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Requirement of Gβγ and c-Src in D2 Dopamine Receptor-Mediated Nuclear Factor-κB Activation

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ABSTRACT

The D2 dopamine receptor (D2R) was examined for its ability to mediate nuclear factor-κB (NF-κB) activation through G proteins. Stimulation of D2R-transfected HeLa cells with its agonist quinpirole induced the expression of a NF-κB luciferase reporter and formation of NF-κB-DNA complex. This response was blocked by pertussis toxin, and by the Gβγ scavengers transducin and β-adrenergic receptor kinase 1 carboxyl-terminal fragment. Unlike G_i-coupled chemoattractant receptors, D2R activated NF-κB without an increase in phospholipase C-β activity, and the response was only slightly affected by the phosphoinositide 3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). In contrast, treatment with genistein and 4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*] pyrimidine abolished the induced NF-κB activation, suggesting involvement of protein tyrosine kinases. Activation of D2R led to phosphorylation of c-Src at Tyr-418, and expression of a kinase-deficient c-Src inhibited D2R-mediated NF-κB activation. The D2R-mediated NF-κB activation was not dependent on epidermal growth factor (EGF) receptor transactivation since 4-(3′-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), an EGF receptor-selective tyrphostin used at 1 μM, blocked EGF-induced NF-κB activation but not the quinpirole-induced response. In addition, the D2R-mediated NF-κB activation was enhanced by over-expression of β-arrestin 1. These results suggest that D2R-mediated NF-κB activation requires Gβγ and c-Src, and possibly involves β-arrestin 1.

The transcription factor nuclear factor κB (NF-κB) regulates the expression of a large number of genes coding for cytokines, growth factors, inducible effector enzymes, and regulators of apoptosis (reviewed in Ghosh et al., 1998). Activation of NF-κB can be induced by a variety of environmental factors such as UV, as well as by cytokines such as TNFα and interleukin-1β. The prototypical NF-κB activation pathway, as seen in cells stimulated with TNFα, involves inducible phosphorylation of IκBα at Ser-32 and Ser-36 and subsequent degradation of this and similar inhibitory proteins. The NF-κB/Rel proteins are then released and translocated to nucleus, where they bind to specific DNA sequences and initiate transcription (reviewed in Karin and Ben-Neriah, 2000). An alternative pathway for NF-κB activation has been described and involves phosphorylation of Tyr-42 by the Src family kinases. Hypoxia and pervanadate are known to activate this pathway, which is characterized by the lack of degradation of the tyrosine-phosphorylated IκBα (Imbert et al., 1996).

A number of G protein-coupled receptors (GPCRs) have been reported to activate NF-κB (Ye, 2001). GPCRs constitute a large family of cell surface receptors, and they play an important role in regulating transcription and cell proliferation. Unlike cytokine receptors such as tumor necrosis factor receptor type 1, GPCRs transduce environmental signals across the plasma membrane through their ability to stimulate guanine nucleotide exchange by heterotrimeric G proteins (Gilman, 1987). Exchange of GDP for GTP results in activation of the Gα subunits and dissociation of the Gβγ subunits. These G proteins can independently activate their downstream effectors including phospholipase C-β (PLC-β) and phosphoinositide 3-kinases (PI3K). Previous studies have shown that stimulation of PLC-β activity by Gα16, a

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ABBREVIATIONS: NF-κB, nuclear factor κB; TNFα, tumor necrosis factor α; GPCR, G protein-coupled receptor; PLC-β, phospholipase C-β; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; IκB, inhibitor of κB; D2R, D2 dopamine receptor; Ab, antibody; mAb, monoclonal Ab; MAP, mitogen-activated protein; MEK, MAP kinase kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadione; EMSA, electrophoresis mobility shift assay; TTBS, Tris-buffered saline/Tween 20; PTX, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; βARK-ct, carboxyl-terminal fragment of β-adrenergic receptor kinase 1; EGFR, epidermal growth factor receptor; RLA, relative luciferase activity; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; Gö 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; PDGFR, platelet-derived growth factor receptor; AG1295, 6,7-dimethyl-2-phenylquinoxaline; KD, kinase-deficient; AG1478, 4-(3′-chloroanilino)-6,7-dimethoxyquinazoline; HA, hemagglutinin; ERK, extracellular signal-regulated protein kinase.

member of the G_q family, leads to NF-κB activation through PKC-α (Yang et al., 2001). The Gβγ subunits, released after activation of the B2 bradykinin receptor, can stimulate NF-κB activation through the PI3K-Akt pathway (Xie et al., 2000; Montaner et al., 2001). Akt is a serine/threonine kinase that promotes NF-κB activation through IκB kinase-dependent and -independent mechanisms (Ozes et al., 1999; Madrid et al., 2001). More recently, it was reported that the protein tyrosine kinase protein-tyrosine kinase2 can mediate NF-κB activation by activated Gαq and Gα13. Protein-tyrosine kinase2 appears to act upstream of PI3K and Akt since its induction of NF-κB activation could be blocked by inhibitors of PI3K (Shi and Kehrl, 2001). It has also been demonstrated that Gα13 could induce NF-κB activation through the small GTPase RhoA (Shepard et al., 2001). Several G_i-coupled receptors have also been reported to activate NF-κB (Ye, 2001); however, the underlying mechanisms remain largely uncharacterized.

The dopamine receptors are classified into D1-like and D2-like subfamilies. Members of the D2-like subfamily inhibit adenylyl cyclase activation, whereas D1-like receptors couple to G_s and stimulate adenylyl cyclase activity (reviewed in Lachowicz and Sibley, 1997). Like other G_i-coupled receptors, D2R was found to mediate a number of signaling events in addition to inhibition of adenylyl cyclase. These include induction of K^+ channel opening and release of arachidonic acid. Purified D2R couples to the heterotrimeric G proteins G_i1, G_i2, and G_i3, and preferably activates G_i2 (Senogles et al., 1990), whereas in the brain, D2R preferentially couples to G_o (Jiang et al., 2001). Several investigators recently reported that dopamine could induce NF-κB activation in PC12, Jurkat, and Chinese hamster ovary cells, and contributes to regulation of apoptosis (Luo et al., 1999; Lee et al., 2001; Panet et al., 2001; Weingarten et al., 2001) and expression of HIV-1 genes (Rohr et al., 1999). However, the specific receptors and the underlying signaling mechanisms for this activity have not been characterized. In this study, we used D2R as a model receptor to study the mechanism of G_i-mediated NF-κB activation. We found that the D2R-mediated NF-κB activation utilizes c-Src and differs from previously reported NF-κB induction pathways by GPCRs.

Materials and Methods

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Calbiochem (San Diego, CA). Human TNFα was purchased from PeproTech, Inc. (Rocky Hill, NJ). The human D2R (long) expression construct was used in this study. Human β-arrestin 1 cDNA was kindly provided by Dr. Basil Rapoport (University of California, San Francisco, CA). The expression construct for β-arrestin 1 was made in pCI vector (Promega, Madison, WI) with an AU5 tag (TDFYLK). The anti-AU5 Ab was obtained from Covance Research Products (Denver, PA). The rabbit polyclonal anti-phosphotyrosine (418) Ab was purchased from BioSource International (Camarillo, CA). The chicken c-Src and its kinase-deficient mutant (c-Src-KD, A430V) were originally obtained from Dr. S. J. Parsons (University of Virginia, Charlottesville, VA) and were described previously (Bushman et al., 1990). Other plasmids were described in a previous publication (Xie et al., 2000).

Cell Culture, Transfection, and Luciferase Reporter Assay. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 50 μg/ml streptomycin. Cells (~50% confluence) in six-well plates were transfected with plasmid expression vectors coding for a 3× κB-directed luciferase reporter (Xie et al., 2000), and D2R and β-galactosidase constructs as indicated. Transient transfection was performed as described (Yang et al., 2001) using the LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instruction. Twenty-four to 28 h after transfection, cells were serum-starved for 16 to 18 h, washed twice with phosphate-buffered saline, and assayed with or without agonist stimulation. Reporter lysis buffer (Promega) was then added to the cells. The expressed luciferase activity was measured in a Femtomaster FB12 luminometer (Zylux Corp., Maryville, TN). Relative luciferase activity was normalized against the coexpressed β-galactosidase activity, determined with luminescence reagent from BD Biosciences Clontech (Palo Alto, CA), to overcome variations in transfection efficiency between samples. Unless otherwise indicated, all luciferase assays were performed with duplicate samples, and two to four independent experiments were conducted. Normalized data were plotted using the Prism software (Version 3.0; GraphPad Software Inc., San Diego, CA).

Inositol Phosphate Production. Twenty-four hours after transfection, HeLa cells were labeled with *myo*-[^{3}H]inositol (3 μCi/ml; Amersham Biosciences Inc., Piscataway, NJ) in inositol-free Dulbecco's modified Eagle's medium. After 24 h, the cells were washed twice with the same medium supplemented with 20 mM HEPES (pH 7.4) and 50 mM LiCl. Thereafter, cells were stimulated with quinpirole (10 μM) or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (100 nM) at 37°C for 45 min. Reactions were stopped by removing the medium and adding 1 ml ice-cold methanol to the samples. Recovery of total inositol phosphates with Dowex AG1-8 was described previously (Yang et al., 1998).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear protein extracts were prepared as described (Kravchenko et al., 1995). Double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. EMSA was performed on 6% acrylamide gels with 0.5× Tris borate-EDTA buffer. Gel autoradiograph was analyzed using the ImageQuant software from Amersham Biosciences Inc.

Immunodetection. Immunoprecipitation of various proteins was performed as described previously (Yang and Leonard, 2001). Briefly, cells were washed with ice-cold Dulbecco's phosphate-buffered saline and harvested using 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM sodium orthovanadate, 20 μg/ml pepstatin A, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Samples were sonicated for 10 s at 4°C and then centrifuged at 14,000*g* to remove insoluble materials. The supernatant was incubated with 20 μl of protein A/G-Sepharose beads for 2 h at 4°C. The mix was centrifuged at 14,000*g* for 1 min. The supernatant was transferred to a fresh tube and incubated with 10 μl of antibody overnight at 4°C. Protein A/G-Sepharose beads were added again, and the mix was incubated for 4 h at 4°C. Immunoprecipitates were washed five times with modified lysis buffer (containing 1 mM sodium orthovanadate), using lysis buffer without detergent for the last wash. Washed immunoprecipitates were dissolved in 50 μl of 2× Laemmli's sample buffer, heated, and analyzed by electrophoresis.

For Western blotting, proteins from whole cell extracts were separated on 10% or 12% acrylamide SDS-polyacrylamide gels by electrophoresis at 50 mA. Proteins were then electrotransferred to nitrocellulose membrane at 100 V for 1 h at 4°C. The membrane was pretreated with 5% nonfat milk in TTBS (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.05% Tween 20) for 1 to 2 h at room temperature. Incubation with primary antibody was done at 4°C in TTBS with 5% bovine serum albumin for 16 h. The membrane was then washed three times with TTBS for 10 min each, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 23°C. After three washes with TTBS, the bound antibody was detected by enhanced chemiluminescence (Pierce, Rockford, IL).

Statistical Analysis. Data are expressed as mean ± S.E.M. The probability of observed difference being a coincidence was examined by paired *t* test (see Figs. 2A and 4B). For multiple comparisons, data were analyzed by one-way analysis of variance (see Figs. 2B, 3, and 6C), with Bonferroni's modification (see Figs. 1A, 5, and 8B). Differences at values of $P < 0.05$ were considered significant.

Results

Stimulation of D2 Dopamine Receptor Induces NF-κB Activation. Previous studies indicated that stimulation of dopamine receptors results in NF-κB activation (Rohr et al., 1999; Lee et al., 2001), but the role of individual dopamine receptors in this activation was not clear. We expressed human D2R (long) in HeLa cells, which lack an endogenous receptor for dopamine. Stimulation of the transfected cells with a D2 agonist, quinpirole (Tsuruta et al., 1981), led to NF-κB-dependent transcription of a luciferase reporter in a dose-dependent manner (Fig. 1A). This effect peaked at 10 μM quinpirole since further increases of agonist concentration to 100 μM did not significantly change the induced NF-κB luciferase reporter activity ($P > 0.05$). Dopamine, an endogenous and nonselective agonist for all dopamine receptors, also induced expression of the NF-κB luciferase reporter in the transfected HeLa cells (data not shown). Using EMSA, it was found that quinpirole-stimulated NF-κB reporter activity was accompanied by induced formation of NF-κB-DNA complex that appeared 20 min after agonist stimulation and peaked at 60 min (Fig. 1B). Addition of antibodies against the p50 and p65 subunits of NF-κB prior to incubation with the radiolabeled NF-κB probe reduced the respective NF-κB-DNA complexes, indicating involvement of these NF-κB/Rel proteins in D2R-mediated NF-κB activation (Fig. 1B).

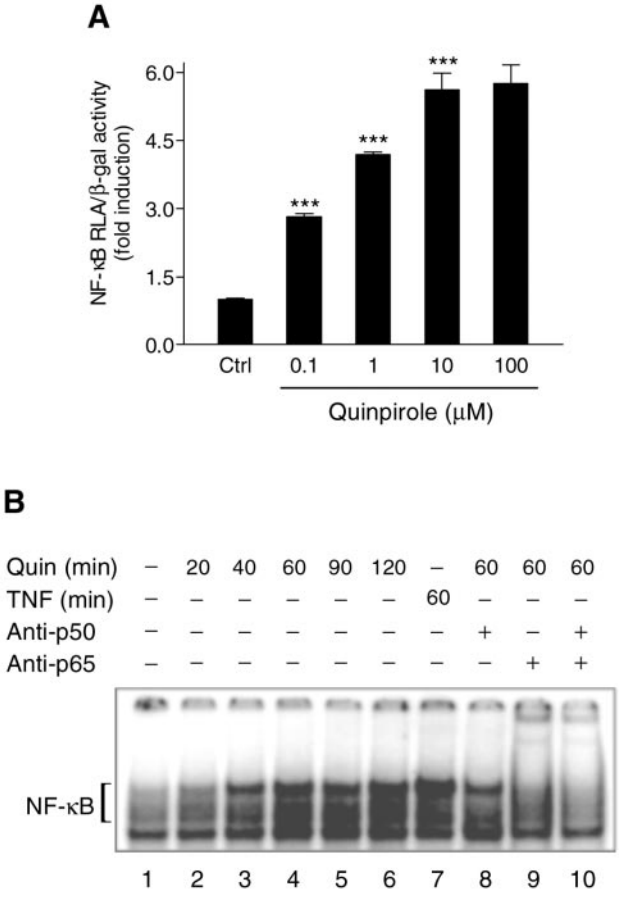

Fig. 1. D2R mediates NF-κB-dependent transcription. A, HeLa cells were transiently transfected with expression constructs for D2R, a κB luciferase reporter (0.2 μg each), and pCMV-β-gal (0.02 μg). After serum starvation, cells were stimulated with quinpirole for 5 h. Luciferase reporter activities were measured, normalized against the β-galactosidase acitivity, and expressed as fold induction over baseline (mean ± S.E.M., $n = 3$). ★★★, $P < 0.001$, when compared with the previous lower ligand concentration. B, formation of NF-κB-DNA complex in response to quinpirole (10 μM) and TNFα (10 ng/ml, lane 7), as detected by EMSA. Supershift was performed by adding anti-p50 and anti-p65 (2.5 μg each; lanes 8–10) prior to ^{32}P-labeled κB probe. The lower portion of the EMSA autoradiograph containing unbound κB probe was removed during figure preparation. Experiments were repeated two more times, and similar results were obtained.

The D2R-Mediated NF-κB Activation Is PTX-Sensitive and Requires Gβγ. We examined the effect of PTX in D2R-mediated NF-κB activation because the receptor is known to couple to the G_i family of G proteins for transmembrane signaling (Senogles et al., 1990). PTX effectively blocked quinpirole-induced expression of the NF-κB luciferase reporter ($P < 0.001$) while having no effect on NF-κB activation induced by phorbol 12-myristate 13-acetate (PMA) (Fig. 2A). This result indicates that D2R-mediated NF-κB activation requires G_i signaling. Cotransfection with the Gβγ scavengers bovine transducin (Gαt) and a carboxyl-terminal fragment of β-adrenergic receptor kinase 1 (βARK-ct) significantly blocked quinpirole-induced NF-κB luciferase reporter expression ($P < 0.001$; Fig. 2B), indicating that PTX-sensitive signaling by D2R requires Gβγ.

A Potential Role of Protein Tyrosine Kinases and c-Src in D2R-Mediated NF-κB Activation. To identify the signaling components involved in D2R-mediated NF-κB activation, we treated the transfected HeLa cells with various pharmacological inhibitors for 30 min before quinpirole stimulation. The protein tyrosine kinase inhibitor genistein completely abolished quinpirole-induced NF-κB reporter expression (Fig. 3). There was no significant inhibition by BAPTA/AM, which buffers the rise of intracellular free calcium, and by calphostin C, which inhibits PKC ($P > 0.05$ versus quinpirole alone). Also, quinpirole stimulation of the D2R-expressing HeLa cells did not lead to accumulation of inositol phosphates (data not shown). Another PKC inhibitor, Gö 6976, produced a small but statistically significant reduction of the NF-κB response. These results indicate that the PLC-β and PKC pathway is not critical to D2R-mediated NF-κB activation. Brami-Cherrier et al. (2002) recently reported that dopamine receptor could stimulate Akt in a PI3K-independent but MAP kinase kinase (MEK)-dependent manner. To investigate the roles of these kinases in NF-κB activation, we treated the transfected cells with the PI3K inhibitor LY294002 and a selective MEK inhibitor, U0126. Both inhibitors produced statistically significant reduction of the NF-κB reporter activity, but the magnitude of inhibition (20–33%) was much less than that of PP1, a Src family protein tyrosine kinase inhibitor (Fig. 3).

The potential involvement of c-Src in D2R-mediated NF-κB activation was next investigated. As shown in Fig. 4A, stimulation of D2R-transfected cells with quinpirole resulted in a time-dependent phosphorylation of c-Src at Tyr-418. Autophosphorylation at Tyr-418 is indicative of c-Src activation (Luttrell et al., 1996). To investigate the relationship between c-Src and D2R-mediated NF-κB activation, a c-Src

expression construct was transfected together with the D2R expression construct. Exogenous expression of c-Src slightly increased the basal level of NF-κB luciferase reporter but strongly enhanced quinpirole-induced NF-κB luciferase activity, suggesting agonist-specific activation through c-Src (Fig. 4B).

Inhibition by PP1 (Fig. 3) indicates the possibility that c-Src or a similar kinase is required for NF-κB activation through D2R. Evidence supporting this notion was obtained by expression of a kinase-deficient (KD) mutant of c-Src, which dose dependently inhibited quinpirole-induced NF-κB luciferase reporter activity in the transfected HeLa cells (Fig. 5). In the control experiment, c-Src-KD did not affect TNFα-induced NF-κB luciferase reporter activity (data not shown).

Potential Mechanisms of Transactivation in D2R-Mediated Activation of c-Src and NF-κB. Previous studies have shown that GPCR-mediated c-Src activation can result from transactivation through EGFR (Gschwind et al., 2001). We stimulated HeLa cells with EGF and observed a 4- to 5-fold induction of NF-κB reporter activity (Fig. 6). The induced response was sensitive to pretreatment with genistein (Fig. 6A), indicating involvement of tyrosine kinase activation through EGFR. To determine whether this mechanism is responsible for the observed NF-κB activation by quinpirole, we pretreated D2R-transfected HeLa cells with the EGFR-selective tyrphostin, AG1478. As shown in Fig. 6B, AG1478 at 1 μM completely blocked EGFR-mediated NF-κB activation ($P < 0.001$) but only slightly affected D2R-mediated NF-κB luciferase reporter expression judged to be statistically insignificant ($P > 0.05$). When AG1478 was used at a higher concentration (10 μM), the quinpirole-induced NF-κB luciferase reporter activity was inhibited by approximately 45%. These results suggest that transactivation through EGFR is at most only partially responsible for the D2R-mediated activation of c-Src and NF-κB.

In addition to EGFR, the receptor for platelet-derived growth factor (PDGF) can also mediate transactivation of GPCRs. Oak et al. (2001) recently reported that D2R and D4R can mediate MAP kinase activation through PDGFR. We examined a potential role of PDGFR-mediated transactivation in quinpirole-induced NF-κB activation. D2R-transfected HeLa cells were stimulated with PDGF (10 ng/ml). Unlike EGF, PDGF does not induce expression of the NF-κB reporter in this system (Fig. 6C). However, when the cells were pretreated with the PDGFR-selective tyrphostin AG1295, a potent inhibition of quinpirole-induced NF-κB reporter activity was observed (Fig. 6C). AG1295 also reduced the NF-κB reporter basal level in HeLa cells ($P < 0.01$), suggesting that the inhibition of quinpirole-stimulated NF-κB activation is mediated through a nonspecific mechanism unrelated to PDGF stimulation of its receptor. Another PDGFR-selective tyrphostin, A9, did not affect D2R-mediated NF-κB activation (data not shown).

Cao et al. (2000) reported that stimulation of the β_3-adren-

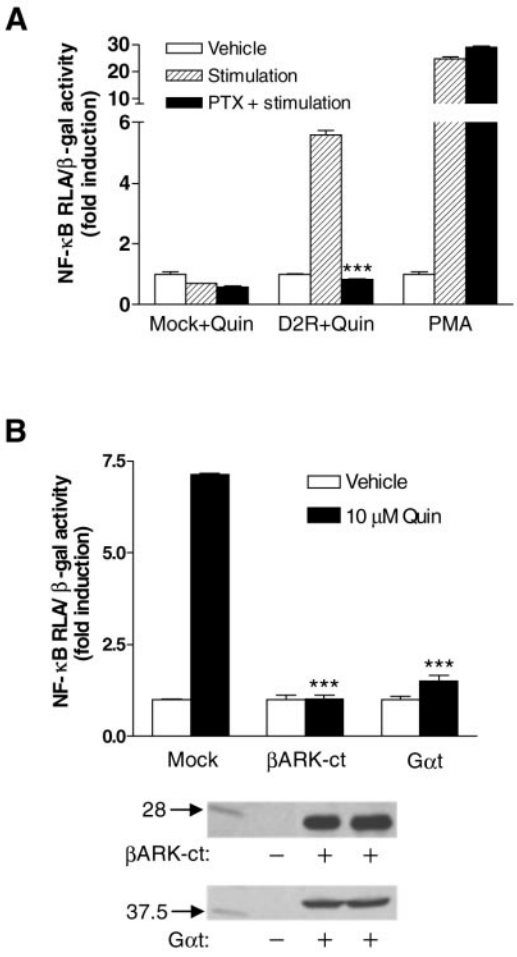

Fig. 2. D2R-mediated NF-κB activation is PTX-sensitive and Gβγ-dependent. A, D2R- or mock-transfected HeLa cells were treated without or with PTX (100 ng/ml, 16 h, solid bars) and then stimulated with quinpirole (10 μM) or PMA (100 nM) for 5 h. PTX inhibition of NF-κB-dependent luciferase expression was observed in quinpirole-stimulated but not PMA-stimulated cells. ★★★, $P < 0.001$ versus PTX-treated samples. B, the Gβγ scavengers βARK-ct and bovine transducin (Gαt) inhibited D2R-mediated expression of NF-κB luciferase reporter. Cells were cotransfected with either βARK-ct or Gαt (0.2 μg each) and, after serum starvation, stimulated with quinpirole for 5 h. ★★★, $P < 0.001$ versus mock. For both A and B, normalized luciferase reporter activities are shown (mean ± S.E.M., $n = 3$). The expression of βARK-ct and Gαt in the transfected cells was determined by Western blotting. Molecular weight markers are indicated by arrows.

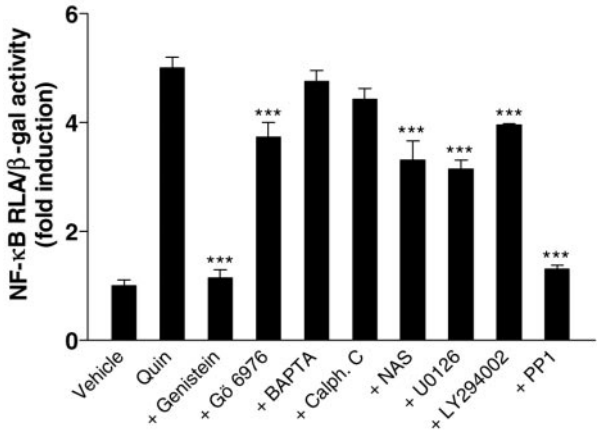

Fig. 3. Effects of pharmacological inhibitors on D2R-mediated NF-κB activation. HeLa cells expressing D2R were serum-starved, treated for 30 min without (first two samples) or with the indicated agents, and then stimulated with 10 μM quinpirole (Quin) for 5 h. Vehicle, no quinpirole stimulation. The concentrations of the agents are: genistein, 100 μM; Gö 6976, 2 μM; BAPTA/AM, 20 μM; calphostin C, 25 nM; *N*-acetylserotonin, 1 mM; U0126, 5 μM; LY294002, 10 μM; and PP1, 10 μM. No significant loss of cell viability was observed during the course of the experiment, as determined by trypan blue exclusion. Changes of normalized luciferase reporter activities are shown (mean ± S.E.M., $n = 3$). ★★★, $P < 0.001$ versus quinpirole only.

ergic receptor activates c-Src through direct recruitment of this tyrosine kinase. This activation is mediated by interaction between the proline-rich PXXP motif in the β_3-adrenergic receptor and the SH3 domain of c-Src. An analysis of the sequence of human D2R (long) reveals two PXXP motifs in the third intracellular loop, suggesting that this mechanism may contribute to D2R-mediated c-Src activation. To examine this possibility, we expressed D2R (C-terminal HA-tagged) together with c-Src in HeLa and HEK293T cells. After immunoprecipitation with the anti-HA Ab, c-Src was detected by Western blotting with a rabbit anti-c-Src Ab. As shown in Fig. 7, no c-Src was detected in the immunoprecipitate from the transfected HeLa cells with or without quinpirole stimulation, although the expression of c-Src was confirmed (Fig. 7, lane 2). In HEK293T cells that over-express the recombinant proteins, a small amount of c-Src was detected in Western blot after immunoprecipitation of D2R (Fig. 7, lanes 7 and 8). However, quinpirole stimulation did not induce additional binding of c-Src (lane 8 versus lane 7), as was observed with agonist-stimulated β_3-adrenergic receptor. No cross-reactivity was observed between the anti-HA Ab and c-Src (data not shown).

Arrestins are proteins that bind phosphorylated GPCRs and facilitate termination of signaling by these receptors. Recent studies demonstrate that β-arrestins serve as adaptors that bring together signaling components following GPCR activation (Miller and Lefkowitz, 2001). This novel function of β-arrestins contributes to β-adrenergic receptor-mediated activation of extracellular signal-regulated protein kinase (ERK) and recruitment of c-Src (Luttrell et al., 1999). To examine a possible role of β-arrestins in D2R-mediated c-Src activation, we cotransfected an expression construct of β-arrestin 1 together with D2R. The transfected cells were then treated with or without quinpirole, and the expression of NF-κB luciferase reporter was determined. We observed no significant induction of the luciferase reporter activity when β-arrestin 1 was expressed alone ($P > 0.05$, Fig. 8B). Cotransfection of both β-arrestin 1 and c-Src expression constructs nearly doubled the basal activity of NF-κB luciferase reporter ($P < 0.01$). It was notable that β-arrestin 1, like c-Src, could enhance quinpirole-induced NF-κB luciferase reporter expression. Furthermore, when both c-Src and β-arrestin 1 were expressed together, an additional enhancement of the reporter gene expression was observed ($P < 0.001$). This action probably requires physical interaction between the two molecules, as confirmed by Western blotting using an anti-FLAG mAb that detected the FLAG-tagged β-arrestin 1 in anti-c-Src immunoprecipitate (Fig. 8A). Taken together, these results support a role of β-arrestin 1 in D2R-mediated NF-κB activation, most likely through Gβγ-dependent recruitment and activation of c-Src.

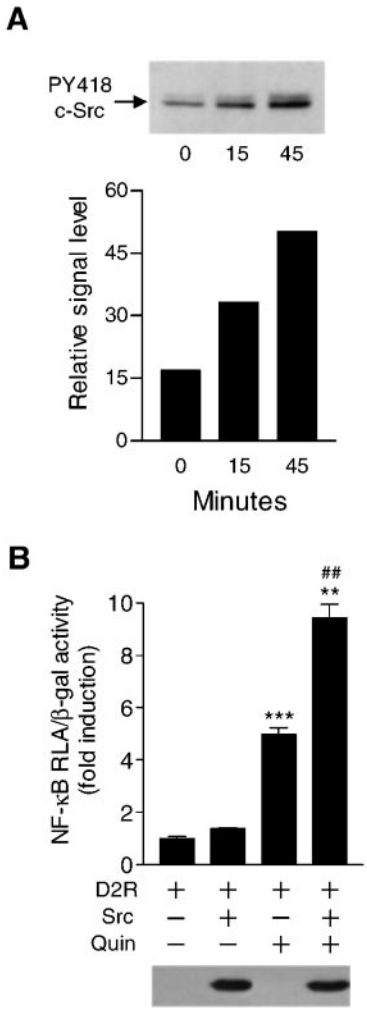

Fig. 4. Potential involvement of c-Src in D2R-mediated NF-κB activation. A, quinpirole-induced tyrosine phosphorylation of c-Src at position 418, as detected by a specific anti-phosphotyrosine Ab. Quinpirole was used at 10 μM, and samples were collected after 0, 15, and 45 min of stimulation. The result of the densitometry analysis is shown below the autoradiograph, which is representative of three experiments. B, expression of c-Src enhances D2R-mediated NF-κB reporter activity. HeLa cells were transfected with the D2R expression vector (0.2 μg), together with (samples 2 and 4) or without (samples 1 and 3) a chicken c-Src expression vector (0.2 μg). Expression of c-Src was determined by Western blotting. Induction of luciferase activity was achieved by stimulation with quinpirole (10 μM for 5 h, samples 3 and 4), and expressed as mean ± S.E.M. ($n = 3$). ★★★, $P < 0.001$, quinpirole versus no quinpirole, without c-Src. ★★, $P < 0.01$, c-Src versus no c-Src, with quinpirole. # #, $P < 0.01$, quinpirole versus no quinpirole, in the presence of c-Src.

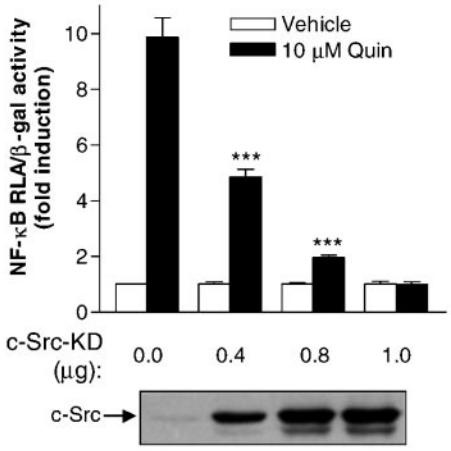

Fig. 5. Inhibition of D2R-mediated NF-κB activation by a kinase-deficient c-Src. HeLa cells were transfected without and with a Src-KD expression construct at three different concentrations, together with D2R and NF-κB reporter genes. Cells were treated with buffer (vehicle, open bars) or with quinpirole (10 μM, closed bars) for 5 h before measurement of luciferase activity. Shown are mean ± S.E.M. of normalized luciferase activities ($n = 3$). ★★★, $P < 0.001$ versus previous lower concentration of c-Src-KD. Extremely significant differences ($P < 0.001$) were observed when each data set was compared with the one lacking c-Src-KD.

Discussion

An increasing number of GPCRs have been found to activate NF-κB when stimulated by a variety of agonists, despite little resemblance between these receptors and cytokine receptors such as the TNFα receptor. Although GPCR-mediated signaling has been studied extensively, the mechanisms used by these receptors to activate NF-κB have just begun to be unraveled (Ye, 2001). In this work, we used D2R to study how agonist stimulation of a G_i-coupled receptor leads to NF-κB activation. The Gβγ-dependent, c-Src-mediated activation of NF-κB represents a previously uncharacterized mechanism by which GPCRs and G proteins regulate NF-κB activation. It differs from the known NF-κB activation mechanisms in that c-Src serves as a downstream effector of Gβγ, and pharmacological inhibitors for protein tyrosine kinase (genistein), and more specifically, for the Src family of protein tyrosine kinase (PP1), are found to be potent inhibitors for the D2R-mediated NF-κB activation. In contrast, inhibitors of conventional PKC and PI3K are not effective on this activation pathway. In addition, the signaling mechanism used by D2R is not entirely dependent on EGFR transactivation (Gschwind et al., 2001). Although EGF could stimulate NF-κB reporter expression and this response was blocked by the tyrosine kinase inhibitor genistein, the use of an EGFR-selective tyrphostin, AG1478, has revealed that EGFR only plays a limited role in D2R-mediated NF-κB activation. AG1478 is highly efficient for inhibition of EGFR-

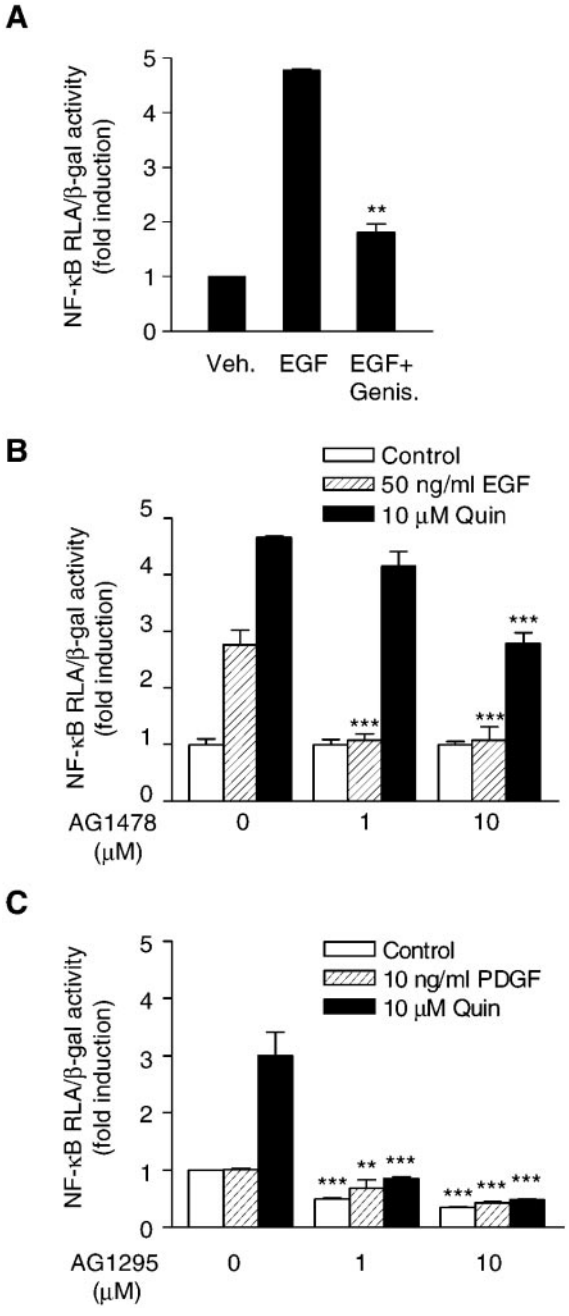

Fig. 6. Effect of blocking EGFR and PDGFR transactivation on D2R-mediated NF-κB activation. A, induction of NF-κB reporter expression in HeLa cells by EGF (50 ng/ml, 5 h) and inhibition by genistein (Genis.) pretreatment (100 μM, 30 min). Veh, untreated control. ⋆⋆, $P < 0.01$ versus EGF sample without genistein, using paired t test. B,) D2R-transfected HeLa cells were treated with AG1478 at the indicated concentrations for 30 min. The cells were then stimulated with EGF (hatched bars) or quinpirole (solid bars) for 5 h. ⋆⋆⋆, $P < 0.001$ versus sample without AG1478 in the same ligand treatment group. C, effect of PDGF on NF-κB reporter expression and inhibition by AG1295. PDGF BB (Sigma-Aldrich) was used at 10 ng/ml for 5 h. Some samples were treated with AG1295 at the indicated concentrations for 30 min before PDGF stimulation. ⋆⋆, $P < 0.01$, and ⋆⋆⋆, $P < 0.001$, versus samples without AG1295 in the same ligand treatment group. For these experiments, expression level of the NF-κB luciferase reporter was normalized against that of the β-galactosidase and shown as mean ± S.E.M. ($n = 3$).

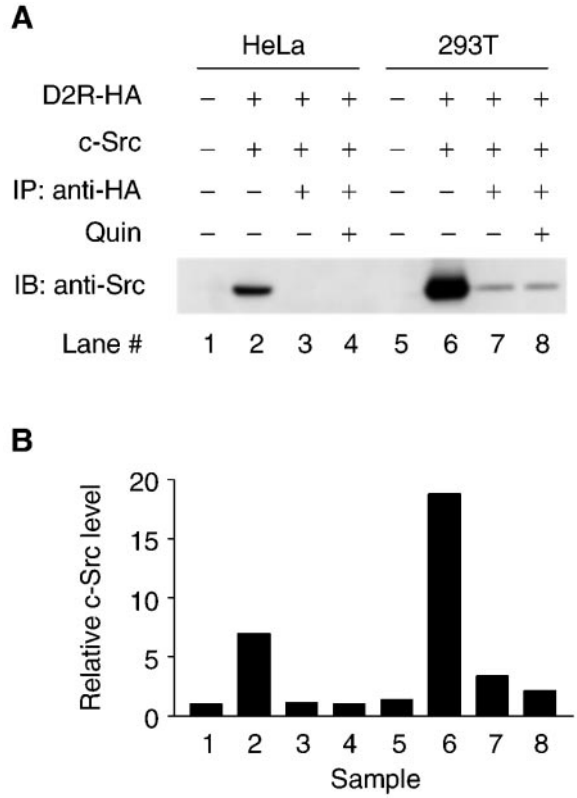

Fig. 7. Association of D2R with c-Src. A, HeLa (left four lanes) and HEK293T cells were transfected with expression vectors for c-Src and a C-terminal HA-tagged D2R. Two samples (lanes 4 and 8) were stimulated with 10 μM quinpirole for 5 h. Equivalent amounts of cell lysate were immunoprecipitated (IP) with an anti-HA mAb. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and Western blotting (IB) was carried out using an anti-Src polyclonal Ab. Some samples (lanes 1–2 and 5–6) were taken before immnoprecipitation to check for expression of the transfected c-Src. The blot shown is representative of three independent experiments with similar results. B, semi-quantification of Western blotting results, with samples corresponding to the lane numbers above.

mediated NF-κB activation at 1 μM, whereas a much higher concentration of AG1478 is required for partial inhibition of the D2R-mediated response. It has recently been shown that PDGFR transactivation contributes to D2R- and D4R-mediated MAP kinase activation in Chinese hamster ovary cells (Oak et al., 2001). Therefore, we examined the possibility that transactivation through PDGFR contributes to D2R-mediated NF-κB activation. PDGF does not induce NF-κB activation in HeLa cells (Fig. 6); thus, it is unlikely that D2R transactivates PDGFR for this function. We have, however, observed a strong inhibitory effect by the PDGFR-selective tyrphostin AG1295. Since AG1295 also reduces the basal level of NF-κB luciferase activity in unstimulated HeLa cells, and the inhibitory effect was not observed with another PDGFR-selective tyrphostin, A9, we believe that the inhibition by AG1295 is not specific for the D2R-mediated response. Iwamoto et al. (2000) reported that AG1295 has inhibitory effect on tyrosine phosphorylation of ERK1/2, PI3-kinase, PLC-γ, and p21 Ras. These findings, combined with the report by Oak et al. (2001), indicate that AG1295 is a broad-spectrum inhibitor for dopamine D2 receptor-mediated signaling, and its effect is probably beyond PDGFR.

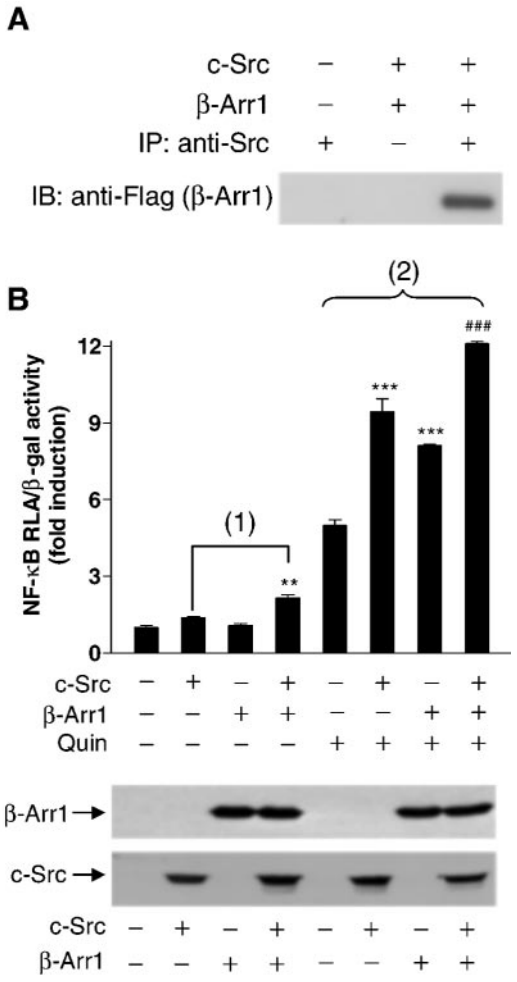

Fig. 8. Expression of β-arrestin 1 potentiates D2R-mediated NF-κB activation. A, association of c-Src with β-arrestin 1. HeLa cells were transfected with expression vectors for c-Src and a FLAG-tagged β-arrestin 1. Immunoprecipitation (IP) was conducted using a polyclonal anti-Src Ab. Western blotting was done with a mAb against FLAG to detect β-arrestin 1. A representative figure from three independent experiments is shown. B, potentiation of NF-κB reporter activity by c-Src and β-arrestin 1. HeLa cells were cotransfected with expression constructs of β-arrestin 1 (β-Arr1) and c-Src in different combinations. After overnight serum starvation, cells were stimulated with quinpirole (10 μM) or with buffer for 5 h. The NF-κB luciferase activities were normalized against β-galactosidase activities and expressed as fold induction (means ± S.E.M., $n = 3$). (1), comparison of NF-κB reporter basal level. ⋆⋆, $P < 0.01$ versus sample without β-arrestin 1. (2), comparison of quinpirole-induced NF-κB reporter activities. ⋆⋆⋆, $P < 0.001$ versus quinpirole alone. # # #, $P < 0.001$ versus sample without β-arrestin 1 within the same group. Lower panels, protein expression levels of c-Src and AU5-tagged β-arrestin 1 as determined by Western blotting.

It was noted that in striatal neurons, dopamine can activate Akt through the MAP kinase kinase MEK but not PI3K (Brami-Cherrier et al., 2002). We therefore investigated the involvement of these signaling components in D2R-mediated NF-κB activation. The PI3K inhibitor LY294002 and the MEK inhibitor U0126 each produced partial inhibition (20–33%) of quinpirole-induced NF-κB reporter expression. Although our results do not completely exclude a role of these kinases in NF-κB activation, they suggest that the PI3K and MEK-ERK pathways are not indispensable for the D2R-mediated response. In comparison, the D2R-induced c-Src pathway plays an essential role in mediating NF-κB activation in the transfected HeLa cells inasmuch as the inhibition by genistein and PP1 nearly completely blocked the quinpirole-induced response. Zhen et al. (2001) recently reported that activation of the D4 dopamine receptor, which also couples to G_i proteins, could lead to NF-κB activation through an Akt-mediated mechanism in stably transfected MN9D cells. Interestingly, this activation was not blocked by inhibition of Src and MEK, which were shown to regulate ERK activation. Therefore, multiple mechanisms may exist in regulating dopamine receptor-mediated NF-κB activation in different cells.

We have found that D2R-mediated NF-κB activation is enhanced by both c-Src and β-arrestin 1. Although the exact mechanism remains to be investigated, this observation is consistent with published reports indicating an association of β-arrestin 1 with c-Src (Luttrell et al., 1999; Miller et al., 2000), which was confirmed by coimmunoprecipitation and Western blotting in this study (Fig. 8B). β-Arrestin 1 serves multiple functions. It interacts with proteins such as activator protein-2 that are necessary for internalization of certain GPCRs through clathrin-coated pits (Goodman et al., 1996). β-Arrestin 1 is also associated with signaling proteins including Raf-1, ERK, and apoptosis signal-regulating kinase 1, in addition to c-Src (reviewed in Miller and Lefkowitz, 2001). Interaction between c-Src and β-arrestin 1 requires the SH2 and SH3 domains of c-Src and a proline-rich fragment of β-arrestin 1 (Luttrell et al., 1999). The biological consequences of β-arrestin 1 and c-Src interaction include promotion of β-adrenergic receptor internalization and activation of MAP kinases by this receptor (Luttrell et al., 1999; Miller et al., 2000). The observation that β-arrestin 1 enhances NF-κB activation by the G_i-coupled D2R may be a new function of β-arrestin 1.

Several G_i-coupled receptors have been found to mediate NF-κB activation, whereas others are less effective in this function (Pan et al., 2000; Ye, 2001). For example, the ability to induce NF-κB activation varies considerably among chemokine receptors (Ye et al., 1996). A recent study has demonstrated that ligand stimulation of the G_i-coupled receptor Edg-1 does not lead to NF-κB activation despite activation of the G_i proteins (Siehler et al., 2001). Our experimental data

also indicate that the G_i-coupled receptor for fMet-Leu-Phe does not mediate NF-κB activation in transfected HeLa cells unless Gα16 is present (Yang et al., 2001). The $G\alpha_i$ proteins are known to inhibit adenylyl cyclase, and activation of downstream effectors by these proteins is mediated primarily through the released Gβγ subunits. Thus, the difference between D2R and formyl peptide receptor in activating NF-κB in the transfected HeLa cells may lie in the available Gβγ proteins that are released following agonist binding. We are currently investigating whether certain specific combinations of Gβγ dimers are preferred for NF-κB activation by the $G\alpha_i$-coupled receptors.

Another possibility for the preferential activation of c-Src (and hence NF-κB) by certain G_i-coupled receptors is that the receptor itself generates a signal independent of, or in addition to, the G protein-mediated signaling. In this regard, it is notable that the G_i-coupled β_3-adrenergic receptor, which apparently lacks phosphorylation site for GRK and does not interact with β-arrestin 1, can activate ERK through direct interaction with c-Src (Cao et al., 2000). This interaction involves the proline-rich motifs (PXXP) located in both the third intracellular loop and the carboxyl-terminal domain of the β_3 receptor (Cao et al., 2000). An analysis of the sequence of human D2R has revealed two PXXP motifs in the third intracellular loop, suggesting that D2R may also use this mechanism to recruit c-Src. We have examined this possibility but could not coimmunoprecipitate c-Src and D2R in transfected HeLa cells. When both proteins were over-expressed in HEK293T cells, there was indeed an association between c-Src and D2R as detected by coimmunoprecipitation and Western blotting. Unlike the interaction with β_3-adrenergic receptor that was stimulated by agonist binding, c-Src association with D2R was weak and was not further induced by quinpirole. Therefore, although the result from this study does not exclude a possible role of this association in NF-κB activation, the receptor-Src interaction itself may not be sufficient for this response. We have found no NF-κB activation in transiently transfected HeLa cells that express the β_3-adrenergic receptor regardless of agonist stimulation (data not shown). Hence, Gβγ may provide an additional signal that is required for NF-κB activation. We have shown that Gβγ scavengers can effectively block quinpirole-induced NF-κB activation. In addition, expression of β-arrestin 1 also resulted in an increase of NF-κB luciferase reporter activity. β-Arrestin 1 is known to interact with the catalytic domain of c-Src (Miller et al., 2000), and it plays an important role in Gβγ-mediated c-Src activation (Luttrell et al., 1996, 1999). Taken together, the results show that D2R-mediated NF-κB activation requires more than a direct interaction between c-Src and the receptor, and possibly involves Gβγ-mediated c-Src activation. The proximal signaling mechanism for D2R-mediated c-Src activation awaits further investigation.

In conclusion, our results provide direct evidence for a c-Src-dependent NF-κB activation by a G_i-coupled receptor. It is likely that β-arrestin 1, and probably D2R itself, plays a role in NF-κB activation by facilitating Gβγ-mediated recruitment and activation of c-Src. NF-κB activation through c-Src was previously demonstrated in cells treated with tyrosine phosphatase inhibitors and under the condition of hyperoxia (Imbert et al., 1996). Results presented in this work demonstrate that GPCRs can also trigger this signaling mechanism and indicate the complexity of cross talks between signaling pathways initiated by different types of receptors that lead to essential cellular functions such as transcriptional regulation through NF-κB.

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References

Brami-Cherrier K, Valjent E, Garcia M, Pages C, Hipskind RA, and Caboche J (2002) Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: a new route to cAMP response element-binding protein phosphorylation. *J Neurosci* **22:**8911–8921.

Bushman WA, Wilson LK, Luttrell DK, Moyers JS, and Parsons SJ (1990) Overexpression of c-src enhances beta-adrenergic-induced cAMP accumulation. *Proc Natl Acad Sci USA* **87:**7462–7466.

Cao W, Luttrell LM, Medvedev AV, Pierce KL, Daniel KW, Dixon TM, Lefkowitz RJ, and Collins S (2000) Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. *J Biol Chem* **275:**38131–38134.

Ghosh S, May MJ, and Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16:**225–260.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56:**615–649.

Goodman OBJ, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, and Benovic JL (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature (Lond)* **383:**447–450.

Gschwind A, Zwick E, Prenzel N, Leserer M, and Ullrich A (2001) Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* **20:**1594–1600.

Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, et al. (1996) Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* **86:**787–798.

Iwamoto H, Nakamuta M, Tada S, Sugimoto R, Enjoji M, and Nawata H (2000) Platelet-derived growth factor receptor tyrosine kinase inhibitor AG1295 attenuates rat hepatic stellate cell growth. *J Lab Clin Med* **135:**406–412.

Jiang M, Spicher K, Boulay G, Wang Y, and Birnbaumer L (2001) Most central nervous system D2 dopamine receptors are coupled to their effectors by Go. *Proc Natl Acad Sci USA* **98:**3577–3582.

Karin M and Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-kappa B activity. *Annu Rev Immunol* **18:**621–663.

Kravchenko VV, Pan Z, Han J, Herbert JM, Ulevitch RJ, and Ye RD (1995) Platelet-activating factor induces NF-kappa B activation through a G protein-coupled pathway. *J Biol Chem* **270:**14928–14934.

Lachowicz JE and Sibley DR (1997) Molecular characteristics of mammalian dopamine receptors. *Pharmacol Toxicol* **81:**105–113.

Lee HJ, Kim SH, Kim KW, Um JH, Lee HW, Chung BS, and Kang CD (2001) Antiapoptotic role of NF-kappaB in the auto-oxidized dopamine-induced apoptosis of PC12 cells. *J Neurochem* **76:**602–609.

Luo Y, Hattori A, Munoz J, Qin ZH, and Roth GS (1999) Intrastriatal dopamine injection induces apoptosis through oxidation-involved activation of transcription factors AP-1 and NF-kappaB in rats. *Mol Pharmacol* **56:**254–264.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, et al. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science (Wash DC)* **283:**655–661.

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

Madrid LV, Mayo MW, Reuther JY, and Baldwin AS Jr (2001) Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem* **276:**18934–18940.

Miller WE and Lefkowitz RJ (2001) Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr Opin Cell Biol* **13:**139–145.

Miller WE, Maudsley S, Ahn S, Khan KD, Luttrell LM, and Lefkowitz RJ (2000) beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis. *J Biol Chem* **275:**11312–11319.

Montaner S, Sodhi A, Pece S, Mesri EA, and Gutkind JS (2001) The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promotes endothelial cell survival through the activation of Akt/protein kinase B. *Cancer Res* **61:**2641–2648.

Oak JN, Lavine N, and Van Tol HH (2001) Dopamine D(4) and D(2L) receptor stimulation of the mitogen-activated protein kinase pathway is dependent on trans-activation of the platelet-derived growth factor receptor. *Mol Pharmacol* **60:**92–103.

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

Pan ZK, Chen LY, Cochrane CG, and Zuraw BL (2000) fMet-Leu-Phe stimulates proinflammatory cytokine gene expression in human peripheral blood monocytes: the role of phosphatidylinositol 3-kinase. *J Immunol* **164:**404–411.

Panet H, Barzilai A, Daily D, Melamed E, and Offen D (2001) Activation of nuclear transcription factor kappa B (NF-kappaB) is essential for dopamine-induced apoptosis in PC12 cells. *J Neurochem* **77:**391–398.

Rohr O, Sawaya BE, Lecestre D, Aunis D, and Schaeffer E (1999) Dopamine stimulates expression of the human immunodeficiency virus type 1 via NF-kappaB in cells of the immune system. *Nucleic Acids Res* **27:**3291–3299.

Senogles SE, Spiegel AM, Padrell E, Iyengar R, and Caron MG (1990) Specificity of receptor-G protein interactions. Discrimination of Gi subtypes by the D2 dopamine receptor in a reconstituted system. *J Biol Chem* **265:**4507–4514.

Shepard LW, Yang M, Xie P, Browning DD, Voyno-Yasenetskaya T, Kozasa T, and Ye RD (2001) Constitutive activation of NF-{kappa}B and secretion of IL-8 induced by the G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus involves G{alpha}(13) and RhoA. *J Biol Chem* **276:**45979–45987.

Shi CS and Kehrl JH (2001) PYK2 links G(q)alpha and G(13)alpha signaling to NF-kappa B activation. *J Biol Chem* **276:**31845–31850.

Siehler S, Wang Y, Fan X, Windh RT, and Manning DR (2001) Sphingosine 1-phosphate activates nuclear factor-kappa B through Edg receptors. Activation through Edg-3 and Edg-5, but not Edg-1, in human embryonic kidney 293 cells. *J Biol Chem* **276:**48733–48739.

Tsuruta K, Frey EA, Grewe CW, Cote TE, Eskay RL, and Kebabian JW (1981) Evidence that LY-141865 specifically stimulates the D-2 dopamine receptor. *Nature (Lond)* **292:**463–465.

Weingarten P, Bermak J, and Zhou QY (2001) Evidence for non-oxidative dopamine cytotoxicity: potent activation of NF-kappa B and lack of protection by antioxidants. *J Neurochem* **76:**1794–1804.

Xie P, Browning DD, Hay N, Mackman N, and Ye RD (2000) Activation of NF-kappa B by bradykinin through a Galpha(q)- and Gbeta gamma-dependent pathway that involves phosphoinositide 3-kinase and Akt. *J Biol Chem* **275:**24907–24914.

Yang M, Buscher R, Taguchi K, Grubbel B, Insel PA, and Michel MC (1998) Protein kinase C does not mediate phenylephrine-induced down-regulation of Madin-Darby canine kidney cell alpha-1B adrenoceptors. *J Pharmacol Exp Ther* **286:**36–43.

Yang M and Leonard JP (2001) Identification of mouse NMDA receptor subunit NR2A C-terminal tyrosine sites phosphorylated by coexpression with v-Src. *J Neurochem* **77:**580–588.

Yang M, Sang H, Rahman A, Wu D, Malik AB, and Ye RD (2001) G alpha 16 couples chemoattractant receptors to NF-kappa B activation. *J Immunol* **166:**6885–6892.

Ye RD (2001) Regulation of nuclear factor kappa B activation by G-protein-coupled receptors. *J Leukoc Biol* **70:**839–848.

Ye RD, Pan Z, Kravchenko V, Browning DD, and Prossnitz ER (1996) Gene transcription through activation of G protein-coupled chemoattractant receptors. *Gene Exp* **5:**205–215.

Zhen X, Zhang J, Johnson GP, and Friedman E (2001) D4 dopamine receptor differentially regulates Akt/nuclear factor-κB and extracellular signal-regulated kinase pathways in D4MN9D cells. *Mol Pharmacol* **60:**857–864.

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