BJP British Journal of Pharmacology

DOI:10.1111/bph.12357
www.brjpharmacol.org

RESEARCH PAPER

Novel roles for β-arrestins in the regulation of pharmacological sequestration to predict agonist-induced desensitization of dopamine D_3 receptors

C Min[1], M Zheng[1], X Zhang[1], M G Caron[2] and K M Kim[1]

[1]*Department of Pharmacology, College of Pharmacy, Drug Development Research Institute, Chonnam National University, Gwang-Ju 500-757, Korea, and* [2]*Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA*

Correspondence
Kyeong-Man Kim, Department of Pharmacology, College of Pharmacy, Chonnam National University, Gwang-Ju 500-757, Korea. E-mail: kmkim@jnu.ac.kr

Keywords
phosphorylation; glycosylation; Gβγ; vasopressin type 2 receptor; small hairpin RNA

Received
11 March 2013
Revised
24 July 2013
Accepted
18 August 2013

BACKGROUND AND PURPOSE

In addition to typical GPCR kinase (GRK)-/β-arrestin-dependent internalization, dopamine D_3 receptor employed an additional GRK-independent sequestration pathway. In this study, we investigated the molecular mechanism of this novel sequestration pathway.

EXPERIMENTAL APPROACH

Radioligand binding, flow cytometry and cell surface biotinylation assay were used to characterize trafficking properties of D_2 and D_3 receptors. Serine/threonine and *N*-linked glycosylation mutants of the D_3 receptor were utilized to locate receptor regions involved in pharmacological sequestration and desensitization. Various point mutants of the D_2 and D_3 receptors, whose sequestration and desensitization properties were altered, were combined with knockdown cells of GRKs or β-arrestins to functionally correlate pharmacological sequestration and desensitization.

KEY RESULTS

The D_3 receptor, but not the D_2 receptor, showed characteristic trafficking behaviour in which receptors were shifted towards the more hydrophobic domains within the plasma membrane without translocation into other intracellular compartments. Among various amino acid residues tested, S145/S146, C147 and N12/19 were involved in pharmacological sequestration and receptor desensitization. Both pharmacological sequestration and desensitization of D_3 receptor required β-arrestins, and functional relationship was observed between two processes when it was tested for D_3 receptor variants and agonists.

CONCLUSIONS AND IMPLICATIONS

Pharmacological sequestration of D_3 receptor accompanies movement of cell surface receptors into a more hydrophobic fraction within the plasma membrane and renders D_3 receptor inaccessible to hydrophilic ligands. Pharmacological sequestration is correlated with desensitization of the D_3 receptor in a Gβγ- and β-arrestin-dependent manner. This study provides new insights into molecular mechanism governing GPCR trafficking and desensitization.

Abbreviations

CRE, cAMP response element; DA, dopamine; GPCR, G protein-coupled receptor

Introduction

The desensitization process of GPCR begins immediately upon agonist binding (Sibley and Lefkowitz, 1985). Uncoupling of the receptor from G proteins occurs very quickly and is believed to be the result of phosphorylation of intracellular domains of the receptor by GPCR kinases (GRKs) (Benovic *et al.*, 1986; 1989), followed by binding with arrestin proteins (Benovic *et al.*, 1987; Lohse *et al.*, 1990; Attramadal *et al.*, 1992). Minutes after agonist exposure, the activated receptors undergo internalization. This process involves movement of receptors from the cell surface to cytosolic compartments. Thus, receptors are sequestered to a compartment where they are unable to interact with hydrophilic ligands in the extracellular environment.

In addition to these biochemical pathways, certain GPCRs can be desensitized or internalized independently of receptor phosphorylation (Qiu *et al.*, 2003; Rasmussen *et al.*, 2004; Jala *et al.*, 2005; Ferguson, 2007; Cho *et al.*, 2010a). Some GPCRs employ a characteristic sequestration pathway called pharmacological sequestration, which is a desensitization via conformation changes of receptor proteins without actual internalization of receptor proteins into intracellular regions (Mostafapour *et al.*, 1996). The pharmacologically sequestered receptor is unable to bind to hydrophilic ligands as occurs during typical sequestration.

Among the five subtypes of dopamine GPCRs, the D_2 and D_3 receptors are the main targets of currently used antipsychotics. Studies of genetically modified mice show that various *in vivo* functions, such as locomotor activity, reward-related activity and autoreceptor functions, are differentially mediated by D_3 receptors and alternatively spliced forms of the D_2 receptor (reviewed in Holmes *et al.*, 2004). Therefore, selective regulation of these receptors is important to widen the therapeutic window of antipsychotics. Nonetheless, the sequence homology between D_2 and D_3 receptor is high, with 46% amino acid homology overall and 78% identity in the transmembrane domains (Giros *et al.*, 1990). Indeed, D_2 and D_3 receptors possess similar pharmacological properties and are involved in the same signalling pathways except that the signalling efficiency of the D_3 receptor is two to five times lower than that of the D_2 receptor (Cho *et al.*, 2010b). These similarities render selective regulation of either receptor difficult.

Previous studies have shown that GRK and β-arrestins exert different regulatory effects on D_2 and D_3 receptor. For example, robust agonist-induced receptor phosphorylation, β-arrestin translocation and receptor internalization occur with the D_2 receptor, whereas these same changes are subtle with the D_3 receptor (Kim *et al.*, 2001; Kabbani *et al.*, 2002; Cho *et al.*, 2007), suggesting that the D_2 receptor would be selectively desensitized. However, that is not the case. Studies have shown that agonist-induced desensitization of the long form of the D_2 receptor does not occur (Ivins *et al.*, 1991) or is observed only after prolonged treatment with agonists of up to 24 h (Zhang *et al.*, 1994; Starr *et al.*, 1995). Similarly, the short form of the D_2 receptor is not desensitized after opening the K^+ channels in AtT-20 neuroendocrine cells or inhibiting cAMP production in HEK-293 cells (Westrich and Kuzhikandathil, 2007; Cho *et al.*, 2010a). Unexpectedly, D_3 receptor undergoes instantaneous desensitization, which is completed within 2 min after agonist treatment (Westrich and Kuzhikandathil, 2007; Zheng *et al.*, 2011). These results suggest that receptor phosphorylation or β-arrestin translocation does not properly explain desensitization of D_2 and D_3 receptors, and that the D_3 receptor might utilize unique regulatory pathways for desensitization, which are distinct from those employed by other GPCRs such as the β_2-adrenergic receptor.

In this study, we were interested in how D_3 receptor, which does not display noticeable agonist-induced receptor phosphorylation and β-arrestin translocation, undergoes homologous desensitization. Thus, we focused on the biochemical and functional characteristics of pharmacological sequestration, which are unique to the D_3 receptor.

Methods

Materials

HEK-293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture media and FBS were obtained from Life Technologies (Grand Island, NY, USA). [^{3}H]-Sulpiride (84 Ci·mmol^{-1}) and [^{3}H]-spiperone (85.5 Ci·mmol^{-1}) ware purchased from PerkinElmer (Fremont, CA, USA). Dopamine (DA), (–)quinpirole, R(+)-7-hydroxy-DPAT, forskolin, sucrose, methyl-β-cyclodextrin (MβCD), antibodies to clathrin heavy chain, FLAG, GFP and agarose beads conjugated to M2 FLAG (DYKDDDDK) antibody were obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO, USA). Antibodies to GRK2, GRK5, actin and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to β-arrestins were provided by Dr. Lefkowitz (Duke University, Durham, NC, USA).

Plasmid constructs

The wild-type (WT) human D_2 and D_3 receptors in pCMV5, S/T mutants and N-terminus chimeric receptor, R132H-D_2R-V2Rt, were described elsewhere (Kim *et al.*, 2001; Cho *et al.*, 2007; Kim and Caron, 2008; Cho *et al.*, 2012). RNA interference plasmids for GRK2, GRK5, β-arrestins and the clathrin heavy chain were published previously (Zhang *et al.*, 2008; Marina-Garcia *et al.*, 2009; Guo *et al.*, 2011). The N-terminus deletion mutants and a point mutant at the *N*-linked glycosylation sites (N12/19Q) were prepared by site-directed mutagenesis. WT or dominant negative forms of GRK4γ were described in a previous publication (Villar *et al.*, 2009). WT or dominant negative forms of GRK6 were provided by Dr. H. Kurose (Kyushu University, Japan). YFP-tagged Gβ1 was provided by Dr. Gautam (Washington University, St. Louis, USA).

Luciferase reporter gene assay

Cellular cAMP was measured by an indirect method (Zheng *et al.*, 2011). Procedure details are described in the Supporting Information.

Internalization and pharmacological sequestration assay

Two different strategies were used to determine internalization of the D_2 or D_3 receptors as described previously (Kim *et al.*, 2001). Procedure details are described in the Supporting Information.

Biotinylation of membrane receptors

Biotin labelling of membrane receptors was conducted using TS biotin ethylenediamine (Biotium, Hayward, CA, USA) according to manufacturer's protocol. Procedure details are described in the Supporting Information.

Statistics

All results are expressed as means ± SEM. Comparisons of dose–response curves between groups were performed using two-way ANOVA with Bonferroni's post-tests. Student's *t*-test was also used to compare some results. A *P*-value of less than 0.05 was considered significant.

Results

The dopamine D_3 receptor undergoes dual pathways of sequestration in response to agonist stimulation

The internalization assay has revealed that D_2 receptor internalization was quantitatively similar regardless of the assay methods employed (Figure 1A). In contrast, internalization of the D_3 receptor was unremarkable by flow cytometry but was found to be increased significantly when assessed by the radioligand binding method using [^{3}H]-sulpiride, a hydrophilic ligand (Figure 1B).

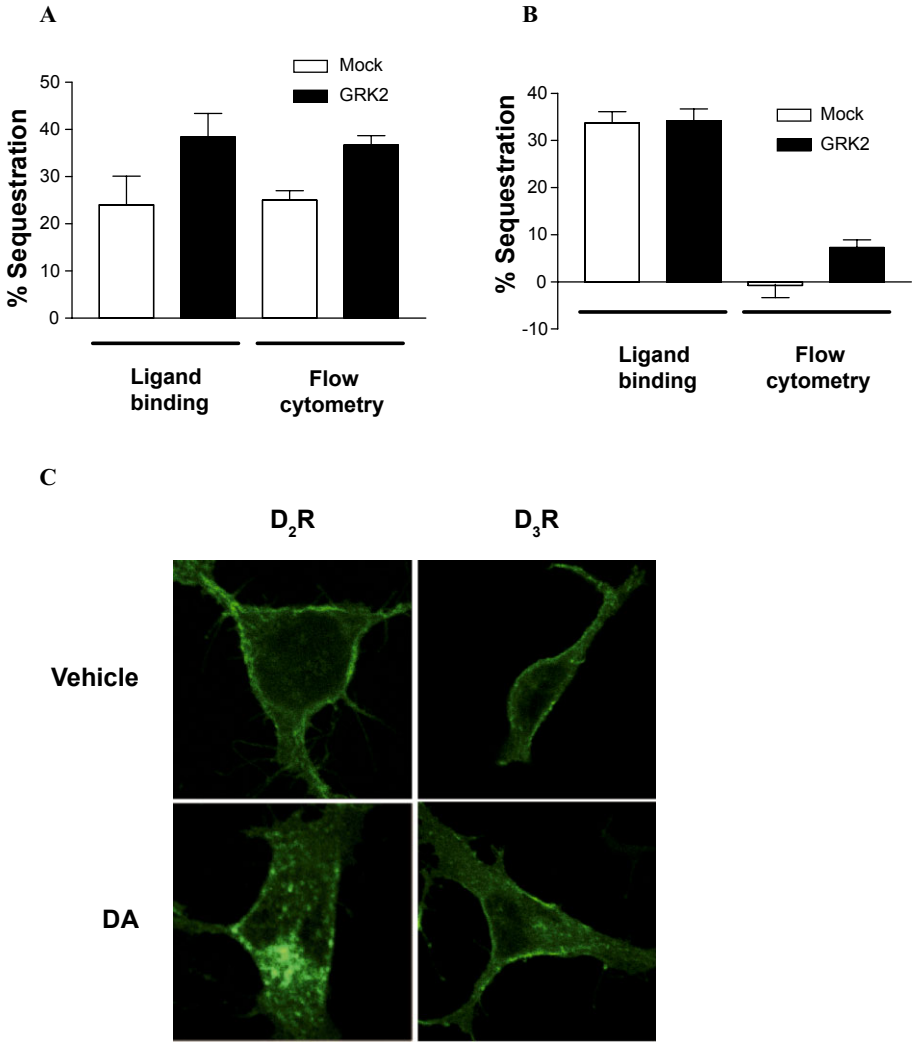

Figure 1

Comparison of agonist-induced internalization of the dopamine D_2 and D_3 receptors. (A, B) Comparison of D_2 and D_3 receptor internalization by flow cytometry and radioligand methods. HEK-293 cells were transfected with 2 μg HA-tagged D_2 receptor-pCMV5 (A) or D_3 receptor-pCMV5 (B) with or without 2 μg of GRK2-pRK5, and the cells were treated with 10 μM DA for 1 h. (C) Cells were transfected with 2 μg of the D_2 or D_3 receptor constructs in pEGFP-N1 together with 2 μg of GRK2. The cells were treated with 10 μM DA for 1 h after 24 h. The cells were examined with TCS SP5/AOBS/tandem laser scanning confocal microscope (Leica, Jena, Germany). All experiments (A–C) were repeated three times.

These results suggest that D_3 receptor is located near the plasma membrane after agonistic stimulation but is in a state where it cannot bind to hydrophilic ligands. Confocal microscopic studies confirmed that the physical movement of the D_3 receptor into cytosol was insignificant compared with that of the D_2 receptor (Kim *et al.*, 2001) (Figure 1C). Therefore, it is likely that internalization of the D_3 receptor measured by ligand binding method accompanies a conformational change that might involve short-distance trafficking of receptor proteins within the plasma membrane through which access to hydrophilic ligands is impaired (pharmacological sequestration).

Pharmacological sequestration could be caused by either abnormally high affinity of agonists to D_3 receptor or changes in affinity to [^{3}H]-sulpiride. Alternatively, it could be caused by physical hiding of ligand binding domain from the cell surface. To clarify this, two different approaches were employed. First, pharmacological sequestration of D_3 receptor could be induced if unwashed agonists compete with radioligands. As shown in Supporting Information Figure S1A, pharmacological sequestration was not observed when hydrophobic [^{3}H]-spiperone was used instead of hydrophilic [^{3}H]-sulpiride to label D_3 receptors. As hydrophobic ligand [^{3}H]-spiperone labels D_3 receptors on the cell surface as well as pharmacologically sequestered D_3 receptors, we expect that pharmacological sequestration will not be detected by [^{3}H]-spiperone binding unless pharmacological sequestration is caused by competition between [^{3}H]-spiperone and unwashed remaining agonist (dopamine, DA). Thus, the results suggest that pharmacological sequestration was not induced by a competition between [^{3}H]-sulpiride and remaining DA. Secondly, pharmacological sequestration could be induced if affinity to [^{3}H]-sulpiride is decreased after agonist pre-exposure. Saturation binding studies showed that affinity to [^{3}H]-sulpiride was not altered after cells were pretreated with DA (Supporting Information Figure S1B). Overall, these results suggest that DA-induced decrease in hydrophilic [^{3}H]-sulpiride binding is more likely to be caused by sequestration of ligand binding domain from the cell surface rather than by changes in ligand affinity to D_3 receptor.

Characterization of pharmacological sequestration of the D_3 receptor

Because pharmacological sequestration can be artificially induced by agonists remaining on cell surface, the cells from the sequestration assay were washed with a pH 2.0 buffer to completely remove the agonists. As shown in Supporting Information Figure S2A, agonist-induced internalization of D_2 receptor was similar between the cells washed with pH 2.0 and pH 7.4 buffers, suggesting that ligand binding properties were intact under these experimental conditions.

The time-course and dose–response relationship were studied as a first step to characterize the pharmacological sequestration of D_3 receptor. Pharmacological sequestration of the D_3 receptor occurred rapidly and reached a near-maximum sequestration in 2 min (Figure 2A). Pharmacological sequestration of the D_3 receptor increased gradually from 1 nM and reached a plateau at about 1 μM DA (Figure 2B). In contrast to near-instantaneous pharmacological sequestration, PKC-mediated internalization of the D_3 receptor (Cho *et al.*, 2007) occurred slowly (Figure 2C) and involved actual translocation of the receptor proteins from the plasma membrane to the cytosol (Figure 2D). Similar results were obtained from the ELISA-based sequestration assay (Supporting Information Figure S2B).

Pharmacological sequestration was not affected by knockdown of the clathrin heavy chain, disruption of caveolae by treatment with 5 mM MβCD or non-specific inhibition of internalization by sucrose treatment (0.45 M for 20 min) (data not shown).

GRKs and β-arrestins are key players involved in the regulation of GPCRs. When these regulator proteins were tested, co-expression of β-arrestin 2, but not GRK2, increased the pharmacological sequestration of D_3 receptor (Figure 3A). As would be expected, knockdown of β-arrestin1/2 inhibited pharmacological sequestration, (Figure 3B), but knockdown of GRK2 had no effects (Supporting Information Figure S3A). In addition, knockdown of GRK5 (Supporting Information Figure S3B) or co-expression of dominant negative mutants of GRK4, GRK5 and GRK6 did not affect pharmacological sequestration of D_3 receptor (Supporting Information Figure S3C). Roles of β-arrestins on pharmacological sequestration of D_3 receptor were not related to changes in ligand affinity. As shown in Figure 3C, knockdown of β-arrestins did not change the agonist affinity of D_3 receptor. These results suggest that β-arrestins are required for pharmacological sequestration, and that its properties are different from conventional GRK/β-arrestin-dependent internalization of GPCRs. Knockdown of β-arrestins, but not that of GRK2, inhibited desensitization of the D_3 receptor (Figure 3D and Supporting Information S3D). These results suggest that β-arrestins are involved both in pharmacological sequestration and in desensitization, and that pharmacological sequestration might be related to desensitization of the D_3 receptor.

Pharmacological sequestration of D_3 receptor involves a conformational change in the receptor proteins

If pharmacological sequestration of the D_3 receptor occurred through translocation of receptor proteins into intracellular compartments, it will not occur at 4°C and should not be reversible at 13°C, a temperature at which intracellular trafficking of receptor proteins is blocked (Mostafapour *et al.*, 1996). However, if pharmacological sequestration of the D_3 receptor occurs through conformational changes of the receptor proteins, it will still occur or revert to a basal position below 13°C. As shown in Figure 4A and B, both pharmacological sequestration and desensitization of the D_3 receptor occurred to a similar extent at 4 and 37°C. To test the effects of temperature on recovery of pharmacological sequestration, cells were treated with 10 μM DA at room temperature for 5 min, washed with ice cold buffer and then incubated with 7.2 nM [^{3}H]-sulpiride at 4, 13 or 29°C. As shown in Figure 4C, pharmacological sequestered D_3 receptor partially and completely returned to the basal state at 13 and 29°C respectively. These results suggest that pharmacological sequestration and receptor desensitization are likely to occur through conformational changes of receptor proteins, which do not accompany a long-distance trafficking to other intracellular compartments.

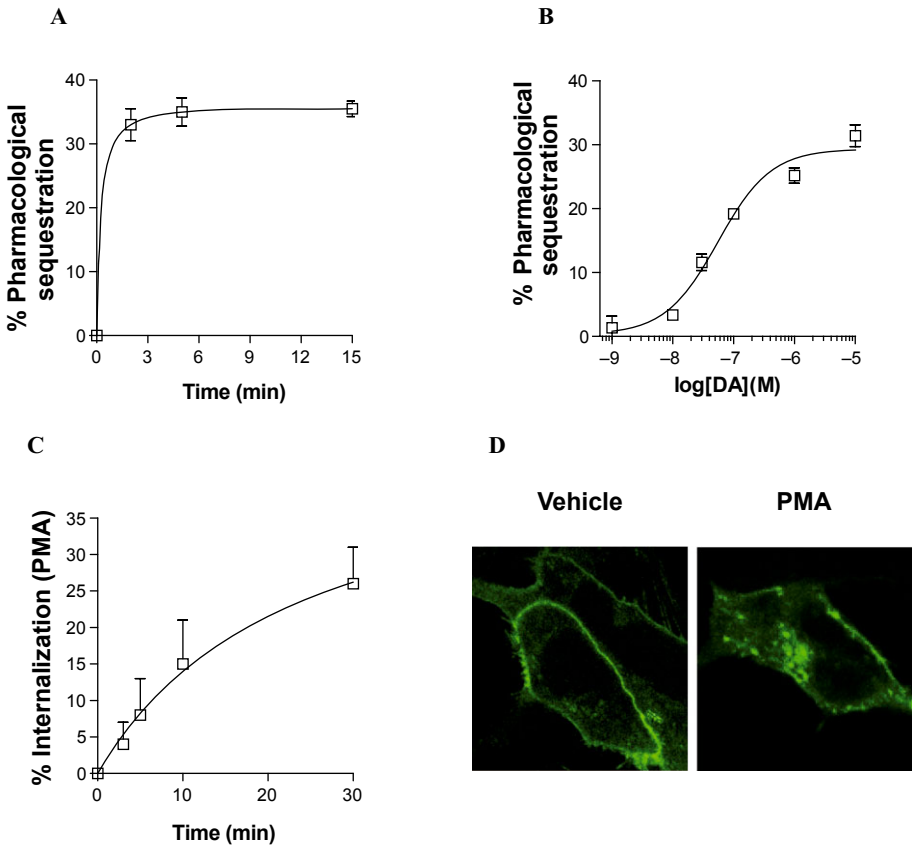

Figure 2

Characterization of pharmacological sequestration of the dopamine D_3 receptor. (A) Cells expressing the D_3 receptor were treated with 10 μM DA for the indicated periods of time. Error bars represent standard deviations. (B) Cells were treated with increasing concentrations of DA (0–10 μM) for 5 min. (C) Cells expressing the D_3 receptor were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for the indicated periods of time. (D) Cells expressing GFP-tagged D_3 receptor were treated with 100 nM PMA for 30 min. Data represent results from three independent experiments with similar outcomes.

It has been suggested that agonist-induced desensitization of the D_3 receptor is mediated by conformational changes through which the receptors are shifted into a more hydrophobic environment (Westrich *et al.*, 2010). To correlate these results with pharmacological sequestration, we used hydrophilic biotin reagent that selectively binds to receptor proteins located on the cell surface. As shown in Figure 4D, the amount of biotinylated D_2 receptor, which represents receptor expressed on cell surface, was not altered by DA treatment for 5 min (lane 1). In contrast, the amount of biotinylated D_3 receptor decreased under the same experimental conditions (lane 2). These results are in agreement with previous results (Westrich *et al.*, 2010). The agonist-induced decrease in the biotinylated D_3 receptor was blocked in β-arrestin1/2-KD cells (lane 3). Together with confocal microscopic studies, these results suggest that pharmacological sequestration of D_3 receptor is not due to the actual movement of receptor proteins from the cell surface to the cytosol. Rather, this sequestration is caused by placement of D_3 receptor in a more hydrophobic environment within the plasma membrane. However, it is not clear at this point whether pharmacological sequestration or desensitization is mediated through conformational changes within the receptor with movement of amino acid residues rather than the whole receptor movement into a more hydrophobic environment within the plasma membrane.

Our results show that β-arrestins act as a mediator for pharmacological sequestration. We were curious whether β-arrestins accelerate the sequestrating process or delay the recovery of the D_3 receptor already sequestered. To clarify this, the time-course of pharmacological sequestration was compared between control and β-arrestin knockdown cells at 4°C. At this temperature, agonist-induced pharmacological sequestration occurs (Figure 4A) but recovery to basal state is blocked (Figure 4C). As shown in Figure 4E, pharmacological sequestration of the D_3 receptor was significantly decreased in β-arrestin knockdown cells compared with control cells. However, recycling of the sequestered D_3 receptor at 37°C occurred to a similar extent in control and in β-arrestin knockdown cells (Figure 4F). These results suggest that β-arrestins play important roles in accelerating the sequestration process rather than retarding the recycling of sequestered receptor to the basal state.

Receptor phosphorylation, pharmacological sequestration and receptor desensitization

As phosphorylation status is an important determinant of the conformation and regulatory properties of GPCRs (Koch

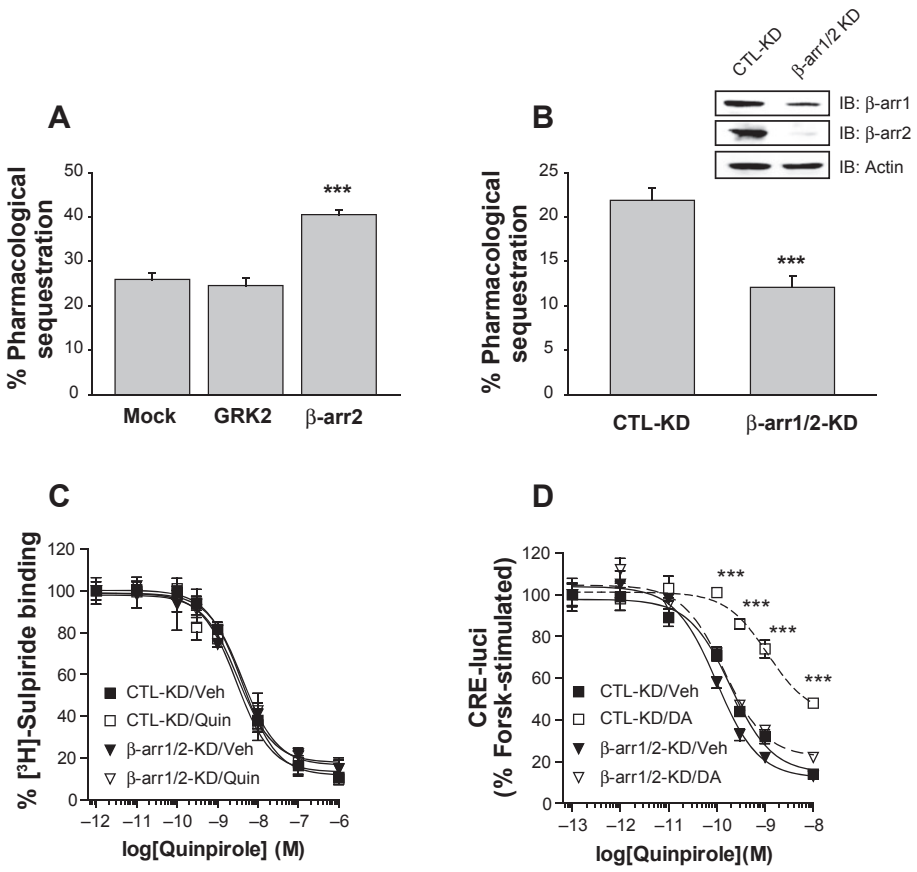

Figure 3

Roles of β-arrestins in pharmacological sequestration and agonist-induced desensitization of the D_3 receptor. (A) Cells were transfected with 2 μg D_3 receptor together with 2 μg Mock plasmid, GRK2-pRK5 or β-arrestin2-pCMV5. ***$P < 0.001$compared with Mock group. Data represent results from two independent experiments with similar outcomes. (B) Cells stably expressing control-shRNA (CTL-KD) or β-arrestin1/2-shRNA (β-arr1/2-KD) were transfected with 2 μg of the D_3 receptor. ***$P < 0.001$ compared with CTL-KD group. Data represent results from three independent experiments with similar outcomes. (C) Effects of lowering cellular levels of β-arrestins on ligand binding properties of D_3 receptors. Cells expressing D_3 receptor were pretreated either with vehicle or with 100 nM quinpirole for 5 min. After being washed, the cells were treated with 7.2 nM [^{3}H]-sulpiride and increasing concentrations of quinpirole for 150 min at 4°C in the presence or absence of 10 μM haloperidol. The cells were washed three times with ice-cold serum-free medium dissolved in 1% SDS and then counted using a liquid scintillation counter. (D) Effects of lowering cellular levels of β-arrestins on agonist-induced desensitization of the D_3 receptor. Cells expressing about 1.2 pmol·mg^{-1} protein of D_3 receptor were pretreated with 10 μM DA for 5 min, and desensitization assay was conducted. ***$P < 0.001$ compared with vehicle-treated group. Data represent results from five independent experiments with similar outcomes.

et al., 1994), the roles of phosphorylation in pharmacological sequestration of the D_3 receptor were studied by mutating serine (S) and threonine (T) residues in the intracellular loops. S/T residues within the second and third intracellular loops of the D_3 receptor were mutated to alanine or valine residues (Figure 5A, Table 1). When the S/T mutants of the third intracellular loop were tested (4–9), these proteins showed similar levels of pharmacological sequestration as that of WT-D_3 receptor. In contrast, pharmacological sequestration of the D_3 receptor was significantly decreased with simultaneous mutations of all S/T residues located within the second intracellular loop (designated as D_3R-IC2; T130V, T142V, S145A and S146A) (Figure 5B). When these S/T residues were individually mutated, pharmacological sequestration was inhibited with S145A or S146A, and this inhibition was stronger with a double-mutation S145/6A (Figure 5B).

As our results suggest that pharmacological sequestration and desensitization are functionally associated (Figure 3), S/T residues responsible for D_3 receptor desensitization were searched using the S/T mutants. Interestingly, the same set of S/T residues were involved in both pharmacological sequestration and receptor desensitization. Receptor desensitization was observed with the S/T mutants of the third intracellular loop (4–9) (data not shown). However, simultaneous mutation of all S/T residues in the second intracellular loop completely abolished desensitization of the D_3 receptor (Supporting Information Figure S4A). When individual S/T residues located within second intracellular loop was analysed, mutation of T130 or T142 did not affect the agonist-induced desensitization of the D_3 receptor (Supporting Information Figure S4B,C); individual mutation of S145 or S146 partially inhibited it (Supporting Information Figure S4D,E); simulta-

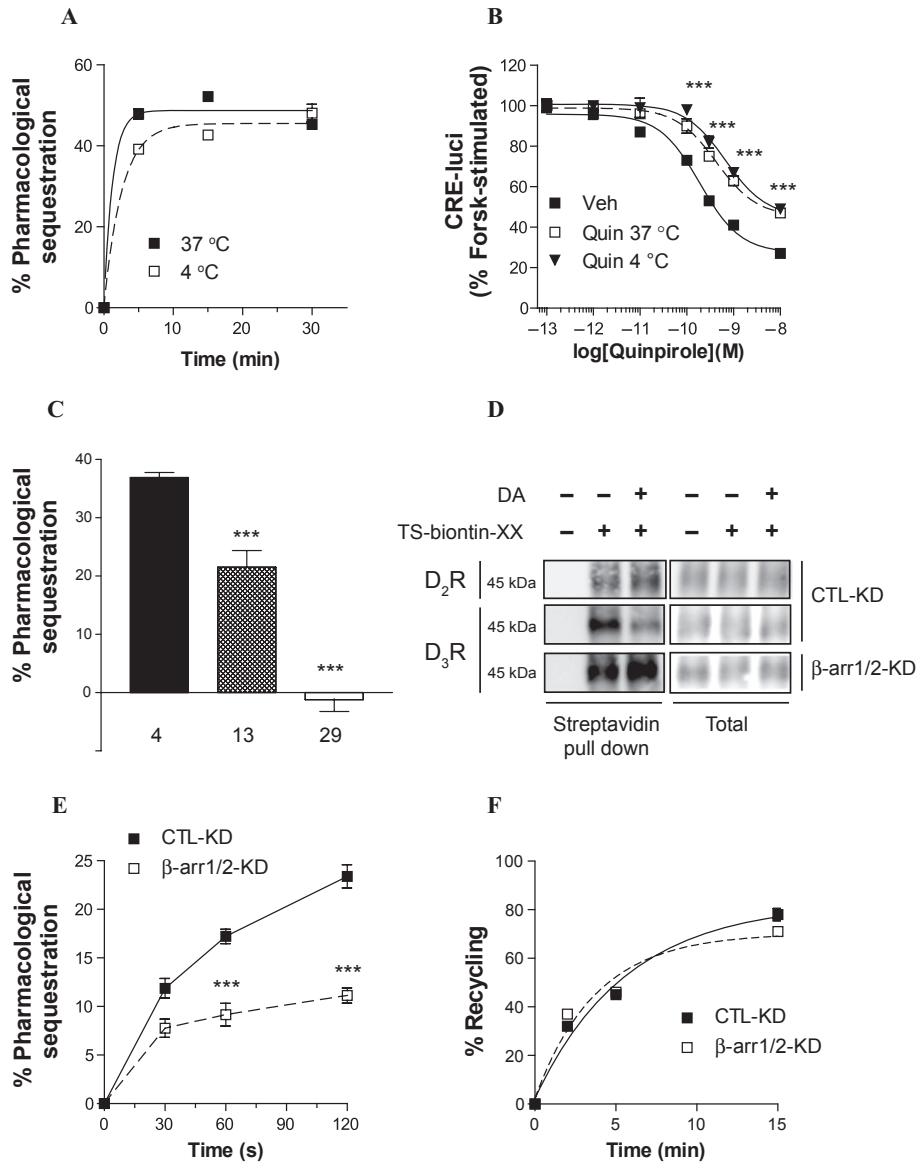

Figure 4

Temperature dependence and trafficking properties of pharmacological sequestration of the D_3 receptor. (A) Comparison of pharmacological sequestration of the D_3 receptor at 4 and 37°C. Cells expressing about 1.4 pmol·mg^{-1} protein of the D_3 receptor were treated with 100 nM quinpirole at 37 or 4°C for 5 min, washed and then treated with 7.2 nM [^{3}H]-sulpiride dissolved in serum-free media for 150 min at 4°C in the presence or absence of 10 μM haloperidol. (B) Comparison of agonist-induced desensitization of the D_3 receptor at 4 and 37°C. Cells expressing about 0.9 pmol·mg^{-1} protein of the D_3 receptor were treated with 100 nM quinpirole for 5 min either at 37 or 4°C. ***$P < 0.001$ compared with vehicle-treated group. (C) Temperature-dependent recovery of pharmacological sequestration. Cells were treated with 10 μM DA for 5 min, washed, and incubated with 7.2 nM [^{3}H]-sulpiride for 150 min at 4, 13 and 29°C. ***$P < 0.001$ compared with 4°C group. (D) Pharmacological sequestration of D_3 receptor determined by biotinylation assay. Cells expressing about 1.3 pmol·mg^{-1} protein of FLAG-tagged D_2 or D_3 receptor were treated with 10 μM DA for 5 min, then treated with 0.2 mM TS-biotin-xx reagents for 10 min at room temperature. Data are representative of three independent experiments with similar outcomes. (E) Comparison of pharmacological sequestration of the D_3 receptor between control and β-arrestin knockdown cells at 4°C. ***$P < 0.001$ compared with CTL-KD group. (F) Comparison of recycling of the pharmacologically sequestered D_3 receptor between control and β-arrestin knockdown cells. Cells were treated with 10 μM DA for 5 min at 37°C, washed with ice cold serum-free media five times and then incubated at 37°C for the indicated period of time. Pharmacological sequestration was determined at each time point. All experiments (A–F) were repeated three times. CRE, cAMP response element.

neous mutations of both residues abolished the desensitization response (Figure 6A, S145/6-D_3R).

The sequence of the second intracellular loop of the D_2 and D_3 receptors is highly conserved, except for few amino acid residues. For example, the D_2 receptor has a ^{147}serine–serine–lysine^{149} sequence, whereas the D_3 receptor has a ^{145}serine–serine–cysteine^{147} sequence (Figure 6, upper panel). As shown in Figure 6B, mutation of C147 to lysine

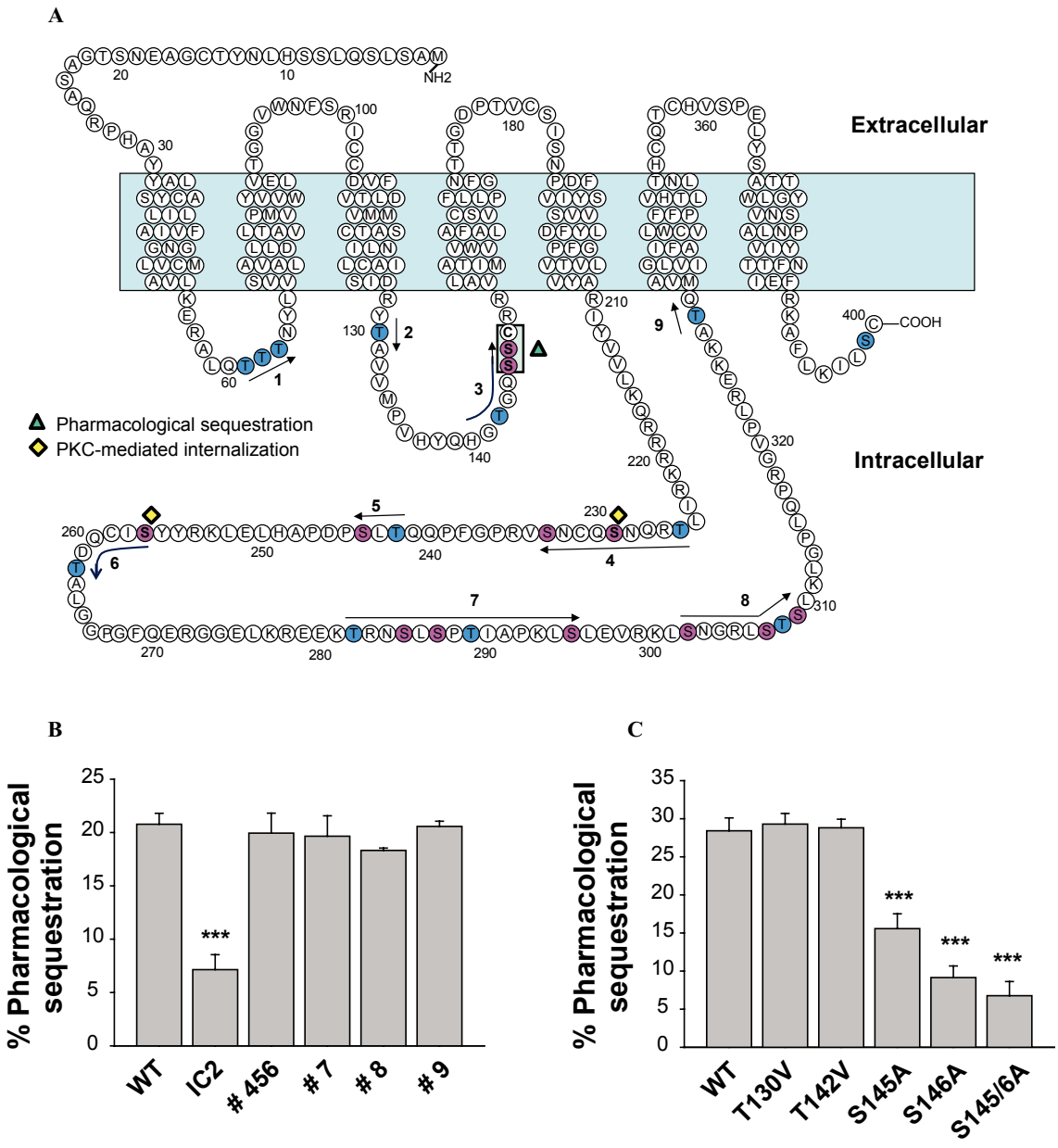

Figure 5

Identification of potential phosphorylation sites responsible for pharmacological sequestration of the D_3 receptor. (A) Diagram of the D_3 receptor and putative phosphorylation sites. The shaded region represents the transmembrane region. (B, C) Pharmacological sequestrations were determined from cells expressing about 0.9 pmol·mg^{-1} proteins of wild-type (WT) or mutant D_3 receptors. ***$P < 0.001$ compared with WT group. Data represent results from two independent experiments with similar outcomes.

(C147K-D_3R) abolished desensitization of the D_3 receptor. Conversely, mutation of the D_2 receptor lysine 149 residue to cysteine (K149C) induced desensitization as was the case in the D_3 receptor (Figure 6C). As expected, pharmacological sequestration of the D_3 receptor was inhibited by the C147K mutation, but this sequestration was increased with the K149C mutation in the D_2 receptor (Figure 6D). These results suggest that two consecutive serine residues at 145/146 and the cysteine residue at 147 form a critical sequence motif, which is involved in agonist-induced pharmacological sequestration and desensitization of the D_3 receptor. A previous study has shown that C147 is responsible for desensitization of the D_3 receptor (Westrich and Kuzhikandathil, 2007). Using TS-biotin, they demonstrated that mutation of C147 abolished conformational change of D_3 receptor, which is needed to shift the receptor to a more hydrophobic environment. This finding is in agreement with our results regarding receptor desensitization (Figure 6B) and pharmacological sequestration, which corresponds to the decrease in biotinylated receptor (Figure 6D).

Table 1

Notation and descriptions for D_3 receptor mutants of possible phosphorylation sites in the intracellular regions

Receptor	Residues mutated	Description
1	T62V, T63V, T64V	Impairs receptor expression
2	T130V	–
3	T142V, S145A, S146A[a]	Individual mutants were prepared S145 and S146 mediates pharmacological sequestration and desensitization
4	T225V, S229A, S233A[a]	Individual mutants were prepared
5	T242V, S244A	–
6	S257A, T262V	Individual mutants were prepared
7	T282V, S285A, S287A, T289V, S296A	–
8	S302A[b], S307A, T308V, S309A[a]	–
9	T328V[b]	–

Values represent the position of amino acid residues starting from the N-terminal region, Met[1].
[a]These regions contain the putative phosphorylation sites for PKC.
[b]These regions contain the putative phosphorylation sites for PKA.

Roles of N-linked glycosylation in pharmacological sequestration of the D_3 receptor

The N-terminus of GPCRs is composed of a variable number of amino acid residues ranging from about 30 residues in the rhodopsin family to more than 600 residues in the metabotropic glutamate receptor family. This domain has several features important for GPCR functions, one of which is critical for trafficking of GPCRs to the cell surface (Rana *et al.*, 2001). A previous study showed that the N-terminus of D_2 receptor controls the receptor conformation, which determines its intracellular trafficking and signalling properties (Cho *et al.*, 2012).

The N-termini of the D_2 and D_3 receptors have disparate sequences (Figure 7, upper panel). N-terminus chimeric receptors between the D_2 and D_3 receptors were utilized to test whether the N-terminus is involved in pharmacological sequestration of the D_3 receptor. Pharmacological sequestration of the chimeric D_3 receptor, in which the N-terminus of the D_3 receptor was replaced with that of D_2 receptor (D_3R-D_2NT), was similar to that of the WT-D_3 receptor, but the sequestration of D_2R-D_3NT was larger than that of the WT-D_2 receptor (Figure 7A), suggesting that the N-terminus is somehow involved in determining pharmacological sequestration. As the N-terminus of the D_3 receptor was sequentially shortened, pharmacological sequestration of the D_3 receptor decreased strongly from ΔN12 (Figure 7B) at which a potential *N*-linked glycosylation site is located. Mutation of N12 and N19 resulted in a decrease in the molecular weight of the D_3 receptor on the SDS gel (Figure 7C) and inhibited pharmacological sequestration (Figure 7D). As expected, agonist-induced desensitization of the D_3 receptor was inhibited in the glycosylation mutant (Figure 7E).

It is not clear how *N*-linked glycosylation on the N-terminus regulates pharmacological sequestration of D_3 receptor. It can be postulated that intact conformation of D_3 receptor is required for pharmacological sequestration to occur. Inhibition of *N*-linked glycosylation probably impairs correct integration of receptors into the plasma membrane, which is needed for maintaining correct receptor conformation.

Differential interaction behaviours of the D_2 and D_3 receptors with β-arrestins

As discussed earlier, β-arrestins were required for pharmacological sequestration and desensitization of the D_3 receptor, and the patterns of interaction with β-arrestins correlated with desensitization. As agonist-induced translocation of β-arrestins does not adequately explain the desensitization of D_3 receptors (Kim *et al.*, 2001; Zheng *et al.*, 2011), constitutive interaction with receptor was examined in more detail. As expected from β-arrestin translocations, D_2 receptor was not bound to β-arrestins in a resting state, but the interaction between these two proteins increased in response to agonistic stimulation (Figure 8A, second and third lanes). In contrast, the D_3 receptor showed constitutive interaction with β-arrestin 2, but agonist-induced interaction between these proteins was not clear (Figure 8A, fourth and fifth lanes). C147K-D_3R or S145/6A-D_3R, which did not undergo agonist-induced desensitization, was not constitutively bound to β-arrestins (Figure 8B). These results suggest that constitutive interaction with β-arrestins, rather than agonist-induced translocation of β-arrestins, could be somehow related to pharmacological sequestration and desensitization of the D_3 receptor. In agreement with that hypothesis, constitutive interaction with β-arrestins, but not agonist-induced translocation of β-arrestin2, was observed with the K149C-D_2 receptor (Figure 8C,D), a point mutant of the D_2 receptor that undergoes agonist-induced desensitization.

To corroborate these results, we designed a mutant D_2 receptor that possesses characteristics similar to D_3 receptor in β-arrestin interaction. First, agonist-induced β-arrestin

```
hD2R-IC2: SIDRYTAVAMPMLYSSKRRVT
hD3R-IC2: SIDRYTAVVMPVHYQHGTGSSCRRVA
```

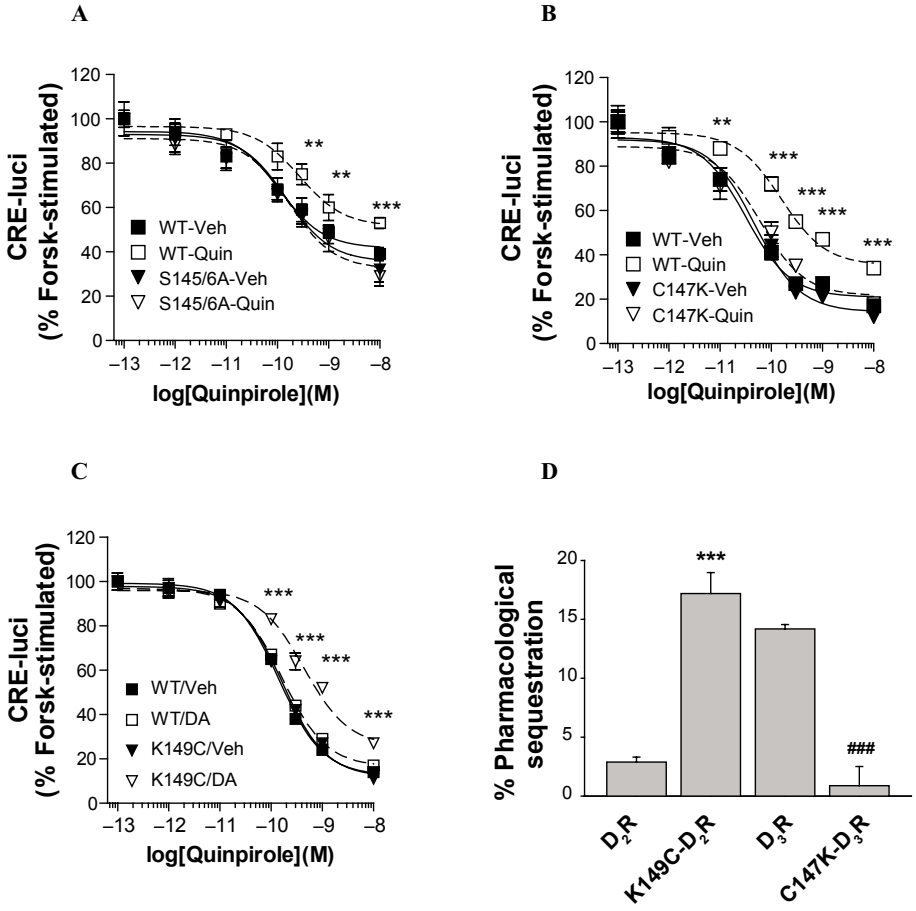

Figure 6

Roles of the serine–serine–cysteine motif within the second intracellular loop in desensitization and pharmacological sequestration of the dopamine D_2 and D_3 receptors. Alignment of the amino acid sequences within the second intracellular loop of the D_2 and D_3 receptors. (A–C) Cells expressing about 1.2 pmol·mg^{-1} protein of the wild-type (WT), S145/6A-D_3 receptor, C147K-D_3 receptor or K149C-D_2 receptor, were treated with 100 nM quinpirole for 5 min. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with other experimental groups. Data represent results from two independent experiments with similar outcomes. (D) Cells expressing about 1.3 pmol·mg^{-1} protein of each receptor were treated with 10 μM DA for 5 min. $^{***}P < 0.001$ compared with D_2R group; $^{###}P < 0.001$ compared with D_3R group. Data represent results from two independent experiments with similar outcomes. CRE, cAMP response element.

translocation was inhibited by mutating an amino acid residue in the DRY motif (R132H), and the affinity for β-arrestins was increased by attaching the C-terminus tail of the vasopressin type 2 receptor, R132H-D_2R-V_2Rt. As expected, R132H-D_2R-V_2Rt showed virtually the same phenotypes as the WT-D_3 and K149C-D_2R receptor in terms of β-arrestin translocation (Supporting Information Figure S5A), constitutive interaction with β-arrestins (Supporting Information Figure S5B), desensitization (Supporting Information Figure S5C) and pharmacological sequestration (Supporting Information Figure S5D). These results again confirm that constitutive interaction with β-arrestins could be important for pharmacological sequestration and desensitization.

Gβγ controls pharmacological sequestration of the D_3 receptor by mediating the interaction between receptor and β-arrestins

Gβγ mediates multiple functions through interaction with pleckstrin homology domain of various proteins (Touhara *et al.*, 1994). Recently, a study has suggested that signalling of D_3 receptor occurs through the Gβγ pathway (Jin *et al.*, 2013). As shown in Figure 9A, pharmacological sequestration of the D_3 receptor was inhibited by co-expression of GRK2-CT, an inhibiting peptide for Gβγ (Koch *et al.*, 1994). In addition, interaction between the D_3 receptor and β-arrestin 2, which is presumably important for pharmacological sequestration,

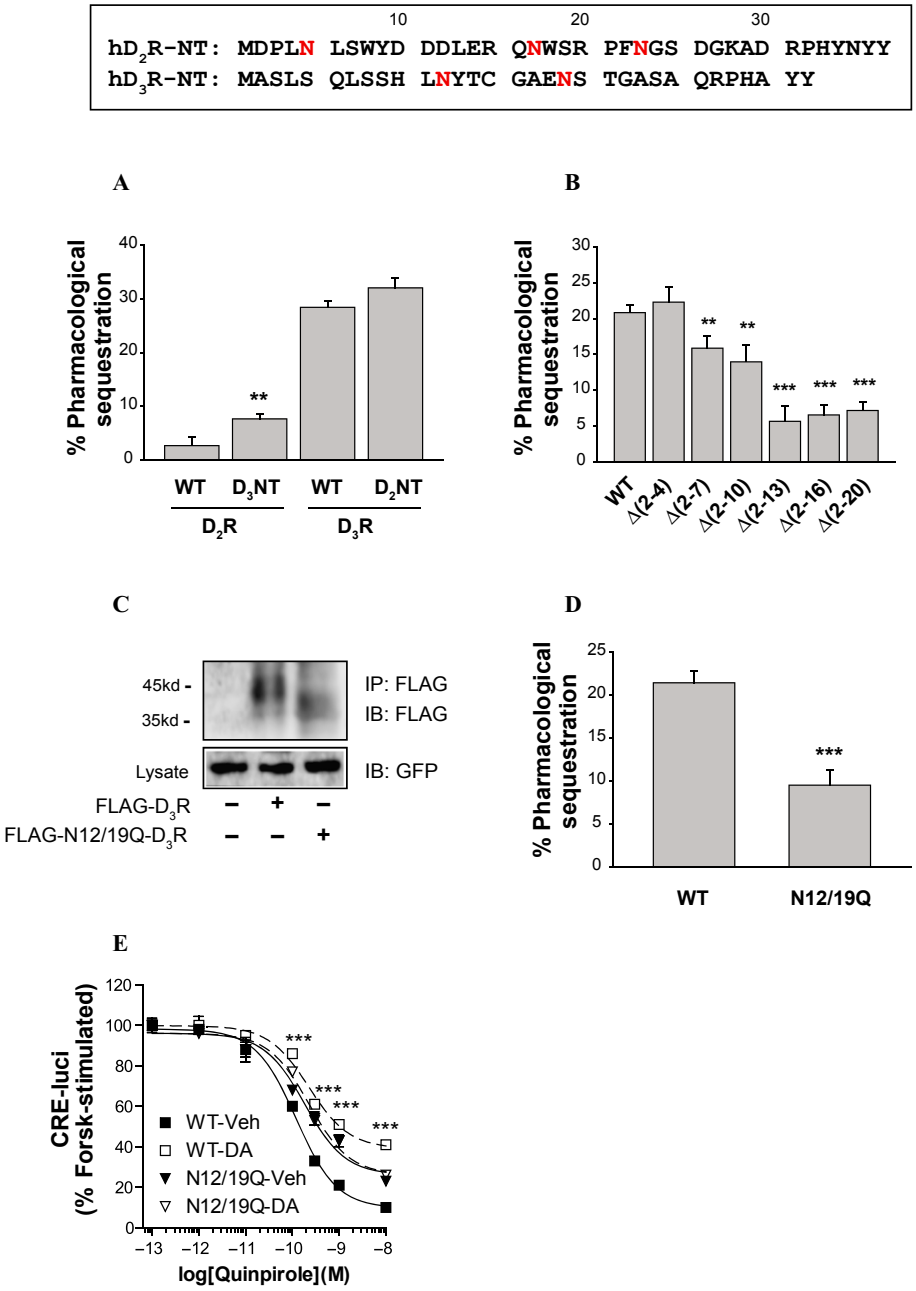

Figure 7

Roles of *N*-linked glycosylation located within the N-terminus in pharmacological sequestration of the D_3 receptor. Alignment of the amino acid sequences within the N-terminal regions of the D_2 and D_3 receptors. Potential glycosylation sites of the D_2 and D_3 receptors are shown in red. (A) Effects of N-terminus switching on pharmacological sequestration of the D_2 and D_3 receptors. Cells expressing each construct at about 1.2 pmol·mg^{-1} protein were treated with 10 μM DA for 5 min. $^{**}P < 0.01$ compared with wild-type (WT)-D_2 receptor. (B) Effects of shortening the N-terminus on pharmacological sequestration of the D_3 receptor. Receptor expression levels were about 0.6 pmol·mg^{-1} protein. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with WT-D_3 receptor. To achieve equal surface receptor expression, cells were transfected with 1 and 10 μg cDNA of WT and deletion mutants, respectively, per 100 mm culture dish. (C) Effects of deglycosylation on motility of the D_3 receptor on SDS-PAGE. Cells expressing FLAG-tagged WT- or N12/19Q-D_3R were immunoprecipitated with FLAG beads, analysed by SDS-PAGE, and immunoblotted with FLAG antibodies. GFP was used as an internal control for equal expression of transfected cDNAs in each experimental group. (D) Effects of deglycosylation on pharmacological sequestration of the D_3 receptor. Cells expressing each construct at about 0.7 pmol·mg^{-1} protein were treated with 10 μM DA for 5 min. $^{***}P < 0.001$ compared with WT-D_3 receptor. To achieve equal surface expression of WT and mutant D_3 receptor, cells were transfected with 1 μg and 10 μg cDNA of WT and mutant receptors, respectively, per 100 mm culture dish. (E) Effects of deglycosylation on agonist-induced desensitization of the D_3 receptor. Cells expressing WT or the N12/19Q-D_3 receptor were treated with 100 nM quinpirole for 5 min to induced desensitization. $^{***}P < 0.01$ compared with the WT/Veh group. All experiments (A–E) were repeated three times.

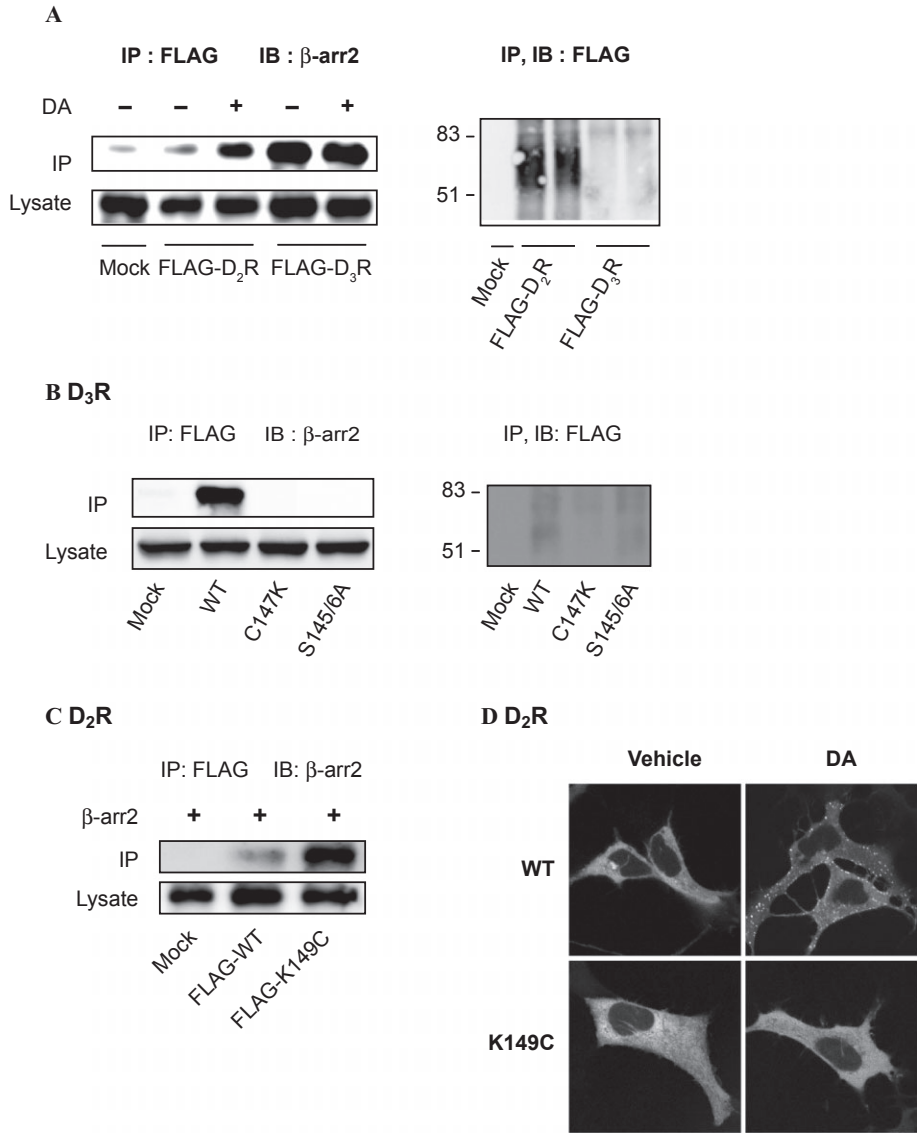

Figure 8

Relationship between β-arrestin binding and pharmacological sequestration of the D_3 receptor. (A) Cells were transfected with 2 μg β-arr2-pCMV5 together with 3 μg FLAG-tagged D_2 or D_3 receptor in pCMV5. Cells were stimulated with 10 μM DA for 5 min. Receptor expression level was maintained at about 1.7 pmol·mg^{-1} protein. Right panel represents immunoprecipitated receptors. Data represent results from three independent experiments with similar outcomes. (B) The receptor expression level was maintained at about 1.3 pmol·mg^{-1} protein. Right panel represents immunoprecipitated receptors. Data represent results from three independent experiments with similar outcomes. (C) Cells were transfected with 2 μg β-arr2-pCMV5 together with 2 μg FLAG-tagged corresponding receptor constructs in pCMV5. Receptor expression level was maintained at about 1.5 pmol·mg^{-1} protein. Data represent results from three independent experiments with similar outcomes. (D) Effects of K149 mutation in the D_2 receptor on agonist-induced β-arrestin translocation. Cells were transfected with 2 μg β-arr2-GFP together with 2 μg of the corresponding receptor constructs, and treated with 10 μM DA for 5 min. Receptor expression levels were maintained about 1.7 pmol·mg^{-1} protein. All experiments (A–D) were repeated three times.

was abolished when GRK2-CT was co-expressed (Figure 9B). Glutathione S-transferase (GST) pull-down study showed that the N-terminus of β-arrestin2 is involved in the interaction with Gβγ (Figure 9C). These results suggest that Gβγ plays important roles in the regulation of D_3 receptor in signalling, trafficking and interaction with adjacent proteins such as β-arrestins.

Pharmacological sequestration is quantitatively correlated with desensitization of D_3 receptor

As our results indicate that pharmacological sequestration is closely related to desensitization of D_3 receptor, we conducted a more thorough examination of the relationship between

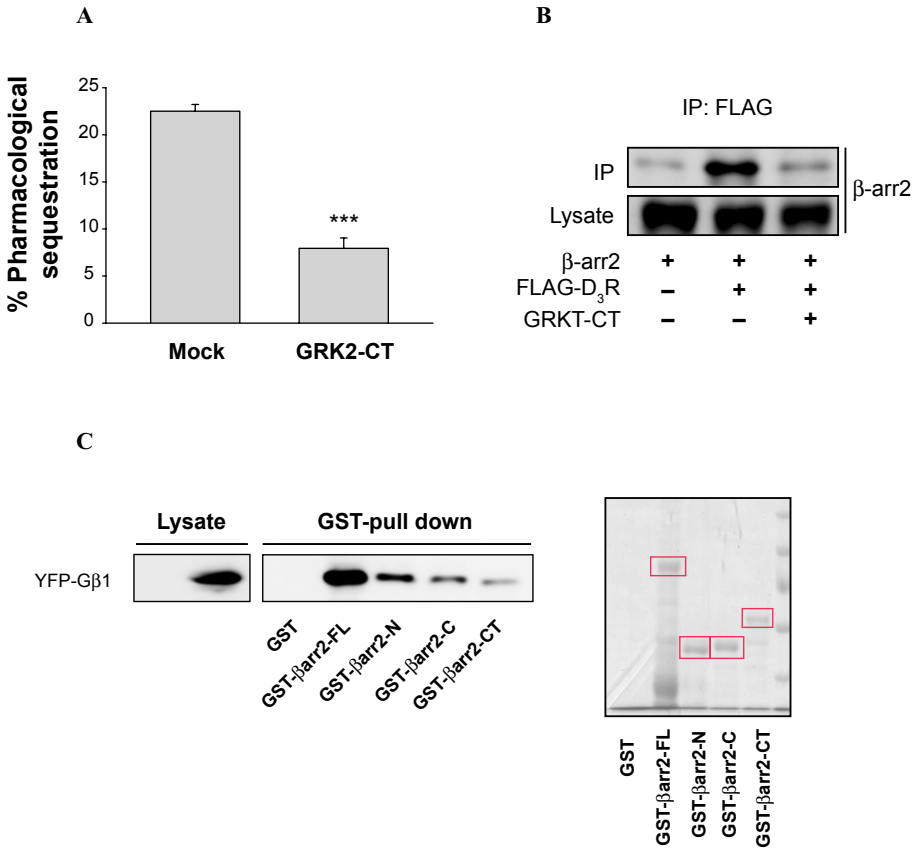

Figure 9

Roles of Gβγ in the pharmacological sequestration. (A, B) Roles of Gβγ in the pharmacological sequestration and interaction between D_3 receptor and β-arrestin2. Cells expressing D_3 receptor were transfected with 4 μg pRK5 or GRK2-CT in pRK5 per 100 mm culture dish. Receptor expression level was maintained at about 1.5 pmol·mg^{-1} protein. ***$P < 0.001$ compared with Mock group. Data represent results from three independent experiments with similar outcomes. (C) GST pull-down assay was conducted using the bacterial lysates containing the GST fusion proteins of the full-length (GST-β-arr2-FL), N-domain (GST-β-arr2-N), C-domain (GST-β-arr2-C) or C-domain plus carboxy tail of rat β-arrestin2 (GST-β-arr2-C-CT) (Zheng *et al.*, 2011) were mixed with the cell lysates of HEK-293 cells transfected with YFP-tagged Gβ1 (Azpiazu and Gautam, 2004). The figure in the right panel shows the SDS-PAGE gel, which contains 'after-wash' of bacterial cell lysates. Data represent results from three independent experiments with similar outcomes.

the two. For this, we employed three different agonists of D_3 receptor and tested whether there is quantitative relationship between their individual abilities to induce pharmacological sequestration and desensitization. As shown in Figure 10A, the three different agonists induced different levels of pharmacological sequestration, with 7-OH-DPAT inducing the greatest sequestration, followed by quinpirole and DA. The same patterns were observed for desensitization using these agonists (Figure 10B), again suggesting that pharmacological sequestration and desensitization has a close functional relationship.

Discussion

According to the working model for the regulation of GPCR, GRK-mediated receptor phosphorylation and subsequent association with β-arrestin causes uncoupling of the GPCR from the G protein (Gurevich and Benovic, 1997; Hausdorff *et al.*, 1990; Lohse *et al.*, 1989). Therefore, the main question addressed in this study was how the D_3 receptor, which rarely undergoes agonist-induced receptor phosphorylation and β-arrestin translocation, is desensitized (Westrich and Kuzhikandathil, 2007; Zheng *et al.*, 2011).

Recent studies on various GPCRs have shown that the working model of homologous desensitization could be different from that established for the β_2-adrenoceptor (Tsao *et al.*, 2001). For example, some GPCRs undergo desensitization or are internalized in a phosphorylation-independent manner (Qiu *et al.*, 2003; Rasmussen *et al.*, 2004; Jala *et al.*, 2005; Zhang *et al.*, 2005; Ferguson, 2007; Mitselos *et al.*, 2008; Cho *et al.*, 2010a; Cho *et al.*, 2010). In addition, a recent study in parathyroid hormone receptor type 1 showed that constitutive interaction between receptor and β-arrestin contributes to prolonged receptor signalling rather than attenuation (Wehbi *et al.*, 2013). These results suggest that the mechanism involved in receptor desensitization can vary for each receptor type and the nature of signal it mediates (Hausdorff *et al.*, 1990).

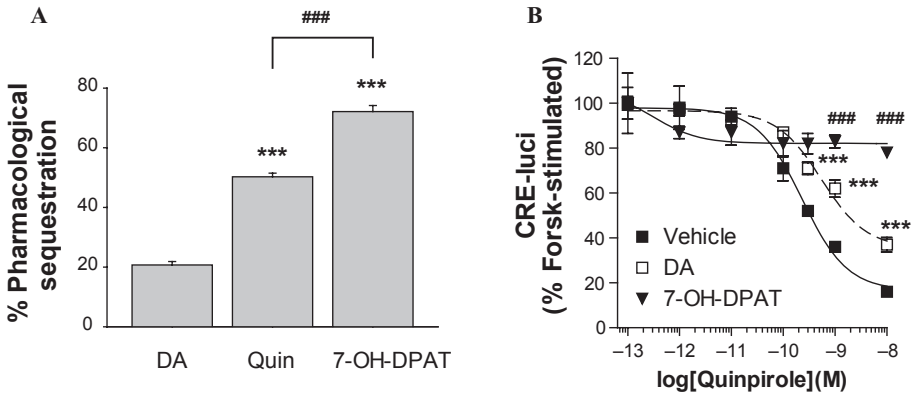

Figure 10

Effects of selective D_3 receptor agonist 7-OH-DPAT on the pharmacological sequestration and desensitization. (A) Cells expressing D_3 receptor were treated with 10 μM DA, quinpirole or 7-OH-DPAT for 5 min respectively. ***$P < 0.001$ compared with DA group. $^{###}P < 0.001$ when 7-OH-DPAT was compared with Quin. Data represent results from three independent experiments with similar outcomes. (B) Cells expressing D_3 receptor were pretreated with vehicle or 10 μM DA or 7-OH-DPAT for 5 min. Desensitization assays were conducted. ***$P < 0.001$ compared with vehicle group. $^{###}P < 0.001$, 7-OH-DPAT was compared with DA. Data represent results from three independent experiments with similar outcomes.

Several studies have shown that one kind of GPCR employs more than one intracellular trafficking pathway; one for rapid desensitization and the other for slow but carefully regulated desensitization and re-sensitization (Roettger *et al.*, 1995a). Some GPCRs have been reported to be sequestered on or near the plasma membrane without a long distance travel to the intracellular domains either as insulation on the membrane (Benovic *et al.*, 1987; Roettger *et al.*, 1995a) or as a result of conformational changes (Mostafapour *et al.*, 1996). Our study shows that the D_3 receptor also uses biochemically distinct intracellular trafficking machineries; a small but actual movement of receptor proteins and impaired binding to hydrophilic ligands, which might involve conformational changes, thereby providing more flexible and dynamic cellular regulation. The D_3 receptor is expressed on virtually all dopaminergic neurons (Gurevich and Joyce, 1999; Diaz *et al.*, 2000), supporting the idea that the D_3 receptor acts as an autoreceptor and requires dynamic and versatile regulation according to cellular needs. Therefore, it is expected that receptors internalized near the plasma membrane would provide a cellular mechanism to rapidly re-sensitize according to cellular needs (Roettger *et al.*, 1995a,b; Ozcelebi *et al.*, 1996). Such a unique mode of regulation allows D_3 receptor to perform highly flexible regulatory functions as an autoreceptor in the synaptic region.

Previous studies showed that SSC motif, which contains S145/S146, is involved in the desensitization of D_3 receptor through conformational changes (Westrich and Kuzhikandathil, 2007; Westrich *et al.*, 2010). SSC motif seems to mediate D_3 receptor desensitization independently of receptor phosphorylation. We have shown that S145 and S146 are not phosphorylation sites for PKC (Cho *et al.*, 2007). In addition, S145 and S146 are unlikely to be phosphorylation sites for GRK as the phosphorylation of D_3 receptor was not increased in response to agonistic stimulation, regardless of co-expression of GRK subtypes (Kim *et al.*, 2001; Cho *et al.*, 2007). Results in Supporting Information Figure S3 also show that GRK2, 4, 5 and 6 has no effect on pharmacological sequestration (Supporting Information Figure S3).

It is challenging that pharmacological sequestration and desensitization of the D_3 receptor are intimately related. For example, the three receptors, WT-D_3R, K149C-D_2R and R132H-D_2R-V_2Rt, exhibited both pharmacological sequestration and desensitization; the WT-D_2 and C147K-D_3R showed neither; β-arrestins were involved in both processes; and deglycosylation blocked both of them, abilities to induce pharmacological sequestration and desensitization coincide among different agonists of D_3 receptor. Thus, these results persistently propose a functional relationship between pharmacological sequestration and desensitization. However, it should be mentioned that the time-courses of these two processes do not exactly coincide. Agonist-induced desensitization is maintained after five washes at room temperature. However, agonist-induced pharmacological sequestration is abolished in the middle of washing. It is conceivable that D_3 receptor exists in three different conformations: basal state, agonist-bound state and dissociated state, of which the latter could be the conformation during desensitization. Even though basal and desensitization states have binding properties similar to hydrophilic ligands, they are in different conformations as reported previously (Westrich *et al.*, 2010). Pharmacological sequestration probably represents conformation state between agonist-bound and desensitization state. Thus, pharmacological sequestration, which accompanies conformational changes, might be a trigger to induce desensitization. Other unidentified cellular processes might be involved in the maintenance of the desensitization state.

It is curious how β-arrestins, which are constitutively bound to the D_3 receptor and do not show an agonist-dependent interaction with the D_3 receptor, contribute to agonist-induced desensitization. From the results in Figure 4E & F, it could be speculated that β-arrestins play certain roles in the initiation of conformational changes of D_3 receptor by which it cannot effectively couple to signalling constituents (Figure 11). Also, the results in Figure 9 suggest that cooperative interaction with Gβγ could be related to novel roles of β-arrestin in pharmacological sequestration. More systemic studies are needed to elucidate the functional roles of

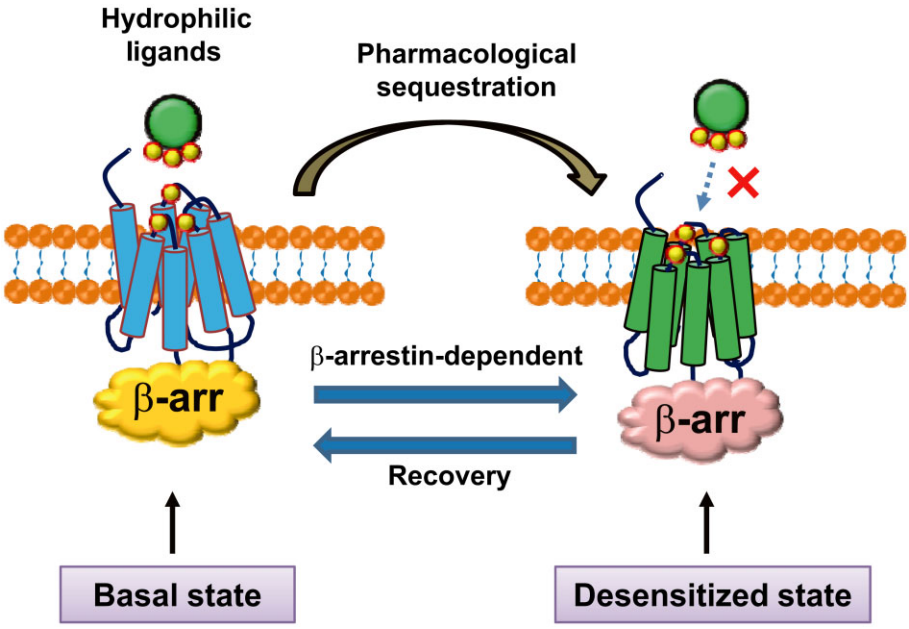

Figure 11

Diagram showing the molecular mechanism involved in pharmacological sequestration and tolerance. The D_3 receptors shown in the left panel represent the receptors at the basal state or recycled state after pharmacological sequestration. The D_3 receptors shown in the right panel represent pharmacologically sequestered and desensitized receptors.

β-arrestins and related pathway proteins play in D_3 receptor desensitization.

The main message delivered in this study is that some GPCRs might be desensitized through pathways distinct from previously established phosphorylation-dependent and GRK2/β-arrestin2-mediated mechanisms. Given that the D_3 receptor possesses regulatory properties different from other GPCRs, such as the β_2-adrenoceptor, the molecular pathway proposed in this study might explain the regulatory properties of other GPCRs whose desensitization cannot be explained by previously established desensitization paradigm of GPCRs. In particular, it is noticeable that pharmacological sequestration could be used to predict agonist-induced desensitization of the dopamine D_3 receptor.

Acknowledgements

This work was funded by KRF-2011-0016396. We thank the Korea Basic Science Institute for the technical support.

Conflict of interest

The authors state no conflict of interest.

References

Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM *et al.* (1992). Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. J Biol Chem 267: 17882–17890.

Azpiazu I, Gautam N (2004). A fluorescence resonance energy transfer-based sensor indicates that receptor access to a G protein is unrestricted in a living mammalian cell. J Biol Chem 279: 27709–27718.

Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ (1986). Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. Proc Natl Acad Sci U S A 83: 2797–2801.

Benovic JL, Kuhn H, Weyand I, Codina J, Caron MG, Lefkowitz RJ (1987). Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proc Natl Acad Sci U S A 84: 8879–8882.

Benovic JL, DeBlasi A, Stone WC, Caron MG, Lefkowitz RJ (1989). Beta-adrenergic receptor kinase: primary structure delineates a multigene family. Science 246: 235–240.

Cho D, Zheng M, Min C, Ma L, Kurose H, Park JH *et al.* (2010). Agonist-induced endocytosis and receptor phosphorylation mediate resensitization of dopamine D(2) receptors. Mol Endocrinol 24: 574–586.

Cho D, Zheng M, Min C, Ma L, Kurose H, Park JH *et al.* (2010a). Agonist-induced endocytosis and receptor phosphorylation mediate resensitization of dopamine D(2) receptors. Mol Endocrinol 24: 574–586.

Cho DI, Zheng M, Kim KM (2010b). Current perspectives on the selective regulation of dopamine D(2) and D(3) receptors. Arch Pharm Res 33: 1521–1538.

Cho DI, Min C, Jung KS, Cheong SY, Zheng M, Cheong SJ *et al.* (2012). The N-terminal region of the dopamine D2 receptor, a rhodopsin-like GPCR, regulates correct integration into the plasma membrane and endocytic routes. Br J Pharmacol 166: 659–675.

Cho EY, Cho DI, Park JH, Kurose H, Caron MG, Kim KM (2007). Roles of protein kinase C and actin-binding protein 280 in the regulation of intracellular trafficking of dopamine D3 receptor. Mol Endocrinol 21: 2242–2254.

Diaz J, Pilon C, Le Foll B, Gros C, Triller A, Schwartz JC *et al.* (2000). Dopamine D3 receptors expressed by all mesencephalic dopamine neurons. J Neurosci 20: 8677–8684.

Ferguson SS (2007). Phosphorylation-independent attenuation of GPCR signalling. Trends Pharmacol Sci 28: 173–179.

Giros B, Martres MP, Sokoloff P, Schwartz JC (1990). [Gene cloning of human dopaminergic D3 receptor and identification of its chromosome]. C R Acad Sci III 311: 501–508.

Guo Q, Subramanian H, Gupta K, Ali H (2011). Regulation of C3a receptor signaling in human mast cells by G protein coupled receptor kinases. PLoS ONE 6: e22559.

Gurevich EV, Joyce JN (1999). Distribution of dopamine D3 receptor expressing neurons in the human forebrain: comparison with D2 receptor expressing neurons. Neuropsychopharmacology 20: 60–80.

Gurevich VV, Benovic JL (1997). Mechanism of phosphorylation-recognition by visual arrestin and the transition of arrestin into a high affinity binding state. Mol Pharmacol 51: 161–169.

Hausdorff WP, Caron MG, Lefkowitz RJ (1990). Turning off the signal: desensitization of beta-adrenergic receptor function. FASEB J 4: 2881–2889.

Holmes A, Lachowicz JE, Sibley DR (2004). Phenotypic analysis of dopamine receptor knockout mice; recent insights into the functional specificity of dopamine receptor subtypes. Neuropharmacology 47: 1117–1134.

Ivins KJ, Luedtke RR, Artymyshyn RP, Molinoff PB (1991). Regulation of dopamine D2 receptors in a novel cell line (SUP1). Mol Pharmacol 39: 531–539.

Jala VR, Shao WH, Haribabu B (2005). Phosphorylation-independent beta-arrestin translocation and internalization of leukotriene B4 receptors. J Biol Chem 280: 4880–4887.

Jin M, Min C, Zheng M, Cho DI, Cheong SJ, Kurose H *et al.* (2013). Multiple signaling routes involved in the regulation of adenylyl cyclase and extracellular regulated kinase by dopamine D(2) and D(3) receptors. Pharmacol Res 67: 31–41.

Kabbani N, Negyessy L, Lin R, Goldman-Rakic P, Levenson R (2002). Interaction with neuronal calcium sensor NCS-1 mediates desensitization of the D2 dopamine receptor. J Neurosci 22: 8476–8486.

Kim KM, Caron MG (2008). Complementary roles of the DRY motif and C-terminus tail of GPCRS for G protein coupling and beta-arrestin interaction. Biochem Biophys Res Commun 366: 42–47.

Kim KM, Valenzano KJ, Robinson SR, Yao WD, Barak LS, Caron MG (2001). Differential regulation of the dopamine D2 and D3 receptors by G protein-coupled receptor kinases and beta -arrestins. J Biol Chem 276: 37409–37414.

Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ (1994). Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. J Biol Chem 269: 6193–6197.

Lohse MJ, Lefkowitz RJ, Caron MG, Benovic JL (1989). Inhibition of beta-adrenergic receptor kinase prevents rapid homologous desensitization of beta 2-adrenergic receptors. Proc Natl Acad Sci U S A 86: 3011–3015.

Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ (1990). beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 248: 1547–1550.

Marina-Garcia N, Franchi L, Kim YG, Hu Y, Smith DE, Boons GJ *et al.* (2009). Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation. J Immunol 182: 4321–4327.

Mitselos A, Peeters TL, Depoortere I (2008). Desensitization and internalization of the human motilin receptor is independent of the C-terminal tail. Peptides 29: 1167–1175.

Mostafapour S, Kobilka BK, von Zastrow M (1996). Pharmacological sequestration of a chimeric beta 3/beta 2 adrenergic receptor occurs without a corresponding amount of receptor internalization. Recept Signal Transduct 6: 151–163.

Ozcelebi F, Rao RV, Holicky E, Madden BJ, McCormick DJ, Miller LJ (1996). Phosphorylation of cholecystokinin receptors expressed on Chinese hamster ovary cells. Similarities and differences relative to native pancreatic acinar cell receptors. J Biol Chem 271: 3750–3755.

Qiu Y, Law PY, Loh HH (2003). Mu-opioid receptor desensitization: role of receptor phosphorylation, internalization, and representation. J Biol Chem 278: 36733–36739.

Rana BK, Shiina T, Insel PA (2001). Genetic variations and polymorphisms of G protein-coupled receptors: functional and therapeutic implications. Annu Rev Pharmacol Toxicol 41: 593–624.

Rasmussen TN, Novak I, Nielsen SM (2004). Internalization of the human CRF receptor 1 is independent of classical phosphorylation sites and of beta-arrestin 1 recruitment. Eur J Biochem 271: 4366–4374.

Roettger BF, Rentsch RU, Hadac EM, Hellen EH, Burghardt TP, Miller LJ (1995a). Insulation of a G protein-coupled receptor on the plasmalemmal surface of the pancreatic acinar cell. J Cell Biol 130: 579–590.

Roettger BF, Rentsch RU, Pinon D, Holicky E, Hadac E, Larkin JM *et al.* (1995b). Dual pathways of internalization of the cholecystokinin receptor. J Cell Biol 128: 1029–1041.

Sibley DR, Lefkowitz RJ (1985). Molecular mechanisms of receptor desensitization using the beta-adrenergic receptor-coupled adenylate cyclase system as a model. Nature 317: 124–129.

Starr S, Kozell LB, Neve KA (1995). Drug-induced up-regulation of dopamine D2 receptors on cultured cells. J Neurochem 65: 569–577.

Touhara K, Inglese J, Pitcher JA, Shaw G, Lefkowitz RJ (1994). Binding of G protein beta gamma-subunits to pleckstrin homology domains. J Biol Chem 269: 10217–10220.

Tsao P, Cao T, von Zastrow M (2001). Role of endocytosis in mediating downregulation of G-protein-coupled receptors. Trends Pharmacol Sci 22: 91–96.

Villar VA, Jones JE, Armando I, Palmes-Saloma C, Yu P, Pascua AM *et al.* (2009). G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. J Biol Chem 284: 21425–21434.

Wehbi VL, Stevenson HP, Feinstein TN, Calero G, Romero G, Vilardaga JP (2013). Noncanonical GPCR signaling arising from a PTH receptor-arrestin-Gβγ complex. Proc Natl Acad Sci U S A 110: 1530–1535.

Westrich L, Kuzhikandathil EV (2007). The tolerance property of human D3 dopamine receptor is determined by specific amino acid residues in the second cytoplasmic loop. Biochim Biophys Acta 1773: 1747–1758.

Westrich L, Gil-Mast S, Kortagere S, Kuzhikandathil EV (2010). Development of tolerance in D3 dopamine receptor signaling is accompanied by distinct changes in receptor conformation. Biochem Pharmacol 79: 897–907.

Zhang LJ, Lachowicz JE, Sibley DR (1994). The D2S and D2L dopamine receptor isoforms are differentially regulated in Chinese hamster ovary cells. Mol Pharmacol 45: 878–889.

Zhang X, Wang F, Chen X, Li J, Xiang B, Zhang YQ *et al*. (2005). Beta-arrestin1 and beta-arrestin2 are differentially required for phosphorylation-dependent and -independent internalization of delta-opioid receptors. J Neurochem 95: 169–178.

Zhang X, Wang F, Chen X, Chen Y, Ma L (2008). Post-endocytic fates of delta-opioid receptor are regulated by GRK2-mediated receptor phosphorylation and distinct beta-arrestin isoforms. J Neurochem 106: 781–792.

Zheng M, Cheong SY, Min C, Jin M, Cho DI, Kim KM (2011). β-Arrestin2 plays permissive roles in the inhibitory activities of RGS9-2 on G protein-coupled receptors by maintaining RGS9-2 in the open conformation. Mol Cell Biol 31: 4887–4901.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12357

Figure S1 Ligand binding properties of dopamine D_3 receptors. (A) Cells expressing D_3 receptor were treated with 10 μM DA for 5 min, washed and then treated with 2 nM [3H]-spiperone dissolved in serum-free media for 1 h at room temperature in the presence or absence of 10 μM haloperidol. The data are representative of two independent experiments, with each point measured in triplicate. (B) Cells transiently transfected with cDNA for the D_3 receptor were treated with either vehicle or 10 μM DA for 5 min, membrane proteins were prepared, and exposed to varying concentrations of [3H]-sulpiride (between 0.25 and 100 nM). Scatchard analysis indicates that the cells pre-exposed to DA display lower B_{max} values than the cells treated with vehicle. Plasma membrane expression of the D_3 receptor in vehicle-treated cells were between 3.3 and 5.0 pmol·mg^{-1} of whole cell protein, while the expression of the D_3 receptor in DA-treated cells varied between 2.0 and 3.1 pmol·mg^{-1}. The K_D of the D_3 receptor in cells treated with vehicle was similar to the K_D of the D_3 receptor pretreated with DA (between 5.2 and 13.2 nM).

Figure S2 Comparison of DA- and PMA-induced sequestration of D_3 receptors. (A) Cells expressing D_2 receptor were treated with 10 μM DA for 1 h, washed three times with either 20 mM pH 7.4 HEPES buffer or low pH buffer (150 mM NaCl, 50 mM acetic acid, pH 2.0) on ice. Data represent results from two independent experiments with similar outcomes. (B) Cells expressing FLAG-tagged D_3 receptor were treated 10 μM DA for 1h or 100 nM PMA for 30 min. Receptor sequestration was determined by ELISA method. Data represent results from two independent experiments with similar outcomes.

Figure S3 Roles of GRK subtypes in pharmacological sequestration of D_3 receptors. (A) Cells stably expressing control-shRNA (CTL-KD) or GRK2-shRNA (GRK2-KD) were transfected with 2 μg of the D_3 receptor cDNA in pcMV5. Cells were treated with either vehicle or 10 μM DA. Cellular levels of GRK2 were determined by antibodies to GRK2, and actin was used as internal control. Data represent results from two independent experiments with similar outcomes. (B) Cells stably expressing control-shRNA (CTL-KD) or GRK5-shRNA (GRK5-KD) were transfected with 2 μg of the D_3 receptor. Cells were treated with either vehicle or 10 μM DA. Cellular levels of GRK5 were determined by antibodies to GRK5, and actin was used as internal control. Data represent results from two independent experiments with similar outcomes. (C) Cells expressing D_3 receptor were transfected with either wild-type or dominant negative mutants of GRK4/5/6. (D) Effects of lowering cellular levels of GRK2 on agonist-induced desensitization of the D_3 receptor. Cells expressing about 1.2 pmol·mg^{-1} protein of D_3 receptor were pretreated with 10 μM DA for 5 min, and desensitization assay was conducted. ***$P < 0.001$ compared with vehicle-treated group. Data represent results from three independent experiments with similar outcomes.

Figure S4 Desensitization properties of the D_3 receptor point mutants at the serine and threonine residues located within the second intracellular loop. (A) Effects of total mutation of all S/T residues located in the second intracellular loop on agonist-induced desensitization of the D_3 receptor. Cells expressing corresponding receptor constructs were treated with 100 pM quinpirole for 5 min. Receptor expression level was maintained at about 1.2 pmol·mg^{-1} protein. The maximum percent of D_3 receptor-mediated inhibition of cAMP production changed from 58.3 to 47.7% (wild-type) and from 61.8 to 61.2% (D_3R-IC2) following quinpirole treatment. **$P < 0.01$, when WT/Quin group was compared with other experimental groups. (B) Effects of T130 mutation on agonist-induced desensitization of the D_3 receptor. The maximum percent of D_3 receptor-mediated inhibition of cAMP production changed from 66.6 to 50.4% (wild-type) and from 60.7 to 50.12% (T134V-D_3R) following quinpirole treatment. (C) Effects of T142 mutation on agonist-induced desensitization of the D_3 receptor. The maximum percent of D3 receptor-mediated inhibition of cAMP production changed from 66.6 to 50.4% (wild-type) and from 64.8 to 58.5% (T142V-D_3R) following quinpirole treatment. (D) Effects of S145 mutation on agonist-induced desensitization of the D_3 receptor. The maximum percent of D_3 receptor-mediated inhibition of cAMP production changed from 76.2 to 52.9 % (wild-type) and from 73.4 to 70 % (S145A-D_3R) following quinpirole treatment. **$P < 0.01$, when wild-type/Quin group was compared with wild-type/vehicle group. #$P < 0.05$, when the S145A-D_3R/Quin group was compared with the S145A-D_3R/vehicle group. (E) Effects of the S146 mutation on agonist-induced desensitization of the D_3 receptor. The maximum percent inhibition changed from 76.2 to 52.9 % (wild-type) and from 68.6 to 63.9 % (S146A) following quinpirole treatment. ***$P < 0.001$ when wild-type/Quin group was compared with the wild-type/vehicle group. #$P < 0.05$ when S146A-D_3R/Quin group was compared with the S146A-D_3R4/vehicle group.

Figure S5 Co-relationship between pharmacological sequestration and desensitization of a D_2 receptor mutant that mimics the D_3 receptor. (A) Comparison between the wild-type D_2 receptor and R132H-D_2R-V2Rt on agonist-induced β-arrestin translocation. Cells were transfected with 2 μg

β-arr2-GFP together with 2 μg of the corresponding receptor constructs in pCMV5. After 24 h, cells were treated 10 μM DA for 5 min. Receptor expression levels were about 1.3 pmol·mg^{-1} protein. (B) Comparison between the wild-type D_2 receptor and R132H-D_2R-V2Rt on the constitutive interaction with β-arrestin. Cells were transfected with 2 μg β-arr2-pCMV5 together with 2 μg FLAG-tagged corresponding receptor constructs in pCMV5. Cell lysates were immunoprecipitated with M2 FLAG affinity gels and immunoblotted with antibodies to β-arr2. Receptor expression level was maintained at about 1.2 pmol·mg^{-1} protein. Data represent results from three independent experiments with similar outcomes. (C) Comparison between wild-type D_2 receptor and R132H-D_2R-V2Rt on agonist-induced desensitization. Cells expressing about 1.4 pmol·mg^{-1} protein of wild-type or R132H-V2Rt D_2 receptor were treated with 10 μM DA for 5 min. Cells were washed three times with serum-free media at room temperature, and dose–response experiments were conducted with increasing concentrations of quinpirole. ***$P < 0.001$ compared with other experimental groups. (D) Comparison between wild-type D_2 receptor and R132H-D2R -V2Rt on pharmacological sequestration. Cells expressing each construct at about 1.4 pmol·mg^{-1} protein were treated with 10 μM DA for 5 min. ***$P < 0.001$ compared with wild-type D_2 receptor. All experiments (AD) were repeated three times.