0026-895X/01/6004-857–864$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 4
904/934973
Mol Pharmacol 60:857–864, 2001
Printed in U.S.A.

D_4 Dopamine Receptor Differentially Regulates Akt/Nuclear Factor-κB and Extracellular Signal-Regulated Kinase Pathways in D_4MN9D Cells

XUECHU ZHEN, JIE ZHANG, GERARD P. JOHNSON, and EITAN FRIEDMAN

Laboratory of Molecular Pharmacology, Department of Pharmacology and Physiology, MCP Hahnemann School of Medicine, Philadelphia, Pennsylvania

Received February 21, 2001; accepted July 12, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The present study was designed to investigate the role of D_4 dopamine receptors in regulating the Akt/nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase (ERK) signaling pathways. The D_4 dopamine receptor agonist PD168077 induced time- and dose-dependent activation of Akt and ERK in D_4MN9D cells that stably express D_4 dopamine receptors. Maximal Akt and ERK stimulation was achieved at 1 μM PD168077. The agonist-mediated stimulations of Akt and ERK were abolished when cells were preincubated with 50 ng/ml PTX or with 1 μM L745,870, a D_4 dopamine receptor antagonist, indicating that activation of the Akt or ERK pathways is mediated by D_4 dopamine receptors and require a pertussis toxin-sensitive G protein. We also detected a time- and dose-dependent activation of NF-κB. Activation of NF-κB by 1 μM PD168077 was attenuated in D_4MN9D cells that were transfected with a kinase-deficient Akt but not in cells transfected with a dominant negative Ras (N17Ras), suggesting that NF-κB activation requires Akt but is independent of Ras. In contrast, the transfection of N17Ras into D_4MN9D cells blunted D_4 dopamine receptor-mediated ERK activation, indicating a Ras-dependent mechanism. Moreover, PP2 (20 nM), an inhibitor of Src, blocked D_4 receptor-mediated SHC phosphorylation and ERK activation. In contrast, transfection of a kinase-dead Akt did not alter D_4 receptor-stimulated ERK. However, PP2 and the mitogen activated protein kinase kinase inhibitor PD98059 did not change D_4 receptor-mediated Akt/NF-κB activation. All these indicate that distinct mechanisms mediate ERK and Akt/NF-κB activation by D_4 dopamine receptor stimulation. We also demonstrated that D_4 receptor-stimulated cell proliferation is mediated by the Src/SHC/Ras/ERK pathway.

G Protein-coupled receptors (GPCRs) play a critical role in regulation of cell proliferation, differentiation, and survival. These receptors transduce signals by coupling to heterotrimeric G proteins. Recent studies have identified members of the mitogen-activated protein kinase (MAPK) family as critical intracellular signaling pathways that are activated by GPCRs. Ras-dependent and-independent mechanisms were demonstrated to be involved in MAPK activation by GPCRs (Garnovskaya et al., 1996; Lopez-Ilasaca et al., 1997; Sugden and Clerk, 1997). The mechanism by which pertussis toxin (PTX)-sensitive Gi-coupled receptors activate MAPK was reported to be Ras-dependent and mediated via Gβγ subunits (Gutkind, 1998). Moreover, the linkage between GPCRs and Ras/MAP kinase activation was shown to require tyrosine phosphorylation of the adaptor protein SHC and its subsequent association with Grb2. Tyrosine kinases such as Src play essential roles in mediating the phosphorylation of SHC by GPCRs (Luttrell et al., 1996, 1997) and the SHC-Grb2/Ras/MAP kinase cascades constitute important mitogenic pathway initiated by GPCRs (Sugden and Clerk, 1997; Gutkind, 1998).

GPCRs were also shown to activate nuclear factor NF-κB and to regulate the expression of inducible genes (Shahrestanifar et al., 1999; Xie et al., 2000). Under basal conditions, NF-κB is a heterodimer composed of 50- and 60-kDa subunits that exist as a complex bound to a member of the IκB family of inhibitory proteins in cytosol. A stimulus triggers the dissociation of NF-κB from IκB; the latter is rapidly degraded. Cytoplasmic NF-κB is then translocated into the nucleus, where it regulates gene transcription. MAP kinases and Akt were both shown to regulate NF-κB activation (Schulze-Ostehoff et al., 1997; Kane et al., 1999; Ozes et al., 1999; Xie et al., 2000). Many GPCRs regulate Akt (Murga et al., 1998 and 2000) and its downstream transcription factor, NF-κB, com-

This work was supported by United State Public Health Service Grants NS29514 and DA11029.

ABBREVIATIONS: GPCR, G protein-coupled receptor; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; MAP, mitogen activated protein; SHC, Src homology 2-containing protein; MBP, myelin basic protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PKI, protein kinase A inhibitor; EMSA, electrophoretic mobility shift assay.

prising a major cellular pathway in the regulation of cell survival and differentiation (Marte and Downward, 1997).

Dopamine receptors are members of the GPCR superfamily. The five cloned dopamine receptors are distinguished based on pharmacological and biochemical characteristics (Sibley et al., 1993; Tang et al., 1994; Missale et al., 1998). Members of the D_2-like dopamine receptor family, D_2, D_3, and D_4, couple to Gi protein. Activation of these dopamine receptors initiates a number of signaling events, including inhibition of adenylyl cyclase and opening of K^+ channels. Stimulation of dopamine receptors was also demonstrated to differentially regulate the MAPKs. Although stimulation of D_2 and D_3 dopamine receptors was shown to activate ERK in vitro and in vivo (Welsh et al., 1998; Cussac et al., 1999; Yan et al., 1999; Cai et al., 2000) and to evoke a mitogenic response through the Ras/ERK pathway (Luo et al., 1998), D_1 dopamine receptor stimulation activates p38 MAPK via a protein kinase A-dependent pathway (Zhen et al., 1998). The D_4 dopamine receptor has garnered attention because of its potential role in schizophrenia and in the psychopharmacology of antipsychotic drugs (Van Tol et al., 1991; Helmeste et al., 1996). However, it remains to be determined whether D_4 dopamine receptors participate in the regulation of the ERK pathway, as do other D_2-like dopamine receptors.

Enhanced NF-κB activation was observed in brains from patients with Parkinson's disease (Hunot et al., 1997), suggesting its possible regulation by the dopaminergic system. However, the regulation and signaling mechanism of Akt and its associated NF-κB cascade by dopamine receptors has not been addressed. In the present study, we employed a stable D_4 dopamine receptor-transfected MN9D cell line (D_4MN9D) to examine the possible role of the Akt/NF-κB and ERK pathways in D_4 dopamine receptor signaling. Our results constitute the first evidence that the D_4 dopamine receptor regulates the Akt/NF-κB and ERK pathways via distinct mechanisms.

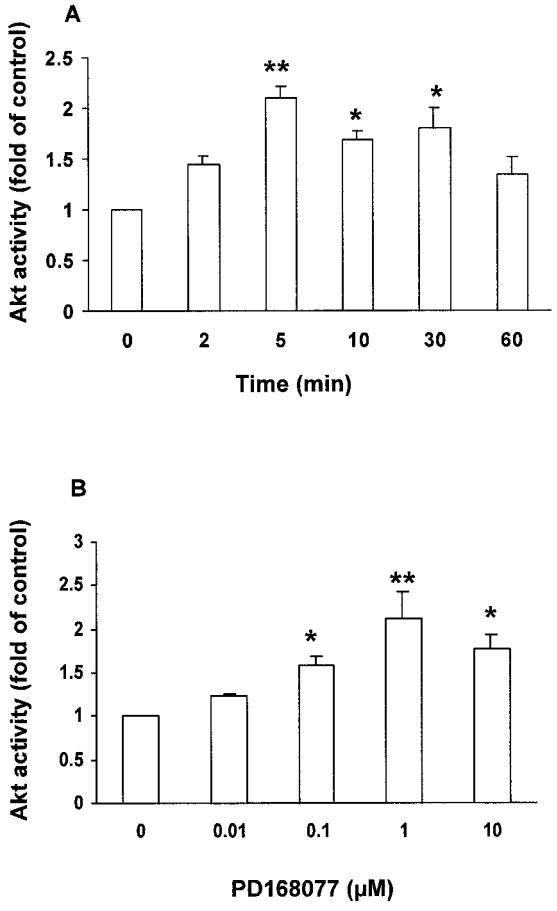

Fig. 1. Time- and dose-dependent activation of Akt by the selective D_4 dopamine receptor agonist, PD168077. D_4MN9D cells were cultured in DMEM containing 10% FBS and allowed to reach 70 to 80% confluence. The medium was replaced with DMEM containing 1% FBS overnight before addition of PD168077 for the indicated times or doses. Akt activity was assessed as described under *Experimental Procedures*. A, time course for Akt activation induced by 1 μM PD168077. B, dose response for PD168077–mediated Akt activation during 5 min of stimulation. The results of at least three experiments are summarized; data are expressed in fold stimulation relative to control. $^*p < 0.05$, $^{**}p < 0.01$.

Experimental Procedures

Materials. PD168077 maleate (a selective D_4 dopamine receptor agonist), L745,870 trihydrochloride (antagonist), and quinpirole (D_2-like receptor agonist) were purchased from Tocris Cookson, Inc. (Baldwin, MO). NF-κB consensus oligonucleotide and T4 kinase were from Promega (Madison, WI). PD98059, LY294002, PP2 and PP3 were obtained from CalbioChem (La Jolla, CA). Myelin basic protein (MBP) and PKI were purchased from Sigma (St. Louis, MO). Electrophoresis reagents were obtained from Bio-Rad (Richmond, CA). Anti-phosphotyrosine antibody, anti-SHC, and Crosstide were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Akt, ERK_2, IκBα, and anti-Grb2 and proteins A/G PLUS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA 12CA5 antibody was from Roche Molecular Biochemicals (Indianapolis, IN). Horseradish peroxidase-linked anti-rabbit secondary antibodies were obtained from Pierce (Rockford, IL). [γ^{32}P]ATP (3000 Ci/mmol) and [^{3}H]thymidine (76 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). LipofectAMINE transfection reagent was obtained from Invitrogen (Carlsbad, CA). Other reagents were purchased from standard laboratory suppliers.

Cell Culture and Cotransfection. Wild-type MN9D cells and D_4 dopamine receptor-transfected MN9D cells (D_4MN9D) were kindly provided by Dr. K. L. O'Malley (Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO). The MN9D cell line is a stable immortalized clonal cell line established by the fusion of the neuroblastoma cell N18TG2 with embryonic mouse mesencephalic dopamine-producing neurons. This cell line does not express native dopamine receptors yet exhibits features of neuronal cells and has been demonstrated to be useful in the study of dopamine receptor-mediated signal transduction (Tang

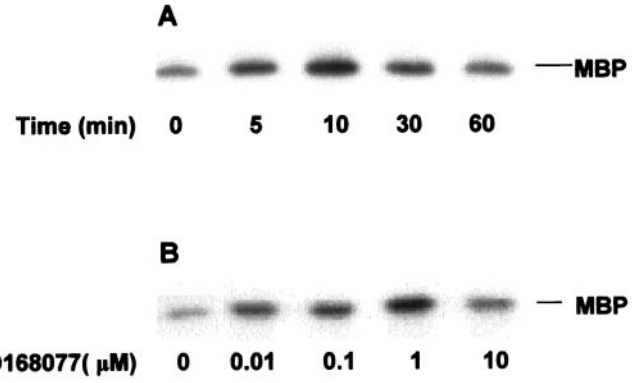

Fig. 2. D_4 dopamine receptor mediates ERK activation in D_4MN9D cells. Cells were treated as described in Fig. 1 for the indicated times (A) or with the indicated concentrations of PD168077 for 10 min (B). ERK activity was assessed using MBP as substrate. Representative autoradiograms of three independent experiments that yielded similar results are shown for each experiment.

et al., 1994; Yamaguchi et al., 1997). Plasmids expressing Myr-Akt (activated Akt) and kinase-deficient Akt-K179 M (KD-Akt) were gifts from Dr. R. N. Pittman (University of Pennsylvania, Philadelphia, PA). D_4MN9D cells were cultured in DMEM as described previously (Yamaguchi et al., 1997). Cells were transfected by the LipofectAMINE method using the indicated amounts of plasmid encoding N17Ras, Myr-Akt, or KD-Akt. After 48 h, cells were treated with various agents and harvested for assays.

Preparation of Cell Lysates. When the cells were 70 to 80% confluent, the medium was replaced with low serum DMEM (1% FBS) and kept overnight before experiments. After incubation with various agents, the reactions were stopped by aspiration of the medium and the cells were washed twice with cold phosphate-buffered saline. For immunoblot and phosphorylation analyses, cells were lysed in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 3 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM PMSF, and proteinase inhibitors). In preparation for the ERK activity assay, cells were lysed in buffer B (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 20 mM β-glycerophosphate, 1 mM EGTA, 20 mM NaF, 3 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% Nonidet P-40). The lysis buffer for the Akt assay was described previously (Murga et al., 2000). Lysates were centrifuged at 12,000*g* for 15 min at 4°C and the protein content in the supernatant was determined. Aliquots of supernatants were used in the immunoprecipitation or immunoblotting experiments as described below.

In Vitro Immune Complex Kinase Assays. For immunoprecipitation, 3 μg of anti-ERK2 or anti-Akt antibodies were added to aliquots (200 μg of protein) of cell lysates and incubated overnight at 4°C before the addition of 15 μl of protein A/G PLUS agarose and incubated for 2 h at 4°C. The immune complex was washed three times with buffer B and twice with the corresponding kinase assay buffer (see below). Aliquots of immunoprecipitates (10 μl) were boiled in an equal volume of 2× sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose, the filter was blotted with anti-ERK2 (which reacts predominantly with ERK2, exhibiting a slight cross-reactivity with ERK1) or Akt antibodies to confirm the equivalence of precipitated enzyme. ERK kinase activity was assessed for 20 min at 30°C in the presence of 50 μM [γ^{32}P]ATP (5 μCi), and 0.2 mg/ml MBP as described previously (Zhen et al., 1998). The reaction was terminated by adding an equivalent volume of 2× sample buffer, boiled for 5 min, and the products were resolved by SDS-PAGE. The gels were stained with Coomassie Blue to confirm equivalence of loaded substrate. Phosphorylated MBP was assessed by autoradiography and quantified by densitometry. Alternatively, the radioactivity incorporated into MBP was determined by scintillation counting (see below). The Akt kinase assay was performed in 20 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 20 μM ATP, 1 mM DTT, 10 μCi of [γ-^{32}P]ATP, and 30 μM Crosstide in the presence of PKI (a protein kinase A inhibitor) for 20 min at 30°C. The reaction was stopped by addition of 200 mM EDTA-bovine serum albumin solution (15 μl) and 20 μl were spotted onto P81 paper and washed extensively in 0.425% phosphoric acid. Radioactivity incorporated into Crosstide was determined by scintillation counting as described previously (Zhen et al., 1998).

Immunoblotting. For the assay of tyrosine phosphorylation of SHC, 0.5 mg of supernatant protein was incubated overnight at 4°C with 3 μg of anti-SHC polyclonal antibody. After the addition of 15 μl of protein A/G PLUS agarose, immunoprecipitates were collected and washed three times with buffer A. The immune complex was resuspended in 40 μl of sample buffer, separated by SDS gel electrophoresis, and transferred to nitrocellulose membranes. The blots were probed with anti-phosphotyrosine antibody. The signals were visualized with the Supersignal Western Blot Detection System (Pierce). In some experiments, membranes containing SHC immunoprecipitates were stripped and reprobed with anti-Grb2 antibody to assess SHC-Grb2 complex formation. For immunoblots, equal amounts of protein were subjected to 10 or 12% SDS-PAGE, blotted, and the membranes were probed with anti-IκBα or anti-HA antibody, respectively.

Nuclear Extraction. Nuclear protein extraction was prepared as described previously (Pan et al., 1999; Shahrestanifar, 1999). Briefly, control or treated cells grown in 100-mm^2 culture dishes were washed in cold phosphate-buffered saline and collected by centrifugation. All procedures were conducted at 4°C. Cells were suspended in buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 2 mM DTT, and 1 mM PMSF, and proteinase inhibitors for 10 min on ice. After adding Nonidet P-40 (to a final concentration of 0.5%) and

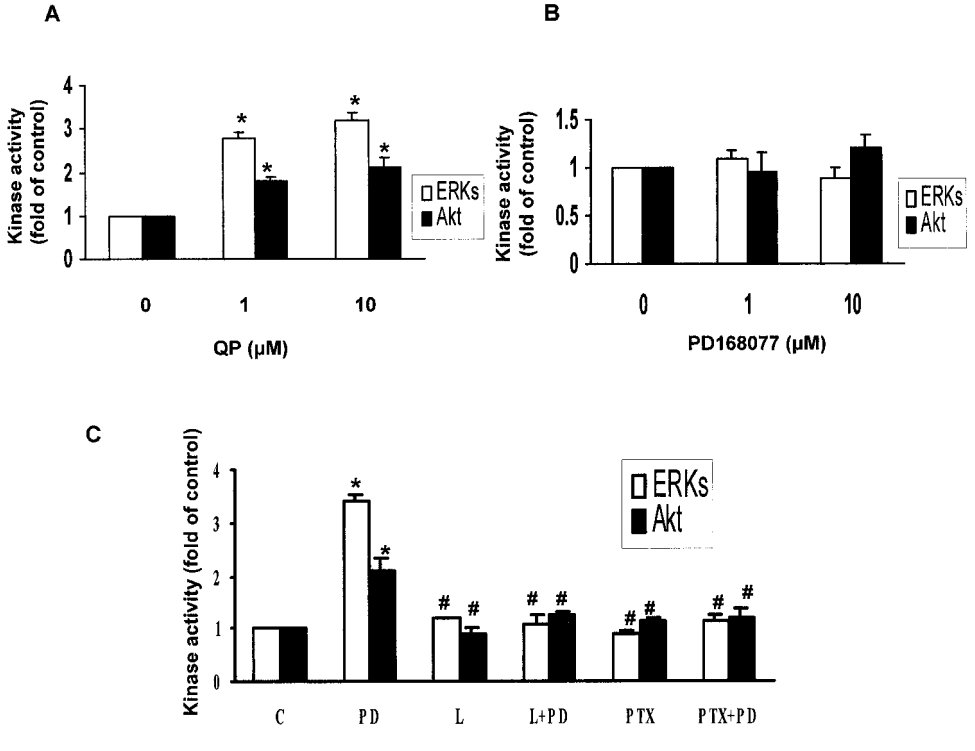

Fig. 3. PTX-sensitive G protein is involved in activation of ERK and Akt by D_4 dopamine receptor stimulation. D_4MN9D (A) and MN9D (B) cells were incubated with the indicated concentrations of quinpirole (QP) (A) or PD168077 (B) for 5 min. ERK and Akt activities were measured in cell lysates. C, D_4MN9D cells were incubated with vehicle (C), 100 ng/ml pertussis toxin (PTX) for 6 h, or 1 μM L745,870 (L) for 30 min before and during incubation with 1 μM PD168077 for 10 min. Akt and ERK activities were assayed. The results summarize at least three independent experiments and are expressed as mean ± S. E.M. * $p < 0.01$ compared with control; # $p < 0.01$ compared with stimulation with PD168077.

incubation for an additional 10 min, the lysate was centrifuged for 5 min at 12,000*g*. The pellet was resuspended in buffer [20 mM HEPES, pH 7.8, 0.42 M NaCl, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, and 10% (v/v) glycerol], and agitated at 4°C for 30 min. After centrifugation at 12,000*g* for 10 min, the protein content of the supernatant (nuclear fraction) was determined by Bradford assay (Bio-Rad) and aliquots were stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA). Double-stranded oligonucleotides containing a specific consensus sequence for NF-κB were labeled with ^{32}P using T_4 polynucleotide kinase and purified with G-50 spin columns. The binding reaction was conducted in 20 μl of reaction buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol) containing 10 μg of nuclear extract and 1 μg of poly(dI.dC), and ^{32}P-labeled oligonucleotide at room temperature for 20 min. After addition of loading buffer, the samples were separated by electrophoresis on 5% polyacrylamide gels in 0.5× Tris/borate/EDTA buffer. The gel was dried and exposed to film at −80°C. Appropriate positive and negative controls were included in the assay to ensure the specificity of the reaction.

Measurement of Cell Proliferation. Cells were seeded in 24-well plates and grown to 70 to 80% confluence. The medium was replaced with fresh medium containing 0.5% FBS and the cells were incubated overnight before the addition of test agents. The incubation continued for 18 h before the addition of [^3H]thymidine (0.5 μCi/ml) and cells were harvested 6 h later. Incorporation of [^3H]thymidine was determined by liquid scintillation counting.

Data Analysis. Unless otherwise indicated, data were analyzed by analysis of variance followed by Newman-Keuls test. $p < 0.05$ was considered significant.

Results

D_4 Dopamine Receptor Regulates Akt and ERK Pathways in D_4MN9D Cells. Subconfluent D_4MN9D cells were treated with the selective D_4 dopamine receptor agonist PD168077, and Akt or ERK activity was determined. A time-dependent activation of Akt was observed; maximal stimulation was achieved at 5 min and maintained for at least 30 min (Fig. 1A). Stimulation of Akt was detected with 0.1 μM PD168077, reached a maximal 2-fold stimulation at 1 μM, and declined at 10 μM (Fig. 1B). The kinetics of ERK stimulation indicates that maximal stimulation by 1 μM PD168077 occurred at 10 min and returned to basal levels by 30 min (Fig. 2A). As was the case for Akt, maximal stimulation of ERK was achieved with 1 μM PD168077 (Fig. 2B). Quinpirole, an agonist of the D_2 dopamine receptor family, also activated ERK and Akt (Fig. 3A) in these cells. In contrast to this agonist-specific effect in D_4MN9D cells, treatment of MN9D cells that do not express the D_4 dopamine receptor with PD168077 did not stimulate either ERK or Akt (Fig. 3B). The results, therefore, suggest that activation of ERK and Akt are mediated by D_4 dopamine receptors in D_4MN9D cells. Indeed, a selective D_4 receptor antagonist, L745,870, completely blocked the stimulatory effects of PD168077 on ERK and Akt in D_4MN9D cells (Fig. 3C). Moreover, PTX attenuated the PD168077-induced stimulation of ERK and Akt, indicating that a PTX-sensitive G protein is involved in D_4 receptor-mediated activation of Akt and ERK (Fig. 3C).

Stimulation of the D_4 Dopamine Receptor Elicits Activation of NF-κB. We next tested whether NF-κB is also activated by stimulation of the D_4 dopamine receptor. Stimulation of the receptor resulted in a time- and dose-dependent activation of NF-κB in D_4MN9D cells (Fig. 4, A and B). Increased NF-κB binding activity in the EMSA was observed at 30 min and achieved maximal stimulation after 120 min of receptor stimulation. Maximal stimulation of NF-κB was achieved at 1 μM PD168077, a dose similar to that observed for Akt activation. In agreement with the increases in NF-κB binding activity noted in the EMSA, decreases in IκBα protein levels were observed upon receptor stimulation (Fig. 4C, PD). Furthermore, pretreatment of cells with 1 μM L745,870 attenuated the PD168077-induced decline in IκBα (Fig. 4C), indicating that activation of NF-κB in D_4MN9D cells is mediated by the D_4 receptor. The data, therefore, indicate that D_4 dopamine receptor stimulation activates NF-κB in a time- and dose-dependent manner.

Requirement of Akt, Not ERKs for D_4 Dopamine Receptor-Mediated NF-κB Activation. NF-κB is an important downstream signaling molecule in the Akt and MAP kinase pathways. We, therefore, sought to determine whether Akt is involved in the activation of NF-κB by the D_4 receptor stimulation. Transiently transfection of plasmids expressing Myr-Akt into D_4MN9D cells enhanced D_4 receptor-stimulated NF-κB activation as assessed by the level of IκBα, while transfection of the KD-Akt into D_4MN9D cells attenuated D_4 dopamine receptor-mediated NF-κB activation (Fig. 5A, left panel). Expression of Akt was monitored using anti-HA antibody (Fig. 5A, right panel). In contrast, inhibition of MEK with PD98059, did not change D_4 receptor-mediated NF-κB activation, Similarly a selective Src inhibitor (PP2) that blocked D_4 receptor-induced ERK activation (see below) did not alter the receptor-activated NF-κB (Fig. 5B). These observations suggest that D_4 dopamine receptor-mediated NF-κB activation requires Akt but not ERK activity.

Ras Is Involved in D_4 Dopamine Receptor-Mediated ERK, but Not Akt and NF-κB Activation. Ras-dependent and -independent mechanisms were reported to regulate the ERK and Akt/NF-κB pathways. To further define the signaling cascades involved in the activation of ERK and Akt/NF-κB by the D_4 dopamine receptor, we tested the role of Ras in these pathways. Transient transfection of the Ras dominant negative N17Ras into D_4MN9D cells induced inhibition of D_4 receptor-mediated ERK activation (Fig. 6A). However,

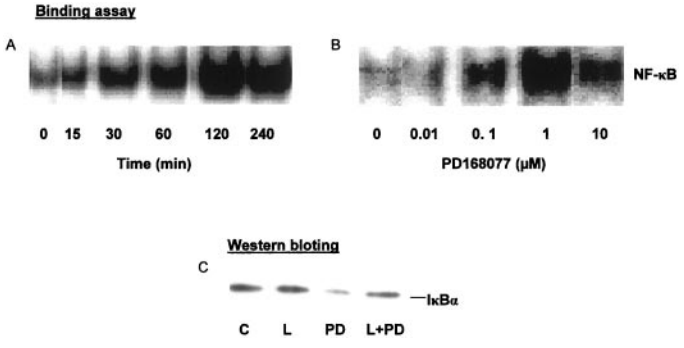

Fig. 4. D_4 dopamine receptor mediates the activation of NF-κB in D_4MN9D cells. D_4MN9D cells were treated for the indicated time periods with 1 μM PD168077 (A) or with various concentrations of PD168077 for 2 h (B), and NF-κB binding activity in nuclear extracts was assessed by EMSA. C, cells were preincubated with vehicle (C) or 1 μM L745,680 (L) for 30 min before and during the addition of 1 μM PD168077 (PD) for additional 2 h. Cells were lysed, and IκBα protein was assessed by Western blotting using anti-IκBα antibody. All experiments were repeated at least three times and a representative autoradiograph or blot is shown.

neither Akt (Fig. 6A) nor NF-κB (Fig. 6B) activation was altered in N17Ras-transfected cells, indicating that D_4 dopamine receptor stimulation activates ERK via a Ras-dependent mechanism, whereas Akt and NF-κB activation seems to be independent of Ras. To further check the possible interaction between ERK and Akt/NF-κB, we measured D_4 receptor-stimulated ERK activation in D_4MN9D cells transiently expressing Myr-Akt, KD-Akt, or empty plasmid. As shown in Fig. 6C, PD168077-mediated ERK activation was not changed by transfection of any of these plasmids, indicating that Akt is not an upstream signaling molecule in the ERK pathway. Similarly, inhibition of ERK by PD98059 exhibited no effect on D_4 receptor-stimulated Akt activation (Fig. 6D). Taken together, D_4 dopamine receptors seem to regulate the ERK and Akt/NF-κB pathways via independent mechanisms in D_4MN9D cells.

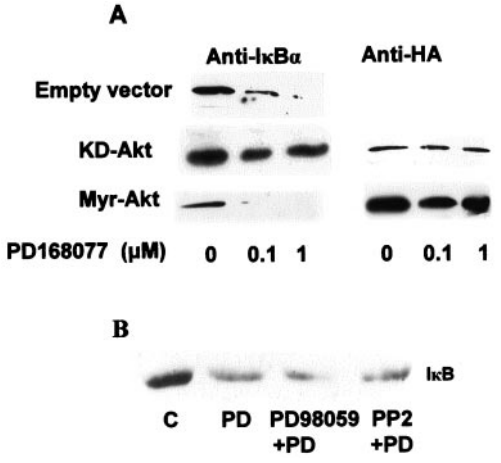

Fig. 5. Requirement of Akt but not ERK or Src for D_4 dopamine receptor-mediated NF-κB activation. A, D_4MN9D cells were transiently transfected with 3 μg of plasmids expressing KD-Akt, Myr-Akt, or with empty vector, and 48 h later, cells were incubated in medium containing 1% serum and stimulated with various concentrations of PD168077 for 2 h. Cells were lysed and NF-κB activation was assessed by measuring the expression of IκBα (left panel, anti-IκBα.). Akt expression was monitored by anti-HA antibody. Representative blots are shown. B, D_4MN9D cells were pretreated with either 10 μM MEK inhibitor PD98059 or 20 nM PP2 for 60 min before and during stimulation of 1 μM PD168077 for an additional 2 h. NF-κB activation was assessed as described above. The experiments were repeated three times with similar results. C, control; PD, PD168077.

Role of Src in D_4 Receptor-Mediated Tyrosine Phosphorylation of SHC and ERK Activation. We have demonstrated that D_4 dopamine receptor stimulation activates ERK via a Ras-dependent but Akt-independent mechanism. To further define the upstream signaling cascades for D_4 dopamine receptor-mediated ERK activation, cells were treated with 1 μM PD168077 and cell lysates were immunoprecipitated with anti-SHC antibody. The immune complex was resolved on SDS-PAGE and blotted with anti-phosphotyrosine antibody. As seen in Fig. 7A, a rapidly increase in tyrosine-phosphorylated SHC (both 52- and 46-kDa isoforms) was observed at 2 min. The 52-kDa SHC band was the predominant type activated in response to D_4 receptor stimulation with PD168077. Moreover, a parallel increase in the association of SHC with Grb2 was also observed when the blots were stripped and reprobed with anti-Grb2 antibody (Fig. 7A, bottom), suggesting that D_4 dopamine receptor-

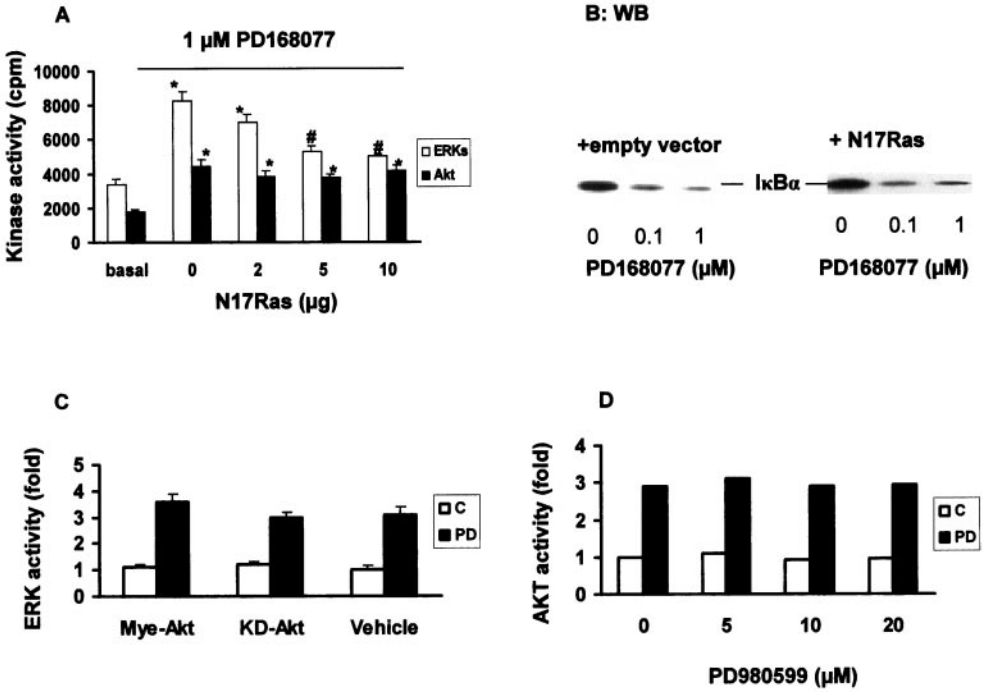

Fig. 6. Requirement of Ras but not Akt for D_4 dopamine receptor-mediated ERK activation in D_4MN9D cells. A, D_4MN9D cells were transiently transfected with various concentrations of dominant negative Ras, N17Ras; the cells were stimulated with 1 μM PD168077 or vehicle for 5 min, lysates were prepared, and ERK and Akt activities were assessed. The results are expressed as mean ± S. E. of three independent experiments. B, D_4MN9D cells transfected with empty vector or N17Ras were stimulated with various concentrations of PD168077 for 2 h. Cell lysates were prepared and blotted with anti-IκBα antibody. C, D_4MN9D cells were transiently transfected with Myr-Akt, KD-Akt, or empty plasmid. The cells were then stimulated with 1 μM PD168077 for 10 min, and ERK activity was then determined in cell lysates. Results are expressed fold change over control and summarized as mean ± S.E. of three independent experiments. D, D4 MN9D cells were preincubated with 10 μM MEK inhibitor PD98059 for 60 min before and during addition of 1 μM PD168077 for 5 min, Akt activity was detected as described in Fig. 1. The experiments were repeated two times with similar results. $^*p < 0.01$, compared with basal control. #$p < 0.01$, compared with PD168077 stimulation. C, control; PD, PD168077.

mediated ERK activation involves SHC-Grb2 association. We next tested the role of the tyrosine kinase Src in D_4 receptor-mediated SHC phosphorylation and ERK activation. PP2, a selective Src inhibitor (20 nM), abolished PD168077-stimulated SHC phosphorylation, whereas PP3 (20 nM), a negative control for PP2, did not affect SHC phosphorylation (Fig. 7B). Moreover, 20 nM PP2 that was sufficient to abolish SHC phosphorylation also blocked PD168077-mediated ERK activation. In contrast, the PI-3 kinase inhibitor LY294002 (20 μM) did not change D_4 receptor-stimulated ERK activity (Fig. 7C). These observations clearly indicate that Src is responsible for D_4 dopamine receptor-stimulated SHC phosphorylation and ERK activation.

ERK Activation Is Responsible for the Mitogenic Action of D_4 Dopamine Receptor Stimulation. The data summarized in Fig. 8 illustrate that the selective MEK inhibitor PD98059 (10 μM), which blocks the activation of ERK, abolished cell proliferation induced by 1 μM PD168077 in D_4MN9D cells. Moreover, selective inhibition of Src by 20 nM PP2, which blocked SHC phosphorylation and ERK activation, also attenuated D_4 receptor-stimulated cell proliferation. In contrast, SN50 (50 μg/ml), a cell-permeable inhibitor of NF-κB translocation, exhibited no effect on D_4 receptor-stimulated cell proliferation in D_4MN9D cells. The results clearly implicate Src kinase-dependent ERK activation in mediating the mitogenic response to D_4 dopamine receptor stimulation.

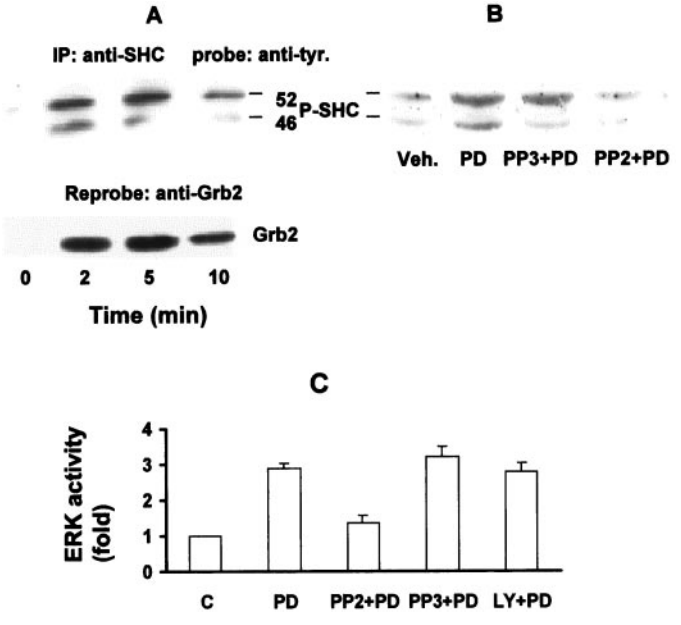

Fig. 7. D_4 dopamine receptor-mediated activation of ERK involves Src-mediated SHC-Grb2 association. A, D_4MN9D cells were incubated with 1 μM PD168077 for the indicated times; cells were lysed and equal aliquots of lysate protein were precipitated with anti-SHC antibody. Immunoblots were performed and tyrosine-phosphorylated SHC was detected with anti-phosphotyrosine antibody (anti-*p*-Tyr). Membranes from A were stripped and reprobed with anti-Grb2 antibody (bottom). B, D_4MN9D cells were preincubated with either PP2 or PP3 for 60 min before and during stimulation with PD168077 for additional 5 min. The tyrosine phosphorylation of SHC was analyzed as described in A. C, D_4MN9D cells were preincubated with either PP2 or PP3 or LY294002 for 60 min before and during stimulation with PD168077 for 10 min. ERK activity was detected. The experiment was repeated three times with similar results. PD, PD168077; LY, LY294002.

Discussion

The data presented demonstrate that stimulation of the D_4 dopamine receptor activates the ERK and Akt/NF-κB pathways via a PTX-sensitive G protein in D_4 dopamine receptor-expressing D_4MN9D cells. Activation of ERK is mediated via Src and Ras-dependent mechanisms and requires the association of the signaling molecules SHC and Grb2. In contrast, activation of NF-κB depends on Akt but does not require Src or Ras, indicating that separate mechanisms mediate ERK and Akt/NF-κB activation by D_4 receptor stimulation. Lastly, this communication demonstrates that activation of the ERK signaling pathway is responsible for cell proliferation in response to D_4 dopamine receptor stimulation.

Ras is a key signaling intermediate in many cellular pathways involved in cell proliferation and differentiation (Marte and Downward, 1997). Stimulation of ERK via pertussis toxin-sensitive G protein-coupled receptors, including angiotensin II, lysophosphatidic acid, and D_2 dopamine receptors were shown to be dependent on Ras and seem to be mediated by G$\beta\gamma$ released from G proteins (Faure et al., 1994; Luttrell et al., 1996, 1997; Gutkind, 1998; Luo et al., 1998). Moreover, it has been demonstrated that the nonreceptor tyrosine ki-

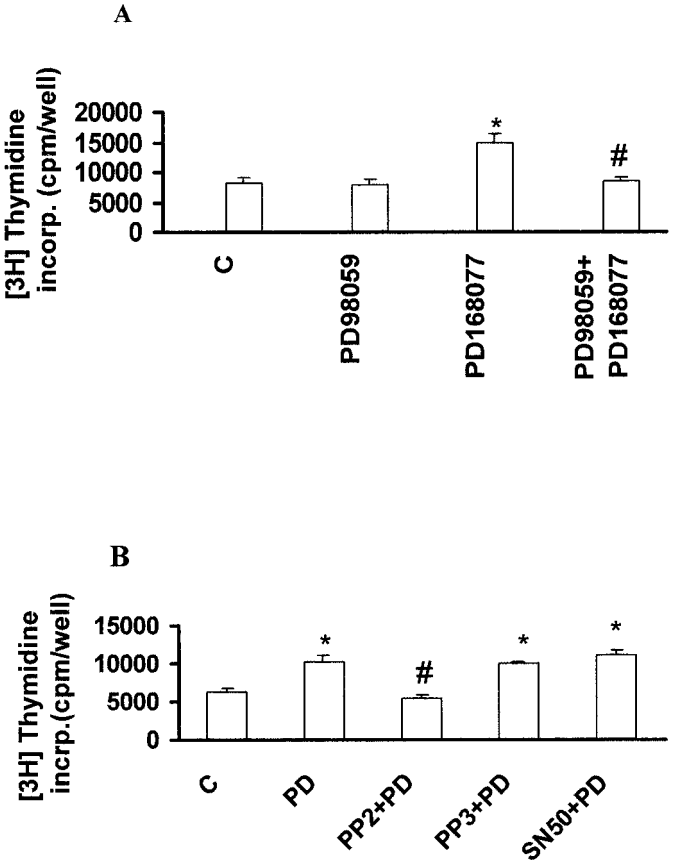

Fig. 8. Src/ERK activation mediates D_4 dopamine receptor-stimulated cell proliferation. A,D_4MN9D cells were cultured in 24-well plates and pretreated with control or 10 μM PD98059 for 2 h before incubation with 1 μM PD168077 for 22 h. B, D_4MN9D cells were pretreated with control or 20 nM PP2, PP3, or SN50 (50 μg/ml) for 60 min before PD168077 stimulation for 22 h. The incorporation of [^{3}H]thymidine into DNA during the last 6 h of the incubation was measured. The data are expressed as mean $\pm$ S.E. and a summary of three experiments with six determinations for each point is presented. $^*p < 0.01$, compared with control; #$p < 0.01$, compared with stimulation with PD168077, Student's *t* test. C, control; PD, PD168077.

nase, Src, links $G\beta\gamma$ to the activation of the Ras/MAPK pathway via phosphorylation of SHC and formation of SHC-Grb2 complex (Gutkind, 1998). In agreement with these observations, our data indicate that Src is also involved in D_4 dopamine receptor-mediated SHC phosphorylation and ERK activation (Fig. 7). Although previous studies implicated PI-3 kinase, activated by the $G\beta\gamma$ complex, in the activation of MAPK in response to Gi-coupled receptor stimulation (Lopez-Ilasaca et al., 1997; Gutkind, 1998), as in D_3 dopamine receptor-stimulated MAPK (Cussac et al., 1999), D_4 receptor-mediated ERK activation does not seem to be mediated through PI-3 kinase because inhibition of PI-3 kinase by LY294002 did not interfere with the activation of the ERK pathway (Fig. 7C). However, notwithstanding the precise mechanism, the activation of the ERK pathway is shared by the three dopamine receptors that constitute the D_2 family of dopamine receptors (Luo et al., 1998; Cussac et al., 1999; Yan et al., 1999; Cai et al., 2000). In addition, blocking Ras function has variable effects on Akt activity (Marte and Downward, 1997). In the present study, a dominant negative Ras had no effect on D_4 receptor-stimulated Akt activation, although ERK activation was attenuated (Fig. 7), clearly indicating that Ras is not upstream in this cascade that leads to Akt activation. Thus, the detailed mechanism underlying Akt activation in D_4MN9D cells remains to be determined.

It is generally believed that receptor-stimulated tyrosine phosphorylation plays an important role in mitogenesis initiated by GPCRs. In the case of the growth factor receptor, binding of the adapter protein SHC to the receptor is believed to be critical for mitogenic signaling. Here we show that the phosphorylation of SHC by Src kinase is responsible for D_4 dopamine receptor-induced ERK activation and cell proliferation (Figs. 7 and 8). It seems, therefore, that the D_4 dopamine receptor shares a common signaling pathway with growth factor receptors in regulating cell proliferation. Indeed, recent evidence indicates that some GPCRs may activate such growth factor receptors as platelet-derived growth factor and epidermal growth factor (Luttrell et al., 1997; Gutkind, 1998). It will be interesting to determine whether D_4 receptor-stimulated SHC phosphorylation and subsequent activation of ERK are associated with *trans*-activation of the platelet-derived growth factor or epidermal growth factor receptors.

In D_4MN9D cells, $G\alpha t_2$ couples to D_4 receptors and regulates cAMP formation, although the cells also express $G\alpha i_2$ and $G\alpha o$ (Tang et al., 1994; Yamaguchi et al., 1997). D_4 dopamine receptor-mediated stimulation of Akt/NF-κB and ERK are sensitive to PTX, which suggests the involvement of $G\alpha i/t_2$ protein in these responses.

In summary, the present data identify ERK and Akt/NF-κB cascades as distinct signal transduction pathways that are activated by D_4 dopamine receptors. These receptors have been implicated in the pathogenesis of neuropsychiatric disorders and in the pharmacology of antipsychotic drugs. Both ERK and Akt/NF-κB pathways play important roles in neuronal plasticity (Impey et al., 1999; Mattson et al., 2000). The present results, therefore, provide new insights into the signaling and function of the D_4 dopamine receptor that may be important in understanding its role in the CNS during health and disease. However, the present findings, obtained in transfected cells that overexpress D_4 dopamine receptor, may not fully reflect the role these pathways play in all physiologic situations. Future studies, therefore, will have to address the question of relevance of these results in physiologic situations in which the density of receptors may differ.

References

Cai GP, Zhen XC, Uryu K, and Friedman E (2000) Activation of extracellular signal-regulated protein kinase is associated with sensitized locomotor response to D_2 dopamine receptor stimulation in unilateral 6-hydroxydopamine-lesioned rats. *J Neurosci* **20:**1849–1857.

Cussac D, Newman-Tancredi A, Pasteau V, and Millan MJ (1999) Human dopamine D_3 receptor mediates mitogen-activated protein kinase activation via a phosphatidylinositol 3-kinase and an atypical protein kinase C-dependent mechanism. *Mol Pharmacol* **56:**1025–1030.

Faure M, Voyno-Yasenetskaya TA, and Bourne HR (1994) cAMP and $\beta\gamma$ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* **269:**7851–7854.

Garnovskaya MN, Van Biesen T, Hawes B, Ramos SC, Lefkowitz RJ, and Raymond JR (1996) Ras-dependent activation of fibroblast mitogen-activated protein kinase by 5-HT1A receptor via a G protein $\beta\gamma$-subunit-initiated pathway. *Biochem* **35:** 13716–13722.

Gutkind JS (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* **273:**1839–1842.

Helmeste DM, Tang SW, Bunney WE Jr, Potkin SG, Jones EG (1996) Decrease in sigma but no increase in striatal dopamine D_4 sites in schizophrenic brains. *Eur J Pharmacol* **314:**R3–R5.

Hunot S, Brugg B, Ricard D, Michel P, Muriel MP, Ruberg M, Faucheux BA, Agid Y, and Hirsch EC (1997) Nuclear translocation of NF-κB is increased in dopaminergic neurons of patients of Parkinson's disease. *Proc Natl Acad Sci USA* **94:**7531–7536.

Impey S, Obrietan K, and Storm DR (1999) Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron* **23:**11–14.

Kane LP, Shapiro VS, Stokoe D, and Weiss A (1999) Induction of NF-κB by the Akt/PKB kinase. *Curr Biol* **9:**601–604.

Lopez-Ilasaca M, Crespo M, Pellici PG, Gutkind JS, and Wetzker R (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI-3 kinase γ. *Science (Wash DC)* **275:**394–397.

Luo Y, Kokkonen GC, Wang X, Neve KA, and Roth GS (1998) D_2 dopamine receptors stimulate mitogenesis through pertussis toxin-sensitive G protein and Ras-involved ERK and SAP/JNK pathways in Rat C6-D_2L glioma cells. *J Neurochem* **71:**980–990.

Luttrell LM, Hawes BE, Biesen TV, Luttrell DK, Lansing TJ, and Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor and $G\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271:**19443–19450.

Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, and Lefkowitz RJ (1997) $G\beta\gamma$ subunit mediate Src-dependent phosphorylation of the epidermal growth factor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J Biol Chem* **272:**4637–4644.

Marte BM and Downward J (1997) PKB/Akt: connecting phosphoinostide 3-kinase to cell survival and beyond. *Trends Biol Sci* **12:**355358.

Mattson MP, Culmsee C, Yu ZF, and Camandola S (2000) Roles of nuclear factor κB in neuronal survival and plasticity. *J Neurochem* **74:**443–456.

Missale C, Nash SR, Robinson SW, Jaber M, and Caron MG (1998) Dopamine receptors: from structure to function. *Physiol Rev* **78:**189–225.

Murga C, Laguinge L, Wetzker R, Cuadrado A, and Gutkind JS (1998) Activation of Akt/protein kinase B by G protein-coupled receptors. *J Biol Chem* **273:**19080–19085.

Murga C, Fukuhara S, and Gutkind JS (2000) A novel role for phosphatidylinositol 3-kinase β in signaling from G protein-coupled receptors to Akt. *J Biol Chem* **275:**12069–12073.

Ozes ON, Mayo LD, Gustin JA. Pfeffer SR, Pfeffer LM, Donner DB (1999) NF-κB activation by tumor necrosis factor requires the Akt serine-threonine kinase. *Nature (Lond)* **401:**82–85.

Pan ZK, Christiansen SC, Ptasznik A, and Zuraw BL (1999) Requirement of phosphatidylinositol 3-kinase activity for bradykinin stimulation of NF-κB activation in cultured human epithelial cells. *J Biol Chem* **274:**9918–9922.

Schulze-Ostehoff K, Ferrari D, Riehemann K, and Wesselborg S (1997) Regulation of NF-κB activation by MAP kinase cascades. *Immunobiol* **198:**35–49.

Shahrestanifar M, Fan X, and Manning DR (1999) Lysophosphatidic acid activates NF-κB in fibroblasts. A requirement for multiple inputs. *J Biol Chem* **274:**3828–3833.

Sibley DR, Monsma FJ Jr, and Shen Y (1993) Molecular neurobiology of dopaminergic receptors. *Int Rev Neurobiol* **35:**391–415.

Sugden PH and Clerk A (1997) Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal* **9:**337–351.

Tang L, Todd RD, Heller A, and O'Malley KL (1994) Pharmacological and functional characterization of D_2, D_3 and D_4 dopamine receptors in fibroblast and dopaminergic cell lines. *J Pharmacol Exp Ther* **268:**495–502.

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, and Civelli O (1991) Cloning of the gene for human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature (Lond)* **350:**610–614.

Welsh GI, Hall DA, Warnes A, Strange PG, and Proud CG (1998) Activation of microtubule-associated protein kinase (ERK) and p70 S6 kinase by D2 dopamine receptors. *J Neurochem* **70:**2139–2146.

Xie P, Browning DD, Hay N, Mackman N, and Ye RD (2000) Activation of NF-κB by bradykinin through a Gαq-and $G\beta\gamma$-dependent pathway that involves phosphoinositide 3-kinase and Akt. *J Biol Chem* **275:**24907–24914.

Yamaguchi I, Harmon SK, Todd RD, and O'Malley KL (1997) The rat D_4 dopamine

receptor couples to cone transducin (Gαt2) to inhibit forskolin-stimulated cAMP accumulation. *J Biol Chem* **272:**16599–16602.

Yan Z, Feng G, Fienberg AA, and Greengard P (1999) D2 dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons. *Proc Natl Acad Sci USA* **96:**11607–11612.

Zhen XC, Uryu K, Wang HY, and Friedman E (1998) D_1 dopamine receptor agonists mediate activation of p38 mitogen-activated protein kinase and c-Jun amino-terminal kinase by a protein kinase A-dependent mechanism in SK-N-MC human neuroblastoma cells. *Mol Pharmacol* **54:**453–458.

Address correspondence to: Dr. Eitan Friedman, Department of Physiology and Pharmacology, The City University of New York Medical School, 138th Street and Convent Avenue, New York, NY 10031. E-mail: friedman@med.cuny.edu