Bromocriptine, acting through the dopamine D2 receptor, provides robust protection against apoptosis induced by oxidative stress in PC12-D2R and immortalized nigral dopamine cells. We now report the characterization of the D2 receptor signaling pathways mediating the cytoprotection. Bromocriptine caused protein kinase B (Akt) activation in PC12-D2R cells and the inhibition of either phosphoinositide (PI) 3-kinase, epidermal growth factor receptor (EGFR), or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress. Co-immunoprecipitation studies showed that the D2 receptor forms a complex with the EGFR and c-Src that was augmented by bromocriptine, suggesting a cross-talk between these proteins in mediating the activation of Akt. EGFR repressin by inhibitor or by RNA interference eliminated the activation of Akt by bromocriptine. D2 receptor stimulation by bromocriptine induced c-Src tyrosine 418 phosphorylation and EGFR phosphorylation specifically at tyrosine 845, a known substrate of Src kinase. Furthermore, Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation. Thus, the predominant signaling cascade mediating cytoprotection by the D2 receptor involves c-Src/EGFR transactivation by D2 receptor, activating PI 3-kinase and Akt. We also found that the agonist pramipexole failed to stimulate activation of Akt in PC12-D2R cells, providing an explanation for our previous observations that, despite efficiently activating G-protein signaling, this agonist had little cytoprotective activity in some experimental models, and the possibility that they may decrease the progression of PD has been proposed (5). However, the mechanisms underlying the agonist-mediated neuroprotection reported in experimental models are poorly understood, and the potential for dopamine agonists to alter the clinical course of this disease remains an area of controversy (6).

Many heptahelical receptors couple to multiple signal transduction pathways, including various heterotrimeric G-protein-second messenger pathways and growth factor receptor-protein kinase cascades (7). The signal for activation of the proximal mediators of signaling such as heterotrimeric G-proteins, receptor kinases, or other protein partners, is an alteration in the receptor’s conformation that occurs following complexing with agonist. Studies in several heptahelical receptors suggest that these proteins exist in multiple, functionally significant conformations that may differ in their relative activation of different signaling pathways (8–12). Studies with several receptors, including the dopamine D2 receptor, suggest that agonists acting at the same receptor select among different active receptor conformations and determine the relative levels of activation of downstream signaling pathways, a hypothesis called agonist-directed signal trafficking (13–16).

We had previously investigated the role of the D2 receptor expressed in the PC12 cell line (PC12-D2R) in modulating the induction of apoptotic cell loss caused by hydrogen peroxide-induced (\(H_2O_2\)) oxidative stress (17). Although the mechanism of neuronal loss in PD is not known, many studies have implicated oxidative stress (reviewed in Refs. 18 and 19). Oxidation of dopamine by auto-oxidation and monoamine oxidase produces reactive oxygen species, including \(H_2O_2\), which can react with ferrous (Fe²⁺) iron to produce hydroxyl radicals, which can damage proteins, nucleic acids, and membrane phospholipids, and induce apoptosis (20). Some animal model and human PD postmortem studies provide evidence that the degeneration of DA neurons occurs via apoptosis (21, 22). We found that activation of the D2 receptor in the PC12-D2R line caused a robust, concentration-dependent increase in cell survival during oxidative stress that required activation of phosphoinositide 3-kinase (PI 3-kinase). Among the agonists studied, we found significant discrepancies in the capacity of individual agonists to phosphatase; DMEM, Dulbecco’s modified Eagle’s medium; CHO, Chinese hamster ovary; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine.
mediate anti-apoptosis and to stimulate G-protein activation, assayed via [35S]GTPγS binding (17).

To elucidate the mechanisms underlying agonist-specific modulation of cell survival, we have now investigated the anti-apoptotic signaling pathway activated by the D2 receptor. We find that D2 receptor-mediated protection against oxidative stress involves a novel c-Src-dependent transactivation of the epidermal growth factor receptor (EGFR) that activates PI 3-kinase/protein kinase B (Akt) and that agonists differ in their capacity to activate this pathway.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—(+)-Bromocriptine methanesulfonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), H2O2, nerve growth factor (NGF) and pertussis toxin (PTX) were purchased from Sigma Chemical Co. (St. Louis, MO). Pramipexole was a gift from Amershams Biosciences (Kalamazoo, MI). Lipofectamine (DMEM), and fetal calf serum were obtained from Invitrogen (Gaithersburg, MD). AG1478, AG1296, k252a, PFP, L2794002, and Wortmannin were obtained from Calbiochem (La Jolla, CA). Epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit was from Amershams Pharmacia Biotech (Piscataway, NJ). Antibodies specific to phospho-Akt, Akt, phospho-tyrosine EGFR, EGFR were from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Src and c-Src antibodies were from Novagen (Madison, WI). Mouse monoclonal antibodies to dopamine D2 receptor, phospho-tyrosine (PY20) antibody, and protein A/G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture and Viability Analysis**—The development and characterization of the PC12-D2R cell line, which is stably transfected with the human D2L receptors, were previously described (17). The cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, and 500 µg/ml G418 in a humidified atmosphere containing 5% CO2 at 37 °C. For differentiation, PC12-D2R cells were plated onto collagen-coated plates in DMEM containing 10% horse serum and 5% fetal bovine serum and allowed to attach overnight. The cells were then induced to differentiate by growing in DMEM supplemented with 1% fetal bovine serum and 100 ng/ml NGF for 10–14 days. Nigral dopamine cell line SN4741 (generous gift from Dr. J. H. Son, Columbia University, New York, NY) was cultured as described previously (23). Cell viability was measured by the MTT method 24 h after various treatments as described (17).

**Transfections and DNA Constructs**—For live cell fluorescence microscopy, PC12-D2R cells (1 × 106) were plated into 60-mm culture dishes and incubated in the media for 24 h. The media was replaced with serum-free DMEM, and a mixture containing 5 µg of the plasmid DNA encoding the pleckstrin homology domain of Akt protein kinase (1–167) tagged with green fluorescent protein (PH-Akt-GFP) (24) (kindly provided by Dr. T. Balle, National Institutes of Health, Bethesda, MD) and 30 µg of LipofectAMINE reagent were gently added to each plate and incubated for 3 h at 37 °C at 5% CO2. The DNA-containing medium was replaced with fresh DMEM-containing medium. When transfection of c-Src (wild type) or dominant negative c-Src (K295R) (24, 25) (both were generous gifts from Dr. J. Burge, Harvard Medical School, Boston, MA) with PH-Akt-GFP was carried out, the DNA concentration used was 1.1. Green fluorescent protein (GFP) plasmid was from Clontech. SN4741 cells were co-transfected with D2r and PH-Akt-GFP or GFP by calcium phosphate method (25).

**Epifluorescence Imaging**—Microscopy of live cells transfected to express PH-Akt-GFP was performed on the Olympus (BX56) upright fluorescent microscope using a water immersion objective lens (×40) fitted with a heated stage and an objective lens heater. Images were collected at 2-min intervals after the addition of the drugs and processed using Adobe Photshop (5.5).

**Immunoblotting and Immuno precipitation**—PC12-D2R cells (1 × 106 cells/100-mm plate) were grown for 24 h, and following respective treatments, the cells were washed twice with ice-cold phosphate-buffered saline and lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal, 0.5% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 µg/ml aprotinin, and mixture of protease inhibitors (Roche Applied Science, GmbH) at 4 °C for 20 min. After centrifugation at 14,000 × g for 20 min at 4 °C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The resolved proteins were electrotransferred to nitrocellulose membranes. Detection of proteins by immunoblotting was conducted using ECL system according to the manufacturer’s recommendations. The blots were then stripped in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50 °C and re-probed with respective antibodies.

For immunoprecipitation, the protein extract was incubated sequentially (2 h for each incubation at 4 °C) with anti-D2 receptor antibody and protein A/G-agarose with gentle agitation. Immunoprecipitates were washed three times with lysis buffer, boiled for 5 min in 5× Laemmli sample buffer, and processed for Western blotting using EGFR or c-Src antibody. The EGFR blots were stripped and re-probed with anti-phospho tyrosine (PY20) antibody.

**RNA Interference**—Custom SMARTPool plus small interfering RNA (siRNA) to target rat EGFR (catalog no. M-004710-00) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA (50 pmol) was co-transfected with PH-Akt-GFP (2 µg) into PC12-D2R cells using trans-TK0 and neural transfection reagents (Mirus, Madison, WI) according to manufacturer’s protocol. For immunofluorescence, 24 h after transfection, cells were serum-starved for 1 h and were treated with bromocriptine for 10 min. The cells were fixed with ice-cold methanol and immunostained for EGFR and visualized using Cy3-conjugated secondary antibody. A nonspecific RNA duplex (Dharmacon, catalog no. D-001206-09-05) was used in control experiments.

**RESULTS**

**Neuroprotection by D2 Receptor Activation Involves PI 3-Kinase/Akt Signaling Cascade**—We have previously reported that the increased cell survival in PC12-D2R cells mediated by D2 receptor activation was completely abolished by inhibitors of PI 3-kinase, suggesting that the D2 receptor may be altering cell survival by activating PI 3-kinase (17). We therefore studied whether PI 3-kinase/Akt signaling was modulated by the D2 receptor when complexed with an agonist that prevents apoptosis in these cells. Activation of PI 3-kinase generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) and thereby stimulates anti-apoptotic proteins (26). The downstream PI 3-kinase target, protein kinase B (Akt), has been reported to be important in mediating survival in many cell types (27). Akt is activated by phosphorylation at Thr308 in the catalytic loop and Ser473 in the C-terminal domain (28, 29).

We first determined whether the anti-apoptotic dopamine agonist bromocriptine induced phosphorylation of Ser473 of endogenous Akt in PC12-D2R cells. As shown in Fig. 1A, Akt phosphorylation was increased 15 min after exposure to bromocriptine. In some cell lines, H2O2 has been reported to activate Akt (30, 31). However, we found that in PC12-D2R cells H2O2 alone had no effect on the phosphorylation of Akt (Fig. 1A). Akt phosphorylation occurs after it is recruited to the plasma membrane through an interaction of its N-terminal pleckstrin homology (PH) domain with PIP3 (32), thereby bringing the enzyme into the proximity of additional PIP3-dependent and -independent protein kinases (33). We studied the redistribution of Akt by D2 receptor signaling using a PH-Akt-GFP fusion protein (24). The localization of PH-Akt-GFP in quiescent PC12-D2R cells was indistinguishable from that of transfected GFP alone. Receptor activation by bromocriptine, however, caused a rapid (<5 min) translocation of the PH-Akt-GFP to ruffled membrane regions (Fig. 1B, top panels). No response to bromocriptine was observed in control PC12-D2R cells expressing GFP alone (Fig. 1B, middle panels) or in the parent PC12 cells, which lack the D2 receptor, expressing PH-Akt-GFP (Fig. 1B, bottom panels). We also tested whether this pathway was active in cells exposed to oxidative stress. As shown in Fig. 1C, the bromocriptine-induced phosphorylation of Akt and translocation of PH-Akt-GFP (Fig. 1D) were unaffected in the presence of H2O2. Thus, D2 receptor stimulation by bromocriptine caused translocation and phosphorylation of Akt in PC12-D2R cells during oxidative stress.

**D2 Receptor Activation of Akt Is PTX-insensitive and PI 3-Kinase-dependent**—The D2 receptor is a member of the rhodop-
Dopamine Cells

To explore whether the D2 receptor coupling to a sin-like heptahelical receptor family, whose classic signaling pathway involves the activation of the G_i/G_o subtype heterotrimeric G-proteins, which can be inactivated by PTX (34), we examined the role of G_i/G_o coupling in activation of PI 3-kinase/Akt pathway. Western blot showing the phosphorylation of Akt by bromocriptine in presence of PTX, but no stimulation of Akt phosphorylation in presence of PI 3-kinase inhibitors LY 294002 or wortmannin. PC12-D2R cells were either left untreated or pretreated with PTX (100 ng/ml; 16 h), wortmannin (100 nM), or LY294002 (20 μM) for 1 h and then stimulated with 1 μM bromocriptine for 15 min. B, effect of PTX or PI 3-kinase inhibitors on translocation of PH-Akt-GFP by bromocriptine (100 nM). PTX (100 ng/ml, 16 h) pretreatment had no effect on the bromocriptine-induced redistribution of PH-Akt-GFP (top panels). Pretreatment of cells with PI 3-kinase inhibitor wortmannin (100 nM, 1 h) completely inhibited the bromocriptine-induced redistribution of PH-Akt-GFP (bottom panels). Arrows indicate localized areas of PH-Akt-GFP following addition of bromocriptine. Panels shown are from one of six independent experiments.

**Fig. 1.** D2 receptor stimulation activates Akt in PC12-D2R cells. A, phosphorylation of Akt by the D2 receptor agonist bromocriptine. PC12-D2R cells were stimulated with 1 μM bromocriptine or H2O2 (200 μM) for the periods of time indicated. After stimulation, cells lysates were prepared and analyzed by Western blotting with anti-phospho-Akt antibody or with anti-Akt antibody. B, translocation of PH-Akt-GFP by D2 receptor stimulation. PC12-D2R cells expressing PH-Akt-GFP (48 h after transfection) were stimulated with the D2 agonist bromocriptine (100 nM), and translocation of PH-Akt-GFP was determined by live cell imaging. Each pair of left (0 min) and right (10 min) panels represents the images captured from the same living cell. Bromocriptine stimulates translocation of PH-Akt-GFP reporter to the membrane within 10 min (top panels). The arrows indicate localized areas of PH-Akt-GFP translocation following addition of bromocriptine. Bromocriptine (100 nM) had no effect on the translocation of EGFP expressed in PC12-D2R cells (middle panels) or PH-Akt-GFP expressed in native PC12 cells, which lack D2 receptors (bottom panels). Panels shown are from one of eight independent experiments. C, oxidative stress had no effect on the phosphorylation or translocation of Akt in the presence of bromocriptine. Western blot of phospho-Akt. Lane 1, control; lane 2, bromocriptine (1 μM); lane 3, bromocriptine plus H2O2 (200 μM). All the incubations were carried out for 15 min. D, bromocriptine-induced translocation of PH-Akt-GFP was unaffected in presence of H2O2. Live cell imaging was carried out following addition of H2O2 (200 μM) and bromocriptine (100 nM) into the medium. Each pair of left (0 min) and right (10 min) panels represents the images captured from the same living cell. Arrows indicate localized areas of PH-Akt-GFP translocation following addition of bromocriptine. The panels shown are from one of six independent experiments.
to PI 3-kinase/Akt observed in PC12-D2R cells was present when the D2 receptor was expressed in a different cellular context, we studied this signaling pathway in the mouse immortalized nigral dopamine cell line SN4741, which expresses tyrosine hydroxylase, the dopamine transporter, and D2 autoreceptors (23). Activation of D2 receptors by bromocriptine in these cells was found to induce phosphorylation of endogenous Akt (Fig. 3A). When the cells were transfected with the PH-Akt-GFP construct, bromocriptine induced redistribution of this reporter (data not shown). The capacity of the activated D2 receptor to induce Akt redistribution in this model was enhanced in cells co-transfected with D2 receptor and PH-Akt-GFP. Bromocriptine caused a translocation of the PH-Akt-GFP protein into discrete regions of the SN4741 cells (Fig. 3B) that was similar to the response observed in differentiated PC12-D2R cells (Fig. 3C). The bromocriptine-stimulated translocation of PH-Akt-GFP in SN4741 cells was eliminated by pretreatment with the PI 3-kinase inhibitor LY290042 (data not shown). Control experiments in which cells were transfected with the D2 receptor and GFP showed no change in the distribution of fluorescence in response to bromocriptine. These results suggest that the D2 receptor can couple to the PI 3-kinase/Akt signaling pathway in dopaminergic neurons.

**D2 Receptor-mediated Neuroprotection and Activation of PI3-Kinase/Akt Involves EGFR Transactivation**—We previously reported that bromocriptine showed significant PI 3-kinase-dependent anti-apoptotic activity in PC12-D2R cells and have demonstrated, as described above, that bromocriptine also induced Akt phosphorylation and translocation. We next attempted to delineate the signal mediators connecting the D2 receptor to PI 3-kinase. It has been reported that the PI 3-kinase/Akt pathway in PC12 cells can be activated by receptor tyrosine kinases (36). The effectiveness of several receptor tyrosine kinase inhibitors on bromocriptine-mediated neuroprotection was evaluated. H2O2 exposure caused significant loss of PC12-D2R cell viability at 24 h, as determined using the MTT metabolism assay, and this cell loss was nearly completely reversed by the D2 receptor agonist bromocriptine (Fig. 4A), consistent with our previous results (17). The effects of various growth factor receptor inhibitors on the capacity of bromocriptine to protect cells against cell death due to H2O2 exposure were studied. AG1296 (200 nM), AG1478 (200 nM), and k252a (50 nM) in the presence of H2O2 and the presence or absence of bromocriptine (100 nM) for 24 h were evaluated. As shown in Fig. 4A, AG1478, a specific inhibitor of EGFR intrinsic tyrosine kinase activity (37), completely abolished the neuroprotection provided by bromocriptine exposure, an effect similar to that observed with inhibition of PI 3-kinase (17). In contrast, inhibition of platelet-derived growth factor (PDGF) receptors by AG1296 or NGF receptor by k252a had no effect on D2 receptor-mediated cell survival.

We next investigated the role of the EGFR in mediating the signaling from the D2 receptor to Akt. Activation of the EGFR by EGF caused a rapid phosphorylation of Akt and a translocation of PH-Akt-GFP in PC12-D2R cells, similar to the response observed with bromocriptine (Fig. 4B). The involvement of EGFR transactivation in D2 receptor stimulation of Akt phosphorylation and translocation was supported by finding a complete inhibition of these responses after pretreatment with AG1478 (Fig. 4C). These results suggest that PI 3-kinase/Akt is one of the downstream effectors of the EGFR and that the D2 receptor activates PI 3-kinase/Akt via transactivation of the EGFR in PC12-D2R cells.

To confirm the role of the EGFR in the activation of PI-3 kinase/Akt by D2 receptors, we reduced the levels of EGFR expression in PC12-D2R cells using RNA interference. After transfection with EGFR-specific or control small interfering RNA (siRNA), cultures were assessed for EGFR protein expression by immunofluorescence. As shown in Fig. 5, EGFR was substantially repressed by 24 h post-transfection in 60–70% of the cells. The involvement of EGFR transactivation in D2 receptor-stimulation of Akt was studied in PC12-D2R cells co-
transfected with PH-Akt-GFP and EGFR siRNA or control siRNA. 24 h after transfection the cells were serum-starved for 1 h, stimulated with bromocriptine, and assessed for the expression of EGFR and the translocation of PH-Akt-GFP. In control siRNA-transfected cells, PH-Akt-GFP translocation was similar to that observed in cells not transfected with siRNA (Fig. 6A and data not shown). However, EGFR repression by siRNA completely blocked the translocation of PH-Akt-GFP by bromocriptine (Fig. 6B). We conclude that EGFR is essential for the translocation of PH-Akt-GFP following D₂ receptor stimulation.

To clarify the mechanisms through which the D₂ receptor transactivates the EGFR, we examined the association between these two membrane proteins. After exposure of cells to vehicle or bromocriptine and preparation of cell extracts, we used a specific anti-D₂ receptor monoclonal antibody (Fig. 7A) to perform immunoprecipitations followed by immunoblotting for the EGFR (Fig. 7B). These studies showed that the EGFR co-immunoprecipitated with the D₂ receptor and the association between these two proteins was augmented in the presence of bromocriptine. Furthermore, the EGFR that complexed with the D₂ receptor in the presence of bromocriptine showed an increase in Tyr phosphorylation. These data indicate that the D₂ receptor and EGFR form a complex and that their association is augmented by bromocriptine. We also examined this complex for the presence of c-Src, which was detected in extracts immunoprecipitated by the D₂ receptor antibody. The presence of c-Src in this complex was also enhanced by exposure of the cells to bromocriptine (Fig. 7C).

The sites of EGFR Tyr phosphorylation induced by bromocriptine were studied using site-specific anti-phosphotyrosine antibodies. We analyzed tyrosine phosphorylation of the EGFR at residues 992 and 1068, which are EGFR autophosphorylation sites (38) and at residue 845 (Tyr845), a known Src phosphorylation site (39). As shown in Fig. 8, incubation of PC12-D₂R cells with EGF increased the phosphorylation of EGFR at residues 992 and 1068, whereas bromocriptine only enhanced phosphorylation of Tyr845 (Fig. 8B). The bromocriptine-mediated phosphorylation of Tyr845 was inhibited by pretreatment with EGFR inhibitor (Fig. 8C).

**EGFR Transactivation Is c-Src-dependent**—The finding that bromocriptine enhanced the association of the D₂ receptor with c-Src and induced phosphorylation of Tyr845, a Src-dependent phosphorylation site of the EGFR (39), led us to study further the role of c-Src in this signaling. Src family kinases have been implicated in the phosphorylation of the EGFR and of PI 3-kinase (39–41). We examined the phosphorylation of Tyr118 in c-Src, which is an autophosphorylation site required for kinase activity of c-Src (42). We exposed cells to bromocriptine (100 nM) for periods up to 30 min. To determine whether c-Src was activated by D₂ receptor stimulation, we performed immunoblotting using an antibody specific for c-Src phospho-Tyr118. Stimulation of the D₂ receptor by bromocriptine caused c-Src to be phosphorylated at Tyr118 (Fig. 9A).
**D₂ Receptor Signaling Cross-talk**

**Fig. 6. Gene silencing of EGFR inhibits D₂ receptor signaling to Akt.** EGFR siRNA inhibited bromocriptine-induced PH-Akt-GFP translocation. PC12-D₂R cells were transfected with EGFR siRNA or control siRNA together with PH-Akt-GFP plasmid DNA. The cells were treated with bromocriptine (100 nM) for 10 min, and PH-Akt-GFP translocation and EGFR expression were examined in these cells. Each set of three vertical panels represents the same field. EGFR immunofluorescence is indicated in red (top panels). PH-Akt-GFP signal is indicated in green (middle panels). The bottom panels are overlays of both EGFR and PH-Akt-GFP signals. A, bromocriptine induced a characteristic ring-like marginalization of PH-Akt-GFP and concentration of PH-Akt-GFP into membrane processes in cells transfected with control siRNA. B, suppression of EGFR expression by EGFR siRNA eliminated the redistribution of PH-Akt-GFP by bromocriptine. Note that these images are from fixed cells and are not identical in appearance to the live cell images shown in Fig. 1. Data shown are representative of three independent experiments.

**Fig. 7. Bromocriptine induced the association of D₂ receptor with EGFR and c-Src.** PC12-D₂R cells were treated with either vehicle or 1 μM bromocriptine for 10 min, and whole cell extracts were prepared. A, equal amount of cell extract, prior to immunoprecipitation, probed with the anti-D₂ receptor monoclonal antibody to establish specificity of the antibody. B, cell extracts were immunoprecipitated with monoclonal anti-D₂ receptor antibody and immunoblotted with antibodies to EGFR (upper panel). Stripping and reprobing with monoclonal anti-phosphotyrosine antibody revealed EGFR tyrosine phosphorylation associated with bromocriptine stimulated D₂ receptor (lower panel). C, immunoblot of D₂ receptor immunoprecipitate stained with anti-c-Src antibody. Note that activation of the D₂ receptor with bromocriptine increased the association of the receptor with both the EGFR and c-Src. All blots shown are representative of two to three independent experiments.

We then examined the effects of the Src family tyrosine kinase inhibitor PP2 on D₂ receptor-mediated Akt phosphorylation and cell survival. PP2 completely inhibited both the capacity of bromocriptine to induce phosphorylation of Akt (Fig. 9B) and to mediate cell survival in the presence of oxidative stress (data not shown). PP2 also prevented the ability of bromocriptine treatment to induce the phosphorylation of c-Src-Tyr418 and EGFR-Tyr441 (Fig. 8C). However, inhibition of the EGFR by AG1478 did not affect the capacity of bromocriptine to induce phosphorylation of c-Src (Fig. 9C), suggesting that the EGFR is downstream of c-Src in D₂ receptor signaling. The role of c-Src in the D₂ receptor signaling was further evaluated using a dominant negative c-Src construct. When co-expressed with PH-Akt-GFP, the dominant negative c-Src kinase (k295R/Y527F) completely abolished translocation of PH-Akt-GFP in response to bromocriptine (Fig. 9D). Thus both pharmacological inhibition and dominant negative studies indicate that c-Src activation is required for signaling from the D₂ receptor through the EGFR to the neuroprotective PI 3-kinase/Akt pathway.

**Translocation and Phosphorylation of Akt by D₂ Receptor Stimulation Are Agonist-specific—**We had previously found that D₂ receptor agonists varied greatly in their capacity to mediate increased survival of PC12-D₂R cells and that their protective efficacy showed no correlation with G-protein activation, as assayed by GTPγS binding. In particular, the efficacy of the agonists bromocriptine and pramipexole for GTPγS binding were indistinguishable, whereas pramipexole was essentially devoid of neuroprotective activity in the PC12-D₂R model. Having implicated the PI 3-kinase/Akt signaling pathway in the neuroprotection mediated by the D₂ receptor when complexed with bromocriptine, we were interested in determining the effects of pramipexole on this pathway. As shown in Fig. 10, pramipexole failed both to induce translocation of PH-Akt-GFP and phosphorylation of Akt in PC12-D₂R cells. These results suggest that specific agonists that interact with the dopamine D₂ receptor differ markedly in their relative activation of classic and growth factor signaling pathways when complexed with the D₂ receptor (see “Discussion”).

**DISCUSSION**

We have delineated a D₂ receptor-activated signaling pathway that mediates neuroprotection by specific D₂ agonists in dopaminergic cell lines. Bromocriptine stimulates the PI 3-kinase/Akt pathway through a PTX-insensitive mechanism involving c-Src and transactivation of the EGFR. Our results suggest that the relative activation of classic G-protein and growth factor signaling pathways by the D₂ receptor is agonist-specific.

Because bromocriptine can induce the activation of the PI 3-kinase/Akt pathway and in many circumstances the modulation of Akt signaling normally occurs via growth factor stimulation, we sought to determine if the effects of bromocriptine on PC12-D₂-R cells were mediated through a growth factor receptor. Here, we report that the bromocriptine-induced activation of Akt within minutes and this activation required the EGFR. We show that EGFR-specific tyrosine kinase inhibitor completely block bromocriptine-induced activation of Akt. Furthermore, EGFR reexpression by siRNA also inhibited the translocation of PH-Akt-GFP by bromocriptine. Inhibitor and dominant negative Src studies show that the activation of the EGFR by the D₂ receptor involves Src. Co-immunoprecipitation studies show that the D₂ receptor complexes with the EGFR and with c-Src and that this association is enhanced by D₂...
activity by mediating phosphorylation of Tyr845, a consensus Src

EGFR (39, 43, 51). c-Src has been reported to influence EGFR

as well as by growth factor receptor stimulation (50), including the

nases can be activated by several heptahelical receptors (43, 48, 49)

bromocriptine-induced EGFR tyrosine phosphorylation. Src ki-

rylation was found to be Src-independent (46, 47). We found

45). In other studies, GPCR-induced EGFR tyrosine phospho-

implicated in GPCR-induced EGFR tyrosine phosphorylation,

Src family kinases have been

Therefore we propose that, in dopaminergic neurons, the D2

receptor transactivates the EGFR through c-Src, which in turn

activates the cytoprotective PI 3-kinase/Akt pathway.

Several mechanisms have been reported for heptahelical receptor activation of Src kinase. The β3 adrenergic receptor interacts with c-Src directly via Pro-rich domains in the receptor (49). Src activation by the β2 adrenergic receptor requires arrestin (48). The D2 receptor has been found to contain non-

canonical SH3 ligands (54). Putative SH3 domains are also present in the D2 receptor, which might potentially mediate an

interaction with Src. Using co-immunoprecipitation studies, we have demonstrated that the D2 receptor and the EGFR form a

complex that includes c-Src. Our inhibitor and dominant neg-

ative Src data further support the involvement of c-Src in

activation of the EGFR. Whether the activation of c-Src by the

D2 receptor occurs directly or requires additional adaptor pro-

teins remains to be determined.

Heptahelical receptors, including D2-class receptors, have

been reported to induce activation of growth factor receptor-

coupled pathways or PI 3-kinase (49, 55–64). The cellular

background in which a receptor is expressed may be important

in determining its signaling potential. Platelet-derived growth

factor receptor (PDGF) transactivation by the D2 and D4 recep-
tors expressed in CHO cells has been reported (60). However, in

contrast to our results for EGFR phosphorylation, the transac-

tivation of the PDGF receptor in CHO cells showed sensitivity

to PTX (60). The D2 receptor expressed in CHO cells mediates

activation of PI 3-kinase via atypical protein kinase C in a

manner also sensitive to PTX (63). In striatal neurons, the D2

agonist has been reported to activate Akt independently of PI

3-kinase activation (65). However, we find in both PC12-D2R

and the dopaminergic SN4741 cells that Akt activation re-

quires PI 3-kinase activity. Our results in the two dopamine

cell lines studied are consistent with the observations of Kihara

et al. (62) in cortical neurons, who also found that bromocrip-
tine activated the PI 3-kinase/Akt pathway.

Our investigations further suggest that the D2 receptor, when

activated by bromocriptine, can couple both to heterotri-

meric Gαi/βi family G-proteins and, simultaneously, to the PI

3-kinase/Akt signaling pathway (Fig. 11). These data led us to

propose that the coupling to heterotrimeric G-protein and the

coupling to PI 3-kinase/Akt may be independent. First, the

G-protein coupling, but not the Akt activation showed PTX

sensitivity. Second, the agonists studied differed in their ca-

pacity to activate each pathway. Bromocriptine activated both

signaling pathways, whereas pramipexole, although quite effi-
cient at simulating GTPγS binding in these cells (17), failed to

activate the PI 3-kinase/Akt signaling pathway (see Fig. 10).

Our data suggest that the anti-apoptotic activity induced by
dopamine agonists in these cells resulted from transactivation

of the PI 3-kinase/Akt pathway. In a previous study, we found

little correlation between the capacity of agonists to confer

protection against oxidative stress and their capacity to acti-

vate classic G-protein signaling (17). Based on these results, we

phosphorylation site in the EGFR (39). Inhibition of either Src or

the EGFR impaired the ability of bromocriptine to cause activation

of Akt in PC12-D2R cells, indicating that activation of both proteins

are required for this signaling. Inhibition of Src kinase also inhib-

ited EGFR phosphorylation at Tyr845, whereas inhibition of the

EGFR did not prevent phosphorylation of c-Src. These results sug-
gest that c-Src is upstream of both the EGFR and Akt. Moreover,
inhibiting either c-Src or the EGFR completely abolished the ca-
pacity of bromocriptine to increase cell survival during oxidative

stress. Although Akt or upstream kinases have been reported to be

a substrate for c-Src phosphorylation (52, 53), in PC12-D2R cells

both c-Src and EGFR phosphorylation were required for Akt ac-

tivation. Therefore we propose that, in dopaminergic neurons, the D2

receptor transactivates the EGFR through c-Src, which in turn

activates the cytoprotective PI 3-kinase/Akt pathway.

Fig. 8. Tyrosine phosphorylation of residue 845 of the EGFR

by bromocriptine. PC12-D2R cells were incubated with 1 μM

bromocriptine for the indicated time points and EGF for 10 min. The cells

were then lysed, and the lysates were resolved by SDS-gel electrophore-

sis on 7.5% gel and immunoblotted. Tyrosine phosphorylation was
detected using site-specific tyrosine antibodies to EGFR from total cell

lysates using the indicated anti-phosphotyrosine antibodies. EGFR lev-
elas were detected using anti-EGFR antibody. A, bromocriptine had no

effect on the phosphorylation of EGFR Tyr992 and Tyr1068. B, bromocript-
tine-induced phosphorylation of EGFR Tyr845. C, phosphorylation of

EGFR Tyr845 by bromocriptine was inhibited by AG1478 pre-treatment

(200 nm, 30 min).

receptor activation. The neuroprotective D2 receptor signaling

pathway we have characterized is summarized in Fig. 11.

Our data indicate that stimulation of c-Src/EGFR family

kinases are required for Akt activation in response to bromo-
criptine in PC12-D2R cells. Src family kinases have been

implicated in GPCR-induced EGFR tyrosine phosphorylation,

and GPCRs can induce association of Src with the EGFR (43–

45). In other studies, GPCR-induced EGFR tyrosine phospho-

rylation was found to be Src-independent (46, 47). We found

that bromocriptine caused activation of the EGFR, and inhibition

of Src kinases had a significant effect on bromocriptine-induced

EGFR tyrosine phosphorylation, implicating Src family kinases in

bromocriptine-induced EGFR tyrosine phosphorylation. Src ki-
nases can be activated by several heptahelical receptors (43, 48, 49)
as well as by growth factor receptor stimulation (50), including the

EGFR (39, 43, 51). c-Src has been reported to influence EGFR
activity by mediating phosphorylation of Tyr845, a consensus Src
propose a model where the specificity of the agonist complexed with the D2-receptor determines the switching of signaling between G-protein and growth factor signaling pathways. Kenakin (13) originally proposed that specific agonists acting at the same receptor might differentially activate downstream signaling pathways, a phenomenon he called agonist-mediated signal trafficking. Signal trafficking could arise as a result of receptors having multiple active conformational states that differ in their activation of specific signaling pathways. Agonists could cause different patterns of signaling by each inducing a different relative distribution of the accessible active states. Many studies suggest that heptahelical receptors exhibit properties consistent with the existence of multiple conformational states. In rhodopsin, for example, the existence of multiple conformers is evident from absorbance changes (9).

**FIG. 9.** Bromocriptine stimulates the activation of the c-Src tyrosine kinase. A, PC12-D2R cells were incubated with 1 μM bromocriptine for the indicated time points. Tyrosine phosphorylation of site-specific tyrosine residues of c-Src was detected from total cell lysates using the anti-phospho Tyr418 antibody. Total c-Src level was detected using anti-Src antibody. B, PP2 treatment inhibits bromocriptine-induced phosphorylation of Akt. PC12-D2R cells were either left untreated or pretreated with 1 μM PP2 for 30 min. The cells were then stimulated with 1 μM bromocriptine for 15 min. The cells were then lysed, and the lysates were resolved by SDS-gel electrophoresis and immunoblotted with either anti-phospho-Akt antibody or anti-Akt antibody. C, inhibition of Src-kinase abolishes bromocriptine-mediated phosphorylation of c-Src and EGFR. D, inhibition of c-Src kinase activity prevents redistribution of PH-Akt-GFP in response to stimulation of the D2 receptor by bromocriptine. PC12-D2R cells were co-transfected with wild type Src or dominant negative Src (K295R/Y527F) and analyzed by epifluorescence microscopy. Redistribution of PH-Akt-GFP occurs in the presence of wild-type c-Src (top panels, see arrows) but not in the presence of dominant negative c-Src (bottom panels).

**FIG. 10.** Differential phosphorylation of endogenous Akt by D2 receptor agonists. A, Western blot showing the differential phosphorylation of Akt by bromocriptine and pramipexole. Lane 1, control; lane 2, bromocriptine; and lane 3, pramipexole. The concentration of bromocriptine and pramipexole used was 1 μM for 30-min incubation. B, graphical representation of Akt phosphorylation in response to bromocriptine and pramipexole. Bromocriptine (1 μM) significantly increased (*, p < 0.05) Akt phosphorylation within 15 min after the addition of the drug, whereas pramipexole (1 μM) showed no effect on the phosphorylation of Akt. Data are mean ± S.E. values from one experiment performed in triplicate, representative of three independent experiments. C and D, in contrast with bromocriptine, pramipexole had no effect on the translocation of PH-Akt-GFP in PC12-D2R cells.

**FIG. 11.** Schematic of signaling pathways modulated by the D2 receptor. Dopamine D2 receptor activates PI 3-kinase/Akt pathway via c-Src and the EGFR. The D2 agonist bromocriptine, but not pramipexole, activates the c-Src/EGFR/PI 3-kinase/Akt pathway.

propose a model where the specificity of the agonist complexed with the D2-receptor determines the switching of signaling between G-protein and growth factor signaling pathways. Kenakin (13) originally proposed that specific agonists acting at the same receptor might differentially activate downstream signaling pathways, a phenomenon he called agonist-mediated signal trafficking. Signal trafficking could arise as a result of receptors having multiple active conformational states that differ in their activation of specific signaling pathways. Agonists could cause different patterns of signaling by each inducing a different relative distribution of the accessible active states. Many studies suggest that heptahelical receptors exhibit properties consistent with the existence of multiple conformational states. In rhodopsin, for example, the existence of multiple conformers is evident from absorbance changes (9).
Multiple receptor conformational states are also evident in single molecule spectroscopy studies of the β2-adrenergic receptor (11) and are supported by the presence of phenotypically different serotonin 5HT2C receptor activation mutants (12). Pharmacological evidence for signal trafficking has been reported in several heptahelical receptors (14, 15, 66, 67). Evidence for signal trafficking at the D2 receptor based on the G-protein sensitivity of binding affinity has been previously reported for D2 receptor expressing SF21 insect cell lines. Notably, the agonist bromocriptine was found to induce a distinct pattern of coupling (15). Our results are consistent with the signal trafficking hypothesis and suggest that agonists acting at the D2 receptor may differ markedly in their capacity to stabilize conformations leading to classic and growth factor signaling.

We implicate the capacity of D2 agonists in transactivating the PI 3-kinase/Akt pathway and in mediating anti-apoptosis in PC12-D2R cells. Furthermore, we find evidence that the effectiveness of an agonist to protect against oxidative stress by activating PI 3-kinase/Akt may differ greatly for specific agonists. Our results support the hypothesis that agonists have a conformationally specific effect at the D2 receptor. Among the agonists studied to date, we find agonists that preferentially activate GTPγS binding and agonists that activate both GTPγS binding and anti-apoptotic signaling. Our results suggest that it may be possible to identify agonists that specifically traffic signaling to the EGFR-PI 3-kinase/Akt pathway in dopamine neurons. Given the central role of the dopamine D2 receptor in brain function, the refined model of conformationally dependent D2 receptor signaling has important implications for the pathophysiology and treatment of brain diseases involving altered dopamine neuronal survival or neurotransmission, such as Parkinson’s disease and schizophrenia.

Acknowledgments—We are thankful to K. Said and I. Ivanova for technical assistance, to Dr. C. W. Olanow, for helpful suggestions and comments, to Dr. T. Bhalla for PH-Akt-GFP, to Dr. J. Burgge for wild competitors, to Dr. T. Bhalla for PH-Akt-GFP, to Dr. J. Burgge for wild competitors, to Dr. T. Bhalla for PH-Akt-GFP, to Dr. J. Burgge for wild competitors, to Dr. T. Bhalla for PH-Akt-GFP, to Dr. J. Burgge for wild competitors, to Dr. T. Bhalla for PH-Akt-GFP, to Dr. J. Burgge for wild competitors.
Agonist-specific Transactivation of Phosphoinositide 3-Kinase Signaling Pathway Mediated by the Dopamine D2 Receptor
Venugopalan D. Nair and Stuart C. Sealfon

J. Biol. Chem. 2003, 278:47053-47061.
doi: 10.1074/jbc.M303364200 originally published online September 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303364200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 41 of which can be accessed free at http://www.jbc.org/content/278/47/47053.full.html#ref-list-1