Activation of phosphatidylinositol-linked novel D₁ dopamine receptors inhibits high voltage activated Ca\(^{2+}\) currents in primary cultured striatal neurons

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**Running Title:** Activation of PI-DA-R inhibits Ca\(^{2+}\) currents in striatum

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Abstract

Recent evidences indicate the existence of a putative novel phosphatidylinositol (PI) -linked D$_1$ dopamine receptor, which mediates excellent anti-Parkinsonian but less severe dyskinesia action. To further understand the basic physiological function of this receptor in brain, the effects of a PI-linked D$_1$ dopamine receptor selective agonist SKF83959 on high voltage activated (HVA) Ca$^{2+}$ currents in primary cultured striatal neurons were investigated by whole-cell patch-clamp technique. The results indicated that stimulation by SKF83959 induced an inhibition of HVA Ca$^{2+}$ currents in a dose-dependent manner in substance-P (SP)-immunoreactive striatal neurons. Application of D$_1$ receptor, but not D$_2$, $\alpha_1$ adrenergic, 5-HT receptor or cholinoreceptor antagonist prevented SKF83959-induced reduction, indicating that a D$_1$ receptor-mediated event, assumed via PI-linked D$_1$ receptor. SKF83959-induced inhibitory modulation was mediated by activation of phospholipase C (PLC), mobilization of intracellular Ca$^{2+}$ stores and activation of calcineurin. Furthermore, the inhibitory effects were attenuated significantly by the L-type calcium channel antagonist nifedipine, suggesting that L-type calcium channels involved in the regulation induced by SKF83959. These findings may help to further understand the functional role of the PI-linked dopamine receptor in brain.

Keywords: SKF83959, dopamine receptor, calcium channel, striatal neurons, calcineurin

Abbreviations: PI, phosphatidylinositol; PLC, phospholipase C; DA, dopamine; GPCRs, G-protein coupled receptors; HVA, high voltage-activated calcium channel;
AC, adenylyl cyclase; DAG, 1, 2-diacylglycerol; [Ca^{2+}]_i, cytosolic Ca^{2+} concentration; PP1, protein phosphatase 1; substance P (SP).

INTRODUCTION

High voltage-activated (HVA) calcium currents are essential to neuronal function, participating in neurotransmitter release, synaptic integration and plasticity, dendritic electrogenesis, neuronal excitability, gene regulation, neuronal survival and differentiation (Pietrobon 2005). They have been subdivided on the basis of their electrophysiological and pharmacological properties into L-, N-, P/Q-, and R- types (Tsien et al. 1995). The multiple modulatory effects on HVA calcium channels play a prominent role both in changing the integrative properties of neurons and in regulating output at a presynaptic level. These modulations include activated heterotrimeric G-protein subunits (Ikeda 1996), phosphorylation by protein kinases (McDonald et al. 1994), dephosphorylation by protein phosphatases (Frace and Hartzell 1993) and the direct action of Ca^{2+} on the channel itself (Lee et al. 1999).

Dopamine (DA) is an important neurotransmitter in brain, and plays a critical role in regulation of locomotor, cognitive, emotional functions and hormone secretion via dopamine receptors (Goldman-Rakic et al. 2004; Missale et al. 1998; Neve et al. 2004). Dopamine has been reported to regulate extensively HVA calcium channels in neurons via activation of classical D_1-like and D_2-like receptors (Hernandez-Lopez et al. 2000; Surmeier et al. 1995). Recently, a novel D_1-like DA receptor-coupled second messenger system was reported. Other than the conventional G_α-adenylyl
cyclase-cAMP pathway, this D$_1$-like DA receptor couples to G$_q$ protein and stimulates phospholipase C$_{\beta}$ (PLC$_{\beta}$), results in hydrolysis of phosphoinositide (PI) (Deveney and Waddington 1995; Ming et al. 2006; Pacheco and Jope 1997; Undie et al. 1994; Zhang et al. 2005). In the recent work from our and other laboratories, it has been shown that the PI-linked D$_1$-like dopamine receptors mediates more effective functions of anti-Parkinsonian symptom but less dyskinesia (Andringa et al. 1999b; Gnanalingham et al. 1995; Zhang et al. 2007; Zhen et al. 2005). However, it remains to be elucidated whether stimulation of novel PI-linked D$_1$-like DA receptor regulates HVA Ca$^{2+}$ currents. SKF83959, a recently identified selective agonist for this putative PI-linked DA receptor (Andringa et al. 1999a; Jin et al. 2003; O'Sullivan et al. 2005; Waddington et al. 2005), provided a powerful tool for exploring the function of this novel signal pathway in brain. In the present study, whole cell patch-clamp technique was employed to investigate the effect of SKF83959 on HVA Ca$^{2+}$ channels in primary cultured rat striatal neurons. The results indicated that SKF83959 stimulation reduced HVA Ca$^{2+}$ currents in striatal neurons via a D$_1$-like dopamine receptor in a dose-dependent manner. The regulation was mediated by intracellular Ca$^{2+}$ stores mobilization via activation of a PLC-IP$_3$ pathway, leading to calcineurin-dependent suppression in L-type Ca$^{2+}$ currents. These data shed new light on the understanding of the novel PI-linked dopamine receptor contribution to the physiological function in striatum.
MATERIALS AND METHODS

Materials

R-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2, 3, 4, 5-tetrahydro-1H-3-benzazepine [R-(+)-SCH23390] was from Tocris Cookson (Ellisville, MO). 6-Chloro-7, 8-dihydroxy-3-methyl-1-(3-methylphenyl)-2, 3, 4, 5-tetrahydro-1H-3-benzazepine (SKF83959), genistein, spiperone, prazosin were purchased from RBI (Natric, MA, USA). 1-[(6-[[((17)-3-methoxyestra-1, 3, 5[10]-trien-17yl)amino]hexyl]-1H-pyrrole-2, 5-dione (U-73122), 1-[(6-[[((17)β)-3-Methoxyestra-1, 3, 5[10]-trien-17-yl)amino]hexyl]-2, 5-pyrrolidinedione (U-73343), 1, 2-bis-(2-aminophenoxy)ethane-N, N, N’, N’-tetraacetic acid (BAPTA), bisindohylnleimide, cyclosporin A, okadaic acid, heparin, thapsigargin were obtained from Sigma (St. Louis, MO, USA). Anti-glutamic acid decarboxylase (GAD) 65/67 antibody and Anti-SP antibody were obtained from Chemicon International (Temecula, CA, USA). Fura-2/AM was obtained from Biotium (Hayward, CA, USA). Other agents were purchased from commercial suppliers. SKF83959 was prepared freshly with distilled water. Other agents were prepared as stock solutions with sterile water except nifedipine, U-73122, U-73343 and Fura-2/AM, which were dissolved in dimethylsulfoxide (DMSO) and stored at -20°C. They were diluted to the final concentrations before application. The final concentration of DMSO was less than 0.05%.

Primary striatal culture
The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. Primary cultures of striatal neurons were prepared as described in previous experiment (Falk et al. 2006). Briefly, striata were dissected from the neonatal Sprague-Dawley rats (day 0-3) of both sexes and rinsed in cold PBS. Tissues were incubated in 0.125% trypsin for 25 min at 37°C and mechanically dissociated using a fire-polished Pasteur pipettes. Cell suspension was centrifuged and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) and F-12 supplement (1:1) (Gibco Invitrogen Corporation) with 10% fetal bovine serum (heat-inactivated, Hycolne), 0.5 mM L-glutamine, 5 U/ml penicillin and 5 µg/ml streptomycin (Sigma). Cells were plated at a density of 10^4-10^5 per 35 mm^2 on poly-D-lysine coated coverslips and kept at 37°C in 5% CO₂. After 24 h, the culture medium was changed to DMEM/F-12 medium supplemented with 2% B27. Astrocytes were minimized by treating the culture with cytarabine (10 µM) on day 3. The neurons were fed with fresh medium twice weekly. Experiments were performed on day 7-9.

**Whole-cell patch-clamp recording**

The procedure for whole-cell patch-clamp recording was described in our previous study (Chen et al. 2002; Lin et al. 2007). The extracellular solution consisted of (in
mM): 110 NaCl, 5 KCl, 10 CaCl2, 1 MgCl2, 11 glucose, 10 HEPES, 5 4-AP, 25 TEA-Cl and 1 µM tetrodotoxin (pH 7.4, 300 mOsm/l). The pipette solution consisted of (in mM): 64 CsCl, 64 CsF, 0.1 CaCl2, 2 MgCl2, 10 HEPES, and 5 Tris–ATP (pH 7.4, 265-270 mOsm/l). The resistance of the recording pipette was in the range of 3–5 MΩ. Recordings were obtained according to standard patch-clamp methods (Hamill et al., 1981) using an EPC10 patch-clamp amplifier (HEKA, Lambrecht, Germany). Data were sampled at 10 kHz and filtered at 3 kHz. Voltage commands and data acquisition were controlled by PULSE/PULSEFIT software (HEKA, Southboro, Germany). Cells with a series resistance > 10MΩ were discarded and series resistance was generally compensated to 60%~80%.

All the recordings were carried out at 20-22 °C under conditions optimized so as to ensure the isolation of Ca2+ currents from other voltage-activated currents. Drug actions were measured only after steady-state conditions reached, which were judged by the amplitudes and time courses of currents remaining constant with further perfusion of drug. All experiments were repeated at least four times using different batches of cells and at least the 3–4 dishes with cells were used for recording in different batches of cells.

**Calcium imaging experiments**

Calcium imaging experiments were performed as described in our previous studies (Ming et al. 2006). Briefly, striatal neurons were washed three times with HEPES-buffered solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10
glucose and 10 HEPES (pH 7.3). Cells were incubated in buffer containing Fura-2/AM (1 µM) for 25 min at 37°C and rinsed three times with HEPES-buffered solution. In Ca^{2+}-free experiments, EGTA (100 µM) replaced CaCl₂. Coverslips were mounted on a recording chamber positioned on the movable stage of an inverted IX-70 Olympus microscope equipped with a calcium imaging system (TILL Photonics GmbH, Gräfelfing, Germany). Loaded cells were illuminated at 340 nm for 150 ms and 380 nm for 50 ms at 1 s intervals using a TILL Polychrome monochromator. Fura-2 fluorescence emission was imaged at 510 nm by an intensified cooled charge coupled device (TILL Photonics GmbH) through an X-70 fluor oil immersion lens (Olympus) and a 460-nm long-pass barrier filter. F340/F380 fluorescence ratios were generated by TILLVISION 4.0 software. Paired F340/F380 fluorescence ratio images were acquired every second for [Ca^{2+}]. The intracellular free calcium concentration is presented as the ratio of the fluorescence signals obtained (340/380 nm). All experiments were repeated at least three times using different batches of cells.

**Immunocytochemistry**

The expressions of GAD and SP were detected according to previous experiments (Henderson et al. 2000; Segal 2003). Striatal cultures were rinsed 3 times for 5 min each in PBS before they were fixed in fresh 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 30 min at room temperature. After 3 more PBS rinses for 10 min each, neurons were permeabilized in 0.5% Triton X-100 in PBS and blocked in 2%
normal goat serum in PBS/Triton X-100. Fixed cells were then incubated overnight at 4°C with anti-GAD 65/67 antibody (1:1000) or anti-SP antibody (1:1000). Cultures were rinsed 3 times for 5 min each in PBS before they were incubated with a biotinylated secondary antibody and then an avidin-biotin peroxidase complex (ABC Kit, Vector). Antibodies labeling were visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen.

**Statistical analysis**

Data analysis was performed with SPSS10.0 software (Inc, Chicago, IL). Dose-response curve was fitted with the Hill equation: \[ I/I_{\text{max}} = \frac{1}{1 + (EC_{50}/C)^n} \], where \( I \) is the current amplitude after administration of SKF83959, \( I_{\text{max}} \) is the control current amplitude, \( C \) is the concentration of SKF83959, and \( n \) is Hill coefficient. Sample statistics are given as means±S.E.M of \( n \) observations. One-way ANOVA followed by post-hoc test was used to evaluate differences. The statistical significance level was set at \( p<0.05 \).

**RESULTS**

**SKF83959 suppressed HVA Ca\(^{2+}\) currents in cultured striatal neurons in a dose-dependent manner**

The whole-cell patch-clamp technique was employed to study the effect of SKF83959 on HVA Ca\(^{2+}\) currents evoked by a voltage step to 10 mV from a holding potential of -80 mV in primary cultured rat striatal neurons. Only medium-sized
neurons with a whole-cell capacitance between 5-10 pF were used in this study. Rapid application of SKF83959 (0.1–100 µM) through a wide-bore, gravity-fed pipette reversibly inhibited Ca\textsuperscript{2+} currents in a dose-dependent manners (Fig. 1C). The EC\textsubscript{50} was 8.77±0.72 µM, and the Hill coefficient was 1.20±0.45 (fitted with Hill equation). A brief (15-30 sec) application of SKF83959 (10 µM) decreased the amplitude of Ca\textsuperscript{2+} currents by 37.6±4.43% with almost completely recovery after washout in most of cells (n=13, p<0.05 vs control, Fig. 1A & B). Dopamine (10 µM) also reversibly reduced HVA Ca\textsuperscript{2+} currents by 25.2±3.14% (Fig. 1A). Because F\textsuperscript{−} can combine with trace amounts of Al\textsuperscript{3+} to produce AlF\textsubscript{3}, which is an activator of heterotrimeric G-proteins to inhibit some phosphatases (Li 2003), we replaced CsF in the pipette solution with equal concentration of CsCl. The effects of SKF839589 (10 µM) on HVA Ca\textsuperscript{2+} currents were not significantly changed by the replacement (n=12, data not shown). In addition, 10 µM SKF839589 decreased the maximum amplitude of HVA Ca\textsuperscript{2+} currents in striatal neurons, but had no effect on the reversal potential (Fig. 1D & E).

It is well known that agonist-induced reductions in Ca\textsuperscript{2+} currents are strongly voltage-dependent (Beech et al. 1992). To determine whether the SKF83959-mediated reduction exhibits voltage-dependent, the ability of a strong depolarizing prepulse to affect the modulation was examined. Prepulse (30 ms) to +100 mV before test pulse (Surmeier et al. 1995) did not alter SKF83959 (10 µM) induced reduction of HVA Ca\textsuperscript{2+} currents (n=7, Fig.1F), indicating that SKF83959 effects were mediated by a voltage-independent mechanism.
The cells in which HVA Ca\(^{2+}\) currents were suppressed by SKF83959 were SP-immunoreactive

More than 90% of the striatal cells are GABAergic. GAD, the synthetic enzyme for GABA, has typically been employed to locate GABAergic cells. Immunocytochemistry experiment revealed that 93% of cells in the striatal culture were GAD-immunopositive (Fig. 2A). Two neuronal subpopulations of the GABAergic striatal cells have been described, one that mainly express DA D\(_1\) receptors and colocalize SP, and another that mainly express DA D\(_2\) receptors and colocalize enkephalin. Among 19 neurons in which HVA Ca\(^{2+}\) currents were inhibited by SKF83959, 15 (79%) neurons were proved to be SP-immunopositive by subsequent immunocytochemical staining with anti-SP antibody (Fig. 2B & C).

**D\(_1\)-like dopamine receptor was involved in SKF83959-induced HVA Ca\(^{2+}\) currents inhibition**

SKF83959 is known to have a high affinity to the D\(_1\)-like dopamine receptor, which also exhibits a moderate affinity to D\(_2\)-like dopamine receptor, adrenergic receptor and 5-HT receptor (Andringa et al. 1999a). To test which kind of receptors was responsible for the modulation produced by SKF83959, we examined the ability of SCH23390, a selective D\(_1\) receptor antagonist and spiperone, a selective D\(_2\) receptor antagonist on the action of SKF83959. Application of SCH23390 (10 µM) or spiperone (10 µM) itself had no effect on HVA Ca\(^{2+}\) currents respectively (data not
shown). However, preincubation of SCH23390 (10 µM) to the cultured striatal neurons prior to SKF83959 for 10 min almost completely eliminated the inhibitory effects induced by 10 µM SKF83959 (Fig. 3A & C). In contrast, application of spiperone (10 µM) did not affect the inhibitory actions of SKF83959 (n=6, Fig. 3 B&C). Application of other receptor antagonists such as the 5-HT1c and 5-HT2 receptor antagonist mesulergine HCl (10 µM), α-adrenoceptor blocker prazosin (10 µM) or anticholinergic scopolamine (10 µM) did not occlude SKF83959-induced reduction of HVA Ca2+ currents (n=6, Fig. 3C). These results suggested that D1 receptor but not other receptors implicated in the SKF83959-induced reduction of HVA Ca2+ currents.

**SKF83959 reduced HVA Ca2+ currents via phospholipase C-IP2 pathway**

SKF83959 is a putative selective PI-linked D1 dopamine receptor agonist that mediates actions via the PLCβ pathway. As depicted in Fig. 4A & C, the inhibitory effects of SKF83959 on HVA Ca2+ currents were blocked by addition of PLC inhibitor U-73122 (10 µM) (n=10). 10 µM U-73122 alone had no significant effect on HVA Ca2+ currents (data not shown). Whereas the inactive analog U-73343 (10 µM) failed to affect SKF83959 modulation (n=8, Fig 4C). When striatal neurons were incubated overnight in pertussis toxin (PTX, 500ng/ml)-containing medium, the amplitude of HVA Ca2+ current produced by SKF83959 (10 µM) was decreased by 31.7±4.03% (n=12). No significant difference was found between PTX group and SKF83959 alone group (40.4±5.12%, n=5, p>0.05, Fig. 4C), which suggested that the
effects of SKF83959 were independent of G_i/o protein activation. The involvement of tyrosine kinase seemed to be unlikely, because preincubation with tyrosine kinase inhibitor genistein (50 µM) had no effect on the reduction of HVA Ca^{2+} currents by SKF83959 (n=5, Fig 4A & C).

Activation of PLC is known to stimulate PI hydrolysis, IP_{3} and 1, 2-diacylglycerol (DAG) production. Subsequently, IP_{3} stimulates the release of Ca^{2+} from endoplasmic reticulum (ER) stores; DAG leads to activation of PKC. To test which pathway was involved in SKF83959-mediated inhibition of HVA Ca^{2+} currents, primary cultured striatal neurons were pretreated with PKC inhibitor bisindolylmaleimide (BIM, 1 µM) for 10 min. The inhibitory effects of SKF83959 were not noticeably altered by BIM. However, when neurons were dialyzed with heparin (10 mg/ml, a competitive antagonist of IP_{3}) in pipette solution, SKF83959-induced reduction of HVA Ca^{2+} currents was significantly diminished (n=5, p<0.05, Fig. 4B & C). After preincubation with 1 µM thapsigargin to deplete the intracellular calcium store in neurons, the inhibition of HVA Ca^{2+} currents by SKF83959 was attenuated from 35.5±3.90% (SKF83959 alone) to 9.79±1.51% (n= 9, p<0.05, Fig. 4C).

*The inhibitory effect on HVA Ca^{2+} currents by SKF83959 was Ca^{2+}-dependent*

Previous studies have shown that HVA Ca^{2+} currents can be suppressed by elevations of intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) (Lukyanetz et al. 1998). It has been demonstrated that SKF83959 elevated [Ca^{2+}]_{i} in primary cultured hippocampal neurons by our previous study (Ming et al, 2006) and acutely isolated striatal neurons
by Tang and co-workers (Tang and Bezprozvanny 2004). To test whether the effects of SKF83959 on HVA Ca$^{2+}$ currents were depended on [Ca$^{2+}$]i, [Ca$^{2+}$]i was measured with fura-2/AM (100 µM) by fluorometric techniques. The results revealed that SKF83959 (10 µM) evoked a rapid [Ca$^{2+}$]i elevation regardless of in Ca$^{2+}$-containing or Ca$^{2+}$-free solution in primary cultured striatal neurons (n=35, Fig. 5A & B), and D$_1$ receptors antagonist SCH23390 reversed these responses (n=38, data not shown). The cells were further dialyzed with high concentration of fast Ca$^{2+}$ chelator BAPTA (15 mM), which is sufficient to maintain [Ca$^{2+}$]i at a nanomolar level. As shown in Fig 5C & D, BAPTA almost abolished SKF83959-mediated inhibitory effect completely (n=9, p<0.05 vs SKF83959 alone). These results revealed that the inhibition of HVA Ca$^{2+}$ currents induced by SKF83959 was Ca$^{2+}$-dependent.

In addition to the intracellular calcium release pathway, the extracellular Ca$^{2+}$ influx also resulted in elevation of [Ca$^{2+}$]i. To test which Ca$^{2+}$ source was implicated in the SKF83959 regulation, extracellular Ca$^{2+}$ was replaced with Ba$^{2+}$. As shown in Fig 5E & F, SKF83959 (10 µM) decreased the amplitude of Ba$^{2+}$ currents by 32.2±2.50% (n=9, p<0.05 vs control current levels). However, there was no significant difference when compared with the Ca$^{2+}$ currents inhibition induced by SKF83959 (n=5, p>0.05), suggesting that SKF83959 effects were independent of extracellular Ca$^{2+}$ influx, but the intracellular Ca$^{2+}$ release.

*Inhibition of calcineurin blocked the PI-linked D$_1$-like receptor modulation of HVA Ca$^{2+}$ currents*
One potential means by which elevations in cytosolic Ca\(^{2+}\) levels could inhibit HVA Ca\(^{2+}\) currents is through a rapid Ca\(^{2+}\)/calmodulin-dependent activation of calcineurin (Lukyanetz et al. 1998; Zhu and Yakel 1997), which negatively modulates Ca\(^{2+}\) currents by dephosphorylation of some sites in Ca\(^{2+}\) channels. In our experiments, 10 µM cyclosporine A (CsA), an inhibitor of calcineurin, induced a slight increase on HVA Ca\(^{2+}\) currents (data not shown). When neurons were preincubated with CsA (10 µM), the inhibitory effects of SKF83959 on HVA Ca\(^{2+}\) currents were dramatically attenuated (n= 9, Fig. 6A & C). In contrast, inhibition of protein phosphatase 1 and 2A by okadaic acid (1 µM) had no effect on the ability of SKF83959 to suppress the HVA Ca\(^{2+}\) currents (n=4, Fig. 6C). Okadaic acid (1 µM) alone significantly enhanced HVA Ca\(^{2+}\) currents (data not shown).

**SKF83959 regulated HVA Ca\(^{2+}\) currents mainly via L-type calcium channels**

To further demonstrate which type of calcium channel was modulated by SKF83959, the effects of SKF83959 on HVA Ca\(^{2+}\) currents in striatal neurons were observed in the presence of 10 µM nifedipine, a dihydropyridine calcium channel blocker. As shown in Fig 6B & C, nifedipine (10 µM) alone reduced the HVA Ca\(^{2+}\) currents amplitude by 52.8±6.33% when applied in the extracellular solution (n=10, \(p<0.05\) via control). Pretreatment with 10 µM nifedipine significantly attenuated SKF83959-mediated inhibitory effect on HVA Ca\(^{2+}\) currents contrast to SKF83959 alone (n=8, \(p<0.05\)).
DISCUSSION

The present study demonstrated that SKF83959 reversibly inhibited HVA Ca\(^{2+}\) currents in a dose-dependent manner in primary cultured striatal neurons. 79% neurons in which HVA Ca\(^{2+}\) currents were suppressed were proved to be SP-immunopositive. The inhibitory modulation was voltage-independent and mediated via D\(_1\) dopamine receptors. We demonstrated that SKF83959-induced reduction in HVA Ca\(^{2+}\) currents was mediated by mobilization of intracellular Ca\(^{2+}\) stores through activation of a PLC-IP\(_3\) pathway, leading to a calcineurin-dependent decrease of L-type Ca\(^{2+}\) channel currents (Fig 7).

Striatal neurons express a complete array of HVA Ca\(^{2+}\) channels (Bargas et al. 1994; Martella et al. 2008), which exerting important roles in neuronal excitability and neurotransmitter receptor-dependent signaling. Dopamine receptors appear to affect the activity of HVA Ca\(^{2+}\) channels. In rat striatal neurons, D\(_1\) receptor agonists increase L-type HVA Ca\(^{2+}\) channels, which is due to phosphorylation of calcium channels by PKA (Liu et al. 1992; Surmeier et al. 1995). In addition, D\(_1\) receptor agonists reduce N- and P-type Ca\(^{2+}\) channels by PKA stimulation of PP1, which, in turn, dephosphorylates and inactives the channels (Surmeier et al, 1995). Unlike other D\(_1\) dopamine receptor agonists, SKF83959 elicits no stimulation of cAMP production, exerting its role via a putative PI-linked D\(_1\) dopamine receptor. Our results detailed the regulation and signaling pathway for SKF83959-induced HVA Ca\(^{2+}\) currents inhibition in primary cultured striatal neurons, thereby providing further support for the importance of PI-linked D\(_1\) dopamine receptor in neuronal function.
In a variety of other neurons, G-protein-coupled receptors (GPCRs)-induced suppression in HVA Ca\(^{2+}\) currents occurs through receptor-activated G-protein directly interact with Ca\(^{2+}\) channels, without the need of any additional intracellular messenger (Beech et al. 1992; Hille 1994). This mechanism is called “membrane-delimited”. However, it seemed that the novel PI-linked D\(_1\) receptors do not rely on the mechanism, since SKF83959 modulation was relatively slow which produced a response in 15-30 sec, while “membrane-delimited” mechanism are typically fast, and completed in 1-2 sec (Brown 1991). Furthermore, the membrane-delimited mechanism is strongly voltage-dependent, whereas the SKF83959 modulation was not affected by depolarizing prepulse. Finally, as discussed below, the SKF83959-induced modulation was blocked by second messengers or phosphatase inhibitors. These lines of evidences suggest cytosolic signaling elements may be involved in the modulation produced by SKF83959.

Distinct signaling pathways may be tailored to particular types of GPCRs to mediate HVA Ca\(^{2+}\) channels. PLC was constitutively expressed in striatal neurons (Hernandez-Lopez et al. 2000). Activation of PLC leads to generation of IP\(_3\) and DAG (Umemori et al. 1997). Inhibition of PLC with U-73122 blocked the SKF83959-mediated reduction of HVA Ca\(^{2+}\) currents. However, U-73122 also acted as an alkylating agent and block Gi/o protein pathways in a manner similar to pertussis toxin (Horowitz LF et al. 2005). Moreover, Gi/o protein coupled pathways can activated PLC (though usually not as well as Gq-coupled pathways). To preclude the effect of Gi/o protein, we employed the PTX, the specific Gi/o protein inhibitor in
this experiment. It was found that PTX had no significant effects on SKF83959 modulation. DAG generated by PLC results in the activation of PKC isoforms (Tanaka and Nishizuka 1994) and PKC was reported to decrease Ca$^{2+}$ currents in other type of neurons (Rane and Dunlap 1986). However, in striatal neurons, this limb of the PI-linked D$_1$ dopamine receptor signaling cascade did not participate in the SKP83959-induced regulation of HVA Ca$^{2+}$ currents as PKC inhibitor bisindolylmaleimide did not affect the SKF83959 effect. In contrast, blockade of IP$_3$ receptors with heparin or depletion of intracellular calcium store with thapsigargin disrupted the SKF83959-mediated modulation. These data indicated that PLC-IP$_3$ pathway contributed to the modulation.

The data presented here further suggest that SKF83959-induced inhibition of HVA Ca$^{2+}$ currents was dependent on Ca$^{2+}$ release from intracellular stores. SKF83959 stimulation induced the release of Ca$^{2+}$ from intracellular stores in striatal neurons. Chelation of intracellular Ca$^{2+}$ reduced SKF83959 regulation and the use of Ba$^{2+}$ as the charge carrier failed to block the effects of SKF83959. Ca$^{2+}$ released from intracellular stores is known to activate calcineurin in a variety of cell types (Day et al. 2002; Hernandez-Lopez et al. 2000; Yan et al. 1999). Calcineurin is a Ca$^{2+}$/calmodulin-regulated protein phosphatase highly expressed in striatal neurons (Mulkey et al. 1994). It has been shown to possess the character of negative feedback regulation of various ligand- and voltage-gated ion channels by dephosphorylation (Lukyanetz et al. 1998). Consistent with these reports, we have shown that inhibitor of calcineurin significantly attenuated the SKF83959-induced modulation of HVA Ca$^{2+}$
currents. Activation of D₂ dopamine receptors and 5-HT₂ receptors have also been reported to decrease HVA Ca\(^{2+}\) channels via the PLC-IP\(_3\)-Ca\(^{2+}\)-calcineurin pathway in neurons (Day et al. 2002; Hernandez-Lopez et al. 2000), whereas SKF83959 exhibits a moderate affinity to both receptors (Andringa 1999). However, our data demonstrated D₁ receptors but not other receptors implicated in the SKF83959 regulation. Although a number of studies suggest that calcineurin is important in Ca\(^{2+}\)-dependent inactivation of voltage-sensitive calcium channels (Armstrong 1989; Lukyanetz et al. 1998) to suppress HVA Ca\(^{2+}\) currents, there are some contradictory reports (Branchaw et al. 1997; Norris et al. 2002; Victor et al. 1997; Zeilhofer et al. 2000). The discrepancy could relate to the differences in neuronal types, in expression of multiple VSCC types, or in experimental regiments.

A large fraction of the SKF83959 modulation results from a targeted suppression in L-type Ca\(^{2+}\) currents. Blockade of L-type Ca\(^{2+}\) currents with nifedipine reduced the effects of SKF83959. A previous study indicated that activation of D₁ receptor enhanced L-type Ca\(^{2+}\) currents by activation of PKA and subsequent phosphorylation of channels. However, our data showed that SKF83959-mediated activation of D₁ receptors selectively reduced L-type Ca\(^{2+}\) channel currents via PLC\(_{\beta}\) pathway. Involvement of distinct signaling pathways may also contribute to the different modulation of L-type Ca\(^{2+}\) currents. Our work first provided direct electrophysiological evidence for the existence of a PLC\(_{\beta}\)-linked D₁-like dopamine receptor which is different from the class D₁ dopamine receptors.

Excessive calcium entry produces deleterious effects and results in cell death. So it
is essential for cells to carefully buffer intracellular calcium and to precisely regulate calcium entry (Tedford and Zamponi 2006). HVA Ca\(^{2+}\) currents activation results in strong increases in the intracellular Ca\(^{2+}\) concentration. Ca\(^{2+}\)-dependent inactivation of HVA Ca\(^{2+}\) currents via activation of calcineurin restricts the entry of Ca\(^{2+}\) into the cytoplasm, which is a negative feedback mechanism between Ca\(^{2+}\) entry and the intracellular Ca\(^{2+}\) concentration (Brehm and Eckert 1978). This mechanism was reported to involve in nicotine-induced neuroprotection (Stevens et al. 2003). Recently, Yu et al confirmed that SKF83959 exhibited excellent neuroprotective effects on H\(_2\)O\(_2\)-induced cortical neurons injury (Yu et al. 2008). Activation of calcineurin by mobilization of intracellular Ca\(^{2+}\) stores, and subsequent L-type Ca\(^{2+}\) currents reduction may be a potent mechanism of SFK83959-mediated neuroprotective effects. On the other hand, both Ca\(^{2+}\) and calcineurin are known to be critical for changes in synaptic strength such as LTP and LTD (Belmeguenai and Hansel 2005; Riedel 1999). Very recently, Rashid et al reported that SKF83959 stimulates Gq/11 through the heteromeric D\(_1\)-D\(_2\) receptor complex, and increases the levels of CaMKII that contributes to synaptic plasticity (Rashid et al. 2007). Thus, although the current studies focused on the regulation of HVA Ca\(^{2+}\) currents, the signal transduction pathways delineated here could also be responsible for effects of SKF83959 on plastic processes.

In conclusion, this is the first demonstration that PI-linked D\(_1\)-like DA receptor agonist SKF83959 inhibited HVA Ca\(^{2+}\) currents in cultured rat striatal neurons by PLC-IP\(_3\)-Ca\(^{2+}\)-calcineurin signal pathway. These results give further support about the
importance of PI-linked D₁ dopamine receptor in neuronal function, which provides a new target for the treatment of neurodegenerative disease.

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**FIG. 1.** SKF83959 reversibly decreased HVA Ca\(^{2+}\) currents in primary cultured striatal neurons. (A) Representative traces of HVA Ca\(^{2+}\) currents under control, application of 10 µM SKF83959 (top trace) and 10 µM dopamine (bottom trace). Voltage protocol was shown on the top. (B) The time-course curve before, during and after 10 µM SKF83959 treatment corresponding to A. (C) The inhibitory effects of SKF83959 on HVA Ca\(^{2+}\) currents were in a dose-dependent manner. The inhibitory effects for each dose of SKF83959 (0.1-100 µM) were normalized to the maximal inhibitory responses. The fit by the Hill equation yielded an EC\(_{50}\) of 8.77±0.72 µM (n=13). (D) Representative recording of HVA Ca\(^{2+}\) currents evoked by a series of depolarizing steps (top). (E) Current-voltage (I-V) relationships under control conditions and 10 µM SKF83959 (n=6). (F) Effect of a strong depolarization on SKF83959-induced reduction of HVA Ca\(^{2+}\) currents. HVA Ca\(^{2+}\) currents were recorded with double pulse voltage protocol (top). A strong depolarizing prepulse (30 ms) to +100 mV was followed by the latter test pulse. The depolarizing prepulse did not relieve the effect of SKF83959 (10 µM) on HVA Ca\(^{2+}\) currents (n=7). *p<0.05, **p < 0.01 compared to control.

**FIG. 2.** Neurons in which HVA Ca\(^{2+}\) currents were inhibited by SKF83959 were SP-immunopositive. (A) The majority of cultured cells (93%) were GAD-immunoreactive. Scale bar, 50 µm. (B) Representative image of striatal neurons with patch-clamp recording. (C) Representative image of the same cell in (B) was subsequently proved to be immunoreactive for SP, in which HVA calcium currents
were inhibited by SKF83959. Scale bar, 50 µm.

**FIG. 3.** D₁-like dopamine receptors mediated SKF83959-induced reduction of HVA Ca²⁺ currents. (A) Representative traces showing the inhibitory effects of SKF83959 were blocked by pretreatment of 10 µM SCH23390 (n=9, left), but not D₂-like dopamine receptor antagonist spiperone (10 µM, n=6, right). (B) Summary data of the results with SCH23390 (10 µM), spiperone (10 µM), prazosin (1 µM), mesulergine HCl (10 µM) and scopolamine (10 µM) preincubation on SKF83959-mediated reduction of HVA Ca²⁺ currents in striatal neurons. **p < 0.01 compared to SKF83959 alone.

**FIG. 4.** SKF83959 reduced HVA Ca²⁺ currents via a phospholipase C-IP₃ pathway in primary cultured striatal neurons. (A) Representative traces showing pretreatment with 10 µM U73122 blocked SKF83959-induced HVA Ca²⁺ currents in striatal neurons (n=10, left), but pretreatment with 50 µM genistein had no effect (n=5, right). (B) Representative traces showing dialysis with 10 mg/ml heparin significantly attenuated the inhibitory effects of SKF83959 (n=5, left), but pretreatment with BIM failed to have the effect (n=3, right). (C) Summary data of pretreatment with U73122, U73343, genistein, heparin, BIM, thapsigargin (TG) and PTX on the inhibitory action of SKF83959. *p<0.05, **p<0.01 compared to SKF83959 alone.

**FIG. 5.** The SKF83959-induced effect was Ca²⁺-dependent. (A) SKF83959 induced
[Ca^{2+}]_{i}} elevation in Ca^{2+}-containing ACSF using calcium imaging technique as described in the Methods and expressed as F340/F380 nm ratio. (B) Summary data of 10 µM SKF83959 on [Ca^{2+}]_{i} in both Ca^{2+}-containing and Ca^{2+}-free ACSF (n=35). **p < 0.01 compared to control. (C) Representative trace showing dialysis with 15 mM BAPTA significantly reduced the inhibitory effects of SKF83959 (n=9). (D) Summary data of BAPTA on the inhibitory modulation of SKF83959. **p < 0.01 compared to SKF83959 alone. (E) Representative trace of HVA Ba^{2+} currents under control condition, application of 10 µM SKF83959 and washout. (F) There was no significant difference between HVA Ba^{2+} and Ca^{2+} currents inhibition produced by SKF83959.

**FIG. 6.** Activation of calcineurin was account for SKF83959-mediated reduction of L-type Ca^{2+} currents. (A) Pretreatment with 10 µM cyclosporine A (CsA) for 30 min greatly decreased the inhibitory actions of SKF83959 (10 µM) on HVA Ca^{2+} currents (n=9). (B) 10 µM nifedipine inhibited HVA Ca^{2+} currents by 52.8±4.57%. While pretreatment with 10 µM nifedipine for 10 min, the inhibitory effects of SKF83959 on HVA Ca^{2+} currents were significantly attenuated in striatal neurons. (C) Summary data of pretreatment with CsA, okadaic acid and nifedipine on the inhibitory action of SKF83959. *p < 0.01, **p < 0.01 compared to SKF83959 alone.

**FIG. 7.** Working model of the PI-linked D_{1} dopamine receptors signaling cascade targeting HVA Ca^{2+} channels in rat striatal neurons.