Dopamine D$_4$ and D$_{2L}$ Receptor Stimulation of the Mitogen-Activated Protein Kinase Pathway Is Dependent on trans-Activation of the Platelet-Derived Growth Factor Receptor

JAMES N. OAK, NATALIE LAVINE, and HUBERT H. M. VAN TOL

Laboratory of Molecular Neurobiology, Centre for Addiction and Mental Health, Toronto, Ontario, Canada; and Departments of Pharmacology, Psychiatry, and Institute of Medical Science, University of Toronto

Received September 8, 2000; accepted March 14, 2001 This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The ability of dopamine D$_4$ and D$_2$ receptors to activate extracellular signal-regulated kinases (ERKs) 1 and 2 was compared using Chinese hamster ovary (CHO-K1) cells transfected with D$_{4L}$, D$_{4R}$, D$_{4R7}$, and D$_{2L}$ receptors. Dopamine stimulation of D$_4$ or D$_{2L}$ receptors produced a transient, dose-dependent increase in ERK1/2 activity. Receptor-specific activation of the ERK mitogen-activated protein kinase (MAPK) pathway was confirmed using the D$_2$-like receptor-selective agonist quinpirole, whereas the specific antagonist haloperidol blocked activation. MAPK stimulation was dependent on a pertussis-toxin-sensitive G protein (G$_{i/o}$). trans-Activation of the platelet-derived growth factor (PDGF) receptor was an essential step in D$_2$ and D$_4$ receptor-induced MAPK activation. PDGF receptor-selective tyrosine kinase inhibitors tyrphostin A9 and AG1295 abolished or significantly inhibited ERK1/2 activation by D$_2$ and D$_{2L}$ receptors. Dopamine stimulation of the D$_4$ receptor also produced a rapid increase in tyrosine phosphorylation of the PDGF receptor-β. The Src-family tyrosine kinase inhibitor PP2 blocked MAPK activation by dopamine; however, this drug was also found to inhibit PDGF-BB-stimulated ERK activity and autophosphorylation of the PDGF receptor-β. Downstream signaling pathways support the involvement of a receptor tyrosine kinase. The phosphoinositide 3-kinase inhibitors wortmannin and LY294002, protein kinase C inhibitors GF109203X and Calphostin C, dominant-negative RasN17, and the MEK inhibitor PD98059 significantly attenuated or abolished activation of MAPK by dopamine D$_2$ and D$_{2L}$ receptors. Our results indicate that D$_2$ and D$_{2L}$ receptors activate the ERK kinase cascade by first mobilizing signaling by the PDGF receptor, followed by the subsequent activation of ERK1/2 by pathways associated with this receptor tyrosine kinase.

Dopamine is the predominant catecholamine neurotransmitter in the central nervous system and is involved in neurological and psychiatric disorders, including Parkinson’s disease, Tourette’s syndrome and schizophrenia. The D$_2$-like dopamine receptors (including D$_2$, D$_3$ and D$_4$) share a high affinity for antipsychotic drugs and couple through pertussis toxin-sensitive G proteins (G$_{i/o}$). Functionally, all D$_2$-like receptors have been shown to inhibit adenylyl cyclase, stimulate the Na$^+/H^+$ antiporter, activate K$^+$-channels, produce Cl$^-$ influx, alter neural morphology and stimulate mitogenes.

Additional intracellular effects of D$_2$ or D$_4$ receptors include arachidonic acid release, phosphoinositide hydrolysis, Ca$^{2+}$-channel inhibition and apoptosis.

It is now established that numerous G protein-coupled receptors can also activate mitogen-activated protein kinases (MAPKs), such as p38, ERK, and c-Jun NH$_2$-terminal kinase (Gutkind, 1998). The mitogenic potential of dopamine D$_2$-like receptors was recognized by Lajiness et al. (1993), who observed that the D$_2$ receptor could stimulate $[^{3}H]$thymidine incorporation in CHO cells by a mechanism that was independent of cAMP inhibition but sensitive to the G$_{i/o}$-inactivator pertussis toxin and the tyrosine kinase inhibitor genistein. This effect was later observed with D$_3$ and D$_4$.

This work was supported by Canadian Institutes of Health Research Grant 14573.

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; SOS, son of sevenless guanine nucleotide exchange factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; Ras, rat sarcoma virus p21 GTPase; Pyk2, proline-rich tyrosine kinase; PI3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; MEM, minimal essential medium; HRP, horseradish peroxidase; tyrphostin A9, [3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-methylene]propanedinitrile; BAPTA-AM, 1,2-bis[2-aminophenoxy]ethane-N,N,N,N’-tetraacetic acid acetoxymethyl ester; PP2, 4-amino-5-(4-chlorophenyl)-7-(l-tuty)pyrazolo[3,4-d]-pyrimidine; HA, hemagglutinin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; MBP, myelin basic protein; haD$_2$, HA epitope-tagged dopamine D$_2$ receptor; haD$_{4}$, HA epitope-tagged dopamine D$_{4}$ receptor; βARK1ct, β-adrenergic receptor kinase 1 carboxyl terminus; PTX, pertussis toxin; DMSO, dimethyl sulfoxide; PDGF-Ry, platelet-derived growth factor receptor-β; PLC, phosphoinoslate C; Shc, SH2-containing adapter protein.
MAPK Activation by Dopamine D4 and D2L Receptors

Materials and Methods

Reagents. CHO-K1 cells were purchased from American Type Culture Collection (Manassas, VA). α-MEM was purchased from Central Media Preparation Service (University of Toronto, Toronto, ON, Canada). Fetal bovine serum, horse serum, Geneticin (G418), T4 DNA ligase, T4 polynucleotide kinase, sequencing oligonucleotides and myelin basic protein were bought from Invitrogen (Carlsbad, CA). All restriction endonucleases, calf intestinal alkaline phosphatase, rabbit polyclonal anti-phosphoERK (Tyr204), mouse monoclonal anti-phosphoERK (E10 (Thr202/Tyr204), rabbit polyclonal anti-phosphoElk1 (Ser383) and anti-rabbit-HRP were obtained from New England Biolabs (Beverly, MA). [H]Sipiperone (95 Ci/mmol) and protein A-Sepharose were from Amersham Pharmacia Biotech (Baie d’Urfe, PQ, Canada). Dopamine, forskolin, pertussis toxin, phorbol 12-myristate 13-acetate (PMA), LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one HCl], and polyclonal rabbit anti-mouse-HRP were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Isobutyl-1-methyl xanthine was obtained from Aldrich Chemical Co. (Milwaukee, WI). Haloperidol, tyrphostin A9, AG1295 (6,7-dimethyl-2-phenylquinoline), AG1417 (N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine), wortmannin, BAPTA-AM, and PDGF-BB were purchased from Sigma/RBI (St. Louis, MO). γ[32P]ATP (3000 Ci/mmoll) and adenosine 3’,-5’-cyclic phosphoric acid, 2’,-O-succinyl [3H]dIodotyrosine methyl ester ([3H]ICAMP) (3300 Ci/mmol) were purchased from PerkinElmer Life Science Products (Mississauga, ON). PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one], GF109203X (bisindolylmaleimide I), 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), G6 6976, and Calphostin C were from Calbiochem (San Diego, CA). Rabbit anti-ERK1 (C-16), rabbit anti-ERK2 (C-14), and rabbit polyclonal anti-PDGFR (958) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-phosphoerostrogen antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY).

D4 and D2L Plasmid Constructs. The vector pcDNA3 (Invitrogen) containing the dopamine D2L or D4 receptor cDNA with an amino-terminal, cleavable signal sequence (MKTIALSYSYFCLVI) and the hemagglutinin (HA) epitope (DYPYDVPDYA) were produced by Dr. R. Vickery (University of California at San Francisco). D2L was excised from pcDNA by a bluntNeoIXhol digest. The plasmid pBCSSHA5 was digested with BamHI to excise the β-opioid receptor. After blunting the vector and a Xhol digest, the D2 sequence was inserted to create pBCSSHA2L. The modified D2L receptor was removed from pBC with a HindIII/Xhol digest and subcloned into pcDNA3 to produce pCSCSSHA2L. Using a partial NeoI/BamHI digest, the dopamine D4.7 receptor was cut from PD4.7 (Asghari et al., 1994) and cloned into the NeoI/BamHI site of plBCSSHA5 to create pBCSSHA2L. The tagged D4 receptor was cut with HindIII/BamHI from pBCSSHA2L and subcloned into pcDNA3. Partial NeoI/Xhol digest of the D4.7 and D4 receptors in pHBluescript (Asghari et al., 1994) resulted in 1.2- and 1.3-kilobase pair fragments that were ligated into pCSCSSHA2L and subcloned into pcDNA3.4c. Cell Culture. Cells were grown in supplemented α-MEM (2.5% fetal bovine serum and 2.5% horse serum) to monolayers at 37°C in a humidified, 5% CO2 atmosphere. Stable CHO-K1 cell lines of pcDNA3 (vector control), pcSSHA4.2, pcSSHA4.4, pcSSHA4.7, and pcSSHA4.7 were transfected by electroporation as described previously (Asghari et al., 1995). Transient transfections and stable transfection of pBK-PDGFβ were carried out with Lipofectamine reagent (Invitrogen) as described by the manufacturer. Individual CHO-K1 clones were grown in media containing 500 µg/ml G418. Radioligand Binding. Cells were resuspended in binding buffer (120 mM NaCl, 50 mM Tris-HCl, 5 mM KCl, 5 mM MgCl2, 1.5 mM CaCl2, 0.5 mM EDTA, pH 7.4) and mechanically homogenized (Pro Scientific Inc., Monroe, CT; maximum, 15 s, on ice). Cell membranes were pelleted by centrifugation (34,000g for 20 min at 4°C) and the membrane pellet was resuspended in binding buffer. After activation, the membranes were filtered through glass fiber filters (Whatman GF/B). Bound radioactivity was measured by liquid scintillation.

These receptors were in contrast to studies with pituitary tumor tissue and cell lines, where the D2 receptor was found to have an antiproliferative role (Florio et al., 1992) and reduced thyrotropin-releasing hormone-induced MAPK activation (Ohmichi et al., 1994). In neuronal MN9D cells, D2, D3, and D4 receptors were shown to alter neural morphology (Swarzenski et al., 1994), whereas others reported both apoptosis and differentiation as a result of D2 receptor signaling in neuronal cells (Coronas et al., 1997). A unifying element of these effects may be MAPK pathways, which are capable of mediating diverse cellular actions. Recent studies have reported that dopamine D2, D3, and D4 receptors can independently stimulate the activity of the MAPKs ERK1/2 or c-Jun N-terminal kinase (Luo et al., 1998; Oldenhof et al., 1998; Welsh et al., 1998; Cussac et al., 1999).

The MAPKs are a group of serine/threonine kinases that are activated by a cascade of protein kinases to induce responses such as proliferation, differentiation, apoptosis, and long-term potentiation. Classically, activation of MAPK pathways have been attributed to the activity of specific receptor factor binding. Activation of growth factors such as EGF or PDGF to receptor tyrosine kinases results in receptor dimerization and autophosphorylation. Binding of Grb2 (either directly or via other adapter proteins) to activated growth factor receptors promotes translocation of the Grb2-associated protein Sos to the membrane. The guanine nucleotide exchange factor Sos catalyzes the activation of Ras by promoting GTP/GDP exchange. Ras participates in the activation of the cytosolic serine/threonine kinase Raf-1 (MAPK kinase), which activates MEK1/2 (MAPK kinase) and ultimately stimulates activation of ERK1/2. Activated ERK1/2 can phosphorylate cytosolic substrates and is translocated to the nucleus, leading to the activation transcription factors.

Among G_{i/o}-coupled receptors, activation of MAPK has been attributed to both Gα and Gβγ, and the mechanism of activation can differ among specific G_{i/o}-coupled receptors and cell lines. Some G protein-coupled receptor seems to activate MAPK by trans-activation of a receptor tyrosine kinase such as the EGF receptor (Daub et al., 1996). Once trans-activated by an as-yet poorly defined mechanism, these receptors can recruit effectors, such as the She/Grb2/Sos pathway of Ras activation, to stimulate MAPK. The Ca^{2+}-dependent kinase Pyk2 (related focal adhesion tyrosine kinase) has been identified as a component of the G_{i/o}-coupled receptor pathway to ERK activation (Dikic et al., 1996). Ras-independent mechanisms of MAPK activation by G_{i/o}-coupled receptors have also been described. In CHO cells, G_{i/o}-coupled lysosphoshatid acid receptors seem to signal through PI3-kinase/PTK to activate MEK in a manner that is unaffected by dominant negative Sos (Takeda et al., 1999).

We have demonstrated that both dopamine D4 and D2L receptors activate the ERK MAPK pathway through G_{i/o}. No observable differences were apparent in the magnitude or duration of MAPK activation by D4 and D2L receptors. Activation of ERK1/2 by dopamine involves trans-activation of the PDGF receptor-β and is also dependent on PKC, PI3-kinase, Ca^{2+}, Ras, and MEK. These results suggest that cross-talk with a growth factor receptor is a required upstream component in the activation of the ERK MAPK pathway by D4 and D2L receptors.
resuspended by homogenization (maximum, 5 s, on ice) in binding buffer. Binding assays were carried out by mixing 25 μg of membrane protein with 1 ml of binding buffer containing [3H]spiperone and unlabeled drugs, in duplicate. After incubation (2 h at room temperature), membranes were harvested onto glass-fiber filters using a combicell harvester (Skatron Instruments Inc., Sterling, VA) and washed with 50 mM Tris-HCl, pH 7.5 (2 × 9 s). Bound radioligand was detected by liquid scintillation counting.

Saturation binding experiments were carried out with 0.01 to 1 nM [3H]spiperone. The receptor Kd (nanomolar) and Bmax (disintegrations per minute) were determined by nonlinear curve fitting using GraphPad Prism v2.0 (GraphPad Software, San Diego, CA). Saturation binding data were fit to the equation

\[ Y = \frac{B_{\text{max}} \times (X - Y)}{K_d + (X - Y)} + (X - Y) \times NS, \]

where X is the total amount of ligand (disintegrations per minute), Y is the total binding (disintegrations per minute), and NS is the nonspecific binding constant. NS was determined experimentally by coincubating with 1 μM haloperidol and fitting the data to the equation

\[ Y = \frac{B_{\text{max}} \times (X - Y)}{K_d + (X - Y)} + (X - Y) \times NS, \]

where X is the logarithm of the concentration, Y is the response, and \( n_H \) is the Hill slope using GraphPad Prism v2.0.

MAPK Assay. Cells stably expressing receptors were plated on six-well plates and grown to ~80% confluence. Cells were treated with 1 μM dopamine and the cAMP concentration in the lysate was determined by [125I]cAMP radioimmunoassay as described previously (Asghari et al., 1995) using a standard curve fit to a sigmoidal dose-response curve (Y = Min + (Max − Min)/(1 + 10LogEC50 − X × nH), where Y is the logarithm of the concentration, X is the response, and nH is the Hill slope) using GraphPad Prism v2.0.

Western Blotting of Phosphorylated PDGFRβ. CHO-K1 cells stably expressing PDGFR receptor-β were transiently transfected with pcSSHAD4.4 and/or treated with drugs and cell lysates were prepared as described previously (Domin et al., 1996). Receptors were immunoprecipitated by incubating with 1 μg of anti-PDGFR-β (3 h at 4°C) followed by addition of 20 μl of 50% protein A-Sepharose (3 h at 4°C). After washing three times with lysis buffer, pellets were resuspended in sample buffer and denatured (95°C for 5 min). Immunoprecipitated proteins were separated with a 4 to 12% SDS-PAGE gel and Western blotting was carried out using anti-phosphotyrosine (1 μg/ml)-anti-mouse-HRP (1:8000).

Results

CHO-K1 Cell Lines Expressing HA-tagged D4 or D2L Receptors. Wild-type human dopamine D4.2, D4.4, D4.7, and D2L receptors were modified by the addition of a cleavable, membrane-targeted signal sequence and an amino terminal HA-epitope tag. Clonal CHO-K1 cell lines expressing 0.78 to 1.6 pmol of receptor/mg of membrane protein were selected (Table 1). All cell lines except the control CHO-pcDNA3 inhibited forskolin-stimulated CAMP in response to 1 μM dopamine (Fig. 1).

Activation of ERK1/2 by haD4 and haD2L Receptors. To determine whether various polymorphic variants of the dopamine D4 receptor can stimulate the MAPK pathway, the

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-haD4.2</td>
<td>0.161 ± 0.016</td>
<td>1.05 ± 0.59</td>
</tr>
<tr>
<td>CHO-haD4.4</td>
<td>0.246 ± 0.071</td>
<td>1.62 ± 0.16</td>
</tr>
<tr>
<td>CHO-haD4.7</td>
<td>0.160 ± 0.029</td>
<td>0.784 ± 0.100</td>
</tr>
<tr>
<td>CHO-haD2L</td>
<td>0.064 ± 0.011</td>
<td>1.30 ± 0.17</td>
</tr>
</tbody>
</table>

- Table 1

ERK1/2 activity was also measured by the in vitro phosphorylation of myelin basic protein (MBP). Cleared cell lysates (see above) were incubated overnight with 1:100 dilutions of anti-ERK1 (C-16) and anti-ERK2 (C-14). After collection with protein-A Sepharose, the beads were washed twice with lysis buffer and twice with MBP kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, and 1 mM DTT). Beads were resuspended in 50 μl of MBP kinase buffer containing 12.5 μg of myelin basic protein, 2 μCi of [γ-32P]ATP, and 20 μM unlabeled ATP and incubated at 30°C for 20 min. A 10-μl aliquot was spotted onto P81 phosphocellulose filter paper (Whatman), washed with cold 0.5% phosphoric acid (5 × 5 min), rinsed with ethanol and dried. The incorporation of 32P into myelin basic protein was measured by liquid scintillation counting.

To detect phosphorylated forms of ERK1/2, cells were scraped after addition of sample buffer, sonicated for 20 s and denatured (95°C for 5 min). Samples were microcentrifuged for 5 min, and 50 μl was loaded onto an 8 to 16% Tris-glycine gel. After SDS-PAGE, Western blotting, and blocking, the polyvinylidene difluoride membranes were incubated with mouse monoclonal anti-phospho-MAPK E10 (Thr202/Tyr204) (1:1000 in blocking buffer). The blots were incubated 1 to 2 h at room temperature, washed with TBS-T (4 × 5 min), and subsequently incubated for 1 h with anti-mouse-HRP (1:4000 blocking buffer without NaN3) at room temperature. Membranes were washed with TBS-T (4 × 5 min) and the phosphorylated forms of ERK1 and ERK2 were detected by chemiluminescence.
activity of immunoprecipitated ERK1/2 was measured by an in vitro Elk1 kinase assay. Dopamine treatment stimulated ERK1/2-dependent phosphorylation of Elk1 in cells expressing haD4.2, haD4.4, haD4.7, and haD2L, but had no effect on CHO-K1 cells transfected with the vector, pcDNA3 (Fig. 2A). Increased ERK activity was also distinguished by an increase in the level of the phosphorylated (pThr202/pTyr204) forms of ERK1/2 (Fig. 2B), whereas the total level of ERK1/2 was unaffected (Fig. 2C). Dopamine stimulation of ERK1/2 phosphorylation and activity was transient, with ERK kinase activity returning to basal levels by 30 min (Fig. 3B), whereas residual ERK2 phosphorylation was still observable after 60 min (Fig. 3A). The three polymorphic variants of the dopamine D4 receptor and the D2L receptor all displayed a similar pattern of transient ERK activation. Dopamine stimulation of CHO-haD4.4 and CHO-haD2L cells (1 μM for 5 min) produced increases of 355 ± 71% (mean ± S.D., n = 12) and 330 ± 105% (n = 13), respectively, in the activity of ERK1/2 as measured by in vitro myelin basic protein kinase activity compared with unstimulated cells.

A dose-response curve of MAPK activation by dopamine in CHO-haD4.4 and CHO-haD2L cells produced EC50 values of 9 and 10 nM (Fig. 4). Dopamine receptor-specific stimulation of ERK activity was confirmed using the D2-like receptor-selective drugs quinpirole and haloperidol. The agonist quinpirole (1 μM) produced a stimulation in ERK activity, and pretreatment of cells for 5 min with the antagonist haloperidol (1–5 μM) abolished dopamine-stimulated ERK activity in CHO-haD2L cells and significantly reduced the activity of this kinase in cells expressing haD4.4 receptors (Fig. 5).

**MAPK Activation by D4 and D2L Receptors Involves G Protein-Coupled Stimulation of Ras and MEK.** To study the mechanism of ERK activation by dopamine D4 and D2 receptors, CHO-haD4.4 and CHO-haD2L cell lines were preincubated with pertussis toxin (100–200 ng/ml), which blocks receptor coupling through Go, or with the MEK inhibitor PD98059 (50 μM) (Fig. 6, A and B). Both pertussis toxin and PD98059 abolished ERK activation by dopamine.

To further characterize the pathway, wild-type CHO-K1 cells were transiently cotransfected with pcSSHAD4.4 or pcSSHAD2L along with the dominant negative constructs βARK1ct or RasN17 (Fig. 7). Coexpression of βARK1ct and RasN17 significantly attenuated the activation of ERK by haD4. Coexpression of haD4L with RasN17 also reduced ERK activation, whereas βARK1ct did not show a statistically significant effect. The effect of haD4 and haD2L receptors on ERK activity was independent of cellular cAMP levels, because pretreatment of cells with 8-bromo-cAMP (0.01 to 1 mM) did not block activation of this MAPK pathway (data not shown).

**Inhibitors of PKC and PI3-Kinase Block Activation of ERK.** To determine whether ERK activation by D4 and D2L receptors is dependent on PKC, CHO-haD4.4 and CHO-haD2L cells were preincubated with the PKC inhibitors GF109203X (bisindolylmaleimide I) (2 μM), Gö 6976 (2 μM), or Calphostin C (1 μM) (Fig. 8). MAPK activation by both haD4 and haD2L receptors was sensitive to the broad spectrum PKC inhibitors GF109203X and Calphostin C, but was not reduced by Gö 6976, which is selective for PKCα- and PKCβ. Pretreatment of cells with the PI3-kinase inhibitor wortmannin (1 μM) and LY294002 (30 μM) also blocked >50% of ERK stimulation by dopamine (Fig. 9).

**Intracellular Ca2+ Is Required for MAPK Activation by Dopamine D4 and D2L Receptors.** Pretreatment of CHO-haD4.4 and CHO-haD2L cells with the intracellular Ca2+ chelator BAPTA-AM (100 μM) abolished dopamine-

---

**Fig. 1.** Inhibition of forskolin-stimulated cAMP by dopamine D4 and D2 receptors. Stably transfected CHO-pcDNA3 (control), CHO-haD4.2, CHO-haD4.4, CHO-haD4.7, and CHO-haD2L cells were treated with 10 μM forskolin to activate adenyl cyclase and stimulate cAMP levels. Cells were treated with dopamine (1 μM) for 15 min before collecting cell lysates, and the cAMP level was measured using a cAMP radioimmunoassay as described under Materials and Methods. The results were normalized by setting forskolin-stimulated cAMP levels at 100% and represented the mean ± S.D. for three to five independent experiments. Groups that show a significant difference in intracellular cAMP levels because of dopamine using a paired t test: *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 2.** Stimulation of ERK1/2 activity and phosphorylation by dopamine D4 and D2L receptors. The activation of MAPK in serum-deprived CHO-pcDNA3 (control), CHO-haD4.2, CHO-haD4.4, CHO-haD4.7, and CHO-haD2L was measured after a 5-min incubation with dopamine (1 μM). A, activity of MAPK after dopamine treatment. Phosphorylated ERK1 and ERK2 (p44 and p42 MAPK) were immunoprecipitated from cell lysates with anti-phospho-MAPK (Thr202/Tyr204) antibody and protein A-Sepharose. MAPK catalytic activity was measured by a kinase assay using a recombinant MAPK substrate, Elk-1-GST. Phosphorylated Elk-1 was detected by Western blotting with anti-phosphoElk-1/anti-rabbit-HRP antibodies. B, phosphorylation of ERK1/2 after dopamine treatment. Phosphorylated MAPK was detected directly from cell lysates by Western blotting with anti-phospho-MAPK (Thr202/Tyr204)/anti-mouse IgG-HRP. C, total ERK1/ERK2 levels in unstimulated and stimulated CHO cells. Total (i.e., unphosphorylated + phosphorylated) ERK1/2 was measured in cell lysates by Western blotting with anti-p44/p42 MAPK antibody/anti-rabbit IgG-HRP. Western blots are from one of three independent experiments that produced similar results.
mediated ERK1/2 phosphorylation (Fig. 10A). This observation was confirmed by an in vitro kinase assay, where it was found that BAPTA-AM was only effective at concentrations above 10 μM (Fig. 10B).

ERK Activation by Dopamine Is Dependent on PDGF Receptor trans-Activation. To test whether growth factor receptor signaling is recruited by dopamine D₄ and D₂L receptor MAPK signaling, cells were preincubated with the selective receptor tyrosine kinase inhibitors AG1478 (EGF receptor), tyrphostin A9 (PDGF receptor), and AG1295 (PDGF receptor). Although AG1478 (1 μM) had no effect on dopamine-stimulated ERK phosphorylation, tyrphostin A9 pretreatment (1 μM) eliminated the response of ERK to dopamine (Fig. 11A). The in vitro kinase assay confirmed that both tyrphostin A9 and a second PDGF receptor tyrosine kinase inhibitor, AG1295 (10 μM) potently blocked ERK stimulation by both haD₄.₄ and haD₂L receptors in CHO-K1 cells (Fig. 11, B and C).

Fig. 3. Time course of MAPK activation by dopamine. A, serum-deprived CHO-haD4.2, CHO-haD4.4, CHO-haD4.7, and CHO-haD2L were treated with dopamine (1 μM) for up to 60 min. After drug incubation, phospho-MAPK (Thr202/Tyr204) level was measured in cell lysates by Western blotting. B, stimulation of ERK1/2 kinase activity in response to dopamine. After treating serum-deprived CHO-haD4.4 (●) and CHO-haD2L (▲) with dopamine (1 μM) for up to 60 min, ERK1/2 was immunoprecipitated and their activity was quantified with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3).

Fig. 4. Dose-response of MAPK activation by dopamine. A, serum-deprived CHO-haD4.2 cells were treated with dopamine (1 pM to 100 μM) for 5 min. After drug incubation, phospho-MAPK (Thr202/Tyr204) level was measured in cell lysates by Western blotting. B, stimulation of ERK1/2 kinase activity in response to dopamine. After treating serum-deprived CHO-haD4.4 (●) and CHO-haD2L (▲) with dopamine (10 pM to 10 μM) for 5 min, ERK1/2 was immunoprecipitated, and their activity was quantified with an in vitro kinase assay using MBP as a substrate. ERK1/2 kinase activity was normalized by setting unstimulated activity at 0% and dopamine-stimulated activity at 100%. Data are presented as mean ± S.D. (n = 3). The EC₅₀ value for dopamine was determined by fitting the data to a sigmoidal dose-response curve using Prism (v. 2.0; GraphPad Software, San Diego, CA).
Transient transfection of CHO-PDGFRβ cells (stably expressing an increased level of PDGF receptor-β) with pc-SSHAD4.4 followed by treatment with dopamine (1 μM for 1–5 min) produced an immediate increase in the tyrosine phosphorylation of PDGF receptor-β (Fig. 12), confirming that dopamine receptors can trans-activate this receptor tyrosine kinase.

The Src-family tyrosine kinase inhibitor PP2 (50 μM) was also found to block ERK activation in response to dopamine (Fig. 13). When PDGF-BB was tested on wild-type CHO-K1 cells, we found that pretreatment with tyrophostin A9, AG1295, and PP2 blocked ERK activation by endogenous PDGF receptor-β (Fig. 14A). In addition, PP2 also strongly suppressed PDGF receptor-β autophosphorylation in response to PDGF-BB (Fig. 14B).

**Discussion**

This study examined the activation of the ERK MAPK pathway by dopamine D4 and D2L receptors to understand and compare the mechanism they use. Dopamine stimulation of both receptors resulted in a rapid, transient, and dose-dependent increase in ERK phosphorylation and activity. The magnitude of activation was similar between D4.4, D4.7, and D2L receptors. The EC_{50} value of ERK activation (9–10 nM) was similar to values reported previously for the inhibition of intracellular cAMP by D4.4 (12 nM) and D2L (5 nM) receptors (Asghari et al., 1995; Guiramand et al., 1995).

ERK activation by both D4 and D2L receptors required a PTX-sensitive G protein (G_{i/o}), whereas sequestering Gβγ with βARK1ct peptide significantly attenuated ERK activation by the D4 receptor. Activation of the ERK MAPK pathway by dopamine D2R, D2L, and D3 receptors has previously been attributed to PTX-sensitive G proteins (Cussac et al., 1999; Luo et al., 1998). The dominant-negative mutant RasN17 and the MEK inhibitor PD98059 also reduced or eliminated activation of ERK by both D4 and D2L receptors, suggesting that dopamine activates the Ras → Raf → MEK → ERK pathway.

Because there has emerged that G protein-coupled receptors can recruit growth factor receptors to facilitate MAPK signaling (Daub et al., 1996), we tested selective inhibitors of EGF receptor and PDGF receptor tyrosine kinase activity. Although EGF receptor-selective AG1478 failed to block dopamine-stimulated ERK phosphorylation, PDGF receptor-selective tyrophostin A9 and AG1295 potently inhibited ERK phosphorylation and kinase activity, indicating that this receptor tyrosine kinase is a required intermediate. CHO-K1 cells are known to endogenously express a low level of the PDGF receptor-β (Duckworth and Cantley, 1997), and the PDGF receptor can act as a scaffold for numerous other kinases and adapter proteins, including Src, SH2 domain-containing phosphotyrosine phosphatase-2, Shc, Grb2/Sos, PLC-γ, PI3-kinase, and Ras-GAP (reviewed by Heldin et al., 1998). The role of trans-activation is supported by a rapid increase in tyrosine phosphorylation of PDGF receptor-β upon stimulation of the D4.4 receptor, demonstrating that activation of the growth factor receptor occurs in a timeframe consistent with its involvement in MAPK signaling by dopamine.

The Src-family tyrosine kinase inhibitor PP2 blocked the activation of MAPK by dopamine and PDGF receptor-β. PP2 also inhibited PDGF-BB-stimulated autophosphorylation of PDGF receptors overexpressed in CHO-K1 cells. These results are in agreement with a recent report by Waltenberger et al. (1999) that indicated that PP1, a Src-family kinase inhibitor closely related to PP2, inhibits the tyrosine kinase

---

**Fig. 5.** Effect of dopaminergic drugs on MAPK activation. A, inhibition of dopamine-stimulated ERK1/2 phosphorylation by haloperidol. Serum-deprived CHO-haD4.4 and CHO-haD2L cells were treated with dopamine (1 μM) in the absence or presence of the D4 antagonist haloperidol (1 μM and 5 μM). After incubating with drugs for 5 min, phospho-MAPK-(Thr202/Tyr204) levels were measured in cell lysates by Western blotting. B, stimulation of ERK1/2 kinase activity by quinpirole and inhibition by haloperidol. Serum-deprived CHO-haD4.4 and CHO-haD2L were treated with media alone (−), dopamine (DA, 1 μM), or quinpirole (QUIN, 1 μM) for 5 min. To measure the effect of the antagonist, cells were preincubated 5 min with haloperidol (HALO, 5 μM) before stimulation with dopamine (1 μM) for 5 min. ERK1/2 was immunoprecipitated from cell lysates and their activity was quantified with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with dopamine-stimulated values as determined by an unpaired t test: *p < 0.05; **p < 0.01; ***p < 0.001.
activity of the PDGF receptor-β but did not block activation of EGF, fibroblast growth factor-1, or insulin-like growth factor-1 receptors. Conversely, tyrphostin A9 blocks Src kinase activity only partially at a concentration of 10 μM (Lakkakorpi et al., 2000), whereas AG1296, a less hydrophobic analog of AG1295, does not inhibit the kinase activity of Src (Kovalenko et al., 1994). Therefore, it seems that the Src kinase inhibitor PP2 acts at the PDGF receptor-β to block MAPK activation by both dopamine and PDGF-BB.

Nonselective PKC inhibitors and the Ca<sup>2+</sup>-chelator BAPTA-AM (100 μM) also abolished MAPK activation. CHO-K1 cells are known to possess PKCα, γ, δ, ε, and ζ isoforms (Tippmer et al., 1994; Shirai et al., 2000). Cussac et al. (1999) proposed that an atypical PKC, PKC ζ, was involved in ERK activation by the D<sub>3</sub> receptor in CHO cells. Because the reported IC<sub>50</sub> value of PKC ζ inhibition by GF109203X is 5.8 μM (Martiny-Baron et al., 1993), it is unlikely that activation of this PKC isoform contributes sig-

---

**Fig. 6.** Inhibition of dopamine-stimulated MAPK activity by PTX and the MEK inhibitor PD98059. A, CHO-haD4.2 and CHO-haD2L cells were preincubated overnight in serum-free media alone, with PTX (200 ng/ml) or for 1 h with PD98059 (50 μM) before stimulation with dopamine (1 μM). After incubating with drugs for 5 min, phospho-MAPK/Thr202/Tyr204 levels were measured in cell lysates by Western blotting. B, CHO-haD4.4 and CHO-haD2L were preincubated overnight in serum-free media alone (Control), with PTX (100 ng/ml), or for 1 h with vehicle (0.5% DMSO) or PD98059 (50 μM) before stimulation with dopamine (1 μM) for 5 min. ERK1/2 was immunoprecipitated from cell lysates by Western blotting. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with control (dopamine-stimulated) values as determined by an unpaired t test are indicated by *p < 0.05; **p < 0.01; ***p < 0.001.

**Fig. 7.** Dominant negative RasN17 and βARK1ct partially block MAPK activation by dopamine D<sub>2</sub> and D<sub>3</sub> receptors. CHO-K1 cells were plated onto six-well dishes and transiently transfected with pcDNA3 alone (1.1 μg) or receptor (pcSSHAD4.4 or pcSSHAD2L) (0.1 μg) plus pcDNA3, RasN17, or βARKct (1.0 μg). After 24 h, cells were transfected to serum-free media, incubated overnight, and subsequently treated with dopamine (1 μM) for 5 min. ERK1/2 was immunoprecipitated from cell lysates and their activity was quantified with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with dopamine-stimulated values as determined by an unpaired t test: *p < 0.05; **p < 0.01; ***p < 0.001.
significantly to ERK activation by D₄ or D₂L receptors; we observed complete inhibition of ERK activation with 2 μM GF109203X. These observations point to the involvement of PKCγ, δ, or ε in ERK activation by D₄ and D₂L. Of these, classical PKCγ is activated by intracellular Ca²⁺, although its sensitivity to Gö 6976 is unknown.

The PI3-kinase inhibitors wortmannin and LY294002 both attenuated dopamine-stimulated ERK activity by approximately 50%, as was previously observed with D₂S and D₃ cells expressed in CHO cells (Welsh et al., 1998; Cussac et al., 1999). PI3-kinases are known to act upstream of Sos in Gβγ-mediated MAPK activation (Hawes et al., 1996), but D₄ and D₂L do not use the same Ras-independent signaling pathway through PI3-kinase and PKCζ that was described for lysophosphatidic acid receptors in CHO cells (Takeda et al., 1999), because we found that both dopamine receptors utilize Ras and a PKC other than PKCζ. However, we do not presently know the identity of the PI3-kinase involved in MAPK signaling by dopamine.

Figure 15 shows a model of pathways leading from D₄/D₂L to ERK activation based on our data. Grb2/Sos recruitment by phosphorylated PDGF receptor-β represents one mecha-
nism for Ras activation by D₄ and D₂L receptors. Activated Shc or SH2 domain-containing phosphotyrosine phosphatase-2 may also facilitate the binding of the Grb2/Sos complex to the PDGF receptor. In addition, PLC-γ and PI3-kinase have all been implicated in the transduction of mitogenic signals by this receptor tyrosine kinase (Duckworth and Cantley, 1997). Both PLC-γ and PI3-kinase can activate PKCε after activation by the PDGF receptor (Moriya et al., 1996), and GF109203X has been shown to fully block mitogenic signaling by PDGF receptor-β (Alimandi et al., 1997). The mechanism by which Gi/C trans-activates the PDGF receptor has not yet been identified.

It has recently emerged that the mechanism of MAPK activation by the β₂-adrenergic receptor seems to involve the formation of a complex between receptor, β-arrestin, dynamin, Src, and the EGF receptor (Maudsley et al., 2000). Activation of MEK requires dynamin-dependent endocytosis, where phosphorylation of dynamin by Src is required (Daaka et al., 1998). This process may involve G protein-coupled receptor endocytosis, as seen with the β₂-adrenergic receptor, although internalization of the α₁A-adrenergic receptor did not occur after EGF receptor trans-activation (Pierce et al.,

Fig. 10. The Ca²⁺-chelator BAPTA-AM blocks MAPK activation by dopamine D₄ and D₂L receptors. A, serum-deprived CHO-haD4.2 and CHO-haD2L cells were preincubated with vehicle (1% DMSO) or BAPTA-AM (100 µM) for 1 h before stimulation with dopamine (1 µM). After stimulation with dopamine for 5 min, phospho-MAPK(Thr202/Tyr204) levels were measured in cell lysates by Western blotting. B, serum-deprived CHO-haD4.4 (■) and CHO-haD2L (▲) were preincubated with vehicle (1% DMSO) or BAPTA-AM (0.1 to 100 µM) before stimulation with dopamine (1 µM) for 5 min. ERK1/2 was immunoprecipitated from cell lysates and their activity was measured with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with control (dopamine-stimulated) values as determined by an unpaired t-test: *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 11. The PDGF receptor tyrosine kinase inhibitors tyrphostin A9 and AG1295 block MAPK activation by D₄ and D₂L receptors. A, serum-deprived CHO-haD4.2 and CHO-haD2L cells were preincubated for 1 h with vehicle (0.1% ethanol), tyrphostin A9 (1 µM), or AG 1478 (1 µM) before stimulation with dopamine (1 µM) for 5 min. Phospho-MAPK(Thr202/Tyr204) levels were measured in cell lysates by Western blotting. B, CHO-haD4.4 (■) and CHO-haD2L (▲) were preincubated for 1 h with vehicle (0.1% ethanol) or tyrphostin A9 (0.01 to 1 µM) before stimulation with dopamine (1 µM) for 5 min. C, CHO-haD4.4 (■) and CHO-haD2L (▲) were preincubated for 1 h with vehicle (1% DMSO) or AG1295 (0.1 to 10 µM) before stimulation with dopamine (1 µM) for 5 min. To measure MAP kinase activity, ERK1/2 was immunoprecipitated from cell lysates and their activity was quantified with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with control (dopamine-stimulated) values as determined by an unpaired t-test: *p < 0.05; **p < 0.01; ***p < 0.001.
In this regard, D$_2L$ receptors are known to undergo agonist-dependent internalization in CHO cells (Itokawa et al., 1996). Although Vickery and von Zastrow (1999) reported that D$_2$ receptors are internalized by a dynamin-independent mechanism, others have found that D$_2$ undergoes dynamin-dependent sequestration when coexpressed with GRK2 or GRK5 (Ito et al., 1999). Because GRK2 is abundantly expressed in CHO cells, the β-arrestin/Src/dynamin-dependent mechanism of trans-activation may be involved. Although we do not observe agonist-stimulated internalization of the D$_4$ receptor (R. Vickery, M. von Zastrow, and H. H. M. Van Tol, unpublished observations), results with the α$_2$-adrenergic receptor have demonstrated that G protein-coupled receptor endocytosis is not required for dynamin-dependent trans-activation (Pierce et al., 2000).

Although β$_2$-adrenergic receptor trans-activation was independent of Ca$^{2+}$ (Maudsley et al., 2000), serotonin 5-HT$_{1A}$-dependent activation of ERK in CHO cells was dependent of Ca$^{2+}$/calmodulin- and clathrin-dependent endocytosis (Della Rocca et al., 1999). In this case, the Ca$^{2+}$-dependent tyrosine kinase Pyk2 seems to mediate EGF receptor activation by bradykinin, presumably through activation of Src and downstream kinases (Blaukat et al., 1999). Therefore, the Ca$^{2+}$-dependence of ERK signaling by D$_4$ and D$_2L$ receptors may originate at the level of PDGF receptor endocytosis or Pyk2 activation.

Although the physiological role of dopamine receptor-mediated trans-activation is not fully understood; however, J. F. MacDonald (Dept. of Physiology, University of Toronto; unpublished observations) found that the D$_4$ receptor uses PDGF receptor-β trans-activation to block NMDA currents in hippocampal slices.

**Fig. 12.** Dopamine D$_{4.4}$ receptors stimulate tyrosine phosphorylation of PDGF receptor-β. CHO-K1 cells stably overexpressing PDGF receptor-β were transiently transfected with pcSSHAD4.4. After 24 h, cells were preincubated overnight in serum-free media and stimulated with dopamine (DA, 1 μM) for 1 to 5 min. PDGF receptors were immunoprecipitated from cell lysates followed by Western blotting with anti-phosphotyrosine/anti-mouse-HRP.

**Fig. 13.** MAPK stimulation by D$_4$ and D$_{2L}$ receptors is blocked by the Src-family tyrosine kinase inhibitor PP2. Serum-deprived CHO-haD4.4 (■) and CHO-haD2L (▲) were preincubated for 1 h with vehicle (0.5% DMSO), or PP2 (1 to 50 μM) before stimulation with dopamine (1 μM) for 5 min. ERK1/2 was immunoprecipitated from cell lysates and their activity was quantified with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with control (dopamine-stimulated) values as determined by an unpaired t test: *p < 0.05; **p < 0.01; ***p < 0.001.

**Fig. 14.** Tyrophostin A9, AG1295 and PP2 inhibit PDGF-BB-stimulated MAPK activation and PDGF receptor-β autophosphorylation. Wildtype CHO-K1 cells were preincubated with vehicle (0.1% EtOH), tyrophostin A9 (1 μM), vehicle (0.5% DMSO), AG1295 (10 μM) or PP2 (50 μM) for 1 h before stimulation with PDGF-BB (10 ng/ml) for 5 min. ERK1/2 was immunoprecipitated from cell lysates and their activity was quantified with an in vitro kinase assay using MBP as a substrate. Data are presented as mean ± S.D. (n = 3). Groups with a statistically significant difference in kinase activity compared with control (dopamine-stimulated) values as determined by an unpaired t test: *p < 0.05; **p < 0.01; ***p < 0.001. B, CHO-K1 cells stably overexpressing PDGF receptor-β were preincubated with vehicle (0.5% DMSO), AG1295 (10 μM), or PP2 (50 μM) for 1 h before stimulation with PDGF-BB (10 ng/ml) for 5 min. PDGF receptor-β was immunoprecipitated from cell lysates followed by Western blotting with anti-phosphotyrosine/anti-mouse-HRP.
pocampal neurons. In addition, the emergence of MAPK pathways as an element of G protein-coupled receptor signaling has also vastly increased the range of cellular processes that dopamine receptors may affect. Quinpirole was recently found to stimulate ERK phosphorylation in rat neurons in a PKC- and Ca²⁺-dependent manner (Yan et al., 1999) and dopamine D₂-like receptor activation has been shown to contribute to long-term depression (LTD) in rat prefrontal cortex through a mechanism involving MAPK (Otani et al., 1999). These findings suggest that dopamine D₂-like receptor activation of MAPK pathways may play an important role in the brain, possibly through involving trans-activation of receptor tyrosine kinases such as the PDGF receptor.

Acknowledgments

We thank Dr. R. Vickery and Dr. M. von Zawrowsky (University of California, San Francisco, California) for constructing pcSSHAD4.7 and pcSSHAD2L, Dr. R. Lefkowitz (Duke University Medical Center, Durham, NC) for providing the plasmids containing RasN17 and βARK1et, and Dr. J. F. MacDonald (University of Toronto, Toronto, Ontario, Canada) for providing pBK-PDGFRβ.

References


Swarzenski BC, Tang L, Oh YJ, O’Malley KL and Todd RD (1994) Morphogenic potentials of D₂, D₃, and D₄ dopamine receptors revealed in transfected neuronal cell lines. Proc Natl Acad Sci USA.


Address correspondence to: Dr. Hubert H. M. Van Tol, Center for Addiction and Mental Health, 250 College St. W., Toronto ON, Canada, M5T 1R8. E-mail: hubert.van.tol@utoronto.ca

MAPK Activation by Dopamine D₄ and D₂L Receptors