	103.40 ± 2.22 (12)
Single mutant			
C2	0.45 ± 0.13 (5)	0.65 ± 0.12 (4)	104.19 ± 4.28 (10)$^+$
C3	0.33 ± 0.11 (3)	0.85 ± 0.17 (3)	130.99 ± 4.60 (11)$^{+,*}$
CT	0.32 ± 0.20 (3)	0.48 ± 0.26 (4)	116.64 ± 4.89 (9)$^{+,*}$
Double mutant			
C2C3	0.37 ± 0.08 (6)	0.57 ± 0.13 (7)	160.38 ± 6.81 (12)*
C2CT	0.36 ± 0.15 (4)	0.68 ± 0.21 (4)	133.72 ± 4.77 (13)$^{+,*}$
C3CT	0.37 ± 0.19 (4)	0.57 ± 0.13 (4)	124.27 ± 5.11 (12)$^{+,*}$
Triple mutant			
C2C3CT	0.40 ± 0.11 (6)	0.49 ± 0.08 (6)	160.23 ± 7.81 (15)*
i3 loop mutant			
C3ETM	0.32 ± 0.10 (3)	0.64 ± 0.17 (3)	154.58 ± 6.15 (9)*
C3T	0.44 ± 0.13 (5)	0.59 ± 0.16 (7)	147.75 ± 5.49 (6)*
C3EM	0.56 ± 0.22 (3)	0.57 ± 0.17 (3)	144.19 ± 4.49 (9)*
C3E	0.37 ± 0.11 (6)	0.54 ± 0.10 (10)	124.68 ± 5.67 (12)$^{+,*}$
C3M	0.39 ± 0.10 (4)	0.66 ± 0.17 (4)	110.43 ± 4.50 (13)$^+$
C3A	0.37 ± 0.15 (3)	0.55 ± 0.09 (3)	131.23 ± 9.57 (11)$^{+,*}$
C3AM	0.37 ± 0.17 (3)	0.54 ± 0.16 (4)	122.40 ± 4.85 (5)$^{+,*}$
C3S	0.49 ± 0.12 (3)	0.39 ± 0.12 (3)	120.77 ± 6.08 (6)$^+$

COS7 cells were transfected with the indicated cannabinoid receptor and $G_{\alpha16}$. Two days post-transfection, binding studies or IP accumulation experiments were performed. The B_{max} and K_d values were calculated as described in Section 2. The IP responses represent the percent of basal level of total IP accumulation in cells stimulated with WIN55,212-2 (10 μM). All results are expressed as mean ± S.E.M. The numbers in parentheses indicate the numbers of independent transfection experiments performed. The B_{max} and K_d values are not significantly different between the chimeras and the wild type CB_1 or CB_2 receptor.

$^+P < 0.05$, significantly different from WT CB_1 (Bonferroni post-hoc *t*-test); $^*P < 0.05$, significantly different from WT CB_2 (Bonferroni post-hoc *t*-test).

3. Results and discussion

As the first step toward understanding receptor–$G_{\alpha16}$ interaction, a series of CB_1/CB_2 chimeras were constructed by substituting different cytoplasmic regions of the CB_2 receptor with corresponding sequences of the CB_1 receptor. Since the ability to activate phospholipase C can be affected by the expression level or binding affinity of the chimeras, their B_{max} and K_d values were determined by [^{3}H]CP55,940 binding studies and were not significantly different from those of the wild type CB_1 or CB_2 receptor (Table 1). Therefore, changes in the level of activation were not due to differences in expression levels or agonist binding affinity of the chimeras.

Numerous studies on receptor–G_α interaction indicate that sequences in the i2 and i3 loops and the intracellular carboxy-terminus are responsible for the selectivity and affinity of receptor-G_α coupling (for reviews, see [14,15]). Therefore, single mutants were constructed with substitution of the i2 loop (C2), the i3 loop (C3) or the intracellular carboxy-terminus (CT) (Fig. 1A). Each of the chimeric receptors was co-expressed with $G_{\alpha16}$ in COS7 cells (Fig. 2A). As reported previously [9], WIN55,212-2 resulted in a concentration-depen-

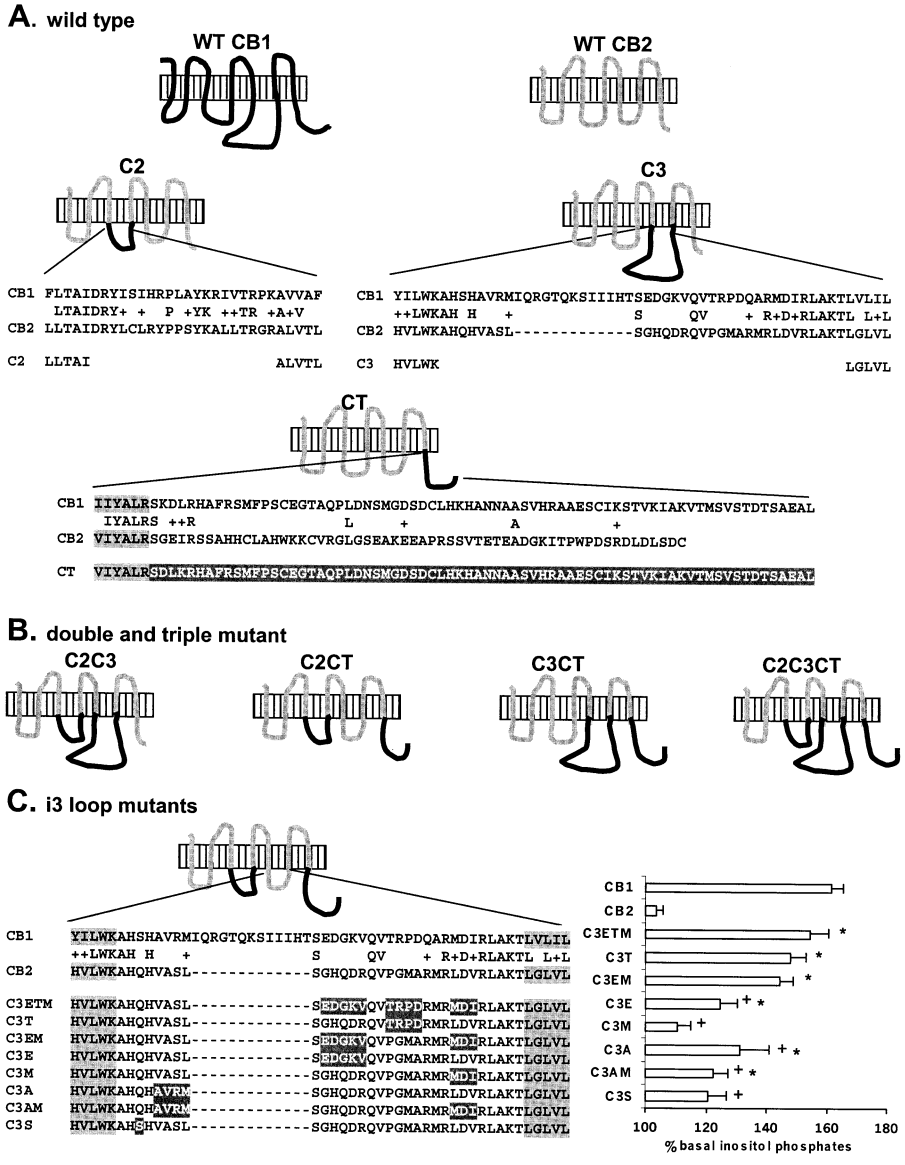
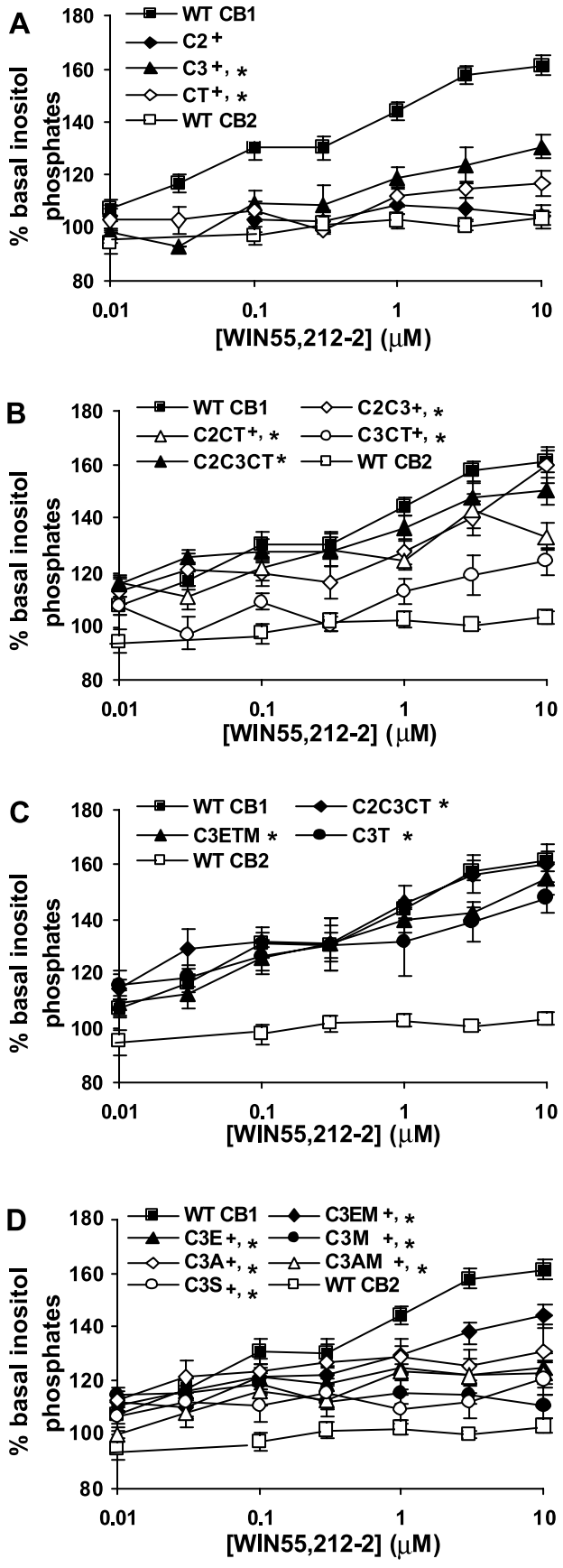


Fig. 1. Amino acid sequences and schematic representations of the CB_1, CB_2 and the CB_1/CB_2 chimeric receptors. A: Amino acid sequences of the CB_1 and CB_2 receptors and the single mutants C2, C3 and CT. Sequences were aligned with the CLUSTALW program. Conserved amino acids between the CB_1 and CB_2 receptors are marked as '+'. Gaps introduced for better alignment of the sequences are hyphenated. Putative transmembrane regions are shaded in light gray. Sequences of the CB_1 receptor in the chimeras are shown in white (with dark gray background). B: Schematics showing the structure of the double mutants C2C3, C2CT, C3CT and the triple mutant C2C3CT. C: Amino acid sequences of the i3 loop chimeras. Putative transmembrane regions are shaded in light gray. Sequences of the CB_1 receptor in the chimeras are shown in white. Bar graph (lower right) shows the stimulation of inositol phosphate formation when COS7 cells transfected with cDNAs encoding the indicated receptor and $G_{\alpha16}$ were stimulated with WIN55,212-2 (10 μM). Results were normalized to percent of basal level of total inositol phosphate accumulation in each experiment and are expressed as mean ± S.E.M. Results from cells co-expressing the indicated chimeric receptor and $G_{\alpha16}$ are significantly different from those co-expressing the CB_1 receptor and $G_{\alpha16}$ (Bonferroni post-hoc t-test, $^+P < 0.05$). Results from cells co-expressing the indicated chimeric receptor and $G_{\alpha16}$ are significantly different from those co-expressing the CB_2 receptor and $G_{\alpha16}$ (Bonferroni post-hoc t-test, $^*P < 0.05$).



dent increase in phospholipase C activation in cells expressing $G_{\alpha 16}$ and the wild type CB_1 but not the wild type CB_2 receptor. The CB_1 receptor did not couple to endogenous or co-expressed $G_{\alpha q}$ or $G_{\alpha 11}$ in COS7 cells, indicating that the phospholipase C activation was mediated by coupling of the CB_1 receptor to the expressed $G_{\alpha 16}$ [6]. In cells co-expressing the single mutants C3 or CT and $G_{\alpha 16}$, WIN55,212-2 produced phospholipase C activation greater than those expressing the CB_2 receptor and $G_{\alpha 16}$, but the level was significantly lower than that of the CB_1 receptor. These results indicate that substitution of a single cytoplasmic region is not sufficient to enable CB_2 receptors to fully activate $G_{\alpha 16}$ like the CB_1 receptor.

A second series of mutants (C2C3, C2CT, C3CT, C2C3CT, Fig. 1B) containing combinations of the cytoplasmic regions from the CB_1 receptor were, therefore, constructed and co-expressed with $G_{\alpha 16}$. The double mutants C2C3, C2CT and C3CT still showed significantly lower levels of phospholipase C activation than the wild type CB_1 receptor (Fig. 2B). However, the triple mutant C2C3CT showed a similar level of phospholipase C activation as the CB_1 receptor.

These findings suggest that all three intracellular regions are required for interacting with $G_{\alpha 16}$. Replacement of only one or two of these three regions enhanced the ability to activate phospholipase C but not to the same extent as the triple mutant that had all three regions replaced. Other studies on CB_1 receptor–$G_{\alpha i/o}$ interaction indicate that cytoplasmic domains of the CB_1 receptor differentially interact with specific $G_{\alpha i}$ and $G_{\alpha o}$ subtypes. Using GTPγS binding, anti-peptide antibody and immunoprecipitation approaches, the amino-terminus of the i3 loop and the carboxy-terminus of the CB_1 receptor were demonstrated to couple to $G_{\alpha i}$ and adenylyl cyclase inhibition. In addition, the carboxy-terminus of the CB_1 receptor was shown to associate with $G_{\alpha i3}$ and $G_{\alpha o}$, but not with $G_{\alpha i1}$ or $G_{\alpha i2}$ [16–18]. Therefore, different cytoplasmic regions of the CB_1 receptors are involved in interacting with $G_{\alpha 16}$, $G_{\alpha i}$ and $G_{\alpha o}$ subunits.

Many studies have shown the involvement of the amino- and carboxy-termini of the i3 loop in G protein coupling [9,14,15]. Amino acid sequences involved in $G_{\alpha q}$ or $G_{\alpha s}$ activation have been mapped to different domains of the i3 loop of the α_{1B}-adrenergic, M_1 muscarinic and V2 vasopressin receptors [9,14,15,19,20]. Therefore, another goal of this study is to examine the sub-domains within the i3 loop for coupling selectivity to $G_{\alpha 16}$. A series of chimeras that have the C2CT

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Fig. 2. Stimulation of inositol phosphate formation in COS7 cells co-expressing the wild type or chimeric receptor and $G_{\alpha 16}$. COS7 cells were co-transfected with cDNAs encoding the indicated receptor and $G_{\alpha 16}$ as described in Section 2. After 48 h, [^{3}H]inositol-labeled cells were incubated with and without (basal level) the indicated concentrations of WIN55,212-2 for 20 min in duplicates. Results were normalized to percent of basal level of total inositol phosphate accumulation in each experiment. Data are expressed as mean ± S.E.M. from at least three independent transfection experiments. Control COS7 cells that were untransfected, vector-transfected or transfected with the receptor alone did not result in significant changes in inositol phosphate accumulation over the same concentrations of WIN55,212-2 (data not shown). Results from cells co-expressing the indicated chimeric receptor and $G_{\alpha 16}$ are significantly different from those co-expressing the CB_1 receptor and $G_{\alpha 16}$ ($^+P < 0.05$, ANOVA). Results from cells co-expressing the indicated chimeric receptor and $G_{\alpha 16}$ are significantly different from those co-expressing the CB_2 receptor and $G_{\alpha 16}$ ($^*P < 0.05$, ANOVA).

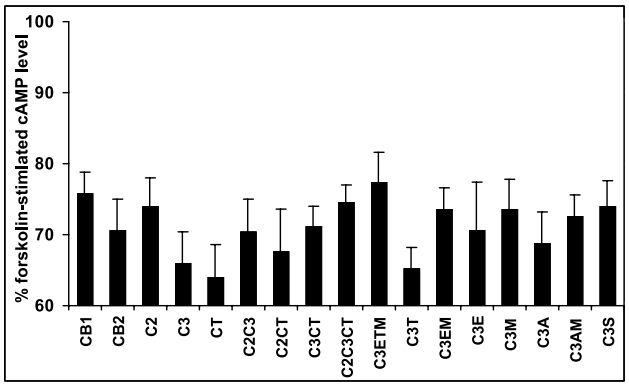

Fig. 3. Inhibition of forskolin-stimulated cAMP formation in wild type and chimeric CB_1/CB_2 receptors. COS7 cells were co-transfected with cDNAs encoding the indicated receptor and $G_{\alpha16}$ as described in Section 2. Two days post-transfection, the cells were stimulated with forskolin in the absence or presence of a maximal concentration of WIN55,212-2 (1 μM). Results were normalized to the percent of forskolin-stimulated cAMP level. Data are expressed as mean ± S.E.M. from at least three independent transfection experiments. The basal and forskolin-stimulated cAMP levels are 9.20 ± 0.83 and 39.91 ± 1.91 pmol/mg protein, respectively. Control cells that were vector-transfected or untransfected did not show any inhibition of forskolin-stimulated cAMP level.

backbone, as well as substitutions of the CB_1 receptor sequences into the i3 loop of the CB_2 receptor at regions that are different between the two receptors were constructed (i3 loop mutants, Fig. 1C). Two of the mutants with substitutions toward the carboxyl half of the i3 loop, C3ETM and C3T, showed phospholipase C activation similar to that of the CB_1 receptor and C2C3CT (Figs. 1C and 2C). Phospholipase C activation mediated by the other i3 loop mutants was still significantly different from the CB_1 receptor (Fig. 2D).

These findings suggested that the substituted amino acids EDGKV and TRPD toward the carboxy-terminus of the i3 loop of C3ETM and C3T are probably involved in interacting with $G_{\alpha16}$ or maintaining a proper conformation for the interaction. In contrast, substitution of sequences towards the N-terminus of the i3 loops did not confer the same degree of activation. The length of the i3 loop has also been suggested to contribute to G_α selectivity [19]. However, the length of the i3 loop in the active mutants C3ETM and C3T is the same as that of the CB_2 receptor, indicating that length is not a contributing factor in this case.

The $G_{\alpha16}$-coupled domains have also been studied in the α_{1B}-adrenergic receptor which can couple to $G_{\alpha q/11}$, $G_{\alpha14}$ and $G_{\alpha16}$ [7,9]. Mutagenesis studies of the i3 loop showed that only deletion of the amino half of the i3 loop, but not other deletions in the same loop, disrupted $G_{\alpha16}$ coupling. The difference between the two receptors may be related to the fact that the α_{1B}-adrenergic receptor can also interact with $G_{\alpha q/11}$ but the CB_1 receptor cannot [6]. In addition, because the amino-terminal mutant in their study also disrupted the coupling to $G_{\alpha q/11}$ and $G_{\alpha14}$ [9], it was not clear whether this mutant was functional.

Both the CB_1 and CB_2 receptors coupled to the inhibition of adenylyl cyclase [11,21]. To exclude the possibility that the decrease in phospholipase C activation was due to improper folding of the intracellular loops, the ability of these mutants to inhibit adenylyl cyclase was tested as an independent function (Fig. 3). All mutants examined were able to inhibit adenylyl cyclase to a similar extent as the wild type CB_1 or CB_2 receptors, indicating that the structure of the chimeras was not grossly altered. The chimeras maintained the ability to couple to adenylyl cyclase as an independent effector, probably through $G_{\alpha i/o}$.

In summary, a gain-of-function approach was used to successfully construct chimeric CB_1/CB_2 receptors that showed enhanced ability to activate phospholipase C. Receptor–$G_{\alpha16}$ interaction depends on the i2 loop, i3 loop and carboxy-terminus. Specific sub-domains within the i3 loop involved in coupling were also identified. Mutagenesis studies of some G protein-coupled receptors (e.g. muscarinic, β-adrenergic and V2 vasopressin receptors) demonstrated that the N- and/or C-termini of the i3 loop can form α-helices, which are suggested to be involved in G_α coupling. However, no structural element or sequence requirement can be generalized for all G protein-coupled receptors [14,15]. Taken together, our results further support the diversity of mechanisms by which receptor–G_α coupling selectivity can be achieved.

Acknowledgements: This work was supported by funding from NIH R29DA09857 to B.Y.H., R29HL54717 and AHA Grant-in-Aid 9951093Z to J.G.D.

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