

Functional Regulation of Dopamine D₃ Receptor through Interaction with PICK1

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Abstract

PICK1, a PDZ domain-containing protein, is known to increase the reuptake activities of dopamine transporters by increasing their expressions on the cell surface. Here, we report a direct and functional interaction between PICK1 and dopamine D₃ receptors (D₃R), which act as autoreceptors to negatively regulate dopaminergic neurons. PICK1 colocalized with both dopamine D₂ receptor (D₂R) and D₃R in clusters but exerted different functional influences on them. The cell surface expression, agonist affinity, endocytosis, and signaling of D₂R were unaffected by the coexpression of PICK1. On the other hand, the surface expression and tolerance of D₃R were inhibited by the coexpression of PICK1. These findings show that PICK1 exerts multiple effects on D₃R functions.

Key Words: Dopamine D₂ receptor, Dopamine D₃ receptor, Dopamine transporter, PICK1, Cell surface expression, Tolerance

INTRODUCTION

Dopamine D₂ and D₃ receptors (D₂R, D₃R) are expressed in dopaminergic neurons together with dopamine transporters (DAT) (Gurevich and Joyce, 1999; Diaz *et al.*, 2000). Functional interactions between D₂R and DATs have been characterized. For example, the stimulation of D₂R activates the uptake activity of DAT (Mayfield and Zahniser, 2001), and the cell surface localization of DAT is facilitated through an interaction between the amino-terminus of DAT and the third intracellular loop of D₂R (Lee *et al.*, 2007).

Among the dopamine receptor subtypes characterized, D₂R and D₃R are important targets for the treatment of various diseases in motor, emotional, and endocrine functions, such as Parkinson's disease, schizophrenia, and pituitary tumors (for review, see ref. Cho *et al.*, 2010b). D₂R and D₃R show different spatial expression patterns within the brain. D₂R is heavily expressed in the regions responsible for motor and endocrine functions (for example, striatum and pituitary glands) whereas D₃R is more predominant in emotional and mental functions areas (for example, nucleus accumbens, olfactory tubercle, and islands of Calleja) (Bunzow *et al.*, 1988; Sokoloff *et al.*, 1990; Gurevich and Joyce, 1999; Cho *et al.*, 2010b). Since current antipsychotics show low selectivity toward the two re-

ceptor types, the treatment of schizophrenia with these agents result in the disturbances of motor and endocrine functions, which are presumably caused by the inhibition of D₂R. Hence, cellular components or cellular events involved in the selective manipulation of D₂R and D₃R could be used as potential targets to separate the desired therapeutic effects from the unwanted side effects of antipsychotics.

PICK1 (protein interacting with C kinase 1) was originally identified by its interaction with PKC α in a yeast two hybrid-screen (Staudinger *et al.*, 1995). A previous study has shown that PICK1 and DAT coimmunoprecipitate from mice striatal membranes and colocalize in cultured dopaminergic neurons (Torres *et al.*, 2001). The uptake activity of DAT was enhanced via an increase in surface expression when PICK1 was co-expressed (Torres *et al.*, 2001). Since D₂R and D₃R are expressed in dopaminergic neurons and functionally related to DAT, it is expected that PICK1 has functional interactions with D₂R and D₃R.

In this study, we tested whether PICK1 exerts any regulatory effects on the D₂R and D₃R.

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MATERIALS AND METHODS

Materials

Human embryonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell culture media and fetal bovine serum were obtained from Life Technologies, Inc. (Carlsbad, CA, USA). Dopamine (DA), (-) quinpirole, haloperidol, PMA, anti-Flag M2 antibodies, anti-Flag-conjugated agarose beads, antibodies for green fluorescence protein (GFP), horseradish peroxidase (HRP)-labeled secondary antibodies, and glutathione beads were purchased from Sigma/Aldrich Chemical (St Louis, MO, USA). [³H]-Sulpiride (87 Ci/mmol) and [³H]-spiperone (85.7 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA).

Plasmid constructs

The mammalian expression constructs for human dopamine D₂ receptor (D₂R) and D₃ receptor (D₃R) have been described previously (Kim *et al.*, 2001; Beom *et al.*, 2004). The PICK1 construct was provided by Dr. Hanley (University of Bristol, Bristol, UK). The GST fusion protein constructs of the 3rd intracellular loops of D₂R and D₃R were described in a previous study (Zheng *et al.*, 2011).

Determination of receptor expression levels

To determine receptor expression levels, cells were incubated with 3 nM [³H]-spiperone with 100 µg of membranes for 1 h at room temperature. Nonspecific binding was determined in the presence of 10 µM haloperidol. Reactions were terminated by rapid filtration over GF/B filters, followed by three washes with ice-cold buffer (100 mM NaCl, 50 mM Tris, pH 7.2).

Endocytosis assay

Endocytosis of D₂R and D₃R were measured based on the hydrophilic properties of [³H]-sulpiride as previously described (Cho *et al.*, 2010a). Briefly, HEK-293 cells expressing D₂R or D₃R were seeded 1 day after transfection at a density of 1.5 × 10⁵ cells/well in 24-well plates. The cells were stimulated with 10 µM DA, 0.1 µM PMA, or 1.0 µM PMA for 0-60 min as indicated. The cells were then incubated with 250 µl of [³H]-sulpiride (final concentration; 2.2 nM for D₂R, 7.2 nM for D₃R) at 4°C for 150 min in the absence and presence of unlabeled competitive inhibitor (10 µM haloperidol). Subsequently, the cells were washed three times with ice-cold serum-free media and 1% SDS was added. The samples were mixed with 2 ml Lefkofluor scintillation fluid and counted on a liquid scintillation counter.

Reporter gene assay

Cellular cAMP was measured by an indirect method which had been used for the determination of D₂R and D₃R signaling as described previously (Cho *et al.*, 2007; Cho *et al.*, 2010a). A reporter plasmid containing the firefly luciferase gene under the control of multiple cAMP responsive elements (CRE) was used with a pRL-TK control vector (Promega, Madison, WI, USA).

Immunoprecipitation

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) on a rota-

tion wheel for 1 h at 4°C. The supernatants were mixed with 35 µl of a 50% slurry of anti-Flag-agarose beads for 2-3 h on the rotation wheel. The beads were washed with a buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40) three times for 10 min each. The immunoprecipitates were analyzed by immunoblotting.

Immunocytochemistry

The HEK-293 cells were plated on cover slips and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature. Next, the cells were incubated with PBS containing 3% fetal bovine serum and 1% bovine serum albumin for 1 h and incubated with anti-Flag antibodies at 1:1000 dilutions for 1 h at room temperature. After three washes, cells were incubated with Alexa 555-conjugated anti-mouse antibodies (Invitrogen, Carlsbad, CA, USA) at 1:500 dilutions. After another three washes with the washing buffer, the cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and visualized with a laser scanning confocal microscope (TCS SP5/AOBS/Tandem, Wetzlar, Germany).

Pharmacological sequestration assay

The pharmacological sequestration of D₃R was conducted as previously described (Min *et al.*, 2013). Briefly, HEK-293 cells expressing the corresponding receptors were stimulated with a vehicle or 1 µM DA for 5 min and washed three times with a low pH buffer (150 mM NaCl, 50 mM acetic acid, pH 2.0), followed by two washes with HEPES buffer (20 mM, pH 7.4) on ice. Subsequently, the cells were incubated with 250 µl of [³H]-sulpiride (7.2 nM) at 4°C for 150 min in the absence or presence of 10 µM haloperidol. The cells were then washed and lysed with 1% SDS, and the remaining radioactivity was counted using a liquid scintillation counter.

Data analyses

The values are expressed as mean ± SEM for the number of independent experiments indicated in the figure legends. Student's *t*-test was used to compare the results. A *p*-value <0.05 was considered significant. Comparisons among experimental groups were performed using ANOVA and Tukey's simultaneous test.

RESULTS

PICK1 forms clusters with dopamine D₂ and D₃ receptors

PICK1 is expressed in dopaminergic neurons and has been shown to colocalize with DATs to increase their uptake activities (Torres *et al.*, 2001). Since dopamine D₂ receptor (D₂R) and D₃ receptor (D₃R) act as autoreceptors in dopaminergic neurons, the functional interactions between PICK1 and D₂R/D₃R were tested. As shown in the left panel of Fig. 1A, PICK1 was distributed throughout the cytosol and near the plasma membrane but not in the nucleus. When D₂R was coexpressed, PICK1 formed extensive clusters on the plasma membrane (Fig. 1A, middle panel). When D₃R was coexpressed, PICK1 also formed some clusters on the plasma membrane, but the majority of the clusters stayed in the cytosol (Fig. 1A, right panel). Consistent with these results, PICK1 and D₂R showed colocalizations on the plasma membrane (Fig. 1B, upper pan-

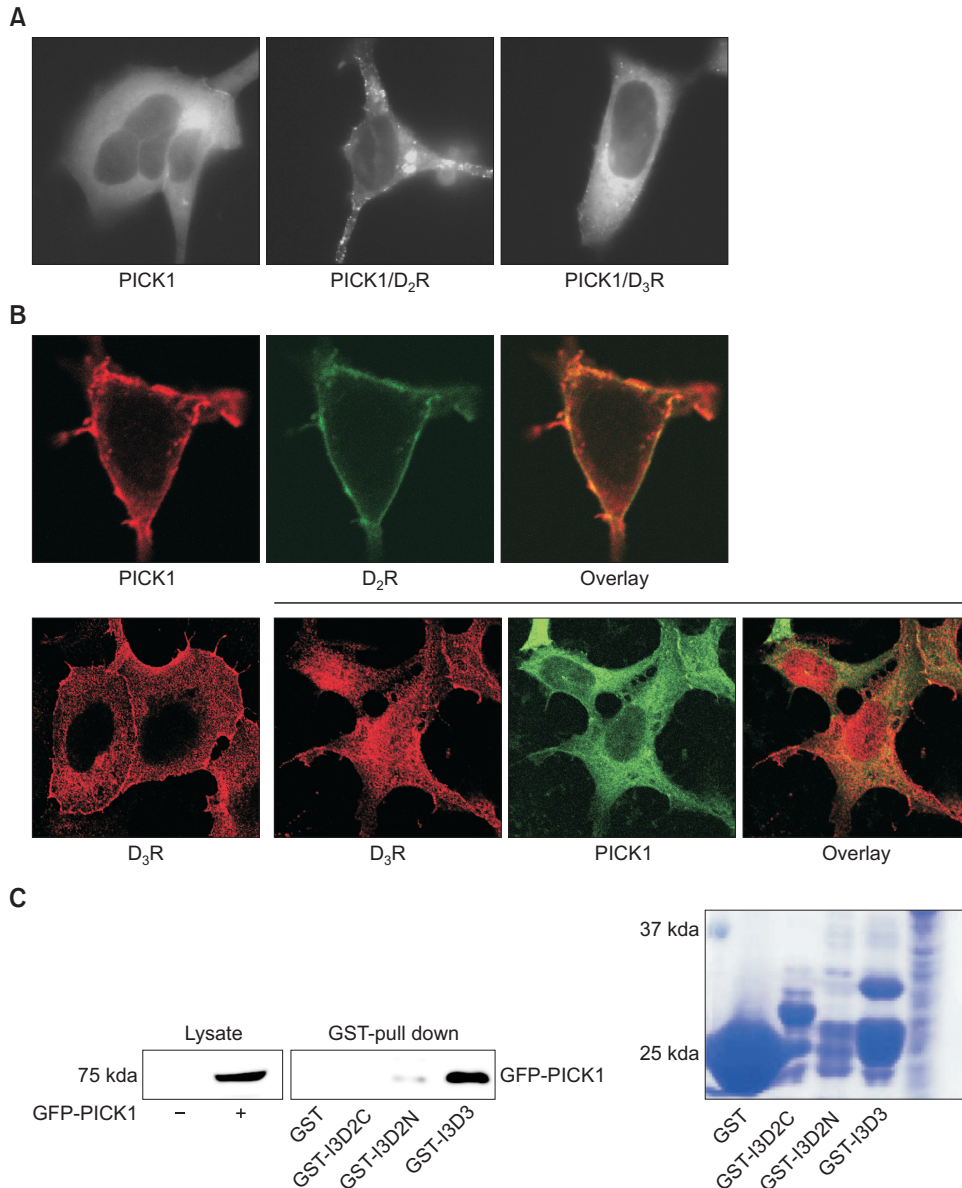


Fig. 1. Interaction between PICK1 and D₂R/D₃R. (A) The effects of D₂R or D₃R on the subcellular distribution of PICK1. HEK-293 cells were transfected with Flag-tagged PICK1 (left panel) or with D₂R (middle panel) or D₃R (right panel) (2.7 and 2.1 pmol/mg protein, respectively). Cells were labeled with anti-Flag antibodies. (B) Colocalization between PICK1 and D₂R or D₃R. (Upper panel) HEK-293 cells were transfected with Flag-PICK1 and D₂R-GFP. Cells were labeled with anti-Flag antibodies. (Lower panel) HEK-293 cells were transfected with Flag-D₃R with or without GFP-PICK1. Cells were labeled with anti-Flag antibodies. (C) GST pull-down assay for the interaction between PICK1 and the intracellular loops of D₂R and D₃R. Constructs for the GST fusion protein with the 3rd intracellular loop of D₂R (I3D2) and D₃R (I3D3) and the functionalities of these constructs were described in a previous study (Zheng *et al.*, 2011). I3D₂C and I3D₂N represent carboxyl region and amino region of I3D₂. Bacterial lysates containing GST-binding agarose beads along with GST-I3D₂ or GST-I3D₃ were incubated with the lysates of HEK-293 cells expressing GFP-PICK1. After washing three times, bound proteins were eluted with SDS sample buffer. Eluents were analyzed on the SDS-PAGE gel and blotted with antibodies against GFP. On the right is an SDS-PAGE analysis of the after-wash of bacterial cell lysates.

el), whereas PICK1 and D₃R colocalized mainly in the cytosol (Fig. 1, lower panel). When the interactions were tested by GST pull-down assays, the 3rd intracellular loop of D₃R showed more abundant interactions with PICK1 than those of D₂R.

Effects of PICK1 on the signaling activities of D₂R and D₃R

Next, we examined the effect of PICK1 coexpression on

the signaling activities of D₂R and D₃R. The coexpression of PICK1 did not affect the signaling of D₂R (Fig. 2A) or D₃R (Fig. 2B, compare Mock/Veh vs. PICK1/Veh).

It was previously reported that D₃R but not D₂R undergoes tolerance in response to pretreatment with agonists (Min *et al.*, 2013) (Fig. 2B, compare Mock/Veh vs. Mock/DA group). The coexpression of PICK1 significantly inhibited the tolerance of

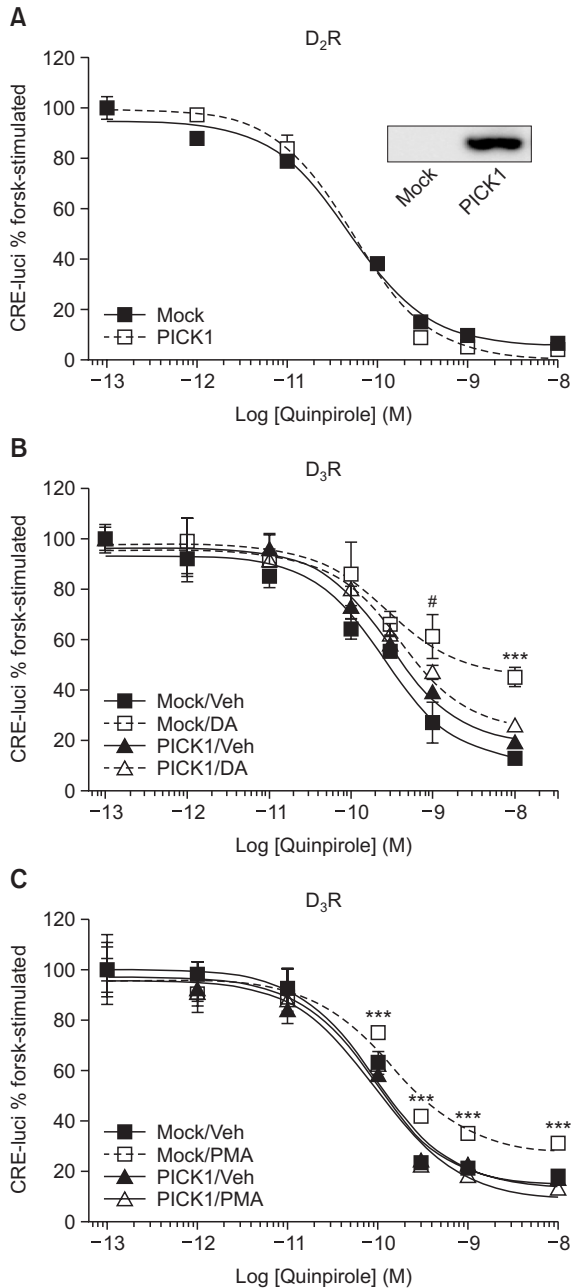


Fig. 2. Effects of PICK1 on the signaling via D_2R and D_3R . (A) HEK-293 cells stably expressing D_2R (1.9 pmol/mg of protein) were transfected with Mock vector or Flag-PICK1. Reporter gene assay was conducted with increasing concentrations of quinpirole. Cell lysates were blotted with anti-Flag antibodies. (B) HEK-293 cells stably expressing D_3R (2.1 pmol/mg of protein) were transfected with either Mock vector or PICK1. Cells were pretreated with either vehicle or 10 μ M DA for 5 min, washed 5 times with warm serum-free media and dose-responses were obtained with increasing concentrations of quinpirole. *** p <0.001 compared with other groups. # p <0.05 when Mock/DA group was compared with PICK1/Veh or PICK1/DA group. (C) HEK-293 cells stably expressing D_3R (2.1 pmol/mg of protein) were transfected with either Mock vector or PICK1. Cells were pretreated with either vehicle or 100 nM PMA for 5 min, washed 3 times with warm serum-free media and dose-responses were obtained with increasing concentrations of quinpirole. *** p <0.001 compared with other groups.

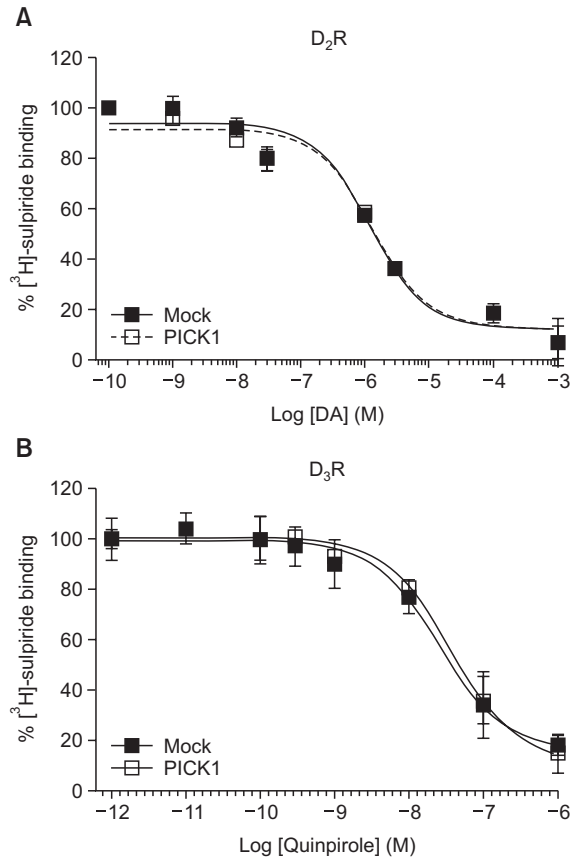


Fig. 3. The effects of PICK1 on the agonist affinity of D_2R and D_3R . (A) HEK-293 cells expressing D_2R (1.9 pmol/mg protein) were labeled with 2.2 nM [3H]-sulpiride with increasing concentrations of DA for 1 h. Cells were washed 3 times with ice-cold serum-free media for 5 min per each wash. Cells were incubated with 1% SDS overnight and radioactivity was measured with liquid scintillation counter. (B) HEK-293 cells expressing D_3R (2.1 pmol/mg protein) were labeled with 7.2 nM [3H]-sulpiride with increasing concentrations of quinpirole for 1 h. K_i value was calculated according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

D_3R (Fig. 2B, compare Veh vs. DA group in Mock- and PICK1-transfected cells). D_3R also undergoes PKC-mediated desensitization (Cho *et al.*, 2007) (Fig. 2C, compare Mock/Veh vs. Mock/PMA), which was blocked by the coexpression of PICK1 (Fig. 2C, compare PICK1/Veh vs. PICK1/PMA).

Effects of PICK1 on the agonist affinity of D_2R and D_3R

Since D_2R and D_3R formed clusters with PICK1, we examined the effects of PICK1 on agonist affinities. Cells were labelled with [3H]-sulpiride and challenged with increasing concentrations of DA or quinpirole. As shown in Fig. 3A, the K_i of D_2R for DA was about 0.98 μ M and this was not affected by the coexpression of PICK1. Similarly, the K_i of D_3R for quinpirole was about 12.7 nM and this was not affected by the coexpression of PICK1 (Fig. 3B).

Effects of PICK1 on the endocytosis and cell surface expression of D_2R and D_3R

Receptor endocytosis is closely related to the regulation of signaling. PICK1 is known to interact with glutamate receptors

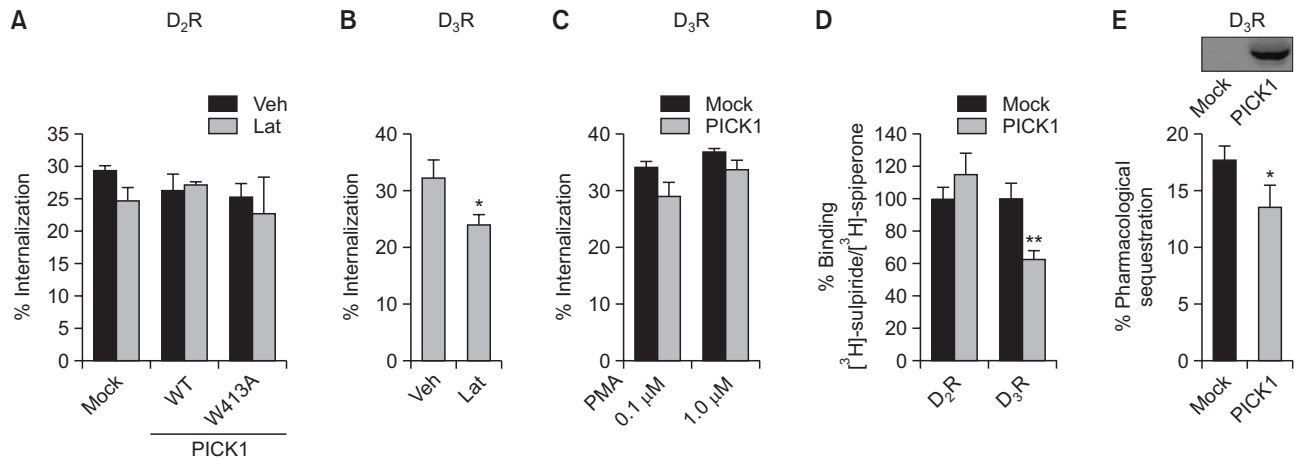


Fig. 4. The effects of PICK1 on the endocytosis and expression of D₂R and D₃R at the cell surface. (A) HEK-293 cells expressing D₂R were transfected with Mock vector, Flag-PICK1, or Flag-W413A-PICK1 plasmid. Cells were treated with either a vehicle or 1 μ M latrunculin A (LatA) for 30 min, followed by 10 μ M DA for 1 h. (B) HEK-293 cells expressing D₃R were treated with either a vehicle or 1 μ M LatA for 30 min, followed by 100 nM PMA for 30 min. * p <0.05 compared with Veh group. (C) HEK-293 cells expressing D₃R were transfected with Mock vector or Flag-PICK1 plasmid. Cells were treated with 0.1 and 1 μ M PMA for 30 min. (D) HEK293 cells expressing D₂R or D₃R were transfected with either Mock vector or Flag-PICK1 plasmid. Cells were labelled with 2.2 or 7.2 nM [³H]-sulpiride for D₂R and D₃R, respectively. Both cell groups were labeled with 2 nM [³H]-spiperone. ** p <0.01 compared with D₃R/Mock group. (E) HEK-293 cells expressing D₃R were transfected with Mock vector or Flag-PICK1 plasmid. Cells were treated with 1 μ M DA for 5 min. * p <0.05 compared with Mock group. Cell lysates were blotted with anti-Flag antibodies.

such as metabotropic glutamate receptors 7 or 3 (Dev *et al.*, 2000; Hirbec *et al.*, 2002) and AMPA receptors (Xia *et al.*, 1999). According to studies on the GluR2 subunit of the AMPA receptor, PICK1 interacts with Arp2/3 complex to increase the internalization of AMPA receptors by inducing actin depolymerization (Rocca *et al.*, 2008). However, this does not seem to be the case with D₂R because latrunculin A (LatA), an inhibitor of actin assembly, did not have any effect. Furthermore, W413A-PICK1, a PICK1 mutant which inhibits Arp2/3 complex-mediated actin polymerization (Rocca *et al.*, 2008), had no effect on the endocytosis of D₂R (Fig. 4A).

In contrast to D₂R which mainly undergoes GRK2-mediated endocytosis, D₃R mainly undergoes PKC-mediated endocytosis (Cho *et al.*, 2007). When cells were treated with LatA, PMA-induced internalization of D₃R was inhibited (Fig. 4B), suggesting that polymerized actin was required for the PKC-mediated internalization of D₃R, which is the opposite to the regulation of glutamate receptors. As expected, PICK1 did not affect the internalization of D₃R (Fig. 4C).

Next, roles of PICK1 were tested for the subcellular distribution of D₂R and D₃R by utilizing the hydrophilicity and hydrophobicity of [³H]-sulpiride and [³H]-spiperone, respectively. As expected from Fig. 1B, coexpression of PICK1 did not affect the subcellular distribution of D₂R but decreased the expression of D₃R on cell surface (Fig. 4D). These results indicate that the alterations of the number of functional receptors at the cell membrane might not be enough to affect the basal signaling of D₃R.

Pharmacological sequestration is defined as a shift of receptors to a more hydrophobic fraction within the plasma membrane without the actual translocation of receptors to cytosolic regions (Mostafapour *et al.*, 1996). A subsequent study has shown that pharmacological sequestration can be used to predict the short-term tolerance of GPCRs, including D₃R (Min *et al.*, 2013). As shown in Fig. 4E, PICK1 moderately in-

hibited the pharmacological sequestration of D₃R, suggesting that PICK1-mediated attenuation of pharmacological sequestration could be one of the factors which are involved in the PICK1-mediated inhibition of D₃R tolerance.

DISCUSSION

At the cell membrane, the function of GPCRs and transporters can be regulated by multiple protein kinases and arresting proteins. The regulation of G protein-coupled receptors (GPCRs) occurs through two different pathways: homologous and heterologous. The homologous pathway involves GRK-mediated receptor phosphorylation followed by an association with arrestin. Arrestins also function as adaptors for the endocytosis of GPCRs at a later stage (Sibley *et al.*, 1987). In comparison, the heterologous desensitization pathway involves the phosphorylation of the receptor proteins by protein kinase A (PKA) or protein kinase C (PKC), which may be activated by other cellular pathways. Thus, in addition to the regulation of receptor functions through receptor phosphorylation and binding with arrestin proteins, this study showed that the functions of D₃R can be regulated through interactions with PICK1.

From Fig. 1A, PICK1 seemed to interact more robustly with D₂R than D₃R. In addition, D₂R and PICK1 formed clusters and colocalized on the plasma membrane (Fig. 1B). On the other hand, the GST pull-down assay showed that the 3rd cytoplasmic loop of D₃R interacted more strongly with PICK1 than those of D₂R. D₂R signaling, endocytosis, agonist affinity, and subcellular localization were not altered by the coexpression of PICK1. On the contrary, some of functional aspects of D₃R were altered by the coexpression of PICK1. The functional significance of the interaction between D₂R and PICK1 in clusters on the plasma membrane is not clear at this time point. More in depth and diverse examinations of receptor functions are

needed to understand their interactions.

Fig. 4D shows that the coexpression of PICK1 caused a 40% decrease in D₃R expression on the cell surface without altering the signaling of D₃R (Fig. 2B). Assuming that the signaling of GPCRs via G proteins occurs on the plasma membrane, these results could be explained by spare receptors. It is possible that an excess number of D₃R are functional on the plasma membrane despite the decreased receptor expression with the coexpression of PICK1.

Desensitization or tolerance of GPCRs represents a decrease in receptor functions through prolonged or repeated exposure to an agonist (Lohse *et al.*, 1989; Hausdorff *et al.*, 1990). According to the current paradigm of GPCR desensitization based on studies of the β_2 adrenergic receptor (β_2 AR), receptor phosphorylation and β -arrestin translocation are key cellular events that explain GPCR desensitization. However, this paradigm does not seem to explain the desensitization of certain GPCRs. For example, receptor phosphorylation (Pao and Benovic, 2002; Ferguson, 2007) or GRK2 (Violin *et al.*, 2008) was not required for the desensitization of certain GPCRs. A more recent study suggested that pharmacological sequestration explains the desensitization of D₃R (Min *et al.*, 2013). In agreement with the previous study, we found that the pharmacological sequestration of D₃R was inhibited by the coexpression of PICK1 (Fig. 4E). Interestingly, PKC-mediated desensitization of D₃R was also inhibited by the coexpression of PICK1. Further studies are required to clarify whether certain cellular events, which are commonly involved in homologous and heterologous desensitization, are regulated by PICK1. In addition, the molecular targets that PICK1 act on to block the receptor tolerance need to be identified.

It was suggested that class I PDZ domains bind to a carboxyl terminal consensus sequence S/T-X-V/I (where X is any amino acid); class II PDZ domains preferentially bind to a carboxyl terminal sequence ϕ -X- ϕ (where ϕ represents any hydrophobic residue) (Songyang *et al.*, 1997). D₂R contains a consensus sequence within the 3rd intracellular loop, ²⁶⁷SP¹²⁶⁹, which is supposed to bind with class I PDZ domain. There are multiple ϕ -X- ϕ motifs within the 3rd intracellular loop of D₂R and D₃R. However, these sequence are not located at the carboxyl terminus, and further studies are needed to confirm whether these sites are responsible for the interaction with PICK1.

Our results showed that PICK1 regulated the surface expression and tolerance of D₃R. Thus, these findings demonstrate that in addition to the regulation of receptor functions through receptor phosphorylation and binding with arresting proteins, the signaling of D₂R and D₃R can be differentially regulated depending on the expression levels of PICK1.

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