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G Protein-Mediated Mitogen-Activated Protein Kinase Activation by Two Dopamine D2 Receptors

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Two isoforms of dopamine D2 receptor, D2L (long) and D2S (short), differ by the insertion of 29 amino acids specific to D2L within the putative third intracellular loop of the receptor, which appears to be important in selectivity for G-protein coupling. We have generated D2L- and D2S-expressing Chinese hamster ovary (CHO) cells, and regulation of the mitogen-activated protein kinase (MAPK) pathway was examined in these cells. Both D2L and D2S mediated a rapid and transient activation of MAPK with dominant activation of p42-kDa MAPK. Pertussis toxin treatment completely abrogated stimulation of MAPK mediated by D2L and D2S, demonstrating that both receptors couple to pertussis toxin-sensitive G proteins in this signaling. Stimulation of MAPK mediated by both D2L and D2S receptor was markedly attenuated by coexpression of the C-terminus of β-adrenergic receptor kinase (βARKct), which selectively inhibits Gβγ-mediated signal transduction. Further analysis of D2L- and D2S-mediated MAPK activation demonstrated that D2L-mediated MAPK activation was not significantly affected by PKC depletion or partially affected by genistein. In contrast, D2S-mediated MAPK activation was potentially inhibited by PKC depletion and genistein was capable of completely inhibiting D2S-mediated MAPK activation. Together, these results suggest that D2L- and D2S-mediated MAPK activation is predominantly Gβγ subunit-mediated signaling and that protein kinase C and tyrosine phosphorylations are involved in these signaling pathways.

The dopamine D2 receptor belongs to the family of seven transmembrane domain G-protein-coupled receptors and is highly expressed in the central nervous system and the pituitary gland (1, 2). 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 (3). Recently, mice lacking in dopamine D2 receptor were created by knocking out the D2 gene (4, 5). 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 (4–6). 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 (7, 8). 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 (7). 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 D2L isoform differs from D2S by the insertion of 29 amino acids in the putative third intracellular loop of the receptor. This loop is involved in the coupling of the receptor to different G proteins. Experiments have shown that the D2 isoforms couple predominantly to the pertussis toxin-sensitive G proteins and ligand activation of both receptors lowers intracellular cAMP levels (9, 10). Thus, it appears that both isoforms have very similar functional properties. However, recent reports have shown that D2L and D2S couple differentially to G-proteins, particularly among Gi subtype proteins, and that their structural difference in the third intracellular loop is believed to provide this distinct specificity for coupling to G-proteins. D2L requires the presence of the α-subunit of the inhibitory G-protein, in particular Gαi2, to inhibit adenylyl cyclase more potently (11, 12). However, it is less clear for D2S and it has been proposed that D2S receptor could couple to Gαi1 and/or Gαi3 and/or Gαo (13, 14). These observations raise the possibility of further differences and increased ability in the coupling of these two do-

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pamine receptors to other effectors, including mitogen-activated protein kinase (MAPK).

The MAPK signaling cascade is a prominent cellular pathway used by many growth factors, hormones and neurotransmitters to regulate diverse physiological functions (15–17). Recently, a number of receptors that couple to heterotrimeric G proteins have been shown to stimulate MAPK activation, including both receptors that couple to Gi and to Gq (18, 19). It has also been observed that the $\beta\gamma$ subunits of G-proteins are able to mediate Ras-dependent MAPK activation (20, 21). 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. The Gqα-mediated pathway utilizes phospholipase C-dependent protein kinase C (PKC) activation, whereas the mechanism of Giα or G$\beta\gamma$-mediated MAPK activation has not been clearly defined.

It has been recently shown that the agonist-stimulated D2 dopamine receptor activates MAPK activity (22, 23). However, it is not known how these two D2 dopamine receptors couple to MAPK signaling pathway and, furthermore, whether there are subtype-related regulations in the signaling pathway utilized by the two D2 receptors in these processes, considering their distinct specificity for coupling to G-proteins.

To characterize the regulation of the G-protein mediated signaling pathway leading to MAPK activation by two isoforms of the dopamine D2 receptor, we expressed mouse D2L and D2S receptors in Chinese hamster ovary (CHO) cells and studied their coupling to MAPK in more detail. In this report, we demonstrate that acute stimulation of D2L and D2S leads to the activation of MAPK and that their MAPK activation is dependent on the $\beta\gamma$ subunits of G-protein. Furthermore, we have found that MAPK activation by two dopamine D2 receptors is regulated by PKC and tyrosine phosphorylations and this, in a differential manner.

MATERIALS AND METHODS

Cell culture and transfection. CHO cells were maintained in F-12 medium supplemented with 10% FBS, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G and 250 mg/ml amphotericin B. Transient transfection of CHO cells was performed using liposome-mediated transfection reagent, DOTAP (Boehringer-Mannheim). Briefly, 60% confluent monolayers in 100-mm culture plates were incubated at 37°C in 8 ml of serum-free medium with transfection mix containing the plasmid DNA encoding the C-terminus of β-adrenergic receptor kinase 1 (βARKct) (24), the plasmid pCH110 carrying the β-galactosidase gene and 45 μl of DOTAP reagent. After 6h, the transfection mixture was then replaced with growth medium. Assays were performed 48 h after transfection. Expression of the βARKct peptide was normalized by measuring β-galactosidase activity (25). The plasmid pRK-βARK1ct (495~689) was kindly provided by Dr. R. J. Lefkowitz (24). In the experiments using PD 98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] (New England Biolabs), cells were preincubated for 30 min with 20 μM PD 98059 prior to dopamine stimulation (26).

Generation of CHO cell lines stably expressing the D2L and D2S dopamine receptors. The full-length dopamine receptors D2L or D2S cDNA were cloned into *Not*I site of the pcDNA3 eukaryotic expression vector (Invitrogen). Each of the subcloned plasmids pcDNA3-D2L and pcDNA-D2S was transfected into CHO cells using DOTAP reagent according to the manufacturer's protocol. Transfection was performed for 6 h at 37°C and terminated by replacing the transfection mixture with culture medium. After 72 h, fresh medium supplemented with 0.5 mg/ml of G418 (Geneticin, Gibco-BRL) was added over the next 2–3 weeks to select stable transfectants expressing neoresistance. G418-resistant colonies were picked and clonal cell lines were obtained by limited dilution. Isolated clones were propagated and total RNA was prepared from them and screened by RT-PCR (reverse transcription-PCR) analysis prior to [^{3}H]spiperone binding assay to identify positive clones, using a pair of oligonucleotide specific for mouse D2 receptor D2R1 and D2R2 (D2R1, 5′-TCTTGCCCACTGCTCTTTGGA-3′; D2R2, 5′-CATTCTTTTCTGGTTTGGCAGGA-3′). Coamplification of the β-actin gene was performed to select the clonal cell line which expresses a relatively high level of D2 receptor. Primers used for the amplification of β-actin cDNA were: B1, 5′-GATGACGATATCGCTGCGCT-3′; B2, 5′-GCTCATTGCCGATAGTGATGACCT-3′. All these oligonucleotides were provided by Genosys (Genosys Biotechnologies, USA). Several clones which express higher levels of D2L and D2S than other clones were selected after hybridization with an internal D2-specific probe (D2R3, 5′-TATACCGGGTCCTCTCTGGGGGGGCT-3′). RT-PCRs yielded products of 399 and 486 bp for the short and long D2 receptor isoforms, respectively, clones with high levels of expression of each isoform of D2 receptor were selected after normalization with respect to the expression of β-actin, then they were subjected to ligand binding assay.

Ligand binding assay. To perform [^{3}H]spiperone binding (4,27) to D2 receptor-transfected CHO cell membranes, cells were first harvested in phosphate-buffered saline and pelleted at 1000*g* for 5 min. After homogenization in 10 mM Tris–HCl (pH 7.5), 5 mM EDTA with a polytron homogenizer (IKA) for 10 s, membranes were isolated by centrifugation at 1000*g* for 10 min. The supernatant was centrifuged at 45,000*g* for 40 min and the pellet was resuspended in the same buffer and centrifuged at the same speed. The final pellet was resuspended in 50 mM Tris–HCl (pH 7.7). Twenty-five micrograms of membrane protein was used for ligand-binding assays with [^{3}H]spiperone (specific activity 2.22–3.33 TBq/mmol, Dupont NEN), as previously described (4, 27). (+)-Butaclamol (1 μM) was used to define non-specific binding. All the binding data were analyzed with the EBDA-Ligand program (Elsevier-Biosoft). The Bmax (maximal binding capacity) value of clones CHOD2L and CHOD2S which were used in this study were found to be 191.4 $\pm$ 0.74 and 176.8 $\pm$ 0.79 fmol/mg protein, respectively.

MAPK activity assay. MAPK activity was assayed by immunodetection of phosphorylated substrate, GST-Elk1 (307~428) fusion protein (New England BioLab) (28). Transfected CHO cells were preincubated overnight in serum-free medium containing pertussis toxin (PTX), phorbol 12-myristate 13-acetate (PMA) or serum-free medium only and then stimulated with Dopamine, PMA or LPA. After stimulation for the indicated time, the cells were washed in ice-cold PBS and then 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 μg/ml leupeptin and 1 mM PMSF. Samples were sonicated 4 times for 5 sec each, centrifuged at 10,000*g* at 4°C for 10 min and the supernatant was collected. Two hundred micrograms of cell lysates was incubated with phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody for 4 h at 4°C, followed by incubation with protein A–Sepharose at 4°C for 1 h. 20 ng of active MAPK (Erk2, New England BioLab) was added to control cell extract as a positive

control. The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$. The kinase reaction was carried out at 30°C for 30 min in kinase buffer with 100 μM ATP and 1 μg of GST-Elk-1 fusion protein. The reaction was terminated by the addition of sample buffer and boiled for 5 min. The proteins were loaded on 10% SDS–polyacrylamide gel and analyzed by immunoblotting with phospho-specific Elk-1 (Ser383) antibody (New England BioLab). Relative densities of the phosphorylated Elk-1 were scanned and quantified using CSC Chemoluminescence Detection Module linked to a Fuji Bio-Imaging analyzer BAS-2500 (Fuji photo film Co., Ltd.).

Immunoblotting analysis. 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 1 h with phospho-specific p44/p42 MAPK antibody or ERK-1/2 (K-23) 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).

RESULTS

To study regulation of the MAPK cascade by D2L/D2S receptors, we used CHO cells stably transfected with mouse cDNA encoding D2L or D2S, respectively. In initial experiments, the expression of each isoform in CHO transfectant expressing D2L (CHOD2L) or D2S (CHOD2S) were analyzed by RT-PCR and further examined by ligand binding assay with the D2 receptor specific-antagonist, [^{3}H]spiperone (see Materials and Methods). We used the clonal cell lines which showed comparable levels of D2 receptor density to permit consistent and comparable analysis of D2L/D2S receptor-mediated responses.

To assess the activation of MAPK, MAPK from the CHO parental cell, CHOD2L and CHOD2S, respectively, was immunoprecipitated with monoclonal antibody against MAPK and its activity was measured by an immunocomplex kinase assay. Immunoprecipitated MAPK phosphorylated the purified recombinant GST-Elk-1 fusion protein (15, 17), a MAPK substrate (Fig. 1a). Recombinant active MAPK was used as a positive control (Fig. 1a). Dopamine (1 μM) stimulation of CHOD2L and CHOD2S cells resulted in a rapid and transient MAPK activation, as measured by subsequent phosphorylation of recombinant Elk-1. Phosphorylation of Elk-1 resulted in a doublet which corresponded to the hyper-and hypophosphorylated Elk-1, as previously described (28). As shown in Fig. 1, however, dopamine induced MAPK activation in CHOD2L (8.24 $\pm$ 0.84-fold over basal) more efficiently than it did in CHOD2S (5.13 $\pm$ 1.89-fold over basal). MAPK activity was maximum at 1 min after stimulation with dopamine. A comparable but partially-decreased activity was shown at 5 min and decreased rapidly thereafter so that at 15 min MAPK activity fell to basal levels (Figs. 1a and 1b) in both cell lines. This activation was comparable to those obtained after stimulation with

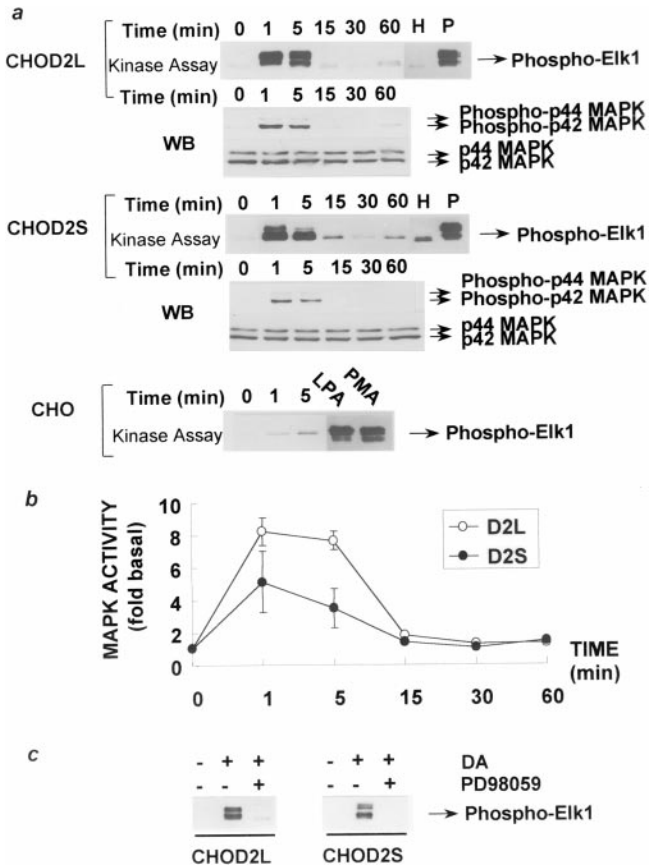

FIG. 1. Time course of MAPK activation by dopamine in CHOD2L and CHOD2S cells and inhibition of MAPK by PD 98059. CHO parental cells and D2L- and D2S-expressing cells (CHOD2L, CHOD2S, respectively) were stimulated with dopamine (1 μM) for the indicated periods of time. For CHO cells, stimulation with LPA (10 μM, 5 min) or PMA (1 μM, 5 min) was also performed. Total cellular extract was immunoprecipitated with a phospho-MAPK antibody. MAPK activity was assayed as described using a GST-Elk-1 fusion protein as the substrate. Phosphorylated Elk-1 is indicated by an arrow. The Thr/Tyr phosphorylation status of MAPK was examined using phosphorylated-Thr202/Tyr204-specific p44/p42 MAPK antibody following dopamine treatments (Phospho-p44/p42 MAPK). Total MAPK (p44/42 MAPK) was analyzed by immunoblotting using Erk1/2 (K-23) antibody as described under Materials and Methods. (a) Representative autoradiogram showing the time course of MAPK activation. Haloperidol (1 μM) was pretreated for 30 min in cells as an antagonist and then dopamine (1 μM) was treated for 1 min (H). Recombinant active MAPK was used as a positive control (P). (b) MAPK activity time course quantification. Relative densities of the phosphorylated Elk-1 were scanned and quantified by CSC chemiluminescence detection module linked to a Fuji Bio-Imaging analyzer BAS-2500. Data are expressed as fold-increase of MAPK activity relative to unstimulated control cells. Values shown represent the mean $\pm$ SE from three independent experiments. (c) Inhibitory effect of PD 98059. The serum-deprived CHOD2L and CHOD2S cells were stimulated with 1 μM dopamine (DA) for 1 min in the absence or presence of 20 μM PD 98059 preincubation for 30 min prior to the dopamine stimulation (26). Cells were lysed and MAPK activity was assayed as described above. Three separate experiments gave similar results.

PMA (Phorbol 12 myristate 13-acetate) or LPA (lysophosphatidic acid) in these cells, which are known to activate MAPK via protein kinase C (PKC) or Gi protein, respectively (Fig. 1a) (18, 24). No activation was observed in non-transfected CHO parental cells (Fig. 1a, lower panel). Haloperidol (1 μM), a D2 receptor-specific antagonist, caused complete inhibition of MAPK activation in CHOD2L and CHOD2S by dopamine, proving that this effect is produced through D2 receptor subtypes (Fig. 1a).

It is well known that the dual phosphorylation of Thr202 and Tyr204 by specific upstream kinases is necessary for full MAPK enzymatic activity (15). The Thr/Tyr phosphorylation status of MAPK was examined using phosphorylated-Thr202/Tyr204-specific p44/p42 MAPK antibody following dopamine treatments. This antibody specifically recognizes the Thr202/Tyr204-phosphorylated active form of p44/p42 MAPK. The MAPK phosphorylation was detected at 1 min after dopamine treatment and returned to its basal level by 15 min (Fig. 1a) showing exact correlation with the time course of MAPK activity stimulated by dopamine. In both cell lines, p42 MAPK was intensely phosphorylated compared to p44 MAPK despite their similar distribution levels in cells (Fig. 1a), indicating that dopamine activates dominantly p42 MAPK in these cells.

The synthetic compound PD 98059 has been characterized as a selective inhibitor of the MAPK pathway by preventing the activation of MEK (MAPK kinase)-1 (26). Pretreatment of cells with 20 μM of PD 98059 for 30 min completely suppressed dopamine-stimulated MAPK activation in CHOD2L and CHOD2S cells (Fig. 1c).

The ability of dopamine to stimulate MAPK activation was clearly mediated by a G-protein coupled pathway, since pretreatment of cells for 16 h with 100 ng/ml pertussis toxin completely abrogated the effect of dopamine on MAPK activation in CHOD2L and CHOD2S cells (Figs. 2a and 2b). PTX alone did not have any effects on basal MAPK activity (data not shown). This inhibition by PTX is also consistent with the selective coupling of D2L and D2S with Gi subfamily proteins (10). Similar to previous reports (24), stimulation via PMA was unaffected by pertussis toxin while the activation of MAPK by LPA was blocked by pertussis-toxin (Figs. 2a and 2b).

A role for $\beta\gamma$ subunits of heterotrimeric G protein ($G\beta\gamma$) in Gi-coupled receptor-mediated MAPK activation has been proposed (20, 21). To determine the role of $G\beta\gamma$ subunits in D2L- and D2S-mediated MAPK activation, we studied the effects of the $G\beta\gamma$ subunit-sequestrant βARKct peptide. βARKct peptide corresponds to the carboxyl terminus of the β-adrenergic receptor kinase containing its $G\beta\gamma$-binding domain, and is reported as a cellular $G\beta\gamma$ antagonist (24). Since expression of βARKct peptide selectively inhibits $G\beta\gamma$-

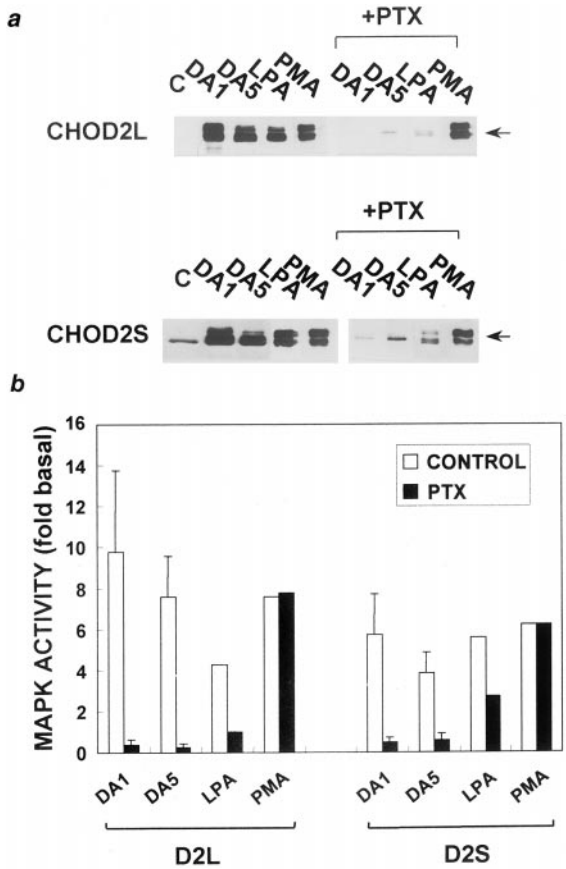

FIG. 2. Effect of pertussis toxin on D2L- and D2S- mediated MAPK stimulation. Cells were incubated overnight in serum-free medium with or without 100 ng/ml pertussis toxin (PTX). They were then stimulated for 1 min (DA1) or 5 min (DA5) with 1 μM of dopamine and for 5 min with LPA (10 μM) or PMA (1 μM). Cells were lysed and MAPK activity was determined as described in the legend to Fig. 1. Phosphorylated Elk-1 is indicated by an arrow. (a) Representative MAPK kinase assay autoradiogram. (b) Quantification of MAPK activity in the presence and absence of PTX. The relative densities of the autoradiograms were measured as described in the legend to Fig. 1. Data indicate mean ± SE from three independent experiments. Values are fold-increase of MAPK activity relative to an unstimulated control sample. Open bar, control; filled bar, pertussis toxin treated.

mediated signal transduction, this peptide can be utilized to distinguish the $G\alpha$ and $G\beta\gamma$ signaling pathways (24). Coexpression of βARKct peptide in CHOD2L and CHOD2S cells markedly affected D2L- or D2S-mediated MAPK activation (Fig. 3). LPA-mediated MAPK activation was βARKct peptide expression-dependent (Fig. 3), as previously reported (24). These results suggest that $G\beta\gamma$ is a principal mediator in MAPK activation in CHOD2L and CHOD2S, providing further evidence of the importance of the $G\beta\gamma$ subunit in Gi-coupled receptor-mediated MAPK activation.

We next examined the effects of the cellular depletion of PKC on MAPK activation mediated by each form of D2 receptor. It has been shown that two iso-

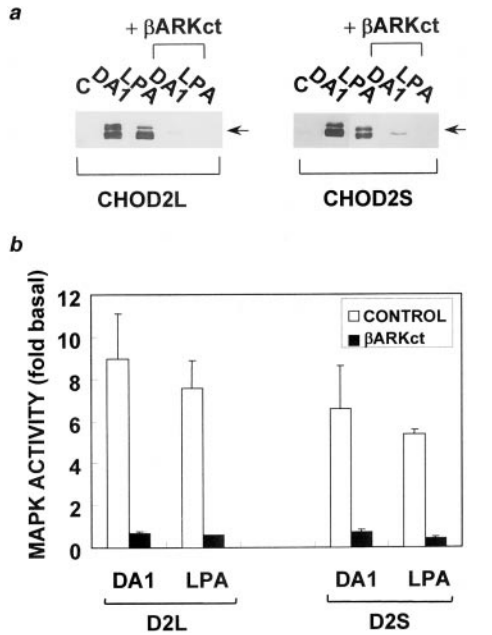

FIG. 3. Effect of βARKct expression on D2L- and D2S-mediated MAPK stimulation. CHOD2L and CHOD2S were transiently transfected with plasmid containing the βARKct cDNA (βARKct) or pRK5 vector alone (control). Transfected cells were incubated for 2 days and then serum-starved overnight. Cells were then stimulated with either 1 μM dopamine for 1 min (DA1) or LPA (10 μM, 5 min) and MAPK activity was determined as described in the legend to Fig. 1. Phosphorylated Elk-1 is indicated by an arrow. (a) Representative autoradiogram showing MAPK assay. (b) Quantification of MAPK activity in the presence and absence of βARK1ct peptide. The relative densities of autoradiograms were determined as described, expression of the βARK1ct peptide was normalized by measuring β-galactosidase activity. Data shown represent the mean ± SE from three independent experiments.

forms of D2 receptor influence the PKC pathway with different sensitivity (29). D2L- and D2S-mediated MAPK activation is distinguished by differences in dependence upon PKC. Figures 4a and 4c shows the effects of cellular PKC depletion in CHOD2L and CHOD2S cells. Following prolonged exposure to PMA, further stimulation with phorbol ester failed to provoke MAPK activation (Fig. 4a), demonstrating that the cells were functionally depleted of PKC activity (30). D2L receptor-mediated MAPK activation was not significantly altered in PKC-depleted cells, whereas the D2S-mediated response was more significantly inhibited by PKC-depletion (by 56%, Figs. 4a and 4c). These data suggest that PKC might be differentially involved in the signaling pathway utilized by D2L and D2S for MAPK activation.

We next assessed a role for protein tyrosine kinases in D2L- and D2S-mediated MAPK activation by pretreating the CHOD2L and CHOD2S cells with genistein, a tyrosine kinase inhibitor (31). As shown in Figs. 4b and 4d, pretreatment with genistein inhibited both D2L- and D2S-mediated MAPK activation. Notably, D2S-mediated MAPK activation was completely attenuated by genistein treatment, whereas D2L-mediated MAPK activation was partially inhibited. LPA-mediated MAPK activation was also affected by genistein, showing a comparable degree of inhibition as observed for D2L (Fig. 4d). These results demonstrated that tyrosine phosphorylation event, as well as PKC, may be differentially involved in D2L- and D2S-mediated MAPK activation and that tyrosine phosphorylation event is indispensable in D2S-mediated MAPK activation.

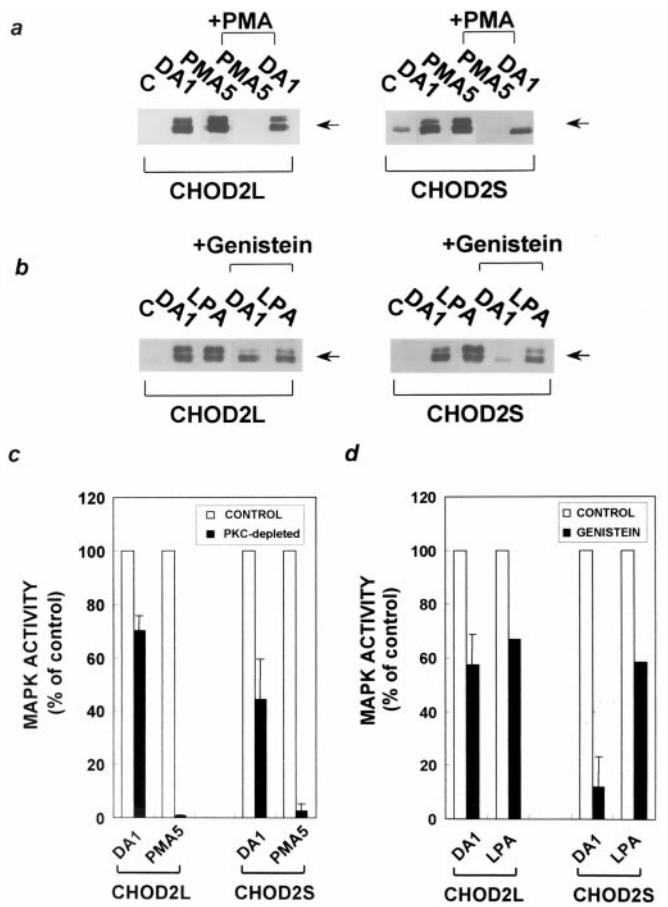

FIG. 4. Effects of PKC depletion and protein tyrosine kinase inhibitor on MAPK activity in CHOD2L and CHOD2S cells. CHOD2L and CHOD2S cells were preincubated overnight in serum-free medium in the presence or absence of PMA (1 μM) to downregulate cellular PKC expression and were then stimulated with 1 μM dopamine for 1 min (DA1) or 1 μM PMA for 5 min (PMA5) (a, c). For protein tyrosine kinase inhibition, serum-starved cells were preincubated for 2h with genistein (100 μM) prior to stimulation with 1 μM dopamine for 1 min, 10 μM LPA for 5 min (b, d). Cell lysates were prepared and then MAPK activity was assayed with GST-Elk-1 as substrate. Phosphorylated Elk-1 is indicated by an arrow. (a and b) Representative autoradiogram showing MAPK assay. (c and d) Quantification of MAPK activity. The relative densities of autoradiograms were determined as described. Data are expressed as the percent of MAPK activity produced by agonist-stimulation in control. Values shown represent mean ± SE from three independent experiments.

DISCUSSION

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. In the present study, using CHO cell lines stably expressing two isoforms of mouse dopamine D2 receptors, we demonstrated that D2L and D2S respectively stimulate MAPK activation in a G$\beta\gamma$ subunits-dependent manner and that their coupling to the MAPK pathway is modulated by PKC and protein tyrosine phosphorylations. Our evidence shows that both D2L and D2S trigger the activation of 42- and 44-kDa MAPK with dominant activation of p42; and this activation is accompanied by Thr/Tyr phosphorylation of these kinases, which corresponds to the activated status of these enzymes. We observed that both D2L- and D2S-mediated MAPK activation is a rapid and transient process as determined by our time course analysis of MAPK activation by dopamine. Both D2L- and D2S-mediated MAPK activation was suppressed by PD 98059, suggesting that MEK-1 is involved as an upstream regulator of MAPK in this pathway.

The MAPK activation by dopamine was totally abrogated by pretreatment of the cells with pertussis toxin, indicating that D2L- and D2S-mediated MAPK activation is dependent on Gi family proteins. Interestingly, the level of MAPK activation was much more pronounced in CHOD2L cells than in CHOD2S cells. This cannot be attributed to differences in the number of receptors between these cells, since the CHOD2L and CHOD2S cells express a comparable level of receptors. Rather, this difference in MAPK activation may imply the differential coupling of D2L and D2S to the MAPK pathway and their different regulation of the MAPK pathway. Indeed, D2L and D2S responded differently to PKC depletion or protein tyrosine kinase inhibitor treatment in this study, suggesting that the two dopamine D2 receptors, D2L and D2S, employ different pathways to activate MAPK or that these two receptors are functionally distinct in terms of efficiency in coupling to the MAPK pathway. It has already been reported that D2L and D2S dopamine receptor isoforms are differentially regulated in other signaling pathways in recombinant CHO cells as well as other cell lines expressing these receptors (29, 32, 33), suggesting that D2L and D2S subtypes are distinct in their coupling to selected second messenger pathways. In addition, several previous studies have demonstrated that D2L-specific insert determines the selectivity of coupling of this isoform to G-proteins (10–13). In a recent study, Welsh *et al.* also observed activation of MAPK by two human dopamine receptors, D2L and D2S (22). However, it is not known if subtype-specific regulation of the MAPK pathway exists by two isoforms of D2 receptors. Altogether, these findings prompted us to examine the MAPK signaling pathway employed by two isoforms of D2 receptor.

We next explored the role of G$\beta\gamma$ subunits in D2 receptor-mediated MAPK activation. It has been shown that G$\beta\gamma$ subunits play a key role in G-protein coupled receptor-mediated MAPK activation (20, 21). In our study, expression of βARKct, which specifically blocks signaling mediated by the G$\beta\gamma$ subunits, does severely affect both D2L- and D2S-mediated MAPK activation. These data demonstrate that the G$\beta\gamma$ subunits are the key mediators in D2L- and D2S-mediated MAPK activation as shown for other G-protein coupled receptors. In particular, MAPK activation via Gi-coupled receptors is proposed to be mediated by G$\beta\gamma$ subunits and occurs as a direct result of Ras activation. Indeed, in COS-7 cells transfected with plasmids encoding Gi-coupled receptors (alpha 2-adrenergic and M2 muscarinic), the activation of Ras and MAP kinase was significantly reduced in the presence of the coexpressed βARKct peptide, the G$\beta\gamma$-subunits antagonist (24). In contrast, Ras-MAPK activation mediated through a Gq-coupled receptor (alpha 1-adrenergic) or the tyrosine kinase epidermal growth factor receptor was unaltered by this G$\beta\gamma$-subunits antagonist (18, 24). The mode of communication used by the G$\beta\gamma$ subunits in MAPK activation still remains an intriguing question. It has been proposed that this interaction may be achieved through the recruitment of other proteins by means of the pleckstrin homology domain interaction with G$\beta\gamma$ subunits, leading to Ras activation (34). Interestingly, a recent paper reported Ras-dependent MAPK activation by D2L receptor in rat C6 cells (23). Taken together, our study strongly supports the hypothesis that the MAPK signaling pathway utilized by D2 receptors involves G$\beta\gamma$-mediated Ras activation.

We have observed that D2L- and D2S-mediated MAPK activation can be affected by PKC depletion and protein tyrosine kinase inhibitor, but with different sensitivity. D2L-mediated MAPK activation is not significantly affected by PKC depletion, while D2S-mediated MAPK activation is potentially attenuated. Genistein treatment has both attenuated D2L and D2S-mediated MAPK activation but with different efficiency. D2L-mediated MAPK activation was inhibited by 43%, showing a comparable level of inhibition observed for LPA by genistein, whereas D2S-mediated MAPK activation was completely blocked by genistein. These data demonstrate that tyrosine phosphorylation is required for both D2L- and D2S-mediated MAPK activation, but distinct mechanisms of MAPK activation may exist for the two D2 receptors and, obviously, the role of tyrosine kinase(s) is crucial for D2S-mediated signaling. Alternatively, though genistein is known to inhibit several classes of protein-tyrosine kinases (31), it is conceivable that D2L and D2S em-

ploy different types of protein tyrosine kinases in their signaling.

It has been proposed that PKC and protein tyrosine phosphorylation may represent the effector system linking the G-protein coupled receptor and the MAPK cascade. In particular, Gq- and Go-coupled receptors were shown to mediate PKC-dependent MAPK activation (18, 34). On the other hand, G$\beta\gamma$ subunits-mediated MAPK activation is known to be dependent on a tyrosine phosphorylation event (21) and this was also observed for certain types of Gi-coupled receptor (21, 35–37). It has been reported that Src family kinases can be an important component in MAPK activation mediated by acetylcholine muscarinic m2 receptor (36) and LPA receptor (37). Furthermore, the release of G$\beta\gamma$ subunits was also shown to promote the tyrosine phosphorylation of Shc to mediate Ras-dependent MAPK activation (35, 39). Interestingly, the D2L receptor, which is known to couple preferentially to the Gαi2 protein (10,11), seems to be relatively independent of PKC or partially dependent on protein tyrosine kinase. It is possible that Gαi2 protein requires other mediators than PKC and tyrosine kinase to stimulate MAPK in D2L-mediated signaling. D2S-mediated MAPK activation, which is PKC-dependent and highly sensitive to tyrosine kinase inhibitor, provides the possibility that D2S couples to multiple G proteins in this pathway. Taken together, these results lead us to propose that the differential choice of effectors and further differences in signaling pathways utilized by two D2 receptors can be derived from their differences in the specificity for coupling G proteins. Future experiments will be required to assess the role of specific G proteins and their interaction with other mediators in D2L- and D2S-mediated MAPK activation. 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 transduction and physiological actions of two dopamine D2 receptors.

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