

## Full-length article

# Cloning, expression, and functional analysis of human dopamine D<sub>1</sub> receptors<sup>1</sup>

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## Key words

cAMP response element-binding protein; alkaline phosphatase; reporter genes; G-protein-coupled receptors; dopamine D<sub>1</sub> receptor; radioligand assay; calcium; fluorescence; screening

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## Abstract

**Aim:** To construct an HEK293 cell line stably expressing human dopamine D<sub>1</sub> receptor (D<sub>1</sub>R). **Methods:** cDNA was amplified by RT-PCR using total RNA from human embryo brain tissue as the template. The PCR products were subcloned into the plasmid pcDNA3 and cloned into the plasmid pcDNA3.1. The cloned D<sub>1</sub>R cDNA was sequenced and stably expressed in HEK293 cells. Expression of D<sub>1</sub>R in HEK293 cells was monitored by the [<sup>3</sup>H]SCH23390 binding assay. The function of D<sub>1</sub>R was studied by the cAMP accumulation assay, CRE-SEAP reporter gene activity assay, and intracellular calcium assay. **Results:** An HEK293 cell line stably expressing human D<sub>1</sub>R was obtained. A saturation radioligand binding experiment with [<sup>3</sup>H]SCH23390 demonstrated that the  $K_d$  and  $B_{max}$  values were  $1.5 \pm 0.2$  nmol/L and  $2.94 \pm 0.15$  nmol/g of protein, respectively. In the [<sup>3</sup>H]SCH23390 competition assay, D<sub>1</sub>R agonist SKF38393 displaced [<sup>3</sup>H]SCH23390 with an  $IC_{50}$  value of 2.0 (1.5–2.8)  $\mu$ mol/L. SKF38393 increased the intracellular cAMP level and CRE-SEAP activity through D<sub>1</sub>R expressed in HEK293 cells in a concentration-dependent manner with an  $EC_{50}$  value of 0.25 (0.12–0.53)  $\mu$ mol/L and 0.39 (0.27–0.57)  $\mu$ mol/L at 6 h/0.59 (0.22–1.58)  $\mu$ mol/L at 12 h, respectively. SKF38393 also increased the intracellular calcium level in a concentration-dependent manner with  $EC_{50}$  value of 27 (8.6–70) nmol/L. **Conclusion:** An HEK293 cell line stably expressing human D<sub>1</sub>R was obtained successfully. The study also demonstrated that the CRE-SEAP activity assay could be substituted for the cAMP accumulation assay for measuring increase in cAMP levels. Thus, both intracellular calcium measurements and the CRE-SEAP activity assay are suitable for high-throughput screening in drug research.

## Introduction

The neurotransmitter dopamine (DA) plays a prominent role in a variety of vital brain functions including motor control, short-term memory, attention and reward<sup>[1–3]</sup>. In the CNS, DA modulates neuronal excitability by regulating ligand- and voltage-gated ion channels<sup>[4]</sup>. The actions of DA are mediated by a family of seven-transmembrane G protein-coupled receptors (GPCRs), i.e., D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub>, D<sub>4</sub>, and D<sub>5</sub>, encoded by five distinct genes<sup>[5]</sup>. These five subtypes have been grouped into two different classes based on phar-

macology and biochemistry: the D<sub>1</sub>-like and D<sub>2</sub>-like receptors. The D<sub>1</sub>-like receptors, D<sub>1</sub> and D<sub>5</sub>, mediate dopamine stimulation of adenylyl cyclase, whereas D<sub>2</sub>-like receptors, D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>, mediate dopamine inhibition of adenylyl cyclase<sup>[5,6]</sup>.

Assays capable of detecting and quantifying GPCR-ligand interactions are valuable tools for both fundamental studies of cell signaling and drug development. Drug screening has mainly relied upon binding assays on membrane preparations using radioligands or assays measuring second messengers or enzymes modulated by these receptors. The abil-

ity to conduct high-throughput screening based upon functional activity of a given GPCR in a cell-based assay offers a more direct way of identifying lead agonists or antagonists<sup>[7-9]</sup>.

In the present study, by using HEK293 cell lines stably expressing human D<sub>1</sub>R, we developed a series of method check character for analyzing GPCR activities in cell lines including CRE-SEAP activity assay and the intracellular calcium assay.

## Materials and methods

**Materials** High glucose Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, G418, penicillin and streptomycin were obtained from Gibco. Restricted enzyme (*Hind*III, *Eco*RI, *Xho*I, *Bam*HI), T4 DNA ligase, reverse transcriptase and buffer were purchased from Promega (USA). Pyrobest DNA polymerase and buffer for PCR were obtained from TaKaRa (Dalian, China). (±)-SKF-38393 hydrochloride (SKF38393) and (+)-Butaclamol hydrochloride (Butaclamol) were obtained from RBI (Natick, MA, USA). Forskolin, Fluo-3/AM, p-(dipropylsulfamoyl)benzoic acid, 3-isobutyl-1-methyl-2,6(1*H*,3*H*)-purine-dione (IBMX), pluronic F-127, 4-methyl-umbelliferyl phosphate (4-MUP), HEPES, Triton X-100, bovine serum albumin leupeptin, pepstatin A, aprotinin and PMSF were purchased from Sigma (USA). [<sup>3</sup>H]SCH23390 was purchased from Amersham (USA). Plasmids pcDNA3, pcDNA3.1(+) and Lipofectamine<sup>2000</sup> were purchased from Invitrogen (USA), and pCRE-SEAP was from BD Biosciences Clontech (USA). cAMP assay kit was purchased from Shanghai Second Medical University (Shanghai, China). Human embryo brain tissue was donated by Huashan Hospital (Shanghai, China). PCR primers were synthesized by Shenyou (Shanghai, China).

**Cloning of the human dopamine D<sub>1</sub>R cDNA** Human brain total RNA was obtained from human embryo brain tissue. The total RNA was reverse-transcribed using oligo-dT18 as a primer. With the cDNA, PCR was carried out using the primers D<sub>1</sub>R-1SE and D<sub>1</sub>R-1AS (Table 1). Gel-purified PCR product was treated with *Bam*HI/*Xho*I and subcloned into the *Bam*HI/*Xho*I site of the mammalian expression vector pcDNA3. With the subcloned D<sub>1</sub>R-pcDNA3 plasmid as a template, PCR was carried out using the primers D<sub>1</sub>R-2SE and D<sub>1</sub>R-2AS (Table 1), which resulted in full length codon sequence of cDNA. Gel-purified PCR product was treated with *Bam*HI/*Eco*RI and cloned into the *Bam*HI/*Eco*RI site of the mammalian expression vector pcDNA3.1(+).

**Production of HEK293 cell lines stably expressing human dopamine D<sub>1</sub>R** HEK293 Cells were transfected with

the D<sub>1</sub>R-pcDNA3.1(+) expression vector, using the Lipofectamine reagent. Cells were treated with selection medium containing G418 (1 g/L) for 3 weeks to select stably transfected cells displaying neomycin resistance. Between 2 and 3 weeks into the selection process, resistant cells began to appear. They were separated out by serial dilution and allowed to grow from single cells. Receptor expression of single cell-derived colonies was tested by the radioligand binding assay.

**Cell culture** Human embryonic kidney (HEK293) cell lines were cultured in DMEM containing streptomycin (100 µg/mL), penicillin G (1000 kU/L) and fetal bovine serum (10%). Cells were incubated at 37 °C in 5% CO<sub>2</sub>. Stably transfected HEK293 cell lines were cultured in DMEM high glucose medium containing streptomycin (100 mg/L), benzylpenicillin (1000 kU/L), G-418 (200 mg/L) and fetal bovine serum (10%). Cells were incubated at 37 °C in 5% CO<sub>2</sub>. For passaging, the cells were detached from the cell culture flask by washing with phosphate-buffered saline (PBS) and brief incubation with trypsin (0.5 g/L)/EDTA (0.2 g/L). The cells were passaged every 3 d.

**Membrane preparation** The D<sub>1</sub> cells were lifted from Petri dishes with a cell scraper. Harvested cells were washed twice with ice-cold PBS and centrifuged at 420×g for 5 min at 4 °C. The cell pellet was resuspended with hypotonic buffer (5 mmol/L Tris-Cl, 2 mmol/L EDTA, pH7.4, leupeptin 1 mg/L, pepstatin A 1 mg/L, aprotinin 1 mg/L, PMSF 1 mmol/L) and sonicated (18 s) three times on ice. The homogenate was centrifuged at 960×g for 10 min at 4 °C. The precipitated nucleic fraction was discarded and the supernatant was centrifuged at 40 000×g for 30 min at 4 °C. The pellet was washed with 50 mmol/L Tris-Cl buffer (pH 7.4) and centrifuged again with the same conditions. Finally, the pellet was resuspended in the same buffer, and protein concentration was determined by the BCA Kit (Pierce) as described previously<sup>[10]</sup>.

**[<sup>3</sup>H]SCH23390 binding assay** Membranes (30–50 µg protein) from D<sub>1</sub> cells were resuspended in 50 mmol/L Tris-Cl (pH 7.4, 120 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl<sub>2</sub> and 1 mmol/L MgCl<sub>2</sub>). Saturation assays were performed at different concentrations of [<sup>3</sup>H]SCH23390 (0.05–6 nmol/L), using butaclamol (10 µmol/L) to obtain nonspecific binding. Competition curves were obtained by using 0.5 nmol/L [<sup>3</sup>H]SCH23390 and different concentrations of dopamine D<sub>1</sub>R agonist SKF38393. The incubation was carried out at 37 °C for 30 min.

Binding assays were stopped by rapid filtration through Whatman GF/B filters, which were immediately washed three times with ice-cold buffer. Filters were then transferred to

Eppendorf tubes, and scintillation liquid was added to measure the radioactivity.

**cAMP accumulation experiment** After scraping the cells off the culture plates, they were washed twice with PBS and resuspended in PBS at a concentration of  $0.5 \times 10^6$ – $1.0 \times 10^6$  cells/mL. Aliquots of 0.2 mL were transferred to test tubes along with the phosphodiesterase inhibitor IBMX (100  $\mu$ mol/L) and different concentrations of dopamine D<sub>1</sub>R agonist SKF38393 added to a final volume of 0.3 mL. The reaction was terminated with 1 mol/L perchloric acid to a final concentration of 0.5 mol/L after a 15 min incubation at 37 °C. Samples were neutralized with 75  $\mu$ L 2 mol/L KOH, and the cAMP content in the supernatants were determined with a protein binding assay<sup>[11]</sup>. cAMP concentrations were calculated using a standard curve according to the protocol of the assay kit.

**CRE-SEAP activity assay** Using the secreted form of human placental alkaline phosphatase (SEAP) as the reporter enzyme transcriptionally regulated by cAMP response elements (CREs)<sup>[12,15]</sup>, CRE-SEAP activity was measured. The pCRE-SEAP plasmid was transfected into D<sub>1</sub> cells using the calcium phosphate precipitation method (Promega). Cells were seeded in 100 m<sup>2</sup> plates, and transfection was performed when cells were 50% confluent. DNA 25  $\mu$ g and 60  $\mu$ L CaCl<sub>2</sub> 2 mol/L were mixed in H<sub>2</sub>O in a total volume of 500  $\mu$ L and then slowly mixed with HEPES buffered saline (HBS). The reaction mixture was incubated at room temperature for 30 min and then evenly added to the cell culture dish containing 10 mL of fresh media. After 12 h, the transfection media was replaced with fresh media, and the transfected D<sub>1</sub> cells were seeded into 24-well plates ( $1.5 \times 10^5$  cells per well) and cultured overnight before experiment<sup>[12]</sup>. The medium was then replaced with 500  $\mu$ L of free serum DMEM containing 100  $\mu$ mol/L IBMX and the different concentrations of SKF38393 then cells were incubated for 6 h and 12 h, respectively. The SEAP assay was subsequently performed (see below).

Following the incubation period of transiently transfected cells, the culture medium was inactivated for 30 min at 65 °C and centrifuged at 15 000 $\times$ g at 4 °C. The supernatant (80  $\mu$ L), was transferred to a new 96-well plate and mixed with 100  $\mu$ L SEAP assay buffer (50 mmol/L Tris/0.1% BSA buffer, pH 8.0) containing 36  $\mu$ mol/L 4-methylumbelliferyl phosphate (4-MUP). The mixture was incubated for 1 h at 37 °C, and the fluorescence intensity was measured at 460 nm using FLUOstar plate reader. Excitation wavelength was 355 nm.

**Intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) measurement** Ca<sup>2+</sup> fluorescence measurements were performed using a NOVOstar plate reader with a pipettor system (BMG

Labtechnologies, Offenburg, Germany). D<sub>1</sub> cells were harvested with 0.05% trypsin/0.02% EDTA and rinsed with high glucose DMEM containing 10% fetal bovine serum, 100 mg/L streptomycin, 1000 U/mL penicillin G. Pelleted cells were resuspended in fresh medium and kept under 5% CO<sub>2</sub> at 37 °C for 1h and vortexed every 15 min. After twice washes with Krebs-HEPES buffer, cells were loaded with 5  $\mu$ mol/L Fluo-3/AM containing 1% pluronic F-127 and 2.5 mmol/L p-(Dipropylsulfamoyl)benzoic acid for 30 min. Then cells were rinsed three times with Krebs-HEPES buffer containing 0.5% bovine serum albumin, diluted, and evenly plated into 96-well plates at a density of  $1 \times 10^4$  cells/well. Microplates were kept at 37 °C for 15 min<sup>[13]</sup>. Buffer alone or different concentrations of SKF38393 were then injected sequentially into separate wells, and fluorescence intensity was measured at 520 nm for 50 s at 0.2 s intervals. The excitation wavelength was 485 nm.

[Ca<sup>2+</sup>]<sub>i</sub> was calculated as follows:  $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$ .  $F_{max}$  refers to the fluorescence intensity measured after permeabilization of the cells with 1% Triton X-100. Ten mmol/L EDTA was added to chelate Ca<sup>2+</sup> and minimum fluorescence intensity was obtained ( $F_{min}$ ). A  $K_d$  value of 324 nmol/L was used for Fluo-3.

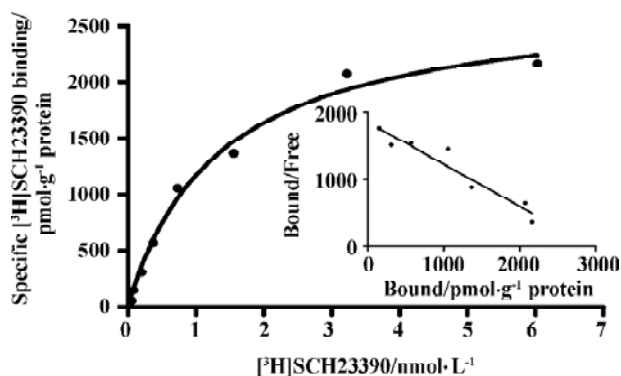
**Data analysis** Experiments were performed in triplicate. All data were expressed as mean $\pm$ SD and analyzed with the GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA, USA). Student's *t*-test was used for statistical analysis.

## Results

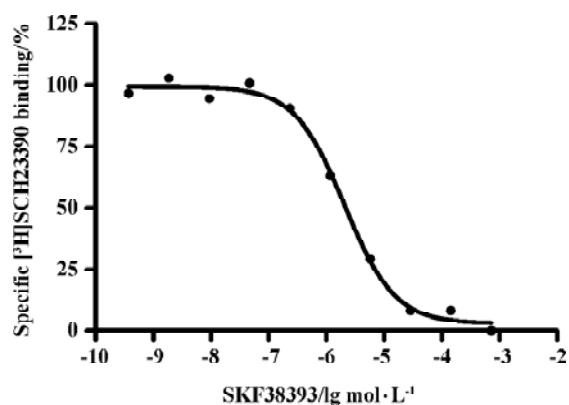
**Cloning of the human dopamine D<sub>1</sub>R cDNA** To obtain the cDNA with full length codon sequence of D<sub>1</sub>R, a reverse transcription reaction was carried out using the total RNA extracted from embryo brain tissue. Using cDNA with the full length codon sequence of D<sub>1</sub>R, a PCR product (1707 bp) was obtained using primers described previously. The PCR product was subcloned into pcDNA3 plasmid and a cDNA (1341 bp) containing the full D<sub>1</sub>R codon sequence was obtained using this subcloned vector as a template. The cDNA was then cloned into the mammalian expression vector pcDNA3.1. Sequence analysis demonstrated that the sequence of the constructed D<sub>1</sub>-pcDNA3.1 expression vector was identical to that of human D<sub>1</sub>R cDNA in the gene bank.

**Selection of cell clones** Colonies of the selected stable integrants were initially analyzed for human D<sub>1</sub>R expression by receptor binding assay using [<sup>3</sup>H]SCH23390. From the transfected HEK293 cell clones, one cell line was obtained with specific [<sup>3</sup>H]SCH23390 binding.

**Binding assays with the D<sub>1</sub>R antagonist [<sup>3</sup>H]SCH-23390** Scatchard analysis revealed a  $K_d$  value of  $1.5 \pm 0.2$  nmol/L) and  $B_{max}$  value of  $2.95 \pm 1.53$  nmol/g of protein (Figure 1). Figure 2 shows a competition of a D<sub>1</sub>R agonist SKF38393 for [<sup>3</sup>H]SCH23390 with an  $IC_{50}$  value of 2.0 (1.5–2.8)  $\mu$ mol/L (means, 95% confidence intervals,  $n=3$ ).

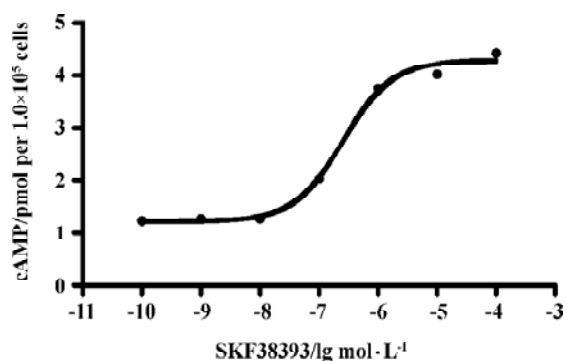


**Figure 1.** Saturation curve of [<sup>3</sup>H]SCH23390 binding to dopamine D<sub>1</sub> receptors in plasma membrane isolated from D<sub>1</sub> cells. Inset: Scatchard plot of the data and analysis gave the  $K_d$  and  $B_{max}$  values indicated in RESULTS. Mean $\pm$ SD from three independent experiments performed in triplicate.



**Figure 2.** Representative competitive inhibition curve of the dopamine D<sub>1</sub> receptor agonist SKF38393 versus the D<sub>1</sub> receptor antagonist [<sup>3</sup>H]SCH23390 in membrane preparations from D<sub>1</sub> cells.  $IC_{50}$  values were expressed as means (95% confidence intervals) obtained from three independent experiments performed in triplicate.

**D<sub>1</sub>R agonist SKF38393 induced accumulation of cAMP** D<sub>1</sub>R agonist SKF38393 induced a concentration-dependent accumulation of cAMP with a  $EC_{50}$  value of 0.25  $\mu$ mol/L (0.12–0.53) (means, 95% confidence intervals,  $n=3$ , Figure 3).



**Figure 3.** The cAMP accumulation induced by the D<sub>1</sub> receptor agonist SKF38393 in D<sub>1</sub> cells. The  $EC_{50}$  value was expressed as a mean (95% confidence intervals) obtained from three independent experiments performed in triplicate.

#### D<sub>1</sub>R agonist SKF38393 induced CRE-SEAP activity

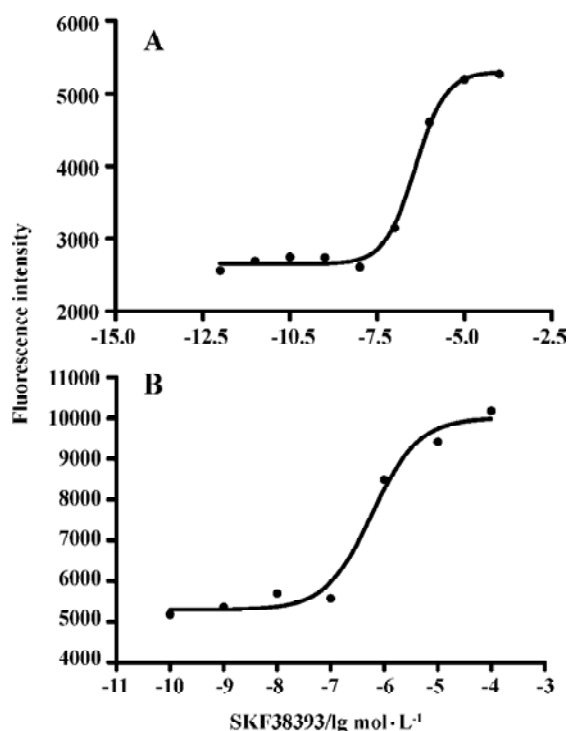
In D<sub>1</sub> cells transiently transfected with pCRE-SEAP reporter vector, SKF38393 induced a concentration-dependent increase in the CRE-SEAP activity.  $EC_{50}$  values at 6 h and 12 h were 0.39 (0.27–0.57) and 0.59 (0.22–1.58)  $\mu$ mol/L (means, 95% confidence intervals,  $n=3$ ), respectively. No significant difference was found between these two values (Figure 4).

**D<sub>1</sub>R agonist SKF38393 induced elevated intracellular calcium level** D<sub>1</sub>R agonist SKF38393 induced a concentration-dependent increase in the intracellular calcium level. The  $EC_{50}$  value was 27 (8.5–70) nmol/L (means, 95% confidence intervals,  $n=3$ ). The SKF38393-induced increase of the intracellular calcium level was completely blocked by butaclamol (100  $\mu$ mol/L), a DA receptor antagonist (Figure 5).

## Discussion

In the present study, a HEK293 cell line stably expressing human D<sub>1</sub>R was constructed. Functional analysis with cAMP accumulation assay, the CRE-SEAP activity assay, and intracellular calcium assay demonstrated that their  $EC_{50}$  values were 0.25 (0.12–0.53)  $\mu$ mol/L, 0.39 (0.27–0.57)  $\mu$ mol/L for 6 h and 0.59 (0.22–1.58)  $\mu$ mol/L for 12 h and 27 (8.6–70) nmol/L, respectively. No significant difference in the  $EC_{50}$  values was found between SKF38393-induced cAMP accumulation and CRE-SEAP activation.

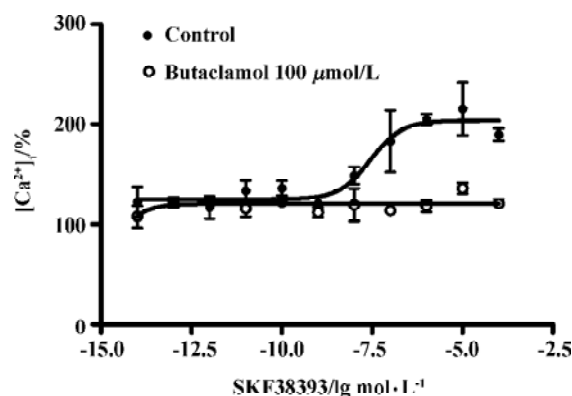
D<sub>1</sub>R can activate adenylyl cyclase, which then results in cAMP production. At present, radioimmunoassay is one of the most widely used methods for the measurement of cAMP accumulation<sup>[14]</sup>. Cell based assays relying on transcriptionally controlled reporter gene have been developed and are suited to monitoring the cellular responses induced by



**Figure 4.** CRE-SEAP activity induced by dopamine D<sub>1</sub> receptor agonist SKF38393. (A) incubation with SKF38393 for 6 h; (B) incubation with SKF38393 for 12 h. EC<sub>50</sub> values were expressed as mean (95% confidence intervals) obtained from three independent experiments performed in triplicate.

GPCRs. Since CRE is a pivotal target in GPCR signaling pathways, it has become one of the most widely used response elements in reporter gene assays. The cAMP response element binding protein (CREB), a transcription factor, is the major regulator of CREs. It can be activated (phosphorylated) by protein kinase A (PKA) and members of calcium/calmodulin kinase (CaMK) family *in vitro*. Both G<sub>os</sub>- and G<sub>oq</sub>-coupled receptors are signaled through CREs [12,15,16]. Since the transcription of CRE-SEAP reporter genes is controlled by CRE-containing promoters, CRE-SEAP activity can be used to monitor the activation of receptors. In the present study, the EC<sub>50</sub> value obtained using CRE-SEAP activity assay is similar to that obtained from cAMP accumulation assay. These results suggest that CRE-SEAP activity assay can be used to estimate the G<sub>os</sub>-coupled receptor function.

G<sub>os</sub>-coupled receptors activate adenylyl cyclase, leading to an increase in cAMP level. cAMP activates PKA, which subsequently phosphorylates different targets. This pathway can lead to an increase in intracellular [Ca<sup>2+</sup>] either from intracellular Ca<sup>2+</sup> stores or from the extracellular matrix [13,17].



**Figure 5.** Concentration-response curves for the effect of SKF38393 on intracellular [Ca<sup>2+</sup>]<sub>i</sub> in D<sub>1</sub> cells. *n*=3. Mean±SD. EC<sub>50</sub> value was mean (95% confidence intervals) obtained from three independent experiments performed in triplicate.

Direct activation of Ca<sup>2+</sup> ion channels by G<sub>os</sub> is also possible [13,18]. We found that a significant difference was observed in the EC<sub>50</sub> value of the intracellular calcium assay and cAMP accumulation assay. While the EC<sub>50</sub> value to induce the cAMP accumulation was 0.25 (0.12–0.53) μmol/L, the EC<sub>50</sub> value to induce an increase in the intracellular calcium was 27 (8.6–70) nmol/L. This is likely to reflect the difference in signaling between intracellular calcium increase and cAMP accumulation. Intracellular calcium signals may be directly transduced, bypassing the adenylyl cyclase pathway and representing transient binding with ligand. Therefore, combination CRE-SEAP activity assay or a cAMP accumulation assay with an intracellular calcium assay will obtain more information on the effect of ligands. Our study demonstrates that the CRE-SEAP activity assay and intracellular calcium assay are suitable for high-throughput screening in drug research.

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