Regulation of MAPK activation by dopamine D2 receptors

Thesis by

Sung Jae Kim

Department of Medical Science

The Graduate School, Yonsei University

Regulation of MAPK activation by dopamine D2 receptors

Directed by Professor Ja-Hyun Baik

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Sung Jae Kim

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This certifies that the Master's Thesis of Sung Jae Kim is approved.

[Thesis Supervisor : Ja-Hyun Baik]

[Thesis Committee Member]

[Thesis Committee Member]

The Graduate School

Yonsei University

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김 성 재

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ABSTRACT

Regulation of MAPK activation by dopamine D2 receptors

Sung Jae Kim

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Ja-Hyun Baik)

Two isoforms of dopamine D2 receptor, D2L (long) and D2S (short), differ by the insertion of 29 amino acid specific to D2L within the putative third intracellular loop of the receptor, which appears to be important in selectivity for G-protein coupling. The tyrosine kinase inhibitors, genistein, herbimycin and PP2, inhibited MAPK activation by two dopamine D2 receptors. Overexpression

of β-arrestin 1 and 2 increased D2S-mediated activation of MAPK, whereas it did not affect the activation of MAPK induced by D2L. Expression of a dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ(K44A) mutant, which blocks conversion of coated pits to vesicles, inhibited the activation of MAPK by D2S, whereas it did not inhibit the activation of MAPK by D2L. Treatment of concanavalin A and monodansylcadaverin, inhibitors of internalization, blocked D2S- but not D2L-mediated MAPK activation. Using confocal microscopy, it was observed that red fluorescent protein-conjugated D2S (RFP-D2S) was more markedly internalized than RFP-D2L. Using green fluorescent protein-conjugated β-arrestin 1 (GFP-β-arrestin 1) and 2, it was observed that GFP-β-arrestin 1 and 2 translocated to the plasma membrane and colocalized with the RFP-D2L and RFP-D2S receptors at 5 min after stimulation with dopamine. At 30 min after stimulation with dopamine, GFP-β-arrestin 1 and 2 were internalized with RFP-D2S receptor but in the case of RFP-D2L, GFP-β-arrestin 1 and 2 remained predominantly in the plasma membrane. These results suggest that D2L-mediated MAPK

activation does not require the receptor internalization, while D2S-mediated MAPK activation requires the receptor internalization.

Key words: Dopamine D2 Receptor, MAP Kinase, Internalization, β-Arrestin, Dynamin

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Ⅰ. Introduction

Dopamine modulates the activities of many neuronal pathways and peripheral organ systems[1]. The importance of proper dopaminergic fuction is evident when any of these systems become compromised such as in Parkinson's disease[2], Schizophrenia[3], or hyperprolactinemia[4]. Molecular cloning of the dopamine receptor

family has revealed five receptor subtypes, D1 through D5. They have been classically divided into two groups based on ligand specificity and effector coupling. D1 receptor and D5 receptor are positively coupled to adenylyl cyclase by the G protein Gs, whereas the D2 receptor, D3 receptor and D4 receptor inhibit this enzyme[5,6].

The dopamine D2 receptor belongs to the family of seven transmembrane domain G-protein-coupled receptors (GPCRs) and is highly expressed in the central nervous system and the pituitary gland[7]. The binding of dopamine to the D2 receptor is crucial for the regulation of diverse physiological functions, such as the control of locomotor activity and the synthesis of pituitary hormones[8]. Recently, mice lacking in dopamine D2 receptor were created by knocking out the D2 gene[9,10]. Absence of D2 receptors leads to animals which show severe impairment in locomotor activity and an abnormal development of pituitary, demonstrating that the D2 receptor plays a dominant role in dopaminergic nervous function[9-11]. Two alternatively spliced transcripts are generated from the D2 receptor gene and code for the D2L (long) and D2S (short) isoforms, which are 444 and 415 amino acids in length, respectively[12,13]. These isoforms exhibit similar

pharmacological characteristics and are expressed in the same cell types, with a ratio that normally favors expression of the longer isoform[12]. The functions and the physiological differences of the subtypes are virtually unknown, in part because their intracellular signaling mechanisms have not been well characterized.

The mitogen-activated protein kinases (MAPKs) are a group of serine/threonine kinase that is activated by a cascade of protein kinases to induce responses such as proliferation, differentiation, apoptosis, and long-term potentiation. This signaling cascade is a prominent cellular pathway used by many growth factors, hormones and neurotransmitters to regulate diverse physiological functions. Classically, activation of MAPK pathways has been attributed to the activity of growth factor receptors. It is now established that numerous GPCRs can also activate MAPK may allow for plasma membrane receptor systems to influence diverse cellular processes, ranging from the regulation of neuronal survival to cell differentiation and gene expression[14]. Although activation of the MAPK pathway by receptors with tyrosine kinase activity is well defined, the mechanisms used by heterotrimeric G-protein coupled receptors to activate this pathway is less clear.

It has been reported that D2L- and D2S-mediated MAPK activation is predominantly $G_{\beta\gamma}$ subunit-mediated signaling. It was also showed that protein kinase C and tyrosine phosphorylations are involved in these signaling pathways[15]. However, it is not clear how these two D2 dopamine receptors couple to MAPK signaling pathway and, furthermore, whether there are subtype-related regulations in this signaling pathway.

Recent studies on the β_2 adrenergic receptor have demonstrated that clathrin/dynamin-mediated receptor internalization may be essential in the activation of the MAPK pathway by GPCRs[16,17]. It has been proposed that stimulation of β_2 adrenergic receptor result in the assembly of a protein complex containing activated c-src and β-arrestin[16]. Formation of the β-arrestin-receptor complex appears to be essential for MAPK activation. Specifically, it has been suggested that the receptor-β-arrestin complex acts as a scaffold binding src, a nonreceptor tyrosine kinase, and the src transduces the signal from the GPCR to ras, activating the MAPK cascade. Components of the MAPK cascade, including raf, MEK, and MAPK, have been identified in isolated endocytic vesicles[16,17].

To characterize the regulation of the G-protein coupled signaling pathway leading to MAPK activation by two isoforms of the dopamine D2 receptor, stable Chinese hamster ovary cell lines expressing two isoforms of dopamine D2 receptor, D2L and D2S was used. In the present study, the regulation of the MAPK pathway by two dopamine D2 receptors in association with agonist-induced receptor internalization was particularly investigated.

Ⅱ. Materials and Methods

1. Materials

β-arrestin 1, β-arrestin 2 were kindly provided by Dr. Robert J. Lefkowitz (Universtity of Duke, NC, USA). A dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ (K44A) mutant were kindly provided by Dr. Eamonn Kelly (University of Bristol, Bristol, UK) and Dr. Jeffrey L. Benovic (University of Thomas Jefferson, PA, USA). pEGFP-C2 and pDsRed1-N1 were purchased from Clontech (Palo Alto, CA, USA). FuGene 6 was purchased from Roche Diagnostics (Indianapolis, IN, USA). Herbimycin A, 4-amino-5 (4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine (PP2) were from Calbiochem (San Diego, CA, USA). Genistein was from RBI (Natick, MA, USA). Concanavalin A, monodansilcadavarin (MDC) was from Sigma (St Louis, MO, USA). Mouse monoclonal anti-phosphoErk (Tyr204), rabbit polyclonal anti-Erk was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse-HRP and anti-rabbit-HRP were from Zymed (South San Francisco, CA, USA). All other biochemicals, including dopamine, were from Sigma (St Louis, MO, USA).

2. Cell culture and transfection

Generation of CHO cell lines stably expressing the D2L and D2S dopamine receptors have been described previously[15]. CHO cells were maintained in F-12 medium supplemented with 10% FBS, 100 mg/ml streptomycin sulfate, 100 units/ml penicillin G and 250 mg/ml amphotericin B. Transient transfections of CHO and HEK cells were performed using the liposome-mediated transfection reagent, fugene 6. Briefly, 70% ~ 80% confluent monolayers in 60-mm culture plates were incubated at 37°C in 3 ml of serum-free medium with transfection mixture containing the plasmid DNA encoding β-arrestin 1, β-arrestin 2, a dominant negative β-arrestin 2 (319-418) mutant, a dominant negative dynamin Ⅰ (K44A) mutant and the plasmid pCH110 carrying the β-galactosidase gene and liposome reagent. After 6h, the transfection mixture was then replaced with growth medium. Assays were performed 48 h after transfection. Expressions of β-arrestin 1, β-arrestin 2, a dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ (K44A) mutant were normalized by measuring β-galactosidase activity.

3. Immunoblotting analysis

Serum-starved transfected cells were stimulated with dopamine for indicated time at 37°C, the media aspirated and the cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin and 1 mM PMSF. Samples were sonicated 4 times for 5 second each, centrifuged at 10,000*g* at 4°C for 10 min and the supernatant was collected. For phospho-specific p44/p42 MAPK (Erk1/Erk2) and total MAPK (Erk1/Erk2) measuring, cell lysates were prepared as in the assay for MAPK activity. The proteins were separated on 10% SDS–polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). The blots were incubated with 5% dried milk powder in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20; also used for all incubations and washing steps) for 30 min. Next, the blots were incubated for 1h with monoclonal anti-phosphoERK (Tyr204), and rabbit polyclonal anti-ERK antibody followed by extensive washing. The blots were subsequently incubated with peroxidase-conjugated anti rabbit-IgG antibody. After washing, signals were visualized

using the Enhanced ChemiLuminescence detection system (ECL, Amersham).

4. Confocal microscopy

For D2L and D2S translocation assay, HEK-293 cells were transfected with the D2L-RFP or D2S-RFP. For the colocalization assay, HEK-293 cells were transfected with D2L-RFP or D2S-RFP and either β-arrestin 1-GFP or β-arrestin 2-GFP. After various treatments, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Cells were then washed with PBS and mounted for fluorescent confocal microscopic evaluation. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscopy by using a Zeiss 100 x oil-immersion lens. Flourescent signals were collected by using a Zeiss LSM sofrware in the line switching mode with dual excitation (488, 568nm) and emission (515-540nm, 590-610nm) filter sets.

Ⅲ. Results

1. Inhibition of dopamine-stimulated MAPK activity by tyrosine kinase inhibitors.

To study regulation of the MAPK cascade by D2L and D2S receptors, CHO cells stably trasnfected with mouse cDNA encoding D2L or D2S were used. To assess the activation of MAPK, phosphorylated-Thr202/Tyr204-specific p44/p42 MAPK antibody was used. This antibody specifically recognizes the Thr202/Tyr204-phosphorylated active form of p44/p42 MAPK.

It has been proposed that stimulation of β_2 adrenergic receptor results in the assembly of a protein complex containing activated c-src and β-arrestin. It had been suggested that formation of the β-arrestin-receptor complex appears to be essential for MAPK activation[16]. It was assessed a role for protein tyrosine kinase including c-src kinase in D2L- and D2S-mediated MAPK activation. The ability of dopamine to stimulation MAPK activation was clearly mediated by a tyrosine kinase pathway, since general tyrosine kinase inhibitor, genistein or herbimycin A significantly attenuated D2L- and D2S-mediated MAPK activation as did the specific Src family inhibitor PP2 (Fig. 1A and 1B).

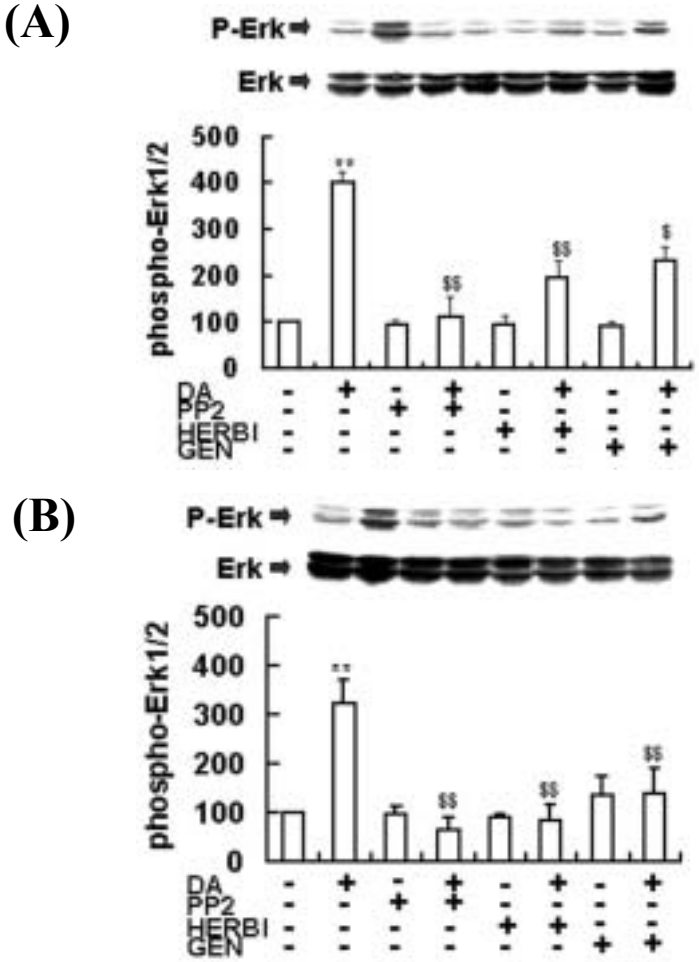

Fig 1. Effect of tyrosine kinase inhibitors on MAPK activity in CHOD2L and CHOD2S cells. Cells were preincubated 30min with PP2 (10 μM), 2h with herbimycin A (1 μM), 2h with genistein (100 μM). Phospho-Erk and total Erk levels were analyzed in cell lysates by immunoblotting. CHOD2L (A) and CHOD2S (B) cells were treated with dopamine (1μM) for 1min**.** Data indicate mean ±S.E. from at least four independent experiments. (***P*<0.01 as compared to dopamine non-stimulated control, $^{\$}P$<0.05, $^{\$\$}P$<0.01 as compared to pGEM transfected control).

2. Effects of β-arrestin 1 and 2 overexpression on dopamine stimulated MAPK activity by D2L and D2S receptors.

Recent studies have confirmed that certain G protein-coupled receptor (GPCR) uses β-arrestin as a clathrin adaptor to mediate internalization of receptor signaling complexes[16,17]. By binding to both the nonreceptor tyrosine kinase, c-src, and to agonist-occupied GPCRs, β-arrestin can confer tyrosine kinase activity upon the receptor[17,18]. To determine the role of β-arrestins in D2L- and D2S-mediated MAPK activation, CHOD2L and CHOD2S stable cell lines were transiently transfected with β-arrestin 1 or β-arrestin 2.

After dopamine treatment for the indicated time, expression of β-arrestin in CHOD2L and CHOD2S cells did not affected D2L-mediated MAPK activation, whereas it significantly increased D2S-medicated MAPK activation (Fig. 2A and 2B).

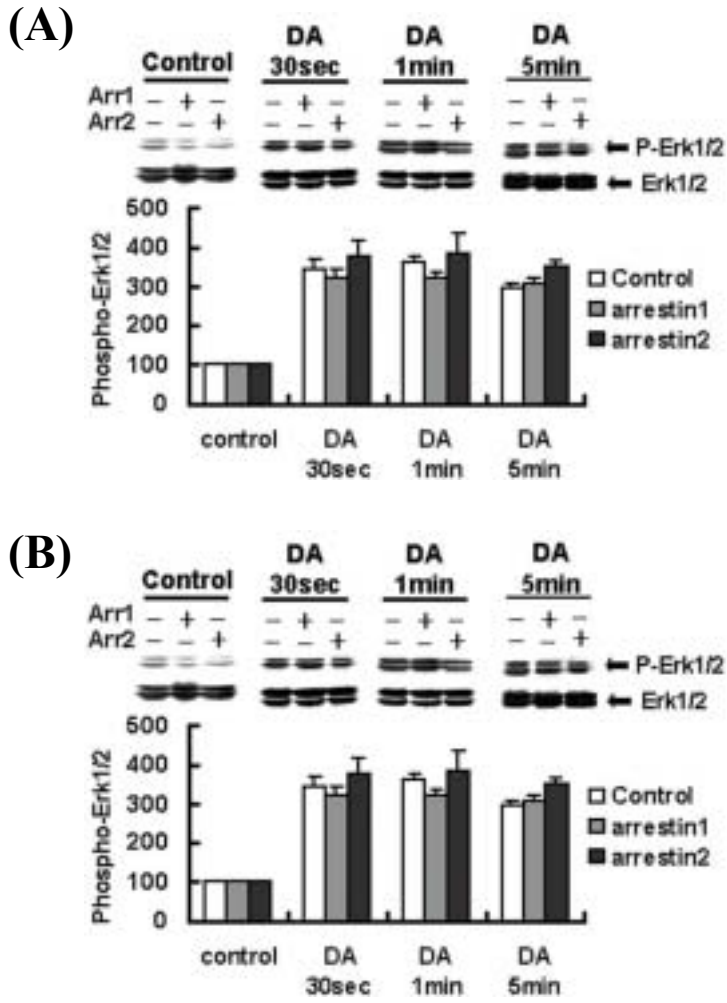

Fig 2. Effects of β-arrestin 1 and β-arrestin 2 in CHOD2L and CHOD2S cells. Cells were transiently transfected with plasmid containing the β-arrestin 1, β-arrestin 2 or pGEM vector alone (control). CHOD2L (A) and CHOD2S (B) cells were treated with dopamine (1μM) for the indicated periods of time. Data indicate mean ±S.E. from at least four independent experiments. (* $P<0.05$ as compared to pGEM transfected control).

3. Effects of a dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ(K44A) mutant on dopamine-stimulated MAPK activity by D2L and D2S receptors.

Recently, it has been reported that GPCR-internalization is regulated by dynamin and β-arrestins[19]. To further analyze the relationship between D2L-/D2S-mediated MAPK activation and internalization induced by treatment of dopamine, the effects of the dominant negative β-arrestin 2 (319-418) mutant and the dominant negative dynamin Ⅰ(K44A) mutant in the D2L- and D2S-mediated MAPK activation were assessed. As shown in Fig. 3A and 3B, transfection of both dominant negative mutants did not affect D2L-mediated MAPK activation. However, D2S-mediated MAPK activation was markedly reduced by transfection of the dominant negative β-arrestin 2 (319-418) mutant and the dominant negative dynamin Ⅰ(K44A) mutant. These results showed that β-arrestin and dynamin are not required for D2L-mediated MAPK activation, while these proteins are required for D2S-mediated MAPK activation.

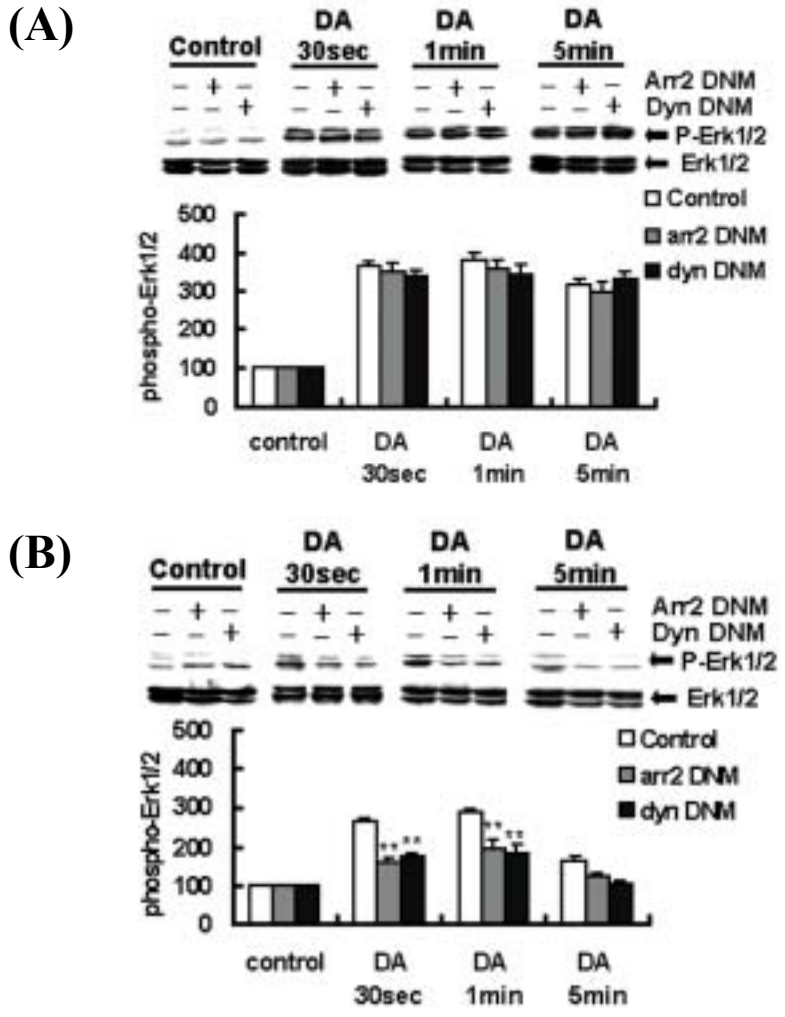

Fig 3. Effects of a dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ (K44A) mutant in CHOD2L and CHOD2S cells. Cells were transiently transfected with plasmid containing the β-arrestin 2 (319-418), dynamin Ⅰ (K44A), or pGEM vector alone (Control). CHOD2L (A) and CHOD2S (B) cells were treated with dopamine (1μM) for the indicated periods of time. Data indicate mean ±S.E. from at least four independent experiments. (**P<0.01 as compared to pGEM transfected control).

4. Effects of internalization inhibitors on dopamine-stimulated MAPK activity by D2L and D2S receptors.

The internalization of GPCRs can be inhibited by diverse agents such as the lectin, concanavalin A[20] (which blocks receptor clustering) and MDC[21] (which prevents clathrin association). Pretreatment of cells with either of these compounds has been shown to inhibit clathrin-mediated GPCR internalization without affecting signal transduction[20]. It was investigated whether D2L- and D2S-mediated MAPK activation still occurs in the presence of these compounds. As shown in Fig. 4 and 5, it was observed that D2L-mediated MAPK activation was not affected in the presence of these compounds, but in the case of CHOD2S, MAPK activation was significantly decreased. These results suggest that agonist-induced internalization may be differentially involved in D2L- and D2S-mediated MAPK activation and that internalization event is indispensable in D2S-mediated MAPK activation.

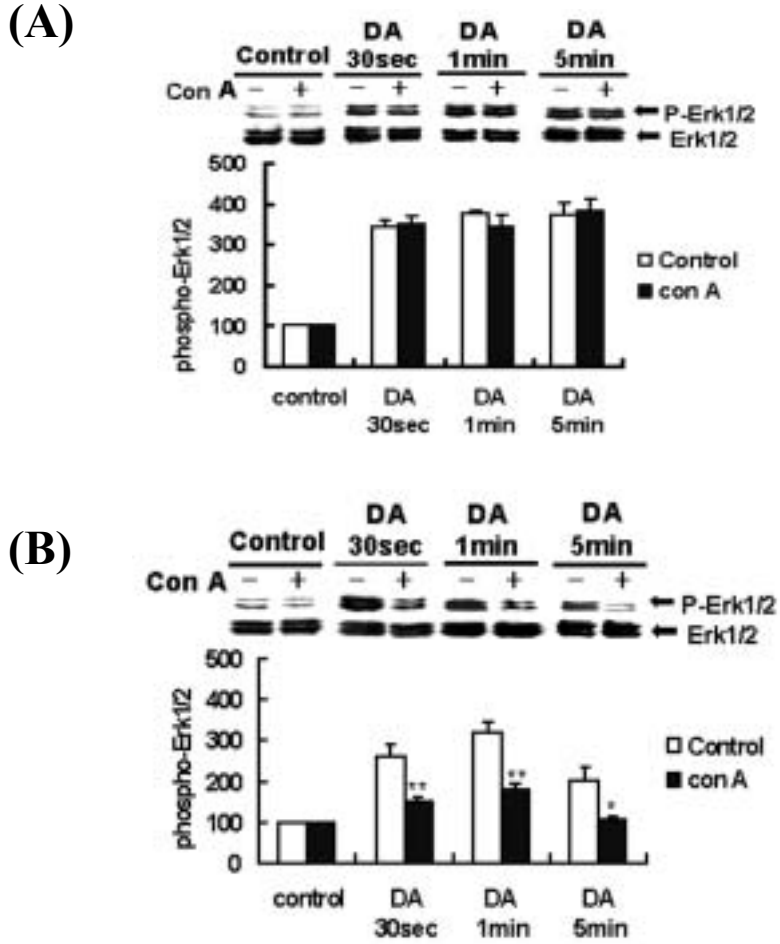

Fig 4. Effect of an internalization inhibitor, concanavalin A on MAPK activity in CHOD2L and CHOD2S cells. Cells were preincubated 30min with con A (0.25μg/ml). Phospho-Erk and total Erk levels were analyzed in cell lysates by immunoblotting. CHOD2L (A) and CHOD2S (B) cells were treated with dopamine (1μM) for the indicated periods of time. Data indicate mean ±S.E. from at least three independent experiments. (**P*<0.05, ***P*<0.01 as compared to con A non-treated control).

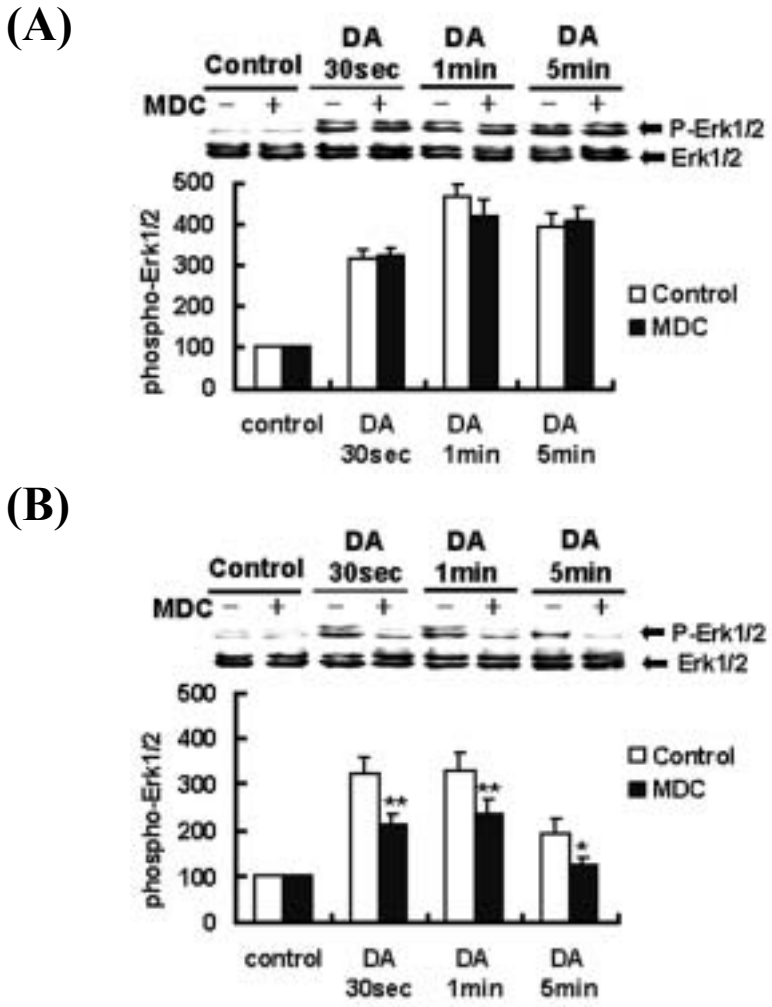

Fig 5. Effect of an internalization inhibitor, monodansylcadaverin (MDC) on MAPK activity in CHOD2L and CHOD2S cells. Cells were preincubated 20min with MDC (300μM). Phospho-Erk and total Erk levels were analyzed in cell lysates by immunoblotting. CHOD2L (A) and CHOD2S (B) cells were treated with dopamine (1μM) for the indicated periods of time. Data indicate mean ±S.E. from at least three independent experiments. (**P*<0.05, ***P*<0.01 as compared to MDC non-treated control).

5. The cellular distribution of D2L-and D2S-RFP after dopamine-stimulation in HEK-293 cells.

To determine the effect of dopamine stimulation on the cellular distribution of D2L and D2S, RFP-D2L and RFP-D2S were transiently expressed in HEK293 cells. As shown in Fig 6, the fluorescence distributions of the receptors were almost exclusively localized to the plasma membrane in unstimulated cells. Until 5 min after stimulation with dopamine, the cellular distribution of RFP-D2L and RFP-D2S was not changed, whereas at 30 min after stimulation with dopamine, RFP-D2L and RFP-D2S each underwent a redistribution to endosomal-like vesicle compartments. Notably, RFP-D2S had more endosomal-like vesicle compartments than RFP-D2L at 30 min after stimulation with dopamine.

6. The cellular distribution of D2L-/D2S-RFP and β-arrestin 1-/2-GFP after dopamine stimulation in HEK-293 cells.

The involvement of β-arrestins in the regulation of D2L and D2S was investigated using a green fluorescence protein-conjugated-arrestins (GFP-β-arrestins) and RFP-D2L/D2S. HEK 293 cells were transiently cotransfected with RFP-D2L or RFP-D2S

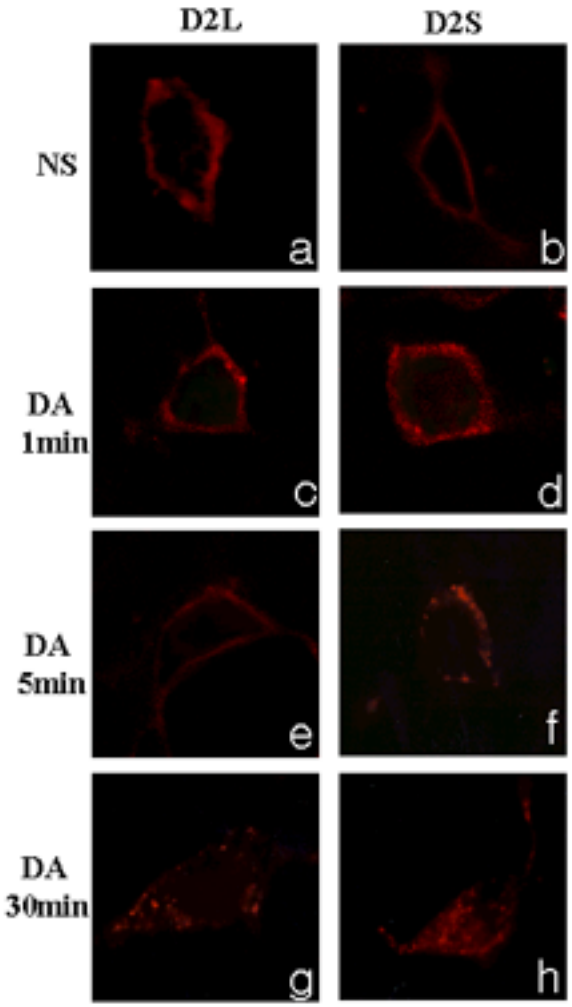

Fig 6. The cellular distribution of D2L- and D2S-RFP after dopamine stimulation in HEK-293 cells. HEK-293 cells were transiently trasfected with plasmid DNA encoding D2L (a, c, e, g)- and D2S (b, d, f, h)-RFP. Cells treated with vehicle (a, b) or dopamine (DA, 10μM) for 1min (c, d), 5min (e, f), and 30min (g, h).

along with the GFP-β-arrestin 1 or GFP-β-arrestin 2. In the absence of ligand, RFP-D2L and RFP-D2S were distributed along the plasma membrane, whereas GFP-β-arrestins were diffused in cytosol and nucleus. The stimulation of D2L and D2S results in the rapid recruitment of β-arrestins from the cytoplasm to agonist-occupied receptor on the plasma membrane at 5 min with stimulation of dopamine (Fig. 7, 8). After 30 min of exposure to dopamine, D2L and β-arrestin each was found predominantly in plasma membrane (Fig. 7). However, D2S and β-arrestin each underwent a dramatic redistribution that both proteins translocated to endosomal-like vesicles (Fig. 8).

7. Effect of expression of a dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ (K44A) mutant on the cellular distribution of D2L-/D2S-RFP after dopamine stimulation in HEK-293 cells.

Fig. 9A. showed that RFP-D2L and RFP-D2S receptors associate with the plasma membrane in cells expressing the dominant negative β-arrestin 2 (319-418) mutant. These results indicated that β-arrestin is required for D2L- and D2S-mediated.

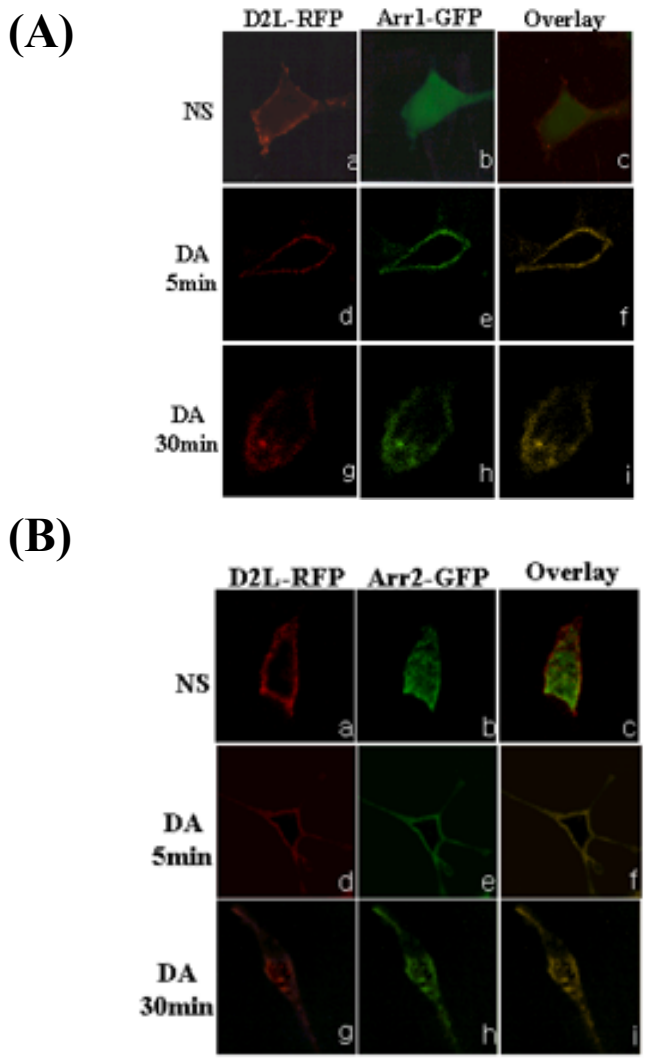

Fig 7. The cellular distribution of D2L-RFP and β-arrestin 1-/2-GFP after dopamine stimulation in HEK-293 cells. HEK-293 cells were transiently trasfected with plasmid DNA encoding D2L-RFP and β-arrestin 1 (A)-/2 (B)-GFP. Cells treated with vehicle (a-c) or dopamine (DA, 10μM) for 5min (d-f) and 30min (g-i). Colocalization of D2L-RFP and β-arrestin 1-/2-GFP is shown in the overlay images (c, f, i).

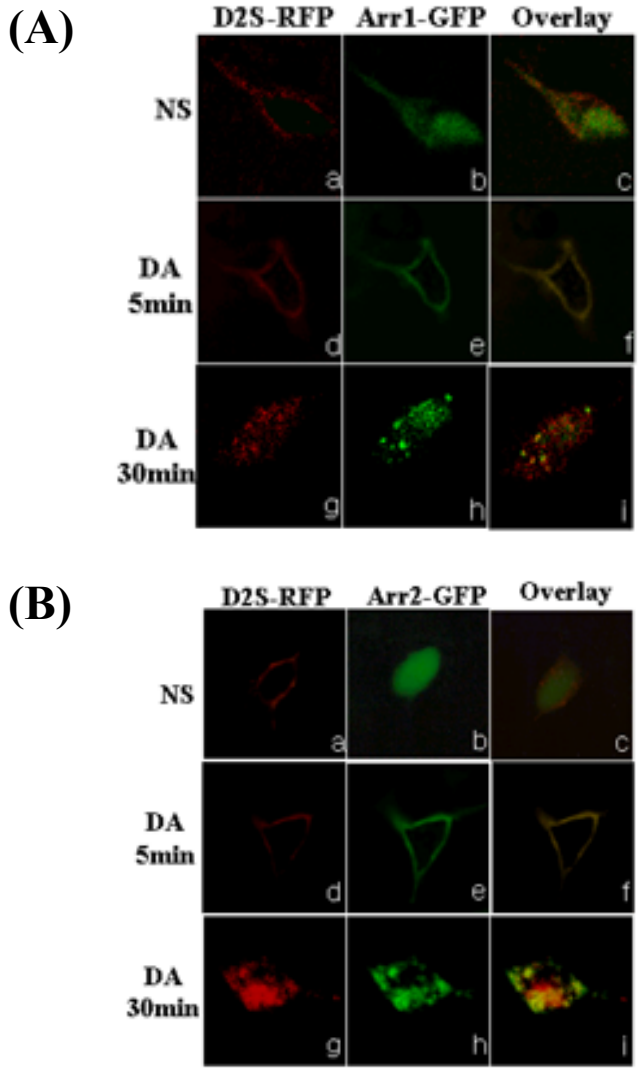

Fig 8. The cellular distribution of D2S-RFP and β-arrestin 1-/2-GFP after dopamine stimulation in HEK-293 cells. HEK-293 cells were transiently trasfected with plasmid DNA encoding D2S-RFP and β-arrestin 1 (A)-/2 (B)-GFP. Cells treated with vehicle (a-c) or dopamine (DA, 10μM) for 5min (d-f) and 30min (g-i). Colocalization of D2S-RFP and β-arrestin 1-/2-GFP is shown in the overlay images (c, f, i).

internalization.

Fig. 9B. showed that RFP-D2S receptor was associated with the plasma membrane in cells expressing the dominant negative dynamin Ⅰ(K44A) mutant, while in the case of RFP-D2L, its internalization was not inhibited by the expression of the dominant negative dynamin Ⅰ(K44A) mutant. These results indicated that dynamin is required for D2S-mediated internalization, while D2L-mediated internalization is dynamin-independent.

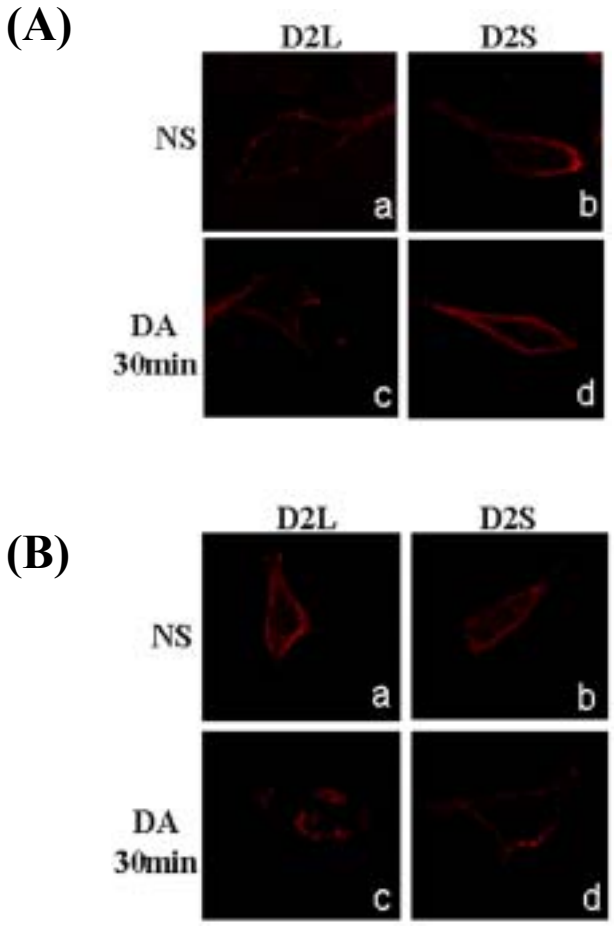

Fig 9. Effect of expression of a dominant negative β-arrestin 2 (319-418) mutant and a dominant negative dynamin Ⅰ (K44A) mutant on the cellular distribution of D2L- and D2S-RFP after dopamine stimulation in HEK-293 cells. HEK-293 cells were transiently trasfected with plasmid DNA encoding D2L (a, c)- or D2S (b, d)-RFP and a dominant negative β-arrestin 2 (319-418) mutant (A) or a dominant negative dynamin Ⅰ (K44A) mutant (B). Cells treated with vehicle (a, b) or 10μM dopamine (c, d) for 30min.

Ⅳ. Discussion

Recently, a unifying theme has emerged where both growth factors and GPCRs utilize protein tyrosine kinase activity and the highly conserved Ras/MAP kinase pathway to control mitogenic signals[22]. Crosstalk between GPCRs and receptor tyrosine kinases (RTKs) is an incredibly complex process, and the exact signaling molecules involved are largely dependent on the cell type and the type of receptor that is activated[23]. Considerable evidence now indicates that certain G-protein coupled receptors can interact with the MAPK signaling pathway, though the molecular basis for this interaction is still poorly understood.

The model for β_2-adrenergic receptor-mediated activation of the MAPK pathway recently proposed by Lefkowitz et al. is thought to represent a general scheme for GPCR stimulation of MAPK[16-18]. It was demonstrated that clathrin/dynamin-mediated receptor internalization may be essential in the activation of the MAPK pathway by GPCRs[16,17]. Specifically, it was suggested that the receptor-β-arrestin complex acts as a scaffold binding src, a nonreceptor tyrosine kinase, and the src transduces the signal from the GPCR to Ras, activating the MAPK cascade. Components of

the MAPK cascade, including Raf, MEK, and MAPK, were identified in isolated endocytic vesicles[16,17]. Similar results were reported for several other GPCRs. MAPK activation by m_1 muscarinic receptor[24], the μ, δ, and κ opioid receptors[25,26], and the proteinase-activated receptor 2[27] were reported to be sensitive to inhibition of endocytosis. While these data conflict with other reports, based on studies on the α_2 adrenergic receptor[28,29], the m_3 muscarinic receptor[30], and B_2 bradykinin receptor[31]. These reports suggested that receptor endocytosis is not universally essential for MAPK activation by GPCR. Therefore, the role of endocytosis in GPCR-mediated MAPK activation is a controversial issue.

To investigate whether receptor internalization is required for the activation of MAPK activation by D2 receptors, it was tested the role of src in the D2L- and D2S-mediated MAPK activation. Both of D2L- and D2S-mediated MAPK activations were suppressed by herbimycin and PP2, suggesting that c-src is involved as an upstream regulator of MAPK in this pathway.

The role of major component of internalization, β-arrestin in D2 receptors-mediated MAPK activation was explored in this study. It was observed that β-arrestin cointernalizes with RFP-D2L/-D2S

receptors. It was also observed that D2L-mediated MAPK activation is not significantly affected by overexpression of β-arrestins and expression of a dominant negative β-arrestin 2 (319-418) mutant, while D2S-mediated MAPK activation is significantly increased by overexpression of β-arrestins. Furthermore, D2S-mediated MAPK activation was significantly reduced by expression of a dominant negative β-arrestin 2 (319-418) mutant. These data demonstrate that β-arrestins are required for D2S-mediated MAPK activation, but these components are not required for D2L-mediated MAPK activation.

A dominant negative dynamin Ⅰ (K44A) mutant is defective in GTP binding and blocks endocytosis at a stage after the initiation of coated vesicle formation but before sequestration into coated pits. It was found that D2S receptor-internalization is inhibited by transfection of the dominant negative dynamin Ⅰ (K44A) mutant in HEK 293 cells, whereas D2L receptor-internalization is less sensitive to the presence of the dominant negative dynamin Ⅰ (K44A) mutant than D2S.

In addition, inhibition of D2L receptor internalization by concanavalin A (con A) and monodansylcardaverin (MDC) did not

affect the ability of the receptor to stimulate MAPK activity, whereas in the case of D2S, pretreatments of con A and MDC reduced MAPK activation. This difference in MAPK activation may imply that agonist-induced internalization differentially involved in D2L- and D2S-mediated MAPK activation and that internalization event is indispensable in D2S-mediated MAPK activation.

Using confocal microscopy, it was observed that dopamine treatment has induced the internalization in both D2L and D2S but with different efficiency. D2S was more markedly internalized than D2L.

These finding suggest that the difference in D2L- and D2S-mediated MAPK activation via internalization may be due to the intrinsic difference between D2L and D2S receptors. D2L and D2S receptors are identical except for an insertion of 29 amino acids in the third intracellular loop of D2L resulting from alternative splicing. Considering these data, it is tempting to speculate that the third intracellular loop of D2 receptors could be involved in the internalization. It has been reported that the third intracellular loops of m_1 and m_2 muscarinic acetylcholine receptors were

involved in internalization[32,33]. Furthermore, it was interesting to note that the β_2-adrenergic receptor is internalized to a greater extent (60%) than the β_1-adrenergic receptor (26%), which contains an additional 24 amino acid residues in the third intracellular loop[34].

Characterization and definition of the molecular basis of these signaling pathways may permit elucidation of the relationship between the structural difference/G protein coupling/downstream signal tranduction and physiolosical actions of two dopamine D2 receptors.

Ⅴ. Conclusion

In the present study, it is investigated whether receptor internalization is required for the activation of MAPK activation-mediated by two isoforms of dopamine D2 receptor, D2L and D2S.

1. **D2L and D2S-mediated MAPK activation involves src-tyrosine kinase pathways.**
2. **Inhibition of internalization blocks D2S- but not D2L-mediated MAPK activation.**
3. **β-Arrestins are required for D2L- and D2S-mediated internalization.**
4. **Dynamin is required for D2S-mediated internalization, while D2L-mediated internalization is dynamin-independent.**

Taken together, these results suggest that D2L-mediated MAPK activation does not require the receptor internalization, while D2S-mediated MAPK activation requires the receptor internalization.

References

1. Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine Receptors: From Structure to Function. Physiological Reviews 1998; 78: 189-225.

2. Lee T, Seeman P, Rajput A., Farley IJ, and Hornykiewicz O. Receptor basis for dopaminergic supersensitivity in Parkinson's disease. Nature 1978; 273: 59-61.

3. Seeman P. Dopamine receptors and the dopamine hypothesis of schizophrenia. Synapse 1987; 1: 133-52.

4. Cunnah D, Besser M. Management of prolactinomas. Clin Endocrinol (Oxf) 1991; 34: 231-5.

5. Kebabian JW. Dopamine-sensitive adenylyl cyclase: a receptor mechanism for dopamine. Adv Biochem Psychopharmacol 1978; 19: 131-54.

6. Sibley DR, Monsma FJ Jr. Molecular biology of dopamine receptors. Trends Pharmacol Sci 1992; 13: 61-9.

7. Hornykiewicz O. Dopamine (3-hydroxytyramine) and brain function. Pharmacol Rev 1966; 18: 925-64.

8. Picetti R, Saiardi A, Abdel ST, Bozzi Y, Baik JH, Borrelli E. Dopamine D2 receptors in signal transduction and behavior. Crit Rev Neurobiol 1997; 11: 121-42.

9. Baik JH, Picetti R, Saiardi A, Thiriet G, Dierich A, Depaulis A, et al. Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. Nature 1995; 377: 424-8.

10. Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, et al. Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. Neuron 1997;19: 103-13.

11. Picetti R, Saiardi A, Abdel ST, Bozzi Y, Baik JH, Borrelli E. Dopamine D2 receptors in signal transduction and behavior. Crit Rev Neurobiol 1997; 11: 121-42.

12. Montmayeur JP, Bausero P, Amlaiky N, Maroteaux L, Hen R, Borrelli E. Differential expression of the mouse D2 dopamine receptor isoforms. FEBS Lett 1991; 278: 239-43.

13. Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, et al. The dopamine D2 receptor: two molecular forms generated by alternative splicing. EMBO 1989; 8: 4025-34.

14. Gutkind, JS. The pathway connecting G-protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J Biol Chem 1998; 273: 1839-1842.

15. Choi EY, Jeong D, Won K, Park, Baik JH. G protein-mediated mitogen-activated protein kinase activation by two dopamine D2 receptors. Biochem Biophys Res Commun 1999; 256: 33-40.

16. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ. Beta-arrestin-dependent formation of beta$_2$ adrenergic receptor-Src protein kinase complexes. Science 1999; 283: 655-61.

17. Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y, et al. The ß(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi- receptor complex with the epidermal growth factor receptor. J Biol Chem 2000; 275: 9572–9580.

18. Miller WE, Maudsley S, Ahn S, Khan KD, Luttrell LM, Lefkowitz RJ. beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-src. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis. J Biol Chem 2000; 275: 11312-9.

19. Mundell SJ, Matharu AL, Pula G, Roberts PJ, Kelly E. Agonist-induced internalization of the metabotropic glutamate

receptor 1a is arrestin- and dynamin-dependent. J Neurochem 2001; 78: 546-51.

20. Pippig S, Andexinger S, Lohse MJ. Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. Mol Pharmacol 1995; 47: 666-76.

21. Ray E, Samanta AK. Dansyl cadaverine regulates ligand induced endocytosis of interleukin-8 receptor in human polymorphonuclear neutrophils. FEBS Lett 1996; 378: 235-9.

22. Dikic I, Blaukat A. Protein tyrosine kinase-mediated pathways in G protein-coupled receptor signaling. Cell Biochem Biophys 1999; 30: 369-87.

23. Lowes VL, Ip NY, Wong YH. Integration of signals from receptor tyrosine kinases and G protein-coupled receptors. Neurosignals 2002; 11: 5-19.

24. Vogler O, Nolte B, Voss M, Schmidt M, Jakobs KH, Koppen CJ. Regulation of Muscarinic Acetylcholine Receptor Sequestration and Function by ß-Arrestin. J Biol Chem 1999; 274: 12333-12338.

25. Ignatova EG, Belcheva MM, Bohn LM, Neuman MC, Coscia CJ. Requirement of Receptor Internalization for Opioid Stimulation of Mitogen-Activated Protein Kinase: Biochemical and Immunofluorescence Confocal Microscopic Evidence. J Neurosci 1999; 19: 56-63.

26. Pierce KL, Maudsley S, Daaka Y, Luttrell LM, Lefkowitz RJ. Dissociation of Functional Roles of Dynamin in Receptor-mediated Endocytosis and Mitogenic Signal Transduction. J Biol Chem 1999; 274: 24575-24578.

27. DeFae KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW. ß-Arrestin-dependent Endocytosis of Proteinase-activated Receptor 2 Is Required for Intracellular Targeting of Activated ERK1/2. J Cell Biol 2000; 148: 1267-1281.

28. DeGraff JL, Gagnon AW, Benovic JL, Orsini MJ. Role of Arrestins in Endocytosis and Signaling of α_2-Adrenergic Receptor Subtypes. J Biol Chem 1999; 274: 11253-11259.

29. Schramm NL, Limbird LE. Stimulation of Mitogen-activated Protein Kinase by G Protein-coupled α_2-Adrenergic Receptors Does Not Require Agonist-elicited Endocytosis. J Biol Chem 1999; 274: 24935-24940.

30. David CB, Angela R, Andrew BT. Activation of the Mitogen-activated Protein Kinase Pathway by a $G_{q/11}$-coupled Muscarinic Receptor Is Independent of Receptor Internalization. J Biol Chem 1999; 274: 12355-12360.

31. Blaukat A, Pizard A, Rajerison RM, Alhenc-Gelas F, Muller-Esterl W, Dikic I. Activation of mitogen-activated protein kinase by the bradykinin B2 receptor is independent of receptor phosphorylation and phosphorylation-triggered internalization. FEBS Lett 1999; 451: 337-41.

32. Maeda S, Lameh J, Mallet WG, Philip M, Ramachandran J, Sadee W. Internalization of the Hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop. FEBS Lett 1990; 269: 386-388.

33. Tsuga H, Kameyama K, Haga T, Kurose H, Nagao T. Sequestration of muscarinic acetylcholine receptor m_2 subtypes: facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. J Biol Chem 1994; 269: 32522-32527.

34. Green SA, Liggett SB. A proline-rich region of the third intracellular loop imparts phenotypic ß$_2$-adrenergic receptor coupling and sequestration. J Biol Chem 1994; 269: 26215-26219.

국문요약

도파민 D2 수용체에 의한 MAPK 활성화의 조절

김 성 재

연세대학교 대학원

의과학 사업단

도파민 D2 수용체는 일곱개의 transmembrane domain을 가진 G-protein coupled 수용체의 그룹으로서, 중추신경계와 뇌하수체에서 다량 발현된다. 본 연구에서는 두 개의 D2 수용체 이성체에 의한 MAPK 활성화를 조사하기 위해서 Chinese Hamster Ovary (CHO) cell에 D2L/D2S를 발현시키는 stable cell line을 이용하여, D2L/D2S에 의한 MAPK 활성화의 조절에 있어서의 internalization의 역할에 대해 조사해 보았다.

D2L 수용체에 유도된 MAPK 활성화는 c-src, β-arrestin이 관여된 경로였으며, internalization 저해제 처리 시에 MAPK 활성화는 변화되지 않았다. 그러나, D2S 수용체에 유도된 MAPK 활성화는 c-src, β-arrestin, dynamin이 관여된 경로였으며, internalization 저해제 처리 시에 MAPK 활성화가 감소하였다. 이런 결과들은, D2L 수용체에 의한 MAPK 활성화는 internalization과 연관되어 있지않으며, D2S 수용체의 MAPK 활성화는 internalization과 연관되어 있음을 시사한다. 중추 신경계의 중요한 생리기능에 관련된 도파민 D2수용체 신호전달기전의 이해를 통해서, 궁극적으로는 파킨슨 증후군과 같은 중추 신경계 질환을 이해하는데 기초가 될 것이다.

핵심되는 말 : 도파민 D2 수용체, MAPK 활성화, Internalization, β-Arrestin, Dynamin