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PKA and ERK1/2 are involved in dopamine D_1 receptor-induced heterologous desensitization of the δ opioid receptor

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Abstract

Aims—Chronic administration of cocaine attenuates delta opioid receptor (DOPR) signaling in the striatum and the desensitization is mediated by the indirect actions of cocaine on dopamine D_1 receptors (D_1R). In addition, DOPR and D_1R co-exist in some rat striatal neurons. In the present study, we examined the underlying mechanism of DOPR desensitization by D_1R activation.

Main methods—NG 108-15 cells stably expressing HA-rat D_1 receptor (HA-D_1R) and Chinese hamster ovary (CHO) cells stably expressing both FLAG-mouse DOPR (FLAG-DOPR) and HA-D_1R were used as the cell models. Receptor binding, [^{35}S]GTPγS binding, receptor phosphorylation and western blot were conducted to examine DOPR affinity, expression, internalization, downregulation, desensitization, phosphorylation and phosphorylated ERK1/2.

Key findings—Pretreatment with either the DOPR agonist DPDPE or the D_1R agonist SKF-82958 for 30 min attenuated DPDPE-stimulated [^{35}S]GTPγS binding to G proteins, demonstrating homologous and heterologous desensitization of the DOPR, respectively. SKF-82958 pretreatment did not affect the level of DOPR or affinity of DOPR antagonist or agonists, nor did it induce phosphorylation, internalization or down-regulation of the DOPR in the CHO-FLAG-DOPR/HA-D_1R cells. Pretreatment of cells with inhibitors of PKA, MEK1 and PI3K, but not PKC, attenuated SKF-82958-induced desensitization of the DOPR. The D_1R agonist SKF-82958 enhanced phosphorylation of ERK1/2, and pretreatment with inhibitors of MEK1 and PI3K, but not PKA and PKC reduced the effect. These results indicate that activation of ERK1/2 and/or PKA, but not PKC, is involved in D_1 receptor-induced heterologous desensitization of the DOPR.

Significance—This study provides possible mechanisms underlying D_1R activation-induced DOPR desensitization.

Keywords

Heterologous desensitization; delta opioid receptor; dopamine D_1 receptor; PKA; ERK1/2

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Authors have no conflicts to disclose.

Introduction

Dopaminergic system interacts with the opioid system, and together they collectively modulate emotional, locomotor, and goal-directed behaviors. These two systems interact at functional, cellular and anatomical levels. In vivo administration of dopaminergic drugs has profound effects on the expression and function of opioid receptors. For example, chronic exposure to cocaine, a non-selective indirect dopaminergic agonist, upregulates mu opioid receptors in brains of rodents and humans (Unterwald et al., 1992; Zubieta et al., 1996). Similar results are found with chronic administration of selective D2 receptor agonist quinpirole (Chen et al., 1993). Kappa opioid receptor binding is also increased after chronic cocaine exposure in rodents and humans (Hurd and Herkenham, 1993; Unterwald et al., 1994). Of note, the brain regions that show the largest increases in opioid receptor levels are those that are innervated by dopaminergic terminals. While repeated cocaine administration increases mu and kappa opioid receptors, neither cocaine (Unterwald et al., 1994; Azaryan et al., 1996) nor selective dopamine receptor agonists (Chen et al., 1993) produce changes in DOPR binding or mRNA levels. Despite stable receptor levels, significant desensitization of DOPR signaling has been demonstrated (Unterwald et al., 1993; Unterwald and Cuntapay, 2000).

Chronic administration of cocaine in rats attenuates DOPR-induced inhibition of adenylyl cyclase activity in striatum (Unterwald et al., 1993), but does not change the number of the DOPR or the levels of four major G proteins (Unterwald et al., 1994; Perrine et al., 2005). DOPR desensitization is mediated by the indirect effects of cocaine on dopamine D_1 receptors. Chronic administration of the D_1R agonist SKF-82958, but not the D2 receptor agonist quinpirole, attenuates DOPR-induced stimulation of [^{35}S]GTPγS binding, indicative of decreased coupling of DOPR with G proteins (Unterwald and Cuntapay, 2000). On an anatomical level, both D_1R and DOPR are found in high levels in the striatum (Mansour et al., 1988; Wamsley et al., 1989) and, more recently, D_1R and DOPR have been shown by immunoelectron microscopy to co-exist in individual neurons in a subset of rat dorsolateral striatal and accumbens neurons (Ambrose et al., 2006; Ambrose-Lanci et al., 2008). Thus, it is likely that D_1R-mediated desensitization of the DOPR occurs at the cellular level in individual neurons.

The functional consequences of cocaine-induced DOPR desensitization have also been investigated. Genetic deletion of DOPR produces a phenotype of increased anxiety- and depression-like behaviors (Filliol et al., 2000), demonstrating the importance of this receptor in regulating mood and emotional behaviors. Chronic administration of cocaine in rats attenuates DOPR signaling with increasing anxiety- and depression-like behaviors (Perrine et al., 2008). The anxiety- and depression-like responses were reduced by acute administration of the selective DOPR agonist SNC80 (Perrine et al., 2008). In addition, abuse of and withdrawal from cocaine in humans causes psychiatric disorders, including psychosis, depression and anxiety (Kampman et al., 2001), and cocaine-induced anxiety and depression are frequently resistant to treatment with classical anxiolytics and antidepressants suggesting a novel pathology.

Although extensive evidence indicates interactions of these two systems, the molecular mechanisms underlying cocaine or D_1R agonist-induced desensitization of the DOPR has not yet been clarified. The complexity and heterogeneity of neurons in the brain preclude the use of brain tissues for such investigations. In this study, we examined the molecular mechanisms underlying the D_1R-mediated heterologous desensitization of the DOPR in clonal mouse neuroblastoma × rat glioma hybrid (NG108-15) cells stably transfected with D_1R and in CHO cells stably transfected with both the DOPR and D_1R.

Materials and methods

Materials

[^{3}H]Diprenorphine (58 Ci/mmol), [^{3}H]SCH23390 (91 Ci/mmol), [^{32}P]orthophosphate (8500–9100 Ci/mmol) and [^{35}S]GTPγS (1000–1200 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). SNC-80 ((+)-4-[(aR)-a-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was provided by National Institute on Drug Abuse (Bethesda, MD). Rabbit polyclonal antibody against the FLAG epitope, GDP, GTPγS, NaF, Na pyrophosphate and kinase inhibitors [PD98059, wortmannin, GF1092036X and Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP-SH)] were obtained from Sigma-Aldrich (St. Louis, MO). The following compounds or reagents were purchased from indicated companies: Geneticin, Mediatech (Herndon, VA); Lipofectamine 2000, Opti-MEM I reduced serum and hygromycin B, Invitrogen (Carlsbad, CA); Naloxone HCl, (±)-chloro-APB HBr (SKF-82958) and fluphenazine dihydrochloride, Research Biochemicals International (Natick, MA); Calyculin A and anti-pERK1/2 antibody, Cell Signaling Technology (Danvers, MA); DPDPE, Chiron Mimotopes Peptide Systems (San Diego, CA); Pansorbin, Calbiochem (San Diego, CA); Complete protease inhibitor cocktail, Roche Diagnostics (Indianapolis, IN).

Generation of the clonal NG 108-15 cells stably expressing HA-rat D_1 dopamine receptor (HA-D_1R)

HA-D_1R in HA-D_1R/pCMV5 (a generous gift from Dr. Marc G. Caron of Duke University) was subcloned into the *Hind*III and *Xba*I sites of the mammalian expression vector pcDNA3.1 (invitrogen, with hygromycin resistant gene). HA epitope tag is located 5′ to the initiation codon of the D_1R. NG108-15 mouse neuroblastoma × rat glioma hybrid cells endogenously expressing ~0.6 pmol DOPR/mg membrane protein were transiently transfected with 10 μg HA-D_1R in pcDNA 3.1 using Lipofectamine 2000 reagent. After two days, cells were cultured in Dulbecco's modified Eagle's medium F12 HAM supplemented with 0.5 mg/ml hygromycin B, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and HAT media supplement for 2 weeks. Then cells were counted, diluted and cultured in 96-well plates at an average of 1 cell/well. The wells containing single cells were monitored. Clonal cells were cultured for about another 2 weeks and transferred into 24-well plates and grown until confluent. [^{3}H]diprenorphine binding (for the native DOPR) and [^{3}H]SCH23390 binding (for the D_1R) were performed on intact cells. The cells expressing both DOPR and D_1R were selected for the further study.

Generation of clonal CHO cells stably expressing both FLAG-mouse DOPR (FLAG-mDOPR) and HA-D_1R

Clonal CHO cells stably expressing FLAG-mDOPR/pcDNA3 (geneticin resistant) established as described previously (Chen et al., 1995) were transfected with HA-D_1R/pcDNA 3.1 (hygromycin resistant) and the clonal CHO cells stably expressing both FLAG-mDOPR/pcDNA3 and HA-D_1R/pcDNA 3.1 were generated and selected with 0.5 mg/ml of geneticin and hygromycin B using the similar procedures. FLAG epitope tag is located 5′ to the initiation codon of the DOPR. The selected double clonal CHO-FLAG-DOPR/HA-D_1R cells were cultured in Dulbecco's modified Eagle's medium F12 HAM supplemented with 0.1 mg/ml geneticin and hygromycin B, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C. Clonal cell lines stably expressing about ~2 pmol/mg protein of each receptor were used to study the molecular mechanism by which dopamine D_1R activation results in desensitization of DOPR function.

Saturation and competition binding assays

CHO cells stably expressing FLAG-DOPR/HA-D_1R were pretreated with vehicle, DPDPE 10 μM or SKF-82958 10 μM in Dulbecco's modified Eagle's F12 HAM medium without serum for 30 min in a humidified 5% CO_2 incubator at 37°C. The cells were then collected and washed twice with cold PBS containing calyculin A 10 nM and membranes were prepared as described previously (Xu et al., 1999) in the presence of 10 mM NaF and 10 mM Na pyrophosphate to inhibit phosphatases Saturation binding of [^{3}H]diprenorphine or [^{3}H]SCH23390 to the NG108-15-/HA- D_1R or the CHO-FLAG-DOPR/HA-D_1R was performed with at least six concentrations of [^{3}H]diprenorphine or [^{3}H]SCH23390 (ranging from 25 pM to 2 nM), and K_d and B_{max} values were determined with the Prism program (GraphPad Software Inc., San Diego, CA). Binding was carried out in 50 mM Tris-HCl buffer containing 1 mM EGTA (pH 7.4) at room temperature for 1 h in duplicate in a volume of 1 mL with ~10–20 μg of membrane protein. Naloxone (for DOPR) or fluphenazine (for D_1R) (10 μM) was used to define nonspecific binding. Competitive inhibition of [^{3}H]diprenorphine binding by drugs was performed with [^{3}H]diprenorphine at a concentration close to its K_d value (~0.3 nM). Binding data were analyzed and K_i values were determined with GraphPad Prism software.

Effect of SKF-82958 treatment on DOPR-mediated [^{35}S]GTPγS binding

Clonal NG108-15-HA-D_1R or CHO-FLAG-DOPR/HA-D_1R cells were pre-incubated in Dulbecco's modified Eagle's F12 HAM medium without serum for overnight. Clonal cells were pretreated with vehicle, DPDPE 10 μM, or SKF-82958 10 μM for 30 min in a humidified 5% CO_2 incubator at 37°C. Clonal cells were then collected and washed three times on ice with ice-cold phosphate-buffered saline (PBS)(pH 7.0) containing calyculin A 10 nM, and membranes were prepared in the presence of 10 mM NaF and 10 mM Na pyrophosphate to inhibit phosphatases. [^{35}S]GTPγS binding was performed as described previously (Zhu et al., 1997). Briefly, membranes (containing 10 μg of proteins) were incubated with 10 μM GDP and ~0.2 nM [^{35}S]GTPγS in the presence or absence of different concentration of DPDPE in a reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA) in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 10 μM GTPγS. After 60 min of incubation at 30°C, bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure and the filter was washed. Radioactivity in filters was determined by liquid scintillation counting. Data were analyzed and EC_{50} values for potency and E_{max} values for efficacy were determined with GraphPad Prism software.

For the experiments to determine effects of kinase inhibitors on SKF-82958-induced desensitization of DOPR, CHO-FLAG-DOPR/HA-D_1R cells were pretreated with vehicle, the PKA inhibitor Rp-cAMP-SH (10 μM), the PKC inhibitor GF 109203X (10 μM), PI3K inhibitor wortmannin (100 nM) and MEK1 inhibitor PD98059 (10 μM) for 20 min, respectively, and then incubated with vehicle or SKF-82958 (10 μM) for 30 min in the presence of calyculin A (10 nM). [^{35}S]GTPγS binding was performed as described above.

Effect of SKF-82958 treatment on phosphorylation of the DOPR

CHO-FLAG-DOPR/HA-D_1R cells were transferred into 60-mm dishes, cultured overnight to confluence and grown in 1 ml/well phosphate-free medium at 37°C for 2 h. [^{32}P]Orthophosphate (0.25 mCi/well) was added and incubated for another 2 h, and medium was aspirated. Cells were incubated with 10 μM DPDPE or 10 μM SKF-82958 for 30 min at 37°C, solubilized for 1 h with solubilization buffer (1% Triton X-100, 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 20 nM calyculin A, and 10% complete protease inhibitor cocktail (pH7.5) and centrifuged at 100,000*g* for 1 h. Immunoprecipitation of FLAG-DOPR was performed with rabbit anti-FLAG polyclonal antibody followed by Pansorbin.

Immunoprecipitated materials were resolved with SDS-PAGE followed by gel drying and autoradiography. Quantitation of receptor phosphorylation was performed with the OptiQuant software program.

Effect of SNC-80 or SKF-82958 treatment on cell surface and total DOPR

Internalized receptors were assessed as we described previously (Li et al., 1999). CHO-FLAG-DOPR/HA-D_1R cells were incubated with DOPR agonist SNC-80 (100 nM) or D_1 R agonist SKF-82958 (10 μM) for 30 min (for internalization) or 24 h (for downregulation) at 37°C and washed three times on ice with cold PBS. Binding was performed on intact cells with [^{3}H]diprenorphine in Kreb's buffer solution (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, and 25 mM HEPES at pH 7.4). Total receptor levels were assessed by binding with 1nM [^{3}H]diprenorphine in the presence or absence of 10 μM naloxone, whereas surface receptors were measured by binding with 1nM [^{3}H]diprenorphine in the presence or absence of 10 μM DPDPE. Binding was performed at 4°C for 3 h. While diprenorphine, which is a hydrophobic ligand, can bind to both cell surface and intracellular receptors, DPDPE, a hydrophilic peptide ligand, binds only to the cell surface receptors. Thus, the difference between the total receptors and the cell surface receptors represents intracellular receptors.

Immunoblot analysis of phosphorylated ERK1/2

CHO-FLAG-DOPR/HA-D_1R cells were cultured in 12-well plate in MEM complete medium for 24 h, washed and incubated with OPTI-MEM for 2 h. Kinase Inhibitors (see Fig. 5) were added to the cells and pre-incubated for 20 min followed by stimulation of D_1R with SKF-82958 for 30 min. The reaction was stopped by washing the cells with PBS followed by the addition of 200 μl/well SDS loading buffer to lyses cells. Cell lysates were boiled for 5 min and 50 μl each of the samples (20 μg total protein/lane) was loaded to 10 % SDS-PAGE for separation. Immunoblotting was performed with anti-pERK1/2 antibody (1:5000).

Statistical Analysis

For comparison of more than two groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *t* test using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). For comparison of two groups, Student's *t* test was performed. $P < 0.05$ was set as the level of significance in all statistical analyses.

RESULTS

Characterization of D_1R and DOPR in clonal NG 108-15-HA-D_1R cells and CHO-FLAG-DOPR/HA-D_1R cells

Receptor expression—Saturation binding of [^{3}H]diprenorphine to the DOPR or [^{3}H]SCH23390 to the D_1R in cell membranes was performed to determine binding affinities and receptor levels. K_d and B_{max} values of [^{3}H]diprenorphine binding to the endogenous DOPR in NG 108-15-HA-D_1R cells were 0.27 ± 0.03 nM and 0.77 ± 0.09 pmol/mg protein, respectively, which are similar to those of the untransfected NG 108-15 cells (Huang et al., 2007). K_d and B_{max} values of [^{3}H]SCH23390 binding to the D_1R were 1.66 ± 0.58 nM and 180 ± 34 fmol/mg protein, respectively.

Stable expression of the D_1R in NG108-15 cells was difficult to maintain; therefore, we established clonal CHO cells stably transfected with the mouse DOPR and the rat D_1R (CHO-FLAG-DOPR/HA-D_1R cells) for mechanistic studies. Similar saturation binding was performed. K_d and B_{max} values of [^{3}H]diprenorphine binding to the DOPR were determined

to be 0.40 ± 0.04 nM and 2.78 ± 0.08 pmol/mg protein (n=3), respectively. K_d and B_{max} values of [^{3}H]SCH23390 binding to the D_1R were calculated to be 0.10± 0.02 nM and 2.17±0.14 pmol/mg protein, respectively. The K_d values are similar to published data (Monsma, Jr. et al., 1990; Zhu et al., 1997).

Receptor signaling—We then carried out functional assays to ensure proper receptor signaling. We found that the DOPR agonist DPDPE dose-dependently increased [^{35}S]GTPγS binding to membranes of NG108-15/HA-D_1R cells and CHO-FLAG-DOPR/HA-D_1R cells (Fig. 1A and 1B). EC_{50} values of DPDPE were determined to be 59.9 ± 15.1 nM and 47.0 ± 10.9 nM, respectively (Table 1 and 2). In addition, the D_1R agonist SKF-82958 dose-dependently increased cAMP level in NG108-15/HA-D_1R cells and CHO-FLAG-DOPR/HA-D_1R cells (data not shown).

Taken together, these results indicate that NG108-15/HA-D_1R cells or CHO-FLAG-DOPR/HA-D_1R cells can be used as cell models to study mechanisms underlying the interactions between DOPR and D_1R.

The D_1R agonist SKF-82958 induced heterologous desensitization of endogenous DOPR

NG 108-15-HA-D_1R cells—NG 108-15-HA-D_1R cells were pretreated with vehicle, 10 μM DPDPE, a selective DOPR agonist, or 10 μM SKF-82958 for 30 min. DPDPE-stimulated [^{35}S]GTPγS binding was performed and its EC_{50} and E_{max} values were determined (Fig. 1A, Table 1). Basal [^{35}S]GTPγS binding did not differ among the control and the treatment groups (control, 17.8 ± 2.5 fmol/mg protein; DPDPE-treated, 15.2 ± 2.5 fmol/mg protein; SKF-82958-treated, 14.5 ± 1.9 fmol/mg protein, n=3) and was subtracted from stimulated [^{35}S]GTPγS binding data. Compared with the control group, pretreatment of cells with DPDPE or SKF-82958 significantly decreased the E_{max} value and increased the EC_{50} value for DPDPE-stimulated [^{35}S]GTPγS binding (P<0.01 and P<0.05, respectively); however, the decrease in E_{max} was less with SKF-82958 pretreatment than with DPDPE pretreatment. It is important to demonstrate D1R activation-induced heterologous desensitization of the DOPR in NG108-15 cells, a neuron-like cell line.

CHO-FLAG-DOPR/HA-D_1R cells—Similar experiments were performed in CHO-FLAG-DOPR/HA-D_1R cells. Cells were pretreated with vehicle, 10 μM DPDPE or 10 μM SKF-82958 for 30 min and DPDPE-stimulated [^{35}S]GTPγS binding was performed on membranes. Basal [^{35}S]GTPγS binding did not differ among the control and the treatment groups (control, 39.0 ± 9.4 fmol/mg protein; DPDPE-treated, 41.9 ± 7.6 fmol/mg protein; SKF-82958-treated, 42.6 ± 7.0 fmol/mg protein, n=3) and was subtracted from the stimulated [^{35}S]GTPγS binding data. Pretreatment with DPDPE or SKF-82958 resulted in a reduction in the E_{max} value of DPDPE by 70.4% or 30.9%, respectively, with no significant change in its EC_{50} value (Fig. 1B and Table 2). When [^{35}S]GTPγS binding was performed with the nonpeptide DOPR agonist SNC80 at 10 μM following SKF-82958 pretreatment, [^{35}S]GTPγS binding was reduced by 23.2% ± 5.7% (mean ± s.e.m.) (n=3) compared with the control. Therefore, the responses of both the peptide agonist DPDPE and the nonpeptide agonist SNC80 were attenuated.

Taken together, these results indicate that in addition to homologous desensitization, the DOPR undergoes heterologous desensitization following activation of the D_1R, in both NG108-15/HA-D_1R cells and in CHO-FLAG-DOPR/HA-D_1R cells. Incubation with DPDPE or SKF-82958 resulted in a significant increase in EC_{50} of DPDPE in NG108-15 cells, but not in CHO cells. This is probably due to the presence of spare DOPRs in the clonal CHO cells.

DPDPE, but not SKF-82958, induced phosphorylation of the DOPR

We examined if D_1R activation caused phosphorylation of the DOPR. CHO-FLAG-DOPR/HA-D_1R cells were treated with 10 μM DPDPE or 10 μM SKF-82958 for 30 min. SKF-82958 did not induce phosphorylation of the DOPR, whereas DPDPE did promote profound DOPR phosphorylation (Fig. 2), indicating that heterologous desensitization of the DOPR induced by D_1R agonist does not result from phosphorylation of the DOPR.

Pretreatment with SKF-82958 for 30 min did not affect affinity or level of the DOPR

CHO-FLAG-DOPR/HA-D_1R cells were pretreated with vehicle, 10 μM DPDPE or 10 μM SKF-82958 for 30 min. Saturation binding of [^{3}H]diprenorphine to the DOPR and [^{3}H]SCH23390 binding to the D_1R were performed and K_d and B_{max} values were calculated. The data summarized in Table 3 shows that pretreatment with DPDPE or SKF-82958 did not affect K_d and B_{max} values of [^{3}H]diprenorphine binding to the DOPR or [^{3}H]SCH23390 binding to the D_1R. Since diprenorphine is an antagonist, we also determined if affinities of the agonists SNC-80 and DPDPE to the DOPR were affected using competitive inhibition of [^{3}H]diprenorphine binding. While pretreatment with DPDPE increased the K_i value of DPDPE, but not of SNC-80, pretreatment with SKF-82958 did not affect K_i values of either agonist (Table 2). These results indicate that the observed heterologous desensitization induced by the D_1R agonist is not due to changes in affinity or expression level of the DOPR.

Effects of pretreatment of SNC-80 or SKF-82958 for 30 min or 24 h on DOPR trafficking

CHO-FLAG-DOPR/HA-D_1R cells were pretreated with vehicle, the DOPR agonist SNC-80 (100 nM) or the D_1R agonist SKF-82958 (10 μM) for 30 min or 24 h. Because of stability issue, the non-peptide selective DOPR agonist SNC-80 was used instead of the peptide agonist DPDPE. Total and cell surface DOPRs were determined by [^{3}H]diprenorphine binding. Pretreatment with SKF-82958 for 30 min or 24 h did not affect total or cell surface DOPRs (Fig. 3A and 3B). In contrast, pretreatment with the DOPR agonist SNC-80 for 30 min significantly reduced cell surface DOPR, without affecting the total DOPR, indicating DOPR internalization (Fig. 3A). In vehicle-treated cells, 76.0 ± 2.6% (n=3) of the total DOPR was present on cell surface.

Pretreatment with SNC-80 for 24 h reduced the total DOPR to 19 ± 1.4% of the control, indicating DOPR downregulation (Fig. 3B). These results demonstrate that the heterologous desensitization induced by the D_1R agonist is not related to internalization and downregulation of DOPR.

Multiple downstream signaling pathways underlying the D_1 receptor-induced heterologous desensitization of DOPR

Heterologous desensitization of seven-transmembrane receptors (7TMRs) often involves kinase activity. Activation of the D_1R has been shown to activate PKA, PKC and extracellular signal-regulated kinase 1/2 (ERK1/2) (Flores-Hernandez et al., 2000; Chao et al., 2002a; Chao et al., 2002b; Zhang et al., 2004; Mangiavacchi and Wolf, 2004; Chen et al., 2004b; Hopf et al., 2005; Rex et al., 2010; Rankin and Sibley, 2010). To examine if these kinases are involved in D_1R-induced desensitization of the DOPR, CHO-FLAG-DOPR/HA-D_1R cells were pretreated for 20 min with vehicle, the PKA inhibitor Rp-cAMP-SH (10 μM), the PKC inhibitor GF 109203X (10 μM), the PI3K inhibitor wortmannin (100 nM) or the MEK1 inhibitor PD98059 (10 μM), and then incubated with vehicle or SKF-82958 (10 μM) for 30 min in the presence of the phosphatase inhibitor calyculin A (10 nM). The concentration of each inhibitor has been reported in the literature to produce significant kinase inhibition (Toullec et al., 1991; Horvath and DeLeo, 2009; Machida et al.,

2011; Allen et al., 2011). Membranes were prepared and DPDPE-induced [^{35}S]GTPγS binding was performed and EC_{50} and E_{max} values were determined. Basal [^{35}S]GTPγS binding did not differ among the control groups and all the treatment groups and was subtracted from the agonist-induced [^{35}S]GTPγS binding. The PKA inhibitor Rp-cAMP-SH, the PI3K inhibitor wortmannin and MEK1 inhibitor PD98059 significantly inhibited D_1R activation-induced desensitization of the DOPR (Table 4 and Fig. 4A, 4B, 4D and 4E). In contrast, the PKC inhibitor GF 109203X did not affect the cross desensitization (Table 4 and Fig. 4A and 4C). These results demonstrate that PKA, PI3K and ERK1/2, but not PKC, signaling pathways play important roles in the heterologous desensitization of DOPR by D_1R activation.

We next investigated whether the involvements of PKA, PI3K and ERK1/2 were through ERK1/2 phosphorylation. As shown as Figure 5, treatment with 10 μM SKF-82958 for 30 min produced a marked increase in ERK1/2 phosphorylation compared with the control. In addition, pretreatment of 10 μM PD98059 (MEK1 inhibitor) and 0.1 μM wortmannin (PI3K inhibitor), but not 10 μM GF109203 (PKC inhibitor) and 10 μM Rp-cAMP-SH (PKA inhibitor), significantly reduced the increase, indicating that MEK1 and PI3K, but not PKC and PKA, pathways were involved in ERK1/2 activation by D_1R. Therefore, inhibition of cross desensitization by MEK1 and PI3K inhibitors, but not PKA inhibitor, is likely due to inhibition of ERK1/2 phosphorylation. Taken together, these data indicate that ERK1/2 and PKA downstream signaling pathways underlie the D_1 receptor-induced heterologous desensitization of DOPR.

Discussion

The present study demonstrates heterologous desensitization of the DOPR by activation of the D_1R in both NG 108-15/HA-D_1R cells and CHO/FLAG-DOPR/HA-D_1R cells. This heterologous desensitization of the DOPR appears to be associated with D_1R-activated ERK1/2 and PKA signaling pathways, but does not involve changes in ligand binding affinity, phosphorylation, internalization or downregulation of the DOPR. These results provide some mechanistic insights for our previous findings that chronic administration of cocaine or a D_1R agonist in the rat attenuates DOPR signaling in the rostral caudate putamen and nucleus accumbens (Unterwald et al., 1993; Unterwald and Cuntapay, 2000). In addition, these observations provide evidence at the cellular level for possible functional consequence of the co-expression of D_1R and DOPR in neurons in the dorsolateral striatum and nucleus accumbens (Ambrose et al., 2006; Ambrose-Lanci et al., 2008).

7TMRs undergo desensitization following prolonged or repeated activation. There are two types of desensitization, homologous and heterologous. In homologous desensitization, only the activated receptor is desensitized, whereas in heterologous desensitization, activation of a 7TMR leads to desensitization of another receptor. The mechanisms underlying homologous desensitization of 7TMRs are similar among receptors [for a review, see (Gainetdinov et al., 2004)]. In contrast, the mechanisms underlying heterologous desensitization are more diverse and involve multiple mechanisms, including phosphorylation and internalization of the 7TMRs, changes in G proteins and depletion of downstream second messengers (Hosey, 1999; Gainetdinov et al., 2004)

PKA-dependent and ERK-dependent pathways are involved in the heterologous desensitization of DOPR by D_1R

PKA pathway—Dopamine D_1-like receptors (D_1R and D_5R) are coupled to $G_{s/olf}$ to activate adenylyl cyclase, increase intracellular cAMP and enhance the activity of PKA. The PKA inhibitor Rp-cAMP-SH blocked desensitization of the DOPR by D_1R activation, indicating that PKA signaling pathway is involved in the reduced coupling of the DOPR and

$G_{i/o}$ following activation of D_1R. D_1R/Gs/cAMP/PKA pathway has been shown to regulate the activity of other receptors, for example, AMPA and $GABA_A$ receptors (Flores-Hernandez et al., 2000; Chao et al., 2002a; Chao et al., 2002b; Mangiavacchi and Wolf, 2004)

ERK1/2 signaling—MAP kinases play important roles in the transduction of a wide variety of extracellular signals. Activation of D_1R was found to activate ERK1/2, but not p38 MAP kinase and c-Jun N-terminal kinases, in SK-N-MC neuroblastoma cells (Zhang et al., 2004; Chen et al., 2004b). We found that the D_1R agonist enhanced phosphorylation of ERK1/2, and inhibitors of MEK1 and PI3K reduced the increase in pERK1/2, indicating that the D_1R agonist activates ERK1/2 in a MEK1- and PI3K-dependent fashion. The finding that pretreatment with the PI3K inhibitor wortmannin or the MEK1 inhibitor PD98059 reduced the heterologous desensitization of DOPR by the D_1R agonist SKF-82958 supports the notion that PI3K and MEK1 are upstream elements of ERK1/2 and involved in the heterologous desensitization of DOPR by D_1R.

Since either the PKA or MEK inhibitor totally abolished D1R activation-induced DOPR desensitization and PKA inhibitor did not affect ERK1/2 phosphorylation, we believe that these two pathways leading to DOPR desensitization are independent of each other.

How PKA and ERK1/2 activation by D_1R activation leads to DOPR desensitization remains to be determined. Since we observed DOPR desensitization following only 30 min activation of the D_1R, it is not likely due to changes at the transcription or translational level. DOPR agonist-induced [^{35}S]GTPγS binding was the functional endpoint; therefore, changes should occur at the receptor or G proteins. However, we found that D_1R activation did not enhance DOPR phosphorylation. Phosphorylation and down-regulation of G proteins have been demonstrated to underline heterologous desensitization of GPCRs (for example, (Yatomi et al., 1992; Green et al., 1992; Seasholtz et al., 1997). We observed that D_1R activation did not affect phosphorylation or levels of $G\alpha_{i2}$ and $G\alpha_{i3}$ (data not shown). The lack of alterations in G proteins is consistent with our previous observations that chronic administration of cocaine did not change the levels of four G proteins α subunits (G_{i1}, G_s, G_{olf} and G_o) in the rat striatum (Perrine et al., 2005). In addition, as [^{35}S]GTPγS is not hydrolyzable, the involvement of RGS proteins seems unlikely.

Role of receptor phosphorylation

Our finding that the D_1R agonist SKF-82958 did not increase phosphorylation of the DOPR indicates that DOPR phosphorylation is not necessary for heterologous desensitization of DOPR by D_1R activation. Heterologous desensitization of 7TMRs may or may not involve receptor phosphorylation [for example, (Grimm et al., 1998; Bundey and Nahorski, 2001; Chen et al., 2004a)].

Role of receptor affinity and trafficking

Our findings that pretreatment of D_1R agonist did not change ligand affinity to DOPR (Tables 2 and 3) or induce internalization and down-regulation (Fig. 3) demonstrate that heterologous desensitization of DOPR by D_1R activation is not attributable to changes of DOPR affinity or trafficking. Our present results are in accord with our previous in vivo findings (Unterwald et al., 1994) that acute or chronic cocaine treatment does not affect [^{3}H]deltorphin or [^{3}H]DPDPE binding to the DOPR in the striatum or nucleus accumbens (Unterwald et al., 1994; Turchan et al., 1999). Receptor internalization and downregulation have been shown to be associated with (Shapira et al., 2003) or not involved in (Grimm et al., 1998; Szabo et al., 2003) heterologous desensitization of 7TMRs.

Here we found that pretreatment with a D_1R agonist for 30 min induced ERK1/2- and PKA-mediated heterologous desensitization of the DOPR, without causing DOPR internalization or down-regulation. Ambrose-Lanci et al. (Ambrose-Lanci et al., 2008) reported that chronic cocaine administration (14 days) followed by 48-h withdrawal resulted in an increase in the percentage of DOPR localized intracellularly in both core and shell of the nucleus accumbens in male rats with no change in DOPR protein expression, indicating DOPR internalization. Perhaps the longer-term activation of D1R, indirectly by cocaine, causes DOPR internalization. Alternatively, activation of D1 receptor by chronic cocaine administration may desensitize the DOPR and lead to a compensatory increase in presynaptic opioid peptides and upon withdrawal the opioid peptides cause DOPR internalization. These results underscore complex interactions between the dopamine receptor and DOPR in NAc.

Conclusion

In summary, these data demonstrate the functional interaction of D_1R and DOPR; activation of D_1R produced heterologous desensitization of DOPR in two cell lines, similar to the previous findings in neurons. Activation of PKA and ERK1/2 pathways by D_1R activation is involved in heterologous desensitization of the DOPR. These data provide important insights into the molecular interactions of dopamine and opioid receptors and shed lights on the underlying mechanism of altered anxiety and mood phenotypes following chronic cocaine exposure.

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Abbreviations

CHO cells	Chinese hamster ovary cells
CHO-FLAG-mDOPR	clonal CHO cells stably transfected with FLAG-mouse δ opioid receptor cDNA in pcDNA3 vector
DPDPE	[D-Pen^2,D-Pen^5]-Enkephalin
CHO-FLAG-DOPR/ HA-D_1R cells	clonal CHO cells stably transfected with FLAG-DOPR cDNA in pcDNA3 vector and HA-D_1R cDNA in pcDNA3.1 vector
NG108-15-HA-D_1R cells	clonal NG108-15 cells stably transfected with HA-D_1R cDNA in pcDNA3.1 vector
Rp-cAMP-SH	Rp-adenosine 3′,5′-cyclic monophosphorothioate
SNC-80	(+)-4-[(aR)-a-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide

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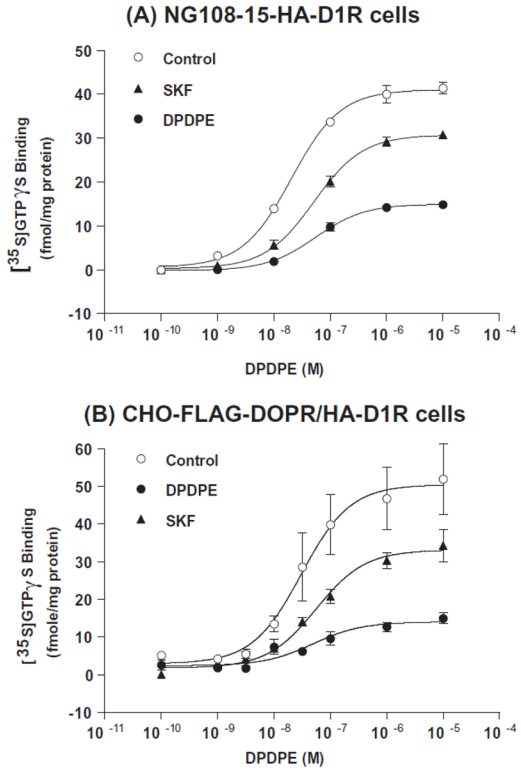

Figure 1. Effects of DPDPE and SKF-82958 pretreatment of (A) NG108-15-HA-D_1R cells and (B) CHO-FLAG-DOPR/HA-D_1R cells on DPDPE-stimulated [^{35}S]GTPγS binding

NG108-15-HA-D_1R cells and CHO-FLAG-DOPR/HA-D_1R cells were pretreated with vehicle, DPDPE 10 μM or SKF-82958 10 μM for 30 min at 37°C. Cells were then collected and washed and membranes were prepared. [^{35}S]GTPγS binding to cell membranes was measured in the absence and presence of increasing concentrations of DPDPE. Data are expressed as mean ± s.e.m. of three independent experiments performed in duplicate. EC_{50} and E_{max} values are shown in Table 1 (NG108-15-HA-D_1R cells) and Table 2 (CHO-FLAG-DOPR/HA-D_1R cells).

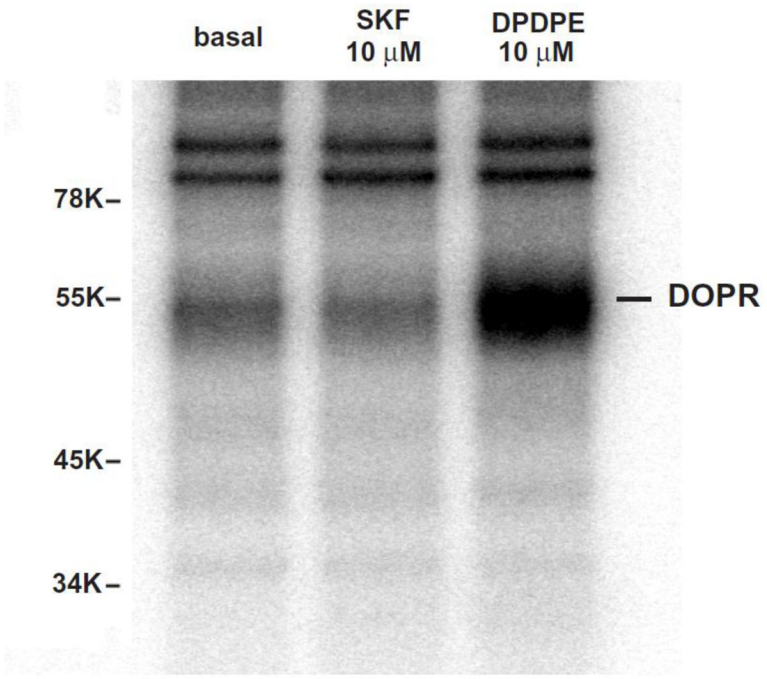

Figure 2. DPDPE, but not SKF-82958, induced phosphorylation of the DOPR in CHO-FLAG-mDOPR/HA-D_1R cells

Cells were grown in 1 ml/well phosphate-free medium in 6-well plates at 37°C for 2 h. [^{32}P]Orthophosphate (0.25 mCi/well) was added and incubated for another 2 h, and medium was aspirated. Cells were incubated with vehicle, 10 μM DPDPE or 10 μM SKF-82958 for 30 min at 37°C, solubilized for 1 h with solubilization buffer and centrifuged at 100,000*g* for 1 h. Immunoprecipitation of FLAG-mDOPR was performed with rabbit anti-FLAG polyclonal antibody followed by Pansorbin. Immunoprecipitated materials were resolved with SDS-PAGE followed by gel drying and autoradiography. This represents one of the three experiments performed. The 54-KDa band is the DOPR.

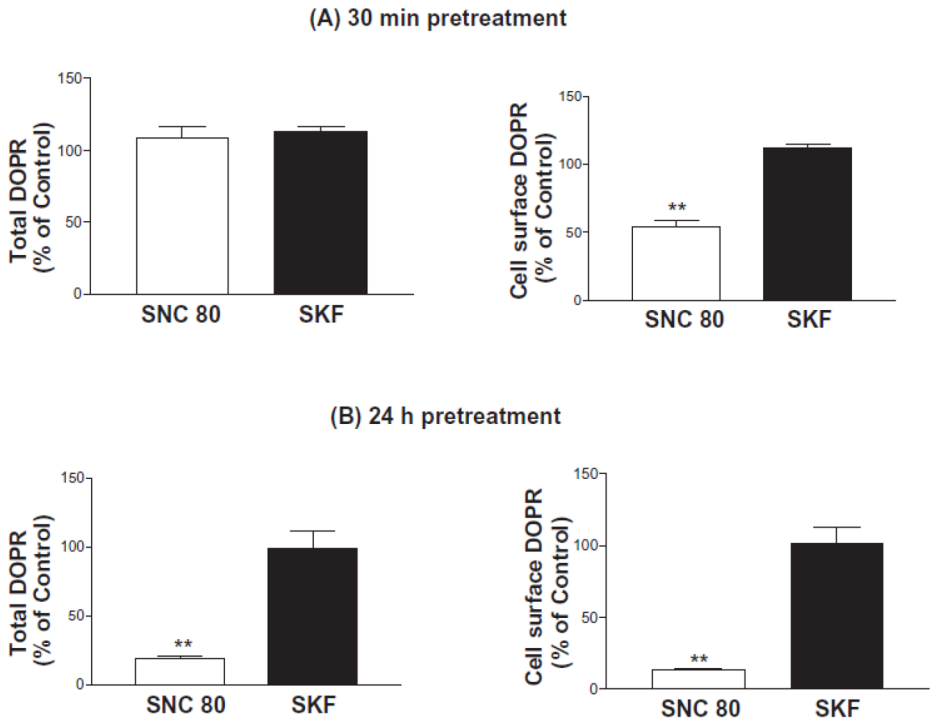

Figure 3. Effects of SKF-82958 treatment on total and cell surface DOPR in CHO-FLAG-mDOPR/HA-D_1R cells

Cells were pretreated with vehicle, 100 nM SNC-80 or 10 μM SKF-82958 for 30 min (A) or 24 h (B) as described in **Methods**. Cells were then chilled and washed and [^{3}H]diprenorphine (~1nM) binding was carried out on intact cells. For total and cell-surface receptors, nonspecific binding was defined as the binding in the presence of 10 μM naloxone and 10 μM DPDPE, respectively. Data were normalized against vehicle-treated cells (100%). The data represent the mean ± s.e.m. of three independent experiments performed in duplicate.

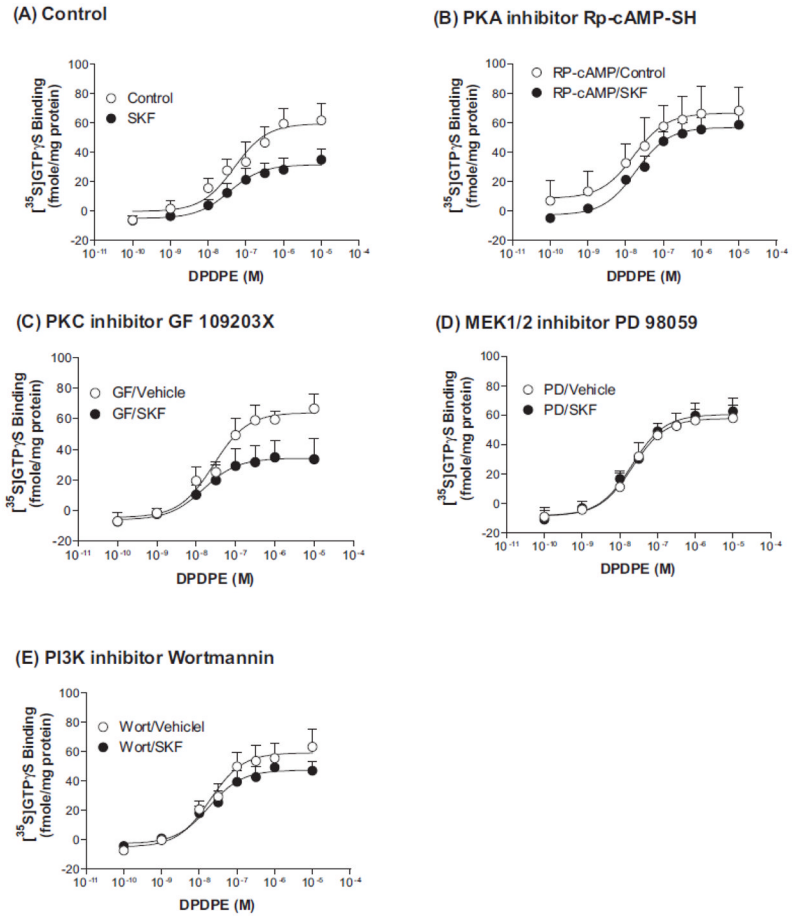

Figure 4. Effects of pretreatment of kinase inhibitors on SKF-82958-induced heterologous desensitization of DOPR

CHO-FLAG-mDOPR/HA-D_1R cells were pretreated with the PKC inhibitor GF109203X (10 μM), the MEK1 inhibitor PD 98059 (10 μM), the PI3K inhibitor wortmannin (100 nM) or the PKA inhibitor Rp-cAMP-SH (10 μM) for 20 min, and then vehicle or 10 μM SKF-82958 and 10 nM calyculin A for 30 min. Membranes were prepared. [^{35}S]GTPγS binding to cell membranes was measured in the presence of increasing concentrations of DPDPE. Each point represents the mean ± s.e.m. of three to four independent experiments performed in duplicate. EC_{50} and E_{max} values are shown in Table 4.

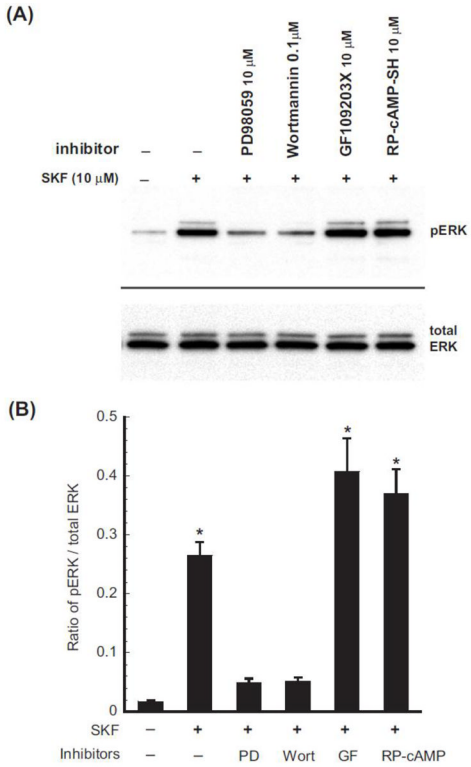

Figure 5. Effects of pretreatment of kinase inhibitors on SKF-82958-induced ERK phosphorylation

CHO-FLAG-mDOPR/HA-D_1R cells were pretreated with the PKC inhibitor GF 109203X), the MEK1 inhibitor PD 98059, the PI3K inhibitor wortmannin or the PKA inhibitor Rp-cAMP-SH at indicated concentrations for 20 min, and then vehicle or 10 μM SKF-82958 for 30 min. Cells were dissolved in 200 μl 4x SDS loading buffer, and 50 μl/lane loaded and resolved by 10% PAGE. Rabbit anti-pERK1/2 was used for ERK phosphorylation. This represents one of the three experiments performed.

Table 1

Effects of DPDPE and SKF-82958 pretreatment on EC_{50} and E_{max} values of DPDPE in stimulating [^{35}S]GTPγS binding to membranes of NG 108-15-HA-D_1R cells

See Fig. 1 legend. Data are expressed as mean ± s.e.m. of three independent experiments performed in duplicate.

	EC_{50} (nM)	E_{max} (fmol/mg protein)
Control	20.9 ± 2.5	41.1 ± 1.6
DPDPE	59.9 ± 15.1 *	15.1 ± 0.2 **
SKF	53.9 ± 9.1 *	30.8 ± 0.6 **

* $P<0.05$,

** $P<0.01$, compared to the control by one-way ANOVA, followed by Dunnett's *t* test.

Table 2

Effects of DPDPE and SKF-82958 pretreatment on K_i values of DOPR agonists in inhibiting [^{3}H]diprenorphine binding and on EC_{50} and E_{max} values of DPDPE in stimulating [^{35}S]GTPγS binding to membranes of CHO-FLAG-DOPR/HA-D$_1$R cells

See Fig. 1 legend. In addition, competitive inhibition of [^{3}H]diprenorphine by DPDPE and SNC-80 binding was conducted and K_i values were determined. Data are expressed as mean ± s.e.m. of three independent experiments performed in duplicate.

Pretreatment	K_i (nM)		EC_{50} (nM)	E_{max} (fmol/mg protein)
	DPDPE	SNC-80	DPDPE	
Control	8.5 ± 1.2	0.83 ± 0.23	39.3 ± 6.5	48.3 ± 6.8
DPDPE	21.8 ± 5.1 *	1.11 ± 0.31	47.0 ± 10.9	14.3 ± 1.1 **
SKF	5.6 ± 0.4	0.59 ± 0.16	60.9 ± 8.9	33.4 ± 2.8 *

* $P<0.05$,

** $P<0.01$, compared to the control by one-way ANOVA, followed by Dunnett's t test.

Table 3

Effects of DPDPE and SKF-82958 pretreatment on K_d and B_{max} values of [^{3}H]diprenorphine binding and [^{3}H]SCH23390 binding to the CHO-FLAG-hDOPR/HA-D_1R

Cells were treated with vehicle, DPDPE 10 μM or SKF-82958 10 μM for 30 min, collected and washed and membranes were prepared. Saturation binding of [^{3}H]diprenorphine to FLAG-hDOR/HA-D_1R was performed and K_d and B_{max} values were calculated as described in the **Method**. Data are expressed as mean ± s.e.m. of three independent experiments performed in duplicate.

	[^{3}H]Diprenorphine		[^{3}H]SCH23390	
	K_d (nM)	B_{max} (pmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Control	0.40 ± 0.04	2.78 ± 0.08	0.10 ± 0.02	2.17 ± 0.14
DPDPE	0.60 ± 0.17	2.50 ± 0.10	0.08 ± 0.01	2.04 ± 0.12
SKF	0.26 ± 0.05	2.65 ± 0.45	0.10 ± 0.02	2.54 ± 0.88

Table 4

Effects of pretreatment with kinase inhibitors on SKF-82958-induced desensitization of DOPR in CHO-FLAG-hDOPR/HA-D_1R cells

EC_{50} and E_{max} values of DPDPE in stimulating [^{35}S]GTPγS binding to membranes of CHO-FLAG-hDOPR/HA-D_1R cells pretreated with or without the inhibitors were calculated. Each value represents the mean ± s.e.m. of three to four independent experiments performed in duplicate.

		EC_{50} (nM)	E_{max} (fmol/mg protein)
Control	vehicle	20.3 ± 2.1	59.6 ± 5.9
	SKF	35.3 ± 10.7	31.6 ± 3.6 *
PKC inhibitor GF 109203X	vehicle	20.3 ± 6.5	57.2 ± 3.8
	SKF	16.7 ± 4.1	34.3 ± 4.7 *
MEK1 inhibitor PD 98059	vehicle	22.6 ± 6.0	57.9 ± 4.4
	SKF	22.2 ± 5.1	60.5 ± 4.2
PI3K inhibitor wortmannin	vehicle	17.1 ± 2.9	60.5 ± 4.7
	SKF	21.3 ± 8.8	47.5 ± 3.2
PKA inhibitor Rp-cAMP-SH	vehicle	20.3 ± 2.1	66.8 ± 7.8
	SKF	17.5 ± 4.8	56.9 ± 3.1

* $P<0.05$, compared to the vehicle control by two tailed t test.