



# Molecular Signature That Determines the Acute Tolerance of G Protein-Coupled Receptors

Chengchun Min, Xiaohan Zhang, Mei Zheng, Ningning Sun, Srijan Acharya, Xiaowei Zhang and Kyeong-Man Kim\*

Pharmacology Laboratory, College of Pharmacy, Chonnam National University, Gwangju 61186, Republic of Korea

## Abstract

Desensitization and acute tolerance are terms used to describe the attenuation of receptor responsiveness by prolonged or intermittent exposure to an agonist. Unlike desensitization of G protein-coupled receptors (GPCRs), which is commonly explained by steric hindrance caused by the  $\beta$ -arrestins that are translocated to the activated receptors, molecular mechanisms involved in the acute tolerance of GPCRs remain unclear. Our studies with several GPCRs and related mutants showed that the acute tolerance of GPCRs could occur independently of agonist-induced  $\beta$ -arrestin translocation. A series of co-immunoprecipitation experiments revealed a correlation between receptor tolerance and interactions among receptors,  $\beta$ -arrestin2, and  $G\beta\gamma$ .  $G\beta\gamma$  displayed a stable interaction with receptors and  $\beta$ -arrestin2 in cells expressing GPCRs that were prone to undergo tolerance compared to the GPCRs that were resistant to acute tolerance. Strengthening the interaction between  $G\beta\gamma$  and  $\beta$ -arrestin rendered the GPCRs to acquire the tendency of acute tolerance. Overall, stable interaction between the receptor and  $G\beta\gamma$  complex is required for the formation of a complex with  $\beta$ -arrestin, and determines the potential of a particular GPCR to undergo acute tolerance. Rather than turning off the signal,  $\beta$ -arrestins seem to contribute on continuous signaling when they are in the context of complex with receptor and  $G\beta\gamma$ .

**Key Words:** GPCR, Acute tolerance, Desensitization,  $G\beta\gamma$ ,  $\beta$ -Arrestin, Dopamine  $D_3$  receptor

## INTRODUCTION

Upon agonist stimulation, most GPCRs undergo conformational changes leading to the dissociation of  $G\alpha$  from  $G\beta\gamma$  (Hepler and Gilman, 1992). Dissociated  $G\beta\gamma$  potentiates receptor phosphorylation by mediating the association of GPCR kinase 2 (GRK2) with the plasma membrane, and activation of GRK2 by affecting the  $K_m$  and stoichiometry of phosphorylation (Benovic *et al.*, 1986; Pitcher *et al.*, 1992; Vickery and von Zastrow, 1999; Zheng *et al.*, 2016). GRK2-mediated receptor phosphorylation increases the affinity of  $\beta$ -arrestins for receptors, and thereby uncouples the receptors from G proteins (Benovic *et al.*, 1987; Lohse *et al.*, 1990) or enhances receptor endocytosis (Ferguson *et al.*, 1996).

Desensitization and tolerance of GPCRs are terms used to describe the attenuation of receptor responsiveness by prolonged or intermittent exposure to an agonist (Lohse *et al.*, 1989; Hausdorff *et al.*, 1990; Williams *et al.*, 2013). Desensitization and acute tolerance of GPCRs have different temporal

resolutions; i.e., desensitization is induced between seconds and minutes after agonist stimulation, whereas acute tolerance is induced between minutes and an hour (Williams *et al.*, 2013). According to the current paradigm based on studies of the  $\beta_2$  adrenergic receptor ( $\beta_2AR$ ), receptor phosphorylation and  $\beta$ -arrestin translocation are suggested to be two key cellular events responsible for GPCR desensitization.  $\beta$ -Arrestins which are recruited to the activated receptors sterically interfere with the interaction between receptors and G proteins (uncoupling), resulting in a decrement of signaling efficiency. However, molecular mechanism of acute tolerance is yet not clear. Acute tolerance occurs over a longer period of time than desensitization, and its molecular mechanism should include desensitization and subsequent cellular events, such as endocytosis and recycling.

In preliminary studies with five GPCRs and related mutants with different tolerance properties, agonist-induced  $\beta$ -arrestin translocation that mediated desensitization did not explain the acute tolerance. The aim of the current study was to under-

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\*Corresponding Author

E-mail: kmkim@jnu.ac.kr  
Tel: +82-62-530-2936, Fax: +82-62-530-2949

stand whether common features or principles exist that can predict or explain the acute tolerance of GPCRs.

## MATERIALS AND METHODS

### Materials

Dopamine (DA), isoproterenol (ISO), (-)-quinpirole (Quin), forskolin, and antibodies against actin and the FLAG epitope were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). [<sup>3</sup>H]-Sulpiride (84 Ci/mmol), [<sup>3</sup>H]-spiperone (85.5 Ci/mmol), and [<sup>3</sup>H]-CGP-12177 (41.7 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Antibodies against green fluorescent protein (GFP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Human embryonic kidney (HEK-293) cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub>.

### DNA constructs

Mammalian expression constructs for the human D<sub>2</sub> receptor (D<sub>2</sub>R, short alternatively spliced form), D<sub>3</sub> receptor (D<sub>3</sub>R), D<sub>4</sub> receptor (D<sub>4</sub>R), β<sub>1</sub> adrenergic receptor (β<sub>1</sub>AR), β<sub>2</sub>AR, GRK2, and β-arrestin2 have been described previously (Kim *et al.*, 2001; Cho *et al.*, 2006). β-Arrestin2-GFP, and the carboxyl terminus tail of GRK2 (GRK2-CT) were also described previously (Kim *et al.*, 2001; Zheng *et al.*, 2011). K149C-D<sub>2</sub>R, S145/6A-D<sub>3</sub>R, C147K-D<sub>3</sub>R, Gβ1, and chimeric receptors between D<sub>2</sub>R and D<sub>3</sub>R (D<sub>3</sub>R-[IL3-D<sub>2</sub>R] and D<sub>3</sub>R-[IL2-D<sub>2</sub>R]) were prepared by site-directed mutagenesis or were described previously (Bizzozero, 1997; Robinson and Caron, 1997; Zheng *et al.*, 2011; Min *et al.*, 2013). All receptors and related constructs were tagged with FLAG or HA epitopes at the N-terminus. Some constructs were tagged with GFP at the carboxyl terminus tail.

### Reporter gene assay

Cellular cAMP was measured by an indirect reporter gene method (Cho *et al.*, 2010a; Zheng *et al.*, 2011). Briefly, a reporter system with a plasmid containing the firefly luciferase gene under the control of multiple cAMP responsive elements, and with a pRL-TK control vector, was used. Transfected cells were seeded in 24-well plates, and each transfection set was organized into three identical groups. For D<sub>2</sub>R, D<sub>3</sub>R, and D<sub>4</sub>R, cells were treated with 2 µM forskolin and Quin (10<sup>-12</sup>-10<sup>-8</sup> M) for 4 h and harvested. Relative luciferase expression was measured using a dual luciferase assay kit (Promega, Madison, WI, USA). For β<sub>1</sub>AR and β<sub>2</sub>AR, cells were treated with increasing concentrations of ISO.

### Induction of tolerance

Tolerance was induced as described previously (Min *et al.*, 2013). Cells expressing the corresponding GPCR were treated with agonist for 1-5 min (first treatment) to trigger tolerance, and washed five times with serum-free medium (5 min per wash) at 37°C to remove all of the receptor-bound agonist. Cells were then re-challenged with agonist to induce the second response. The occurrence of tolerance was determined by comparing the amplitudes of the second response from the cells pre-treated with vehicle or agonist in the first treatment

step.

### Immunoprecipitation

Immunoprecipitation was conducted as described previously (Zheng *et al.*, 2016). Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) for 1 h at 4°C with gentle mixing. A slurry of agarose beads coated with anti-FLAG antibodies (25 µL) was mixed with the supernatant for 2-3 hr on a rotation wheel at 4°C. Beads were washed with washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40) three times for 5 min each at 4°C. The resulting immunoprecipitates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane was blotted with antibodies to corresponding target proteins.

### Confocal microscopy

One day after the transfection, cells were seeded onto the 35-mm confocal dishes, which contain a 1-cm well in the center sealed by glass coverslip on the bottom, and they were allowed to recover for 1 day. Then, the cells were examined with a TCS SP5/AOBS/Tandem laser scanning confocal microscope (Leica, Jena, Germany).

### Statistical analysis

All results are expressed as means ± SEM. Dose-response curves with more than three experimental groups were compared via two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests. Student's *t*-test was used for some results. *p*-values of less than 0.05 were considered significant.

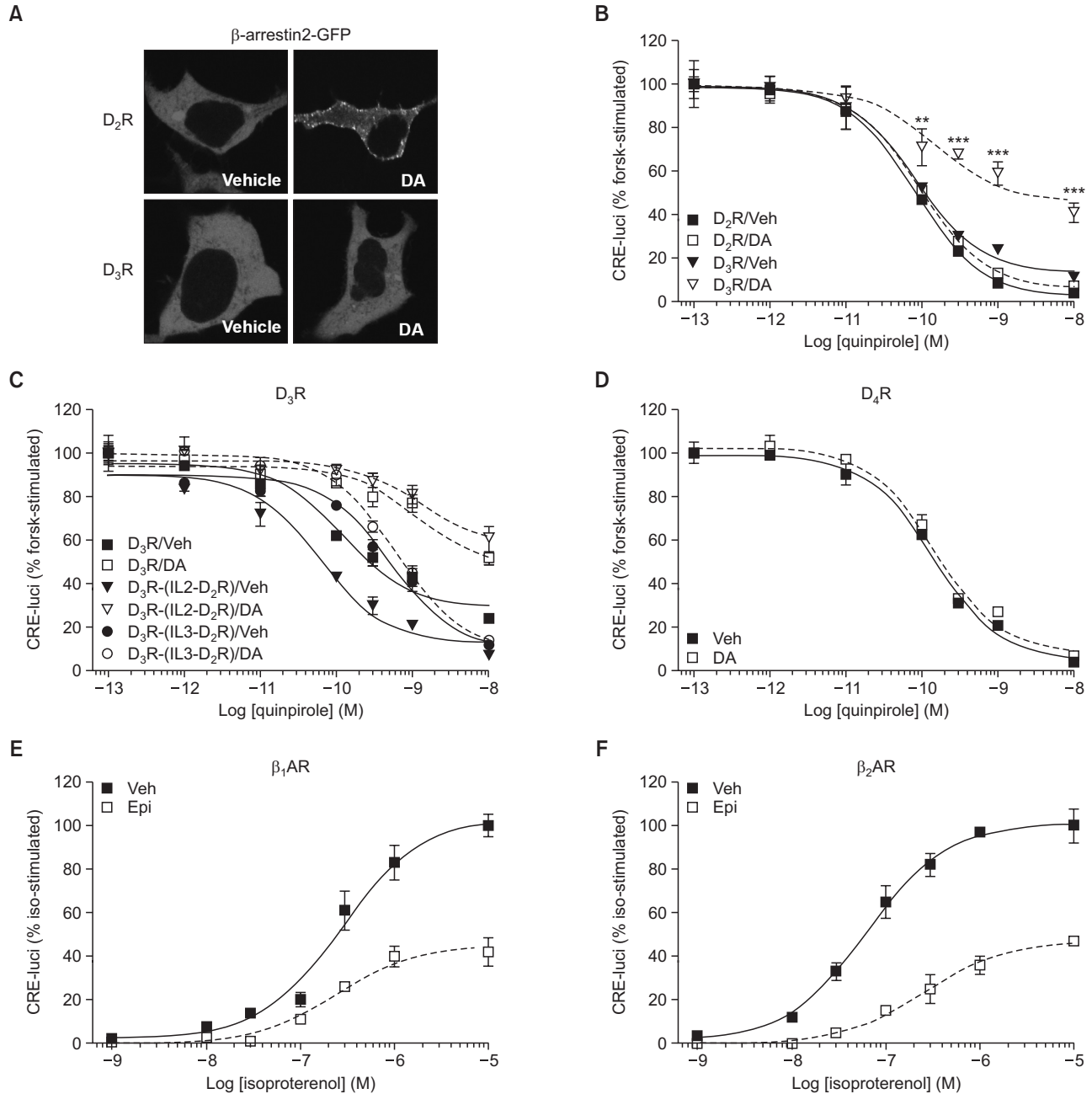
## RESULTS

### Rapid desensitization and acute tolerance are caused by different molecular mechanisms

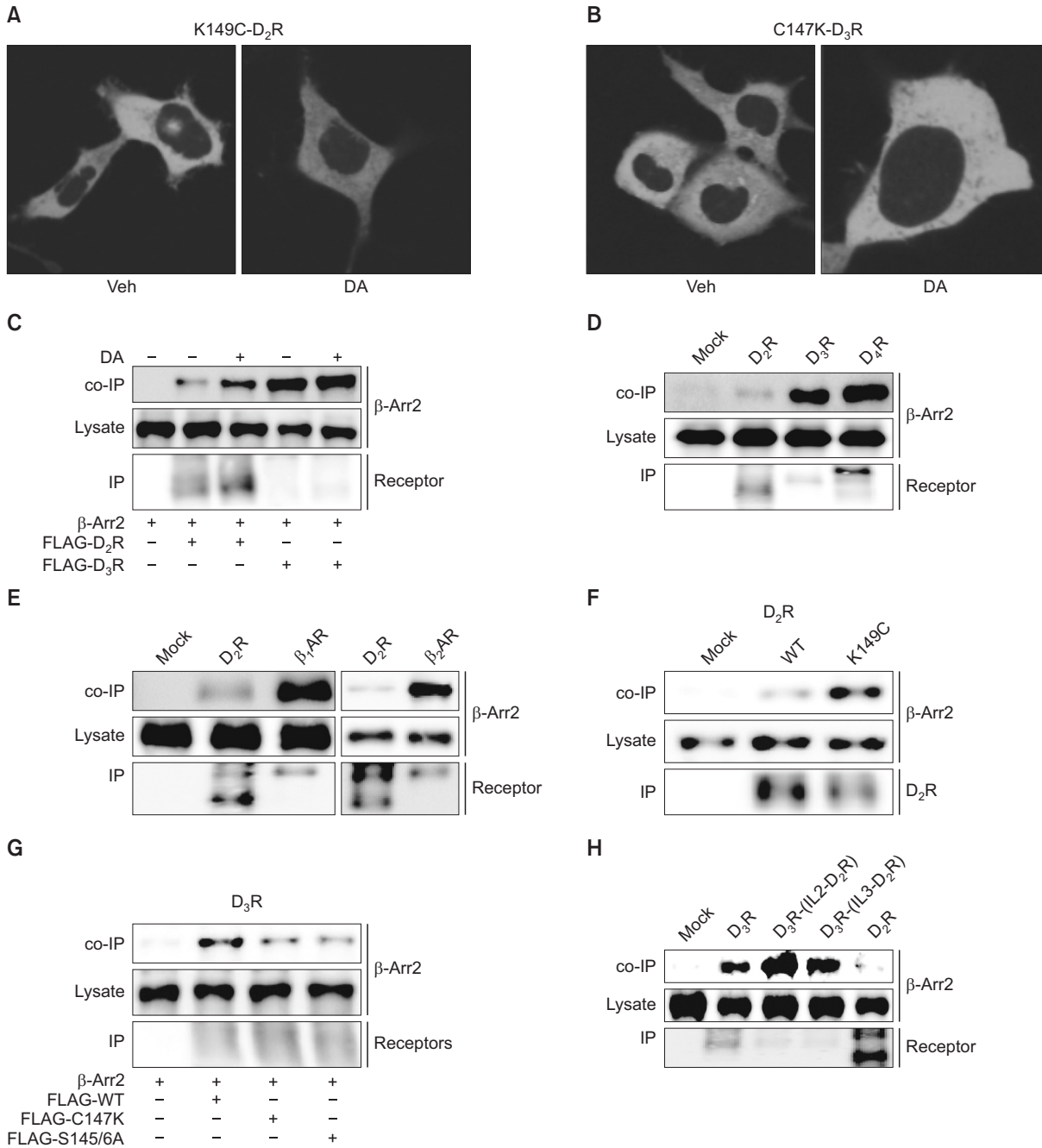
Dopamine D<sub>2</sub> and D<sub>3</sub> receptors (D<sub>2</sub>R, D<sub>3</sub>R) show high homology in amino acid composition (46% overall amino acid homology and 78% identity in the transmembrane domain) (Giros *et al.*, 1990) and share most of their signaling pathways (Cho *et al.*, 2010b). Nevertheless, D<sub>2</sub>R and D<sub>3</sub>R display different intracellular trafficking properties and employ different regulatory mechanisms for given signaling pathways (Cho *et al.*, 2007). It was reported that D<sub>2</sub>R but not D<sub>3</sub>R is phosphorylated in response to agonist stimulation (Kim *et al.*, 2001). Similarly, β-arrestin translocation was observed in cells expressing D<sub>2</sub>R but not D<sub>3</sub>R (Fig. 1A), suggesting that D<sub>2</sub>R but not D<sub>3</sub>R would undergo desensitization. If the same molecular mechanism is involved in the development of desensitization and acute tolerance, D<sub>2</sub>R but not D<sub>3</sub>R is expected to undergo tolerance. As shown in Fig. 1B, D<sub>3</sub>R but not D<sub>2</sub>R showed tolerance.

Results in Fig. 1A and 1B suggest that molecular mechanisms other than β-arrestin translocation could be involved in the development of acute tolerance of GPCRs. To determine whether this observation is generalizable, five different GPCRs (D<sub>2</sub>R, D<sub>3</sub>R, D<sub>4</sub>R, β<sub>1</sub>AR, β<sub>2</sub>AR) were selected. In addition, five mutant receptors of D<sub>2</sub>R and D<sub>3</sub>R, in which the tolerance properties or β-arrestin translocation propensity were altered, were utilized. Using these receptors, the correlation between β-arrestin translocation and their acute tolerance was evaluated.

D<sub>3</sub>R (Fig. 1B), β<sub>1</sub>AR (Fig. 1E), β<sub>2</sub>AR (Fig. 1F), a point mutant



**Fig. 1.** Characterization of acute tolerance of various GPCRs. (A) Visualization of  $\beta$ -arrestin2-GFP translocation in response to agonist activation of D<sub>2</sub>R or D<sub>3</sub>R in HEK-293 cells. Cells stably expressing D<sub>2</sub>R or D<sub>3</sub>R (2.5 and 3.3 pmol/mg protein, respectively) were transfected with 2  $\mu$ g  $\beta$ -arrestin2-GFP per 100 mm culture dish. Cells were stimulated with 10  $\mu$ M DA for 5 min. (B) Determination of tolerance development in cells expressing D<sub>2</sub>R and D<sub>3</sub>R. HEK-293 cells expressing D<sub>2</sub>R or D<sub>3</sub>R were pre-treated with either serum-free medium containing 100  $\mu$ M ascorbic acid (Veh) or 10  $\mu$ M DA dissolved in Veh for 5 min. Cells were washed five times with warm (37°C) serum-free medium and treated with increasing concentrations of Quin. The cellular levels of cAMP were then measured using the CRE-luciferase reporter gene assay. "Forsk" represents forskolin. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  when the D<sub>3</sub>R/Veh group was compared with other experimental groups. (C) Cells expressing D<sub>3</sub>R, D<sub>3</sub>R-[IL2-D<sub>2</sub>R], or D<sub>3</sub>R-[IL3-D<sub>2</sub>R] were treated with 10  $\mu$ M DA for 5 min to induce tolerance. The results for the Veh-treated group significantly differed from those for the DA-treated groups of D<sub>3</sub>R or D<sub>3</sub>R-[IL2-D<sub>2</sub>R] expressing cells at treatment concentrations between  $10^{-10}$ - $10^{-8}$  M ( $p < 0.001$ ). (D) Cells expressing D<sub>4</sub>R were treated with 10  $\mu$ M DA for 5 min to induce tolerance. (E, F) Cells expressing either  $\beta_1$ AR (E) or  $\beta_2$ AR (F) were treated with vehicle (Veh) or 10  $\mu$ M epinephrine (Epi) for 5 min, washed three times with serum-free media at 37°C, then treated with increasing concentrations of isoproterenol to obtain dose-response curves. The Veh group of  $\beta_1$ AR was significantly different from the Epi group at treatment concentrations between  $3 \times 10^{-7}$ - $3 \times 10^{-5}$  M ( $p < 0.001$ ). The results for the Veh group of  $\beta_2$ AR significantly differed from those for the corresponding Epi group at treatment concentrations between  $10^{-8}$ - $3 \times 10^{-5}$  M ( $p < 0.001$ ). For (C-F) receptor expression levels were maintained between 1.5 and 2.5 pmol/mg protein.

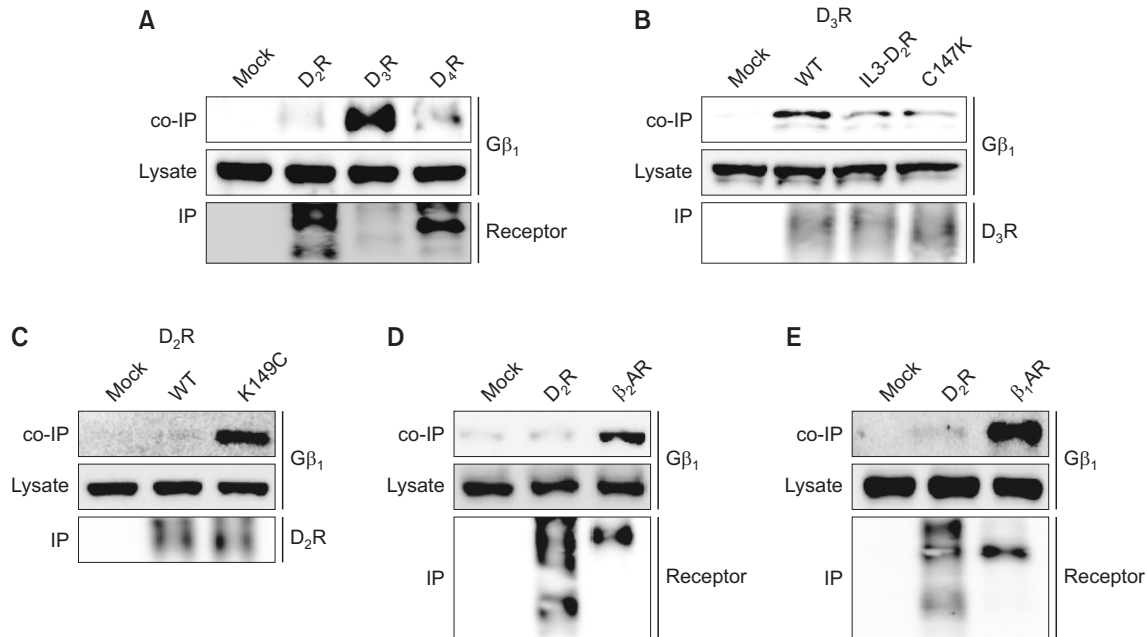


**Fig. 2.** Relationship between receptor/β-arrestin interactions at the basal state and receptor tolerance. (A) HEK-293 cells were transfected with K149C-D<sub>2</sub>R together with GFP-β-arrestin2. Cells were treated with 10 μM DA for 5 min. Receptor expression level was 2.5 pmol/mg protein. (B) HEK-293 cells were transfected with C147K-D<sub>3</sub>R together with GFP-β-arrestin2. Cells were treated with 10 μM DA for 5 min. Receptor expression level was 2.4 pmol/mg protein. (C-H) Cell lysates were immunoprecipitated with FLAG beads. Samples were analyzed on SDS-PAGE. Immunoprecipitates were immunoblotted with antibodies against FLAG; co-immunoprecipitates and lysates were immunoblotted with antibodies against β-arrestin2. All data represent results from three to seven independent experiments with similar outcomes. Receptor expression levels were maintained between 2.1 and 2.5 pmol/mg protein. (C) Cells were transfected with FLAG-tagged D<sub>2</sub>R or D<sub>3</sub>R along with β-arrestin2. Cells were treated with 1 μM DA for 5 min. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.45, D<sub>2</sub>R/DA (-); 2.71 ± 1.45, D<sub>2</sub>R/DA (+); 7.21 ± 3.15, D<sub>3</sub>R/DA (-); 6.82 ± 2.92, D<sub>3</sub>R/DA (+). The D<sub>2</sub>R/DA (-) group is significantly different from the D<sub>2</sub>R/DA (+) (*p* < 0.05, *n* = 7) and D<sub>3</sub>R groups (*p* < 0.001, *n* = 6). (D) Cells were transfected with FLAG-tagged D<sub>2</sub>R, D<sub>3</sub>R, or D<sub>4</sub>R along with β-arrestin2. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.19, D<sub>2</sub>R; 7.2 ± 3.1, D<sub>3</sub>R; 8.8 ± 3.5, D<sub>4</sub>R. D<sub>2</sub>R is significantly different from other groups (*p* < 0.01, *n* = 3). (E) Cells were transfected with FLAG-tagged D<sub>2</sub>R, β<sub>1</sub>AR, or β<sub>2</sub>AR along with β-arrestin2. (Left panel) Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.13, D<sub>2</sub>R; 6.53 ± 2.06, β<sub>1</sub>AR (*p* < 0.01, *n* = 3). (Right panel) Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.12, D<sub>2</sub>R; 7.59 ± 2.33, β<sub>2</sub>AR. D<sub>2</sub>R is significantly different from β<sub>2</sub>AR group (*p* < 0.01, *n* = 3). (F) Cells were transfected with FLAG-tagged D<sub>2</sub>R or K149C-D<sub>2</sub>R along with β-arrestin2. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.12, WT-D<sub>2</sub>R; 5.57 ± 1.69, K149C-D<sub>2</sub>R (*p* < 0.01, *n* = 3). (G) Cells were transfected with FLAG-tagged WT-D<sub>3</sub>R, C147K-D<sub>3</sub>R, or S145/6A-D<sub>3</sub>R at the ratio of 1:6:7, along with β-arrestin2. Ratios of immunoprecipitation which were normalized to WT-D<sub>3</sub>R, are: 1.0 ± 0.25, WT; 0.29 ± 0.04, C147K; 0.18 ± 0.03, S145/6A. The WT group is significantly different from the C147K (*p* < 0.05, *n* = 3) and S145/6A groups (*p* < 0.01, *n* = 3). (H) Cells were transfected with FLAG-tagged D<sub>2</sub>R, D<sub>3</sub>R-[IL2-D<sub>2</sub>R], D<sub>3</sub>R-[IL3-D<sub>2</sub>R], or D<sub>2</sub>R along with β-arrestin2. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.14, D<sub>2</sub>R; 7.29 ± 2.31, D<sub>3</sub>R; 10.25 ± 3.25, D<sub>3</sub>R-[IL2-D<sub>2</sub>R]; 8.21 ± 2.59, D<sub>3</sub>R-[IL3-D<sub>2</sub>R]. D<sub>2</sub>R is significantly different from the other groups (*p* < 0.01, *n* = 3).

**Table 1.** Relationship between  $\beta$ -arrestin translocation and tolerance of GPCRs

Receptor	$\beta_1$ AR	$\beta_2$ AR	D <sub>2</sub> R	D <sub>3</sub> R	D <sub>4</sub> R	K149C-D <sub>2</sub> R	C147K-D <sub>3</sub> R	D <sub>3</sub> R-(IL2-D <sub>2</sub> R)	D <sub>3</sub> R-(IL3-D <sub>2</sub> R)
Tolerance	+++	++++	-	+++	+/-	+++	-	++++	+
$\beta$ -Arrestin translocation	+	++++	+++	-	+/-	+	-	-	+

+: Represents the extent of agonist-induced  $\beta$ -arrestin translocation or tolerance.



**Fig. 3.** Relationship between receptor/ $G\beta\gamma$  interactions at the basal state and receptor tolerance. (A-E) Cell lysates were immunoprecipitated with FLAG beads. Samples were analyzed on SDS-PAGE. Immunoprecipitates were immunoblotted with antibodies against FLAG; co-immunoprecipitates and lysates were immunoblotted with antibodies against GFP. All data represent results from three to four independent experiments with similar outcomes. Receptor expression levels were maintained between 2.1 and 2.5 pmol/mg protein. (A) Cells were transfected with FLAG-tagged D<sub>2</sub>R, D<sub>3</sub>R, or D<sub>4</sub>R along with yellow fluorescent protein-tagged  $G\beta_1$  (YFP- $G\beta_1$ ). Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are:  $1.0 \pm 0.21$ , D<sub>2</sub>R;  $12.17 \pm 3.73$ , D<sub>3</sub>R;  $2.31 \pm 0.53$ , D<sub>4</sub>R. The D<sub>3</sub>R group is significantly different from the D<sub>4</sub>R ( $p < 0.05$ ,  $n = 3$ ) and D<sub>2</sub>R groups ( $p < 0.01$ ,  $n = 3$ ). (B) Cells were transfected with FLAG-tagged D<sub>3</sub>R, D<sub>3</sub>R-[IL3-D<sub>2</sub>R], or C147K-D<sub>3</sub>R at the ratio of 1:1:7 along with YFP- $G\beta_1$ . Ratios of immunoprecipitation which were normalized to WT-D<sub>3</sub>R, are:  $1.0 \pm 0.18$ , WT;  $0.33 \pm 0.15$ , IL3-D<sub>2</sub>R;  $0.30 \pm 0.16$ , C147K. The WT group was significantly different from the other groups ( $p < 0.01$ ,  $n = 3$ ). (C) Cells were transfected with FLAG-tagged D<sub>2</sub>R or K149C-D<sub>2</sub>R along with YFP- $G\beta_1$ . Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are:  $1.0 \pm 0.25$ , WT;  $9.87 \pm 3.01$ , K149C ( $p < 0.01$ ,  $n = 3$ ). (D) Cells were transfected with FLAG-tagged D<sub>2</sub>R or  $\beta_2$ AR along with YFP- $G\beta_1$ . Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are:  $1.0 \pm 0.22$ , D<sub>2</sub>R;  $5.63 \pm 1.57$ ,  $\beta_2$ AR ( $p < 0.01$ ,  $n = 3$ ). (E) Cells were transfected with FLAG-tagged D<sub>2</sub>R or  $\beta_1$ AR along with YFP- $G\beta_1$ . Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are:  $1.0 \pm 0.33$ , D<sub>2</sub>R;  $7.14 \pm 2.02$ ,  $\beta_1$ AR ( $p < 0.01$ ,  $n = 3$ ).

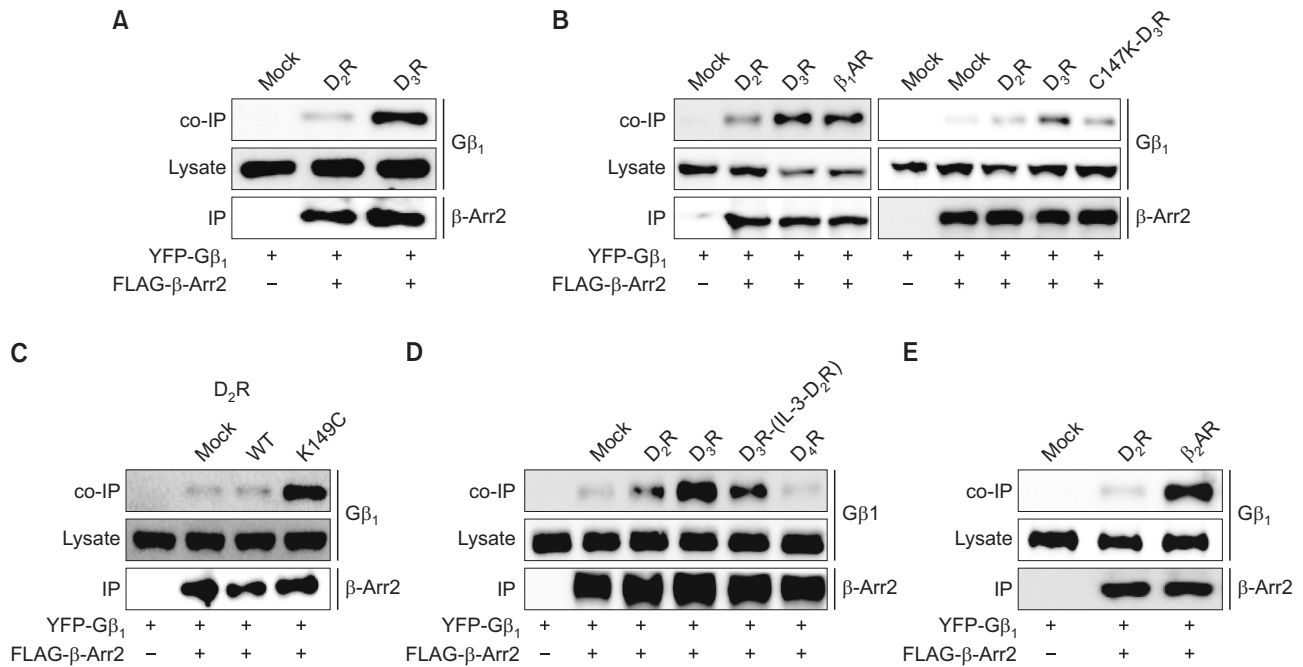
of D<sub>2</sub>R within the second intracellular loop (K149C-D<sub>2</sub>R) (Min *et al.*, 2013), and a chimeric D<sub>3</sub>R whose second intracellular loop was replaced with that of D<sub>2</sub>R, D<sub>3</sub>R-[IL2-D<sub>2</sub>R] (Fig. 1C), all showed tolerance. D<sub>2</sub>R showed a noticeable agonist-induced  $\beta$ -arrestin translocation (Fig. 1A), but K149C-D<sub>2</sub>R showed negligible translocation (Fig. 2A). In addition, only negligible  $\beta$ -arrestin translocation was reported in cells expressing  $\beta_1$ AR (Shiina *et al.*, 2000) and D<sub>3</sub>R-[IL2-D<sub>2</sub>R] (Kim *et al.*, 2001).

D<sub>2</sub>R (Fig. 1B), dopamine D<sub>4</sub> receptor (D<sub>4</sub>R) (Fig. 1D), a point mutant of D<sub>3</sub>R within the second intracellular loop (C147K-D<sub>3</sub>R) (Min *et al.*, 2013), and a chimeric D<sub>3</sub>R in which the third intracellular loop was replaced with that of D<sub>2</sub>R (D<sub>3</sub>R-[IL3-D<sub>2</sub>R]) (Fig. 1C), did not show tolerance. Of these receptors that did not undergo tolerance, D<sub>2</sub>R still mediated noticeable agonist-induced translocation of  $\beta$ -arrestin (Fig. 1A). However, only negligible  $\beta$ -arrestin translocation was observed or

reported in cells expressing C147K-D<sub>3</sub>R (Fig. 2B), D<sub>4</sub>R (Cho *et al.*, 2006), or D<sub>3</sub>R-[IL3-D<sub>2</sub>R] (Kim *et al.*, 2001). These results are summarized in Table 1, and overall suggest that the agonist-induced translocation of  $\beta$ -arrestin, which is responsible for the development of desensitization of GPCRs, is not a determining factor for the acute tolerance of the GPCRs that were tested in this study. Thus, certain cellular processes other than  $\beta$ -arrestin translocation are likely to play a role in the development of tolerance.

### The basal interaction between receptors and $\beta$ -arrestin2 does not determine acute tolerance of GPCRs

Our results showed that agonist-induced  $\beta$ -arrestin translocation does not properly explain acute tolerance, although  $G\beta\gamma$  and  $\beta$ -arrestins are still involved in the acute tolerance of D<sub>3</sub>R (Min *et al.*, 2013). Thus, we conducted a series of co-



**Fig. 4.** Relationship between Gβ<sub>γ</sub>/β-arrestin2 interactions at the basal state and receptor tolerance. (A-E) Cell lysates were immunoprecipitated with FLAG beads. Samples were analyzed on SDS-PAGE. Immunoprecipitates were immunoblotted with antibodies against FLAG; co-immunoprecipitates and lysates were immunoblotted with antibodies against GFP. Receptor expression levels were maintained between 1.5 and 2.0 pmol/mg protein. (A) Cells were transfected with D<sub>2</sub>R or D<sub>3</sub>R along with FLAG-β-arrestin2 and YFP-Gβ<sub>1</sub>. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.37, D<sub>2</sub>R; 4.67 ± 1.29, D<sub>3</sub>R (*p*<0.01, *n*=4). (B) Cells were transfected with D<sub>2</sub>R, D<sub>3</sub>R, β<sub>1</sub>AR, or C147K-D<sub>3</sub>R at the ratio of 0.7:1:1:7, along with FLAG-β-arrestin2 and YFP-Gβ<sub>1</sub>. (Left panel) Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.21, D<sub>2</sub>R; 3.80 ± 1.31, D<sub>3</sub>R; 3.50 ± 1.52, β<sub>1</sub>AR. The D<sub>2</sub>R group is significantly different from other groups (*p*<0.05, *n*=3). (Right panel) The immunoprecipitate/lysate ratios in the right panel, normalized to D<sub>2</sub>R, are: 1.0 ± 0.30, D<sub>2</sub>R; 3.71 ± 1.18, D<sub>3</sub>R; 1.73 ± 0.32, C147K-D<sub>3</sub>R. The D<sub>3</sub>R group is significantly different from other groups (*p*<0.05, *n*=3). (C) Cells were transfected with D<sub>2</sub>R or K149C-D<sub>2</sub>R along with FLAG-β-arrestin2 and YFP-Gβ<sub>1</sub>. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.27, WT; 7.78 ± 2.48, K149C (*p*<0.01, *n*=3). (D) Cells were transfected with D<sub>2</sub>R, D<sub>3</sub>R, D<sub>3</sub>R-[IL3-D<sub>2</sub>R], or D<sub>4</sub>R along with FLAG-β-arrestin2 and YFP-Gβ<sub>1</sub>. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.33, D<sub>2</sub>R; 6.67 ± 1.32, D<sub>3</sub>R; 1.89 ± 0.42, D<sub>3</sub>R-[IL3-D<sub>2</sub>R]; 0.17 ± 0.21, D<sub>4</sub>R. The D<sub>3</sub>R group is significantly different from other groups (*p*<0.01, *n*=3). (E) Cells were transfected with D<sub>2</sub>R or β<sub>2</sub>AR along with FLAG-β-arrestin2 and YFP-Gβ<sub>1</sub>. Ratios of immunoprecipitation which were normalized to D<sub>2</sub>R, are: 1.0 ± 0.27, D<sub>2</sub>R; 6.59 ± 2.07, β<sub>2</sub>AR (*p*<0.01, *n*=3).

immunoprecipitation experiments and attempted to correlate acute tolerance with interactions among the receptors, Gβ<sub>γ</sub>, and β-arrestin2.

The interaction between receptors and β-arrestin2 was examined initially. The receptors that underwent tolerance (D<sub>3</sub>R, β<sub>1</sub>AR, β<sub>2</sub>AR, K149C-D<sub>2</sub>R, and D<sub>3</sub>R-[IL2-D<sub>2</sub>R]) showed a stronger basal interaction with β-arrestin2 (Fig. 2C, 2E, 2F, 2H) than the receptors that did not undergo tolerance (D<sub>2</sub>R, C147K-D<sub>3</sub>R, and S145/6-D<sub>3</sub>R) (Fig. 2C, 2G). However, some receptors that did not undergo tolerance (D<sub>4</sub>R and D<sub>3</sub>R-[IL3-D<sub>2</sub>R]) still showed abundant interactions with β-arrestin2 (Fig. 2D, 2H), suggesting that the basal interaction between receptors and β-arrestin2 does not properly explain the tolerance of some GPCRs.

**Stable interaction between receptor and Gβ<sub>γ</sub> is observed in the cells that express the GPCRs which are prone to undergo acute tolerance**

When the interaction between receptors and Gβ<sub>γ</sub> was tested, all receptors that underwent acute tolerance (D<sub>3</sub>R, K149C-D<sub>2</sub>R, β<sub>2</sub>AR, and β<sub>1</sub>AR) showed a stronger basal interaction with Gβ<sub>γ</sub> than did receptors that did not undergo acute tolerance (D<sub>2</sub>R, D<sub>4</sub>R, D<sub>3</sub>R-[IL3-D<sub>2</sub>R], and C147K-D<sub>3</sub>R) (Fig. 3).

These results suggest that the interaction between receptors and Gβ<sub>γ</sub> could be closely related to the development of receptor tolerance.

**Stable interaction between Gβ<sub>γ</sub> and β-arrestin is observed in the cells that express the GPCRs which are prone to undergo acute tolerance**

Next, interaction between Gβ<sub>γ</sub> and β-arrestin2 was tested. There was a stronger interaction between Gβ<sub>γ</sub> and β-arrestin in cells expressing the GPCRs that showed acute tolerance (D<sub>3</sub>R, β<sub>1</sub>AR, K149C-D<sub>2</sub>R, D<sub>3</sub>R-[IL2-D<sub>2</sub>R], β<sub>2</sub>AR) than in cells expressing GPCRs that did not undergo acute tolerance (D<sub>2</sub>R, C147K-D<sub>3</sub>R, D<sub>4</sub>R) (Fig. 4). The results shown in Fig. 3 and 4 suggest that the presence of β-arrestin2 in the receptor-Gβ<sub>γ</sub> complex determines the ability of a particular GPCR to undergo tolerance. These results are summarized in Table 2.

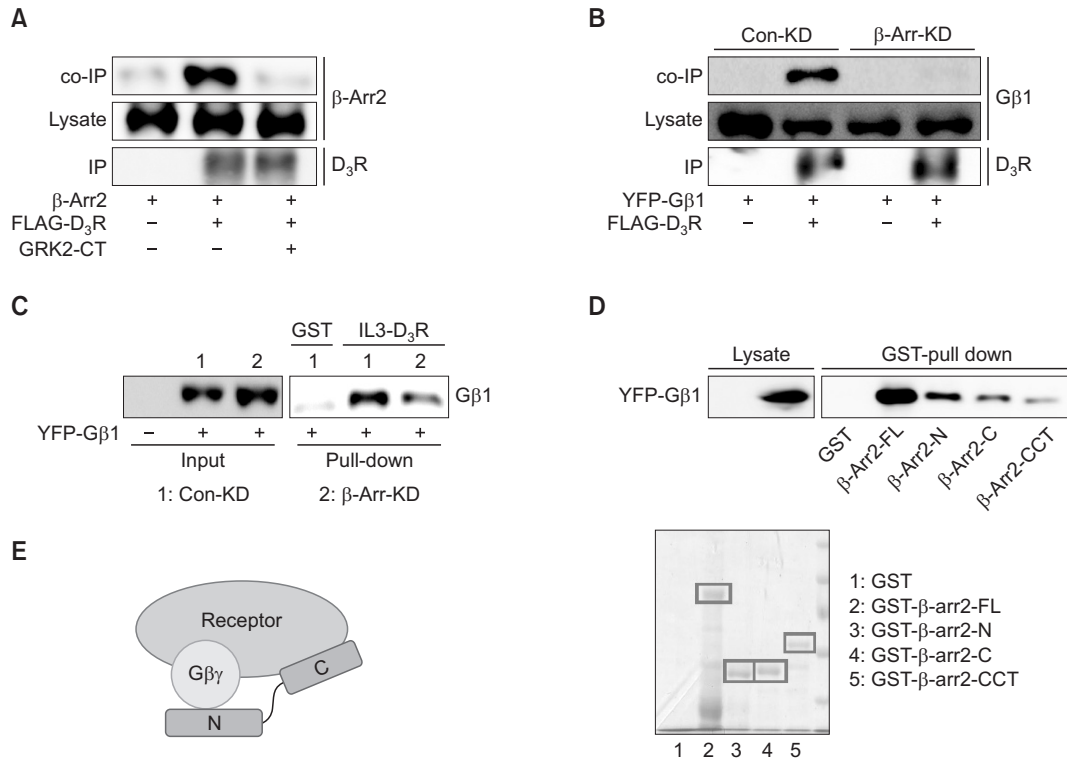
**The N domain of β-arrestin2 is involved in its interaction with D<sub>3</sub>R in collaboration with Gβ<sub>γ</sub>**

Because the simultaneous interaction of each GPCR with Gβ<sub>γ</sub> and β-arrestin was required for acute tolerance, we investigated whether each pair of proteins from the receptor-Gβ<sub>γ</sub>-β-arrestin complex could interact independently, or whether all

**Table 2.** Relationship between receptor/ $\beta$ -arrestin, receptor/ $G\beta\gamma$ , and  $G\beta\gamma/\beta$ -arrestin interactions at basal state and tolerance of GPCRs

Receptor	$\beta_1$ AR	$\beta_2$ AR	K149C-D <sub>2</sub> R	D <sub>3</sub> R	D <sub>2</sub> R	D <sub>4</sub> R	C147K-D <sub>3</sub> R	D <sub>3</sub> R-(IL3-D <sub>2</sub> R)
Tolerance	+++	++++	+++	+++	-	+/-	-	+
Receptor/Arrestin	+++	++++	+++	+++	+	++++	-	++++
Receptor/ $G\beta\gamma$	+++	++++	+++	+++	-	+/-	-	-
$G\beta\gamma$ /Arrestin	+++	++++	+++	+++	+	+/-	-	+

+: Represents the extent of agonist-induced tolerance, and immunoprecipitation between receptor and  $G\beta\gamma$  or between  $G\beta\gamma$  and  $\beta$ -arrestin at basal state.



**Fig. 5.** Mutual requirement of  $G\beta\gamma$  and  $\beta$ -arrestin2 for the interaction and tolerance of D<sub>3</sub>R. (A) Cells expressing D<sub>3</sub>R were transfected with  $\beta$ -arrestin2 and/or GRK2-CT. Cell lysates were immunoprecipitated with FLAG beads. Cell lysates were immunoprecipitated with FLAG beads. Samples were analyzed on SDS-PAGE. Immunoprecipitates were immunoblotted with antibodies against FLAG; co-immunoprecipitates and lysates were immunoblotted with antibodies against  $\beta$ -arrestin2. The ratios of immunoprecipitation are: 5.49  $\pm$  1.32, Mock; 1.02  $\pm$  0.29, GRK2-CT ( $p < 0.01$ ,  $n = 3$ ). (B) Con-KD and  $\beta$ -arrestin1/2-KD cells expressing FLAG-D<sub>3</sub>R were transfected with YFP-Gβ1. Cell lysates were immunoprecipitated with FLAG beads. Immunoprecipitates were immunoblotted with antibodies against FLAG; co-immunoprecipitates and lysates were immunoblotted with antibodies against GFP. The ratios of immunoprecipitation are 5.71  $\pm$  1.28, Con-KD; 0.51  $\pm$  0.03,  $\beta$ -Arr-KD ( $p < 0.01$ ,  $n = 3$ ). (C) A GST pull-down assay was conducted to determine the requirement of  $\beta$ -arrestin for the interaction between the third cytoplasmic loop of D<sub>3</sub>R (IL3-D<sub>3</sub>R) and  $G\beta\gamma$ . For production of GST fusion proteins, 0.5 mM of IPTG was added to the bacterial cultures containing each plasmid for 2 hrs. Bacterial cell lysates containing GST or GST-IL3-D<sub>3</sub>R (Zheng *et al.*, 2011) were incubated with cell lysates from control knockdown (Con-KD) and  $\beta$ -arrestin1/2-KD cells that expressed YFP-Gβ1. Lower panel shows an SDS-PAGE analysis of the afterwash of bacterial cell lysates. The ratios of pull-down are 3.39, Con-KD; 1.0,  $\beta$ -Arr-KD. The data represent results of two independent experiments with similar outcomes. (D) Interaction between  $\beta$ -arrestin2 and  $G\beta\gamma$  was determined by a GST pull-down assay. Bacterial lysates containing the GST fusion proteins of the full-length (GST- $\beta$ -arr2-FL), N-domain (GST- $\beta$ -arr2-N), C-domain (GST- $\beta$ -arr2-C), or C-domain plus carboxyl tail of rat  $\beta$ -arrestin2 (GST- $\beta$ -arr2-CCT) (Zheng *et al.*, 2011) were mixed with the cell lysates of HEK-293 cells transfected with YFP-Gβ1. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed with SDS-PAGE and blotted with antibodies to GFP (GST pull-down part). The figure in the lower panel shows the SDS-PAGE gel which contains 'after-wash' of bacterial cell lysates. The ratios of pull-down are 1.0, N-domain; 0.3, C-domain; 0.1, CCT domain. The data represent results of two independent experiments with similar outcomes. (E) Diagram showing the topology of the protein complex formed by D<sub>3</sub>R,  $G\beta\gamma$ , and  $\beta$ -arrestin.

three binding partners were required. To test this possibility, interactions between D<sub>3</sub>R and  $G\beta\gamma$  or  $\beta$ -arrestin2 were determined in the absence of either  $\beta$ -arrestin or  $G\beta\gamma$ , respectively. When cellular  $G\beta\gamma$  was sequestered by co-expression

of GRK2-CT, the interaction between D<sub>3</sub>R and  $\beta$ -arrestin was abolished (Fig. 5A). In the reverse situation (Fig. 5B), the interaction between D<sub>3</sub>R and  $G\beta\gamma$  was abolished when cellular  $\beta$ -arrestins were knocked down (Min *et al.*, 2013). These results

were also confirmed by GST pull-down assays. Interaction between the third intracellular loop of D<sub>3</sub>R and Gβγ was inhibited when the expression of cellular β-arrestins was lowered (Fig. 5C). Overall, these results suggested that Gβγ and β-arrestin are mutually required for their interaction with D<sub>3</sub>R. In agreement with these results, receptors are able to associate with β-arrestins and Gβγ through distinct residues (Bornancin and Parker, 1997; Mahon *et al.*, 2006). In addition, β-arrestins can interact with Gβ subunits (Yang *et al.*, 2009) through residues that do not necessarily contribute to the association between Gβ and GPCRs (Shenoy and Lefkowitz, 2005; Mahon *et al.*, 2006), thus allowing β-arrestins to associate simultaneously with GPCRs and the Gβγ dimer.

To determine the regions of β-arrestin2 that interact with Gβγ, β-arrestin2 was divided into three fragments, comprising its N-, C-, and CT-domains (Zheng *et al.*, 2011), and GST pull-down assay was conducted for the interaction with Gβ1. As shown in Fig. 5D, the N-domain of β-arrestin2 and C-domain to a less extent interacted with Gβ1. These results suggested that the N domain of β-arrestin2 mainly interacts with D<sub>3</sub>R in a Gβγ-dependent manner (Fig. 5E).

**Strengthening the interaction between Gβγ and β-arrestin enhances the acute tolerance of receptors**

Our results suggested that basal interactions of each GPCR tested with Gβγ, as well as the interaction between Gβγ and β-arrestin, were correlated with acute tolerance. It was noticeable that D<sub>4</sub>R and D<sub>3</sub>R-[IL3-D<sub>2</sub>R], which did not undergo acute tolerance (Fig. 1C, 1D), had an abundant interaction with β-arrestin2 (Fig. 2D, 2H) but a weak interaction with Gβγ (Fig. 3A, 3B). In addition, a weak interaction was observed between Gβγ and β-arrestin2 in cells expressing these receptors (Fig. 4D). Therefore, we wanted to determine whether enforcement of the receptor/Gβγ/β-arrestin complex by strengthening the interaction between Gβγ and β-arrestin endowed GPCRs with the propensity to undergo acute tolerance. For this determination, we prepared a DNA sequence encoding the fusion protein Gβ1-Arr3 where β-arrestin2 was attached to the carboxyl tail of Gβ1.

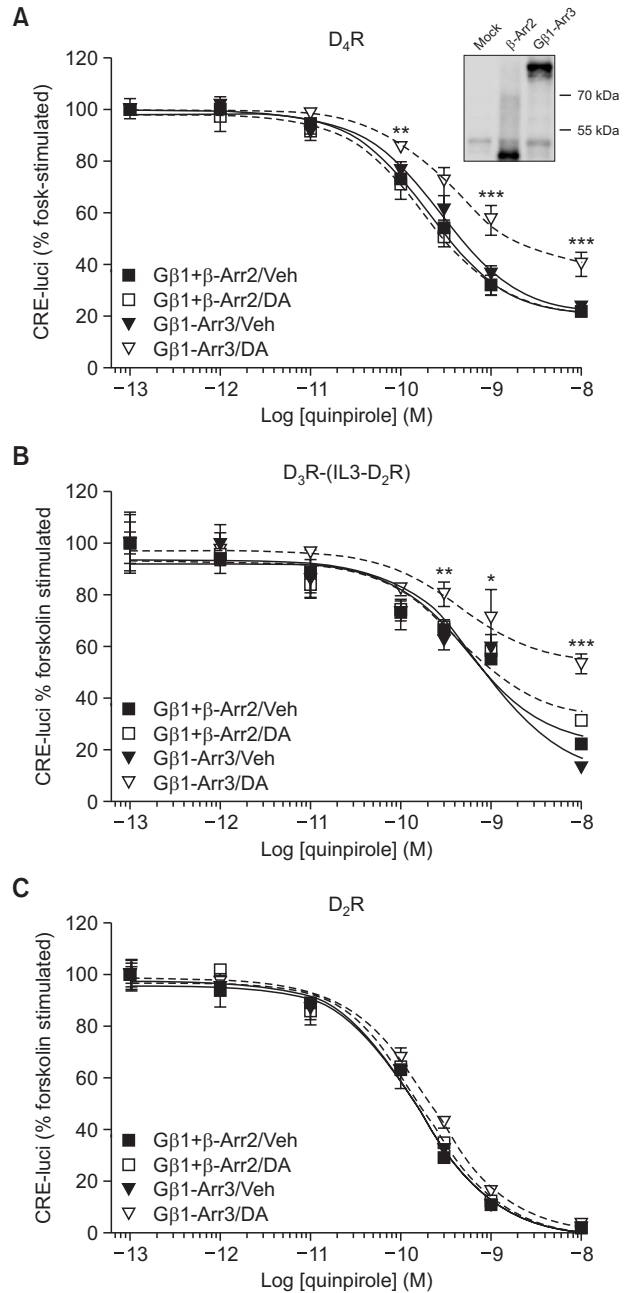
Expression of Gβ1-Arr3, but not simultaneous expression of Gβ1 and β-arrestin2 in β-arrestin1/2-knockdown cells, rendered D<sub>4</sub>R prone to acute tolerance (Fig. 6A). Similar results were obtained with D<sub>3</sub>R-[IL3-D<sub>2</sub>R] (Fig. 6B). When this strategy was applied to D<sub>2</sub>R, which neither underwent tolerance nor was heavily bound to β-arrestin2, receptor tolerance was not induced (Fig. 6C). Thus, it could be postulated that the stable complex with Gβγ and β-arrestin is required for the development of receptor tolerance.

Overall, these results showed that the simultaneous interaction between the receptors, and both Gβγ and β-arrestin may be related to their acute tolerance properties.

**DISCUSSION**

Rapid desensitization and acute tolerance are usually distinguished based on the different time points when functional changes appeared after agonist treatment. Thus, proper establishment of time windows is needed to correctly determine the molecular mechanisms and functional consequences of rapid desensitization and acute tolerance.

While GRK2-mediated receptor phosphorylation and sub-



**Fig. 6.** Effects of strengthening the interaction between Gβ1 and β-arrestin2 on the tolerance development of D<sub>4</sub>R and D<sub>2</sub>R. Tolerance of all GPCRs was induced by pretreatment with 10 μM DA for 5 min. (A) Tolerance development of D<sub>4</sub>R. β-Arrestin1/2-KD cells were transfected with D<sub>4</sub>R along with Gβ1 and β-arrestin2, either separately or as a fusion protein (Gβ1-Arr3). \*\**p*<0.01, \*\*\**p*<0.001 when the Gβ1-Arr3/DA group was compared with other experimental groups. Cell lysates were blotted with antibodies against β-arrestin2. (B) Tolerance development of D<sub>3</sub>R-[IL3-D<sub>2</sub>R]. β-Arrestin1/2-KD cells were transfected with D<sub>3</sub>R-[IL3-D<sub>2</sub>R] along with Gβ1 and β-arrestin2, either separately or as a fusion protein (Gβ1-Arr3). \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001 when the Gβ1-Arr3/DA group was compared with Gβ1-Arr3/Veh group. (C) Tolerance development of D<sub>2</sub>R. β-Arrestin1/2-KD cells were transfected with D<sub>2</sub>R along with Gβ1 and β-arrestin2, either separately or as a fusion protein (Gβ1-Arr3).



sequent  $\beta$ -arrestin translocation toward activated receptors are believed to be responsible for GPCR desensitization, recent studies of  $\beta_2$ AR suggest that this paradigm might need reinterpretation or correction. For example, knockdown of GRK2 or GRK6 resulted in a partial decrement of  $\beta_2$ AR tolerance, but did not affect the agonist-induced association between  $\beta_2$ AR and  $\beta$ -arrestin2 (Nobles *et al.*, 2011). In addition, another study showed that GRK2 was not involved in the rapid desensitization of  $\beta_2$ AR (Violin *et al.*, 2008). Thus, it is clear that  $\beta$ -arrestins are involved in both rapid desensitization and acute tolerance. On the other hand, more data are required to generalize the roles of GRK2 in the rapid attenuation of GPCR signaling.

Results in the current study show that  $\beta$ -arrestins are required for the interaction between  $G\beta\gamma$  and  $D_3$ R, and that formation of a stable  $\beta$ -arrestin-receptor complex does not prevent, but rather favors, continuous signaling of  $D_3$ R.  $\beta$ -arrestins might have different roles in the regulation of GPCR signaling if they are in different context; for example, associated with  $G\beta\gamma$ . For example, recent studies examining the parathyroid hormone receptor (PTHr) and vasopressin type 2 receptor (Feinstein *et al.*, 2013; Wehbi *et al.*, 2013) have shown that these receptors form a ternary complex with  $\beta$ -arrestin2 and  $G\beta\gamma$ , which enables more persistent signaling of the receptors through the  $G\alpha$  subunit.

Tolerance represents a gradual decrement of response to a drug treatment following its repeated use, thus increasingly larger doses are required to obtain similar therapeutic effects obtained by administration of initial dose. Compared to rapid desensitization, detailed molecular mechanism of acute tolerance is not clear. Thus, the present study has revealed that potentialities to undergo tolerance can be predicted. GPCRs have the tendency to undergo tolerance if they are predisposed to mediate the formation of a stable protein complex with  $\beta$ -arrestins and  $G\beta\gamma$ .

## CONFLICT OF INTEREST

The authors state no conflict of interest exists.

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