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Full-length article

Requirement of PSD-95 for dopamine D_1 receptor modulating glutamate NR1a/NR2B receptor function[1]

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Key words

D1 dopamine receptor; NMDA; PSD-95; signal transduction; calcium

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Abstract

Aim: To elucidate the role of scaffold protein postsynaptic density (PSD)-95 in the dopamine D_1 receptor (D_1R)-modulated NR1a/NR2B receptor response. **Methods:** The human embryonic kidney 293 cells expressing D_1R (tagged with the enhanced yellow fluorescent protein) and NR1a/NR2B with or without co-expression of PSD-95 were used in the experiments. The Ca^{2+} influx measured by imaging technique was employed to monitor *N*-methyl-*D*-aspartic acid receptors (NMDAR) function. **Results:** The application of dopamine (DA, 100 μmol/L) did not alter glutamate/glycine (Glu/Gly)-induced NMDAR-mediated Ca^{2+} influx in cells only expressing the D_1R/NR1a/NR2B receptor. However, DA increased Glu/Gly-induced Ca^{2+} influx in a concentration-dependent manner while the cells were co-expressed with PSD-95. D_1R-stimulated Ca^{2+} influx was inhibited by a selective D_1R antagonist SCH23390. Moreover, pre-incubation with either the protein kinase A (PKA) inhibitor H89, or the protein kinase C (PKC) inhibitor chelerythrine attenuated D_1R-enhanced Ca^{2+} influx induced by the *N*-methyl-*D*-aspartic acid (NMDA) agonist. The results clearly indicate that D_1R-modulated NR1a/NR2B receptor function depends on PSD-95 and is subjected to the regulation of PKA and PKC. **Conclusion:** The present study provides the first evidence that PSD-95 is essential in D_1R-regulated NR1a/NR2B receptor function.

Introduction

The dopamine (DA) D_1 receptor (D_1R) and the glutamate (Glu) NMDA receptor (NMDAR) represent two functionally and structurally diverse receptor classes. D_1R belongs to G-protein-coupled receptor family and couples to Gαs proteins. NMDAR are ligand-gated ion channels composed of multiple subunits (NR1, NR2A-D, and NR3A-C)[1]. A remarkable property of NMDAR is its high permeability to Ca^{2+}. Alteration of NMDAR-mediated Ca^{2+} influx is reported to be involved in schizophrenia and in excitotoxic neuronal death associated with brain disorders such as stroke, epilepsy, and trauma[2].

D_1R and NMDAR are co-localized in several brain structures, including caudate-putamen, nucleus accumbens, hippocampus, and prefrontal cortex[3–5]. D_1R and NMDAR are constitutively interacting in cells as revealed by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) studies[6]. The direct physical interaction is mediated through the C terminal of the respective protein[7]. In addition to the direct interaction at the receptor level, previous studies have also shown that the downstream signal molecules mediated by D_1R is also found to regulate NMDAR function. For instance, D_1R stimulated protein kinase A (PKA) was demonstrated to phosphorylate the NR1 subunit of NMDAR[8] and to enhance NMDA-mediated excitability[9]. Furthermore, the activation of D_1R was also found to enhance NMDA currents via protein kinase C (PKC)-dependent mechanisms[10]. However, the direct D_1R-NMDAR interaction was also reported to result in the inhibition of NMDAR-mediated currents[8], indicating that the D_1-NMDAR interaction is rather a diverse and complex event.

D_1R and NMDAR are presented at high concentrations in the postsynaptic density (PSD)[6]. PSD is a highly organized subcellular fraction in which NMDAR co-exists with scaffolding proteins such as PSD-95 and other signaling proteins[11]. PSD-95 comprises three primary decidual zone (PDZ) domains, a Src-homology three domain, and a domain homologous to guanylate kinase[12]. It is known that PSD-95 utilizes those domains for protein interaction to recruit signaling proteins and to mediate its assembly with other components of the PSD.

Recent information indicates that PSD-95 can regulate membrane trafficking and intracellular signaling of a number of neurotransmitter receptors including NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and 5-hydroxytryptamine 2A (5-HT_{2A}) receptors via physical interactions[13–15]. As D_1R and NMDAR are presented at high density in the PSD[6], it is conceivable that PSD-95 may also regulate D_1/NMDA receptor interaction. To elucidate the role of PSD-95 in D_1R-modulated functions of the NR1a/NR2B receptor, HEK293 cells expressing D_1R and NR1a/NR2B receptors were employed to study the role of PSD-95 in the D_1R-modulated NR1a/NR2B receptor-mediated Ca^{2+} influx. Our study demonstrated that PSD-95 was required in D_1R modulating NR1a/NR2B receptor function, and this modulatory effect depended on PKA and PKC.

Materials and methods

Materials Fura-2 acetoxymethyl ester (Fura-2 AM) was from Molecular Probe (Eugene, OR, USA). 3-Isobutyl-1-methyl-2,6 (1H, 3H)-purinedione (IBMX) and Pluronic-127, monoclonal anti-D_1 DA receptor antibody produced in rat clone 1-1-F11 s.E6, monoclonal anti-c-myc antibody produced in mouse clone 9E10, anti-mouse IgG (whole molecule)-TRITC antibody produced in goats, anti-HA antibody produced in rabbits, leupeptin, pepstatin A, aprotinin, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma (St Louis, MO, USA). Normal mouse IgG-HRP (horseradish peroxidase) was from Santa Cruz (Santa Cruz, CA, USA), anti-mouse IgG (H+L)-AP was from Promega (Madison, WI, USA), and [^{3}H]SCH23390 was from Amersham (Cleveland, OH, USA). *l*-Glutamate acid sodium salt and glycine (Gly) were from Sino-American Biotech (Beijing, China). (±)-SKF-38393 hydrochloride, DA, H89, 8-Br-cAMP, and chelerythrine were from RBI (Natick, MA, USA). The cAMP assay kit was from the Shanghai University of Traditional Chinese Medicine (Shanghai, China). Other reagents were obtained as indicated in the text.

Cell culture and transfection Human embryonic kidney 293 (HEK293) cells, a generous gift from Dr Gang PEI (Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China), were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% newborn calf serum (SiJiQing, Hangzhou, China), penicillin (100 U/mL), and streptomycin (100 U/mL). 2.5×10^4 cells were plated on poly-*L*-lysine-coated glass coverslips. Transfections were performed while cells reached 80% confluence. NR1a, NR2B, D_1-enhanced yellow fluorescent protein (EYFP), and PSD-95 were delivered at a ratio of 1:1:1:1 by the calcium-phosphate transfection method. Ketamine 1 mmol/L was added to the culture dish to prevent excitotoxicity during transfection. If not indicated specifically, the cells used for the experiments were harvested 24 h after transfection. NR1a and NR2B cDNA were generous gifts from Dr John WOODWARD (Medical University of South Carolina, Charleston, USA), cDNA for PSD-95 were from Dr Morgan SHENG(Harvard University, Boston, MA, USA), and D_1-EYFP was constructed by Dr You HE in our Laboratory.

Assays of cAMP content After 18–24 h transfection with plasmid-encoded D_1-EYFP receptors, the cells were reseeded into a 96-well plate (1×10^4 cells/well) for 12 h. The cells were pre-incubated with 50 μL serum free DMEM containing 500 μmol/L IBMX prior to D_1R agonist *R*-(+)-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7,8-diol hydrochloride (SKF38393) stimulation for an additional 10 min. The reaction was then terminated on ice by adding 100 μL 1 mol/L trichloroacetic acid. Following the addition of 20 μL 2 mol/L K_2CO_3, the sample was centrifuged for 5 min at 12000×*g*. The supernatant was kept (diluted in 1:10) for determining cAMP content. All experiments were performed in duplicate, and each experiment was repeated at least 3 times.

Internalization assays Cells expressing D_1-EYFP were treated either with 10 μmol/L DA or vehicle for 30 min in serum-free DMEM and then washed 3 times with ice-cold phosphate-buffered saline (PBS). The cells were fixed in 4% paraformaldehyde for 20 min at room temperature before 3 washes of PBS. The receptor internalization was then observed with a Leica SP2 confocal microscope (Leica microsystem, Heideberg, Germany). The excitation wavelength for yellow fluorescent protein (YFP) is at 514 nm.

Calcium imaging Transfected cells were incubated with Fura-2 AM (4 μmol/L with 0.025% Pluronic-127) for 45 min in extracellular solution (135 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 10 mmol/L glucose, and 5 mmol/L HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), pH 6.8). After 3 washes with Fura-free extracellular solution (pH 7.2), the dishes were mounted onto the stage of an Olympus

BX51WI upright microscope (Tokyo, Japan). The cells were perfused continuously at a flow rate of 1.2–1.5 mL/min. Glu/Gly were applied via a computer-controlled Y-tube (outer φ=100 μm) situated 80 μm above the cells. The cells were exposed to alternating 340 nm and 380 nm light every 2 s during and immediately after Glu/Gly application. The signals were acquired via a charge coupled device (CCD) camera (CoolSNAP HQ, Roper Scientific, Duluth, GA, USA). To minimize UV exposure, images were taken every 15–60 s between drug applications. Ratio images were generated with MetaFlour software from Universal Imaging (West Chester, PA, USA). Intracellular calcium concentrations were determined by the ratio of the intensity of 340/380. NMDAR-dependent increases in intracellular calcium were calculated by subtracting the average baseline value from the peak value obtained during Glu/Gly application.

Data analysis All data were expressed as mean±SEM. Unless otherwise indicated, the statistical significance was determined using the least significant difference (LSD) test following ANCOVA with SPSS 11.0 (SPSS, Chicago, USA). The covariate was the average baseline NMDA response before perfusion of the antipsychotic drugs.

Results

Establishing and characterizing D_1R-NR1a/NR2B receptor co-expression in HEK293 cells The HEK293 cells were co-transfected with D_1-EYFP and the NR1a/NR2B receptor. To verify the success of transfection, the cells were locally perfused with Glu/Gly (100/10 μmol/L) for 3 s. This treatment induced reproducible Ca^{2+} influx (Figure 1A, upper panel). The application of 1 μmol/L MK-801 significantly blocked Glu/Gly-induced Ca^{2+} influx (Figure 1A, lower panel). Employing confocal microscopy, we observed the D_1-EYFP receptor location on the cell membrane (Figure 1B, upper left). The application of 10 μmol/L DA to the cells induced significant internalization of D_1R (Figure 1B, upper right). Moreover, SKF38393 resulted in an increase in cAMP accumulation in a dose-dependent manner (Figure 1B, lower panel). The mean EC_{50} value of SKF38393 was 0.16 μmol/L, similar to a previous report[16]. Thus, D_1R and the NR1a/NR2B receptor were functionally expressed in HEK293 cells.

D_1R activation did not affect NR1a/NR2B-mediated Ca^{2+} influx in HEK293 cells that expressed D_1R and NR1a/NR2B We next tested whether D_1R activation could modulate NR1a/NR2B receptor-mediated Ca^{2+} influx in HEK293 cells expressing both the D_1R and NR1a/NR2B receptor. Glu/Gly-induced Ca^{2+} influx in the presence of DA (100 μmol/L) is shown in Figure 2A, 2B. The results clearly indicated that the activation of D_1-EYFP did not alter NR1a/NR2B-mediated Ca^{2+} influx in our system. To exclude the possibility that the EYFP tag interrupts the interaction between the D_1R and the NR1a/NR2B receptor, non-tagged D_1R-NR1a/NR2B receptor co-transfected cells were tested. Again, the activation of D_1R did not alter NR1a/NR2B-mediated Ca^{2+} influx as well in the cells (Figure 2A, 2B, right). Thus, it is clear that D_1R is unable to modulate NR1a/NR2B receptor function in our *in vitro* co-expression system. In order to test whether the lack of D_1R modulation on NR1a/NR2B-mediated response as described earlier was due to insufficient PKA activation by D_1R in the system, the cell permanent PKA

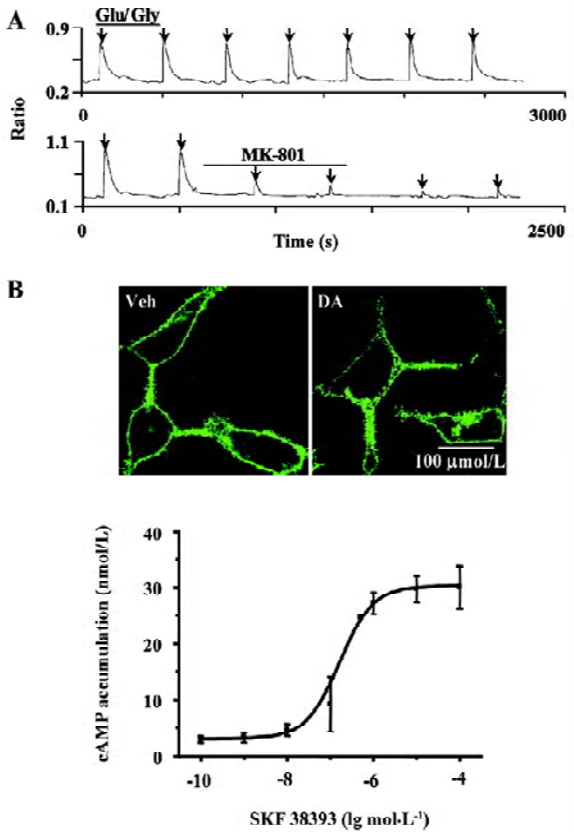

Figure 1. Functional studies of D_1-EYFP and NR1a/NR2B receptors expressed in HEK293 cells. (A) traces of Glu/Gly (100/10 μmol/L) induced Ca^{2+} influx in cells expressing NR1a/NR2B receptors. Upper panel, Glu/Gly induced Ca^{2+} influx was highly reproducible when applied every 400 s (tested for seven paradigms). Lower panel, MK-801 (1 μmol/L), a specific NMDAR antagonist, blocked Glu/Gly-induced Ca^{2+} influx. (B) functional expression of D_1-EYFP receptors in HEK293 cells. Upper left panel, D_1-EYFP receptors were localized in the cell membrane. Veh, vehicle; upper right panel, D_1R internalization induced by 10 μmol/L DA for 30 min. Scale bar: 100 μm; lower panel: dose response curve for SKF38393-stimulated cAMP. Cells were treated with different concentrations of SKF38393 for 10 min. The data shown is summarized from 3 independent experiments and expressed as mean±SEM.

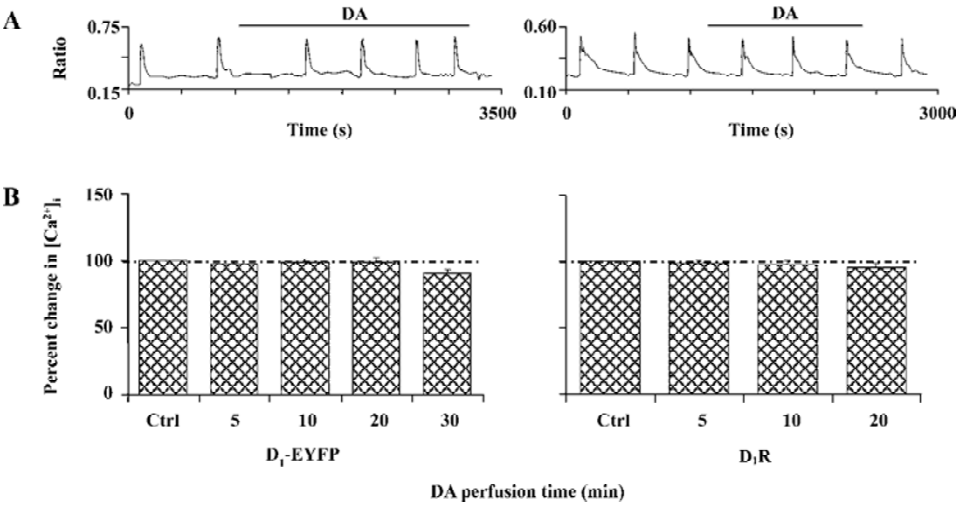

Figure 2. D_1 receptor activation did not significantly alter NR1a/NR2B-mediated Ca^{2+} influx in the D_1R and NR1a/NR2B co-expressing HEK293 cells. (A) traces showing the lack of an effect of DA (100 μmol/L) on Glu/Gly-induced Ca^{2+} influx in cells co-expressing either D_1-EYFP (left) or D_1R (right) with NR1a/NR2B receptors. (B) bar graph is the summary of the results from A (n=20, from 3 and 2 independent experiments, respectively). The cell response prior to DA application was used as the control.

activator 8-Br-cAMP was used. Incubation of the cells with 10 μmol/L 8-Br-cAMP elicited no effect on the Glu/Gly-induced Ca^{2+} influx in HEK293 cells expressing both the D_1R and the NR1a/NR2B receptor (Figure 3). Taken together, our data indicate that the activation of D_1R or PKA fails to modulate NR1a/NR2B receptor-mediated Ca^{2+} influx in HEK293 cells expressed with the D_1R and the NR1a/NR2B receptor.

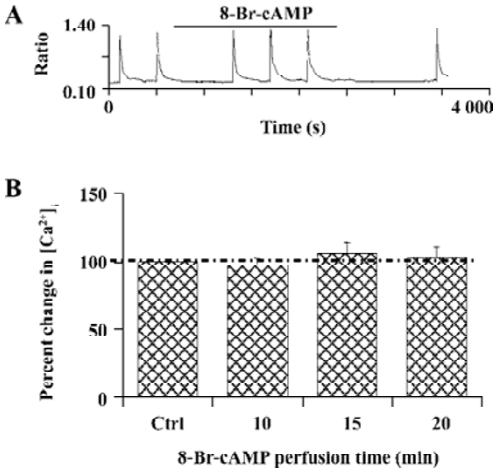

Figure 3. Activation of PKA was not sufficient to alter NR1a/NR2B receptor function in cells expressing D_1-EYFP/NR1a/NR2B receptors. (A) representative trace showing the lack of effect of the PKA activator 8-Br-cAMP (10 μmol/L) on Glu/Gly-induced Ca^{2+} influx in cells. (B) bar graph is the summary of the results from A (n=8, from 2 independent experiments). The cell response prior to 8-Br-cAMP application was used as the control.

PSD-95 is required for D_1R modulation of NR1a/NR2B receptor function Since it has been shown that the scaffold protein PSD-95 is associated with NMDAR and is involved in the regulation of receptor function, we wondered whether D_1R activation could modulate NR1a/NR2B receptor function when PSD-95 is presented in our expression system. Interestingly, after co-expression with PSD-95, activation of D_1R by DA led to an enhancement in NR1a/NR2B receptor function in a concentration-dependent manner. DA 10 μmol/L or 100 μmol/L induced a 34.0%±5.3% ($F_{1,37}$=64.95, n=20, P< 0.01) or a 48.6%±10.3% ($F_{1,97}$=67.58, n=50, P<0.01) increase in Ca^{2+} influx, respectively (Figure 4A). As expected, the enhanced NR1a/NR2B receptor function induced by DA was blocked by 5 μmol/L SCH23390, a selective D_1R antagonist (DA *vs* DA+SCH23390, $F_{1,65}$=3.71, n=67, P<0.01, independent-samples t-test, Figure 4B,C), indicating a D_1R-mediated event. This result demonstrated clearly that PSD-95 was required for the modulatory effect of D_1R on NR1a/NR2B receptor function.

PKA is involved in the modulation of NR1a/NR2B receptor function by D_1R activation The above result reveals an important role of PSD-95 involved in D_1R-modulated NR1a/NR2B receptor function. As we know, D_1R activation can lead to an increase in cAMP formation and PKA activation. We next tested whether PKA is involved in D_1R-enhanced NR1a/NR2B receptor-mediated Ca^{2+} influx in the cells which were co-expressed with PSD-95. After bath application of PKA selective inhibitor H89 (5 μmol/L), NR1a/NR2B receptor-mediated Ca^{2+} influx was significantly attenuated; an average 38.7%±4.1% of inhibition was observed ($F_{1,2}$=66.40, n=13, P<0.01, compared to that in the absence of H89, Figure 5), indicating that NR1a/NR2B receptor-mediated Ca^{2+} influx is subjected to PKA regulation. Furthermore, H89 not only completely abolished the DA-enhanced Ca^{2+} influx mediated by NMDA agonists, but also resulted in an additional inhibition while DA was presented. To check if H89-mediated inhi-

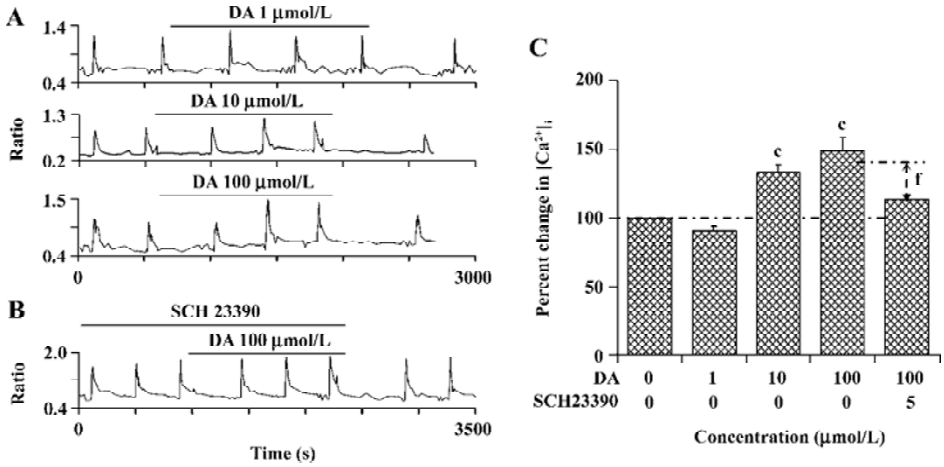

Figure 4. D_1 receptor-modulated NR1a/NR2B receptor function requires the presence of PSD-95. (A) representative trace showing that DA concentration-dependently (1 μmol/L, 10 μmol/L, and 100 μmol/L) increased the Ca^{2+} influx induced by Glu/Gly in HEK293 cells expressing PSD-95, D_1-EYFP, and NR1a/NR2B. (B) representative trace showing the blockade of DA-enhanced Ca^{2+} influx (100 μmol/L) by the D_1R selective antagonist SCH23390 (5 μmol/L). (C) summary of results from A and B (n=15–50, from 2–12 independent experiments). ^cP<0.01 *vs* control. ^fP<0.01 *vs* DA (100 μmol/L), n=20.

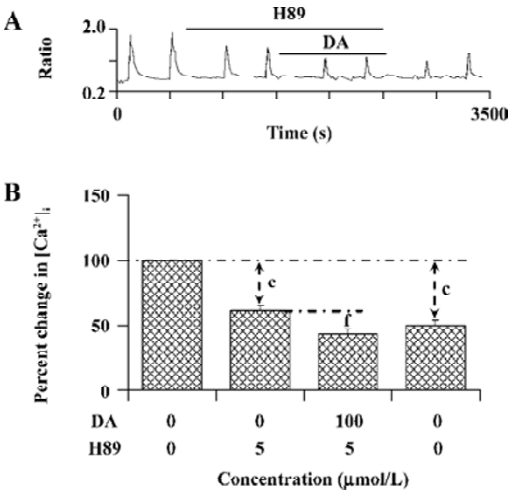

Figure 5. Involvement of PKA for the interaction between D1-EYFP and NR1a/NR2B receptors in the PSD-95 co-expressed cell. (A) representative trace showed that the PKA inhibitor H89 (5 μmol/L) inhibited NR1a/NR2B receptor-mediated Ca^{2+} influx. In the presence of H89, DA application resulted in a further inhibition on Ca^{2+} influx induced by Glu/Gly. (B) bar graph presents summarized results (n=13, from 3 independent experiments). ^cP<0.01 *vs* non DA and non H89 treatment. ^fP<0.01 *vs* H89 and non DA.

bition is reversible, H89-treated cells were washed out for 10 min prior to the reapplication of Glu/Gly. However, NR1a/NR2B receptor-mediated Ca^{2+} influx was unable to recover from the inhibition (Figure 5A).

PKC is involved in the modulation of NR1a/NR2B receptor function induced by D_1R activation As D_1R may also activate PKC via a phospholipase C (PLC)-mediated mechanism and NMDAR was reported to be phosphorylated by PKC, the role of PKC was explored. NR1a/NR2B receptor-mediated Ca^{2+} influx was not altered by selective PKC inhibitor chelerythrine (4.0%±2.4% of inhibition, n=15, Figure 6). However, chelerythrine (5 μmol/L) indeed attenuated the DA-enhanced NR1a/NR2B receptor-mediated Ca^{2+} influx (15.8%±3.4%, $F_{1,27}$=24.89, n=15, P<0.01) with less potency than that of H89. Thus, it indicates that PKC also contributed to the D_1R modulation of NR1a/NR2B receptor function.

Discussion

The present study demonstrates that in HEK293 cells co-transfected with the D_1R and NR1a/NR2B receptor, D_1R activation enhances NR1a/NR2B-mediated Ca^{2+} influx only in the presence of PSD-95. Moreover, when PKA or PKC activity was inhibited, the D_1R-modulated NR1a/NR2B receptor function was also significantly attenuated. To our knowledge, this is the first evidence that D_1/NR1a/NR2B receptor interaction depends on PSD-95.

PSD-95 is a scaffold protein abundant in PSD. PSD-95 can recruit signaling proteins and mediate assembly with other components of the PSD. It is known that PSD-95 plays an important role in mediating neurotransmitter receptor functions such as NMDA and 5-HT_{2A} receptors[13–15]. The present result indicates that D_1R activation fails to modulate NR1a/NR2B receptor-mediated Ca^{2+} influx unless PSD-95 is

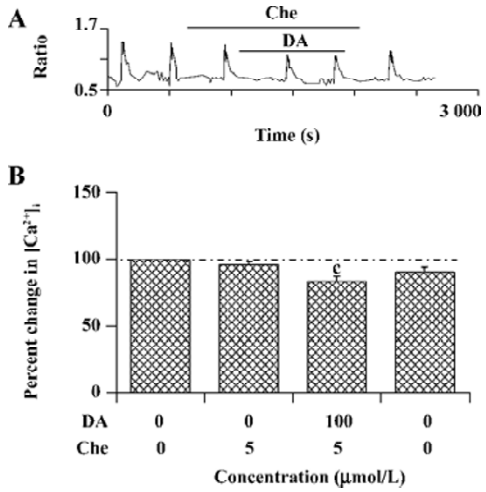

Figure 6. PKC contributed to the functional interaction between D_1-EYFP and NR1a/NR2B receptors in PSD-95 expressed cells. (A) representative trace showed that the PKC inhibitor chelerythrine (Che, 5 μmol/L) had no effect on Glu/Gly-induced Ca^{2+} influx, but it prevented DA (100 μmol/L) from the enhancing effect on Ca^{2+} influx induced by Glu/Gly. (B) bar graph presents the summary (n=15, from 3 independent experiments). $^cP<0.01$ *vs* non DA and non Che treatment.

co-expressed with the D_1R and the NR1a/NR2B receptor. Thus, it is clear that PSD-95 is required for D_1R-modulated NR1a/NR2B receptor function. Other studies also support the important role of scaffold proteins in the regulation of NMDAR function in transfected HEK293 cells. For instance, the PKA-modulated NMDA current was significantly enhanced while the cells were co-expressed with yotiao, another scaffold protein enriched in the PSD[17]. We here found that the physical interaction between PSD-95 and D_1R was essential for the functional expression of D_1R-modulated NMDAR function. It appears that PSD-95 acts as a core component in recruiting D_1R, NMDAR, and other signal transduction molecules to form a multiple protein complex that allows the interaction between D_1R and NMDAR. Indeed, it has been suggested that PSD-95 facilitates the dynamic regulation of phosphoprotein (such as PKA or PKC) and sequentially attaches them to the substrate (for example, the receptors). In this manner, the signals can be efficiently transduced from one kinase to the next[18]. It remains unknown how D_1R and PSD-95 interact, and furthermore, how this physical interaction regulates NR1a/NR2B receptor function.

Both PKA and PKC are found to be involved in the D_1R modulation of NR1a/NR2B receptor function in the presence of PSD-95 in our system. Previous findings also suggested that an anchored pool of PKA was required for the augmentation of NMDAR-induced currents in HEK293 cells[17]. In neurons, the activation of the D_1R has been shown to enhance the NMDA current via PKA- and PKC-dependent mechanisms[9,10]. It has also been reported that PKA, but not PKC, phosphorylates NMDAR or receptor-associated proteins, thereby inducing a conformational change of the receptor[19]. It appears that the role of PKA and PKC in D_1R-modulated NMDAR function varied according to the cell system employed[19].

A previous report of HEK293 cells co-expressed with D_1R and NMDAR indicated that the activation of D_1R could lead to an inhibition of the NMDAR current[7]. It is worthy to note that the PKA and PKC inhibitors were used throughout the experiments in this study in order to exclude the potential post-translational effect of D_1R-stimulated PKA or PKC on NMDAR function. Thus, the observed inhibitory effect of D_1R activation on the NMDA current is more likely a result of the conformation change due to the direct physical association between the two receptors in their system. Indeed, it appears that there are two potential mechanisms for D_1R-modulated NMDAR function. The confirmation change resulted from the physical interaction between the two receptors inhibiting the NMDAR function[7], whereas post-translational modification such as PKA or PKC-mediated phosphorylation enhanced NMDAR function[9,10]. The present data demonstrated that D_1R- enhanced NR1a/NR2B Ca^{2+} influx was observed only in the presence of PSD-95, which was indeed subjected to the regulation of PKA or PKC, indicating that post-translational mechanisms play an essential role in D_1R-modulated NMDAR function.

In summary, the present results demonstrate that the D_1R activation of NR1a/NR2B receptor function requires the presence of PSD-95. The activation of D_1R enhances NR1a/NR2B receptor-mediated Ca^{2+} influx and is dependent on PKA and PKC (Figure 7). It appears that PSD-95 is a core molecule in

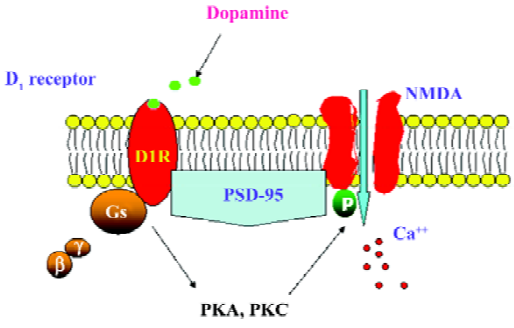

Figure 7. Interaction of D_1R with PSD-95 in regulating NMDA receptor function. Present data suggests that D_1R may directly interacts with PSD-95 to regulate NMDA receptor function, and this modulatory effect is also dependent on PKA and PKC.

recruiting the two receptors to form a functional complex.

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