Activation of phosphoinositide 3-kinase by D₂ receptor prevents apoptosis in dopaminergic cell lines

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Whereas dopamine agonists are known to provide symptomatic benefits for Parkinson's disease, recent clinical trials suggest that they might also be neuroprotective. Laboratory studies demonstrate that dopamine agonists can provide neuroprotective effects in a number of model systems, but the role of receptor-mediated signalling in these effects is controversial. We find that dopamine agonists have robust, concentration-dependent anti-apoptotic activity in PC12 cells that stably express human D_{2L} receptors from cell death due to H₂O₂ or trophic withdrawal and that the protective effects are abolished in the presence of D₂-receptor antagonists. D₂ agonists are also neuroprotective in the nigral dopamine cell line SN4741, which express endogenous D₂ receptors, whereas no anti-apoptotic activity is observed in native PC12 cells, which do not express detectable D₂ receptors. Notably,

the agonists studied differ in their relative efficacy to mediate anti-apoptotic effects and in their capacity to stimulate [³⁵S]guanosine 5'-[γ-thio]triphosphate ([³⁵S]GTP[S]) binding, an indicator of G-protein activation. Studies with inhibitors of phosphoinositide 3-kinase (PI 3-kinase), extracellular-signal-regulated kinase or p38 mitogen-activated protein kinase indicate that the PI 3-kinase pathway is required for D₂ receptor-mediated cell survival. These studies indicate that certain dopamine agonists can complex with D₂ receptors to preferentially transactivate neuroprotective signalling pathways and to mediate increased cell survival.

Key words: dopamine agonist, G-protein, neuroprotection, Parkinson's disease, signal transduction.

INTRODUCTION

Parkinson's disease (PD) is characterized by preferential degeneration of dopamine (DA) neurons in the substantia nigra pars compacta. Inhibition of oxidative phosphorylation, excitotoxicity and generation of reactive oxygen species are considered important mediators of neuronal death in PD [1]. Recent studies suggest that apoptosis may play a role in the loss of DA neurons in PD [2]. The major executioners of apoptosis, caspases, are activated in dopaminergic substantia nigra neurons from PD patients [3,4]. One experimental model for PD uses the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ('MPTP') to lesion DA neurons. Chronic administration of MPTP has been found to activate caspases [5,6] and to induce apoptosis in the substantia nigra pars compacta of mice [7].

Laboratory studies demonstrate that DA agonists can protect DA neurons in a variety of tissue culture and *in vivo* models of PD [8]. In the clinic, DA agonists have long been employed as an adjunct to levodopa therapy in advanced PD patients who experience motor complication [9]. Prospective double-blind clinical trials have also demonstrated that DA agonists can provide symptomatic benefits for early PD patients with a reduced risk of motor complications compared with levodopa [10,11]. Recent clinical trials have reported that, in comparison with levodopa, DA agonists delay the rate of decline in neuroimaging surrogate markers of nigrostriatal function [12]. These clinical trials raise the possibility that DA agonists may slow the rate of disease progression and are neuroprotective in PD. There is, however, uncertainty as to the mechanisms responsible for these effects and how they might be protective in PD. Proposed mechanisms include levodopa sparing, direct anti-oxidant effects,

stimulation of auto-receptors and inhibition of subthalamic nucleus-mediated excitotoxicity [8]. In addition, some *in vitro* and *in vivo* studies have noted that the protective effects of DA agonists were eliminated when they were co-administered with D₂-receptor antagonists, suggesting that D₂-receptor activation may contribute to the neuroprotective effects observed in these models [13,14].

In order to clarify the contribution of the D₂ receptor to DA-agonist-mediated neuroprotection and to investigate the underlying mechanisms, we studied the effects of DA agonists in a PC12 cell line model system in the presence and absence of DA D₂ receptors. In these experiments, PC12 cells were induced to undergo apoptosis by either oxidative stress or trophic-factor withdrawal. We found that certain DA agonists, but not all, could induce a robust increase in cell survival via activation of the D₂ receptors. Furthermore our results implicate phosphoinositide 3-kinase (PI 3-kinase) in receptor-mediated cell survival and suggest a dissociation between neuroprotective signalling pathways and the G-protein activation classically associated with D₂-receptor signalling.

MATERIALS AND METHODS

Chemicals

[³H]Spiperone (specific radioactivity 99.0 Ci/mmol) and [³⁵S]guanosine 5'-[γ-thio]triphosphate ([³⁵S]GTP[S]; specific radioactivity 1250 Ci/mmol) was from NEN (Boston, MA, U.S.A.). Bromocriptine, pergolide, quinpirole, R(+)-7-hydroxy-2-(N,N-di-n-propylamino)tetraline (7-OH-DPAT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Abbreviations used: PD, Parkinson's disease; DA, dopamine; PI 3-kinase, phosphoinositide 3-kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; NGF, nerve growth factor; ERK, extracellular-signal-regulated kinase; [³⁵S]GTP[S], [³⁵S]guanosine 5'-[γ-thio]triphosphate; 7-OH-DPAT, R(+)-7-hydroxy-2-(N,N-di-n-propylamino)tetraline; DMEM, Dulbecco's modified Eagle's medium; MPP⁺, 1-methyl-4-phenylpyridinium.

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and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). LipofectAMINE™ and Dulbecco's modified Eagle's medium (DMEM) were obtained from Life Technologies (Gaithersburg, MD, U.S.A.). Pramipexole was a gift from Pharmacia (Kalamazoo, MI, U.S.A.). Protein kinase inhibitors PD98059, SB203580, LY294002 and wortmannin were from Calbiochem (La Jolla, CA, U.S.A.). Antibodies specific to phospho-extracellular-signal-regulated kinase (ERK)1/2, ERK1/2, phospho-p38 kinase and p38 kinase were from Cell Signalling Technology (Beverly, MA, U.S.A.). The anti-active caspase-3 antibody was from Promega (Madison, WI, U.S.A.) and anti-rabbit Cy3-conjugated antibody was from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

Cell culture

PC12 cells [15] were maintained in DMEM supplemented with 10 % horse serum/5 % fetal bovine serum in a humidified atmosphere containing 5 % CO₂ at 37 °C. For differentiation, PC12 cells were plated on to 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 nerve growth factor (NGF) for 10–14 days. Nigral DA cell line SN4741 (a generous gift from Dr J. H. Son, Columbia University, New York, NY, U.S.A.) was cultured as described in [16].

Cell-viability assay

The MTT reduction assay is one of the most widely used assays for determining cell viability [17]; it detects living cells, but not dead ones, and the signal generated is dependent on the degree of activation of the cells [18]. PC12 or PC12-D₂R (PC12 cells stably transfected with the human DA D₂ receptor) cells were plated on 96-microwell cell-culture plates (4 × 10³ cells/well in 100 µl of medium) and grown for 24 h. Thereafter, H₂O₂ was added either with or without other compounds at the indicated concentrations, and cells were incubated for another 24 h. When the immortalized dopaminergic cell line SN4741 was used the number of cells per well in a microwell plate was 1 × 10⁴ and incubated with 50 µM H₂O₂ at 37 °C for 18 h. MTT solution (10 µl; 5 mg/ml in PBS) was added to the wells, containing 100 µl of medium, and the plates were incubated for 4 h. Thereafter, 100 µl of a solubilization solution (0.1 M HCl in absolute isopropanol) was added and incubated overnight to dissolve the water-insoluble formazan salt. Quantification was then carried out with an ELISA reader at 570 nm using a 655 nm filter as a reference. Data are expressed as a percentage of the untreated controls, and values represent the means ± S.E.M. from eight microwells from each of four independent experiments (*n* = 32).

Apoptosis analysis

Apoptosis was measured by nuclear DNA staining, caspase-3 immunocytochemistry and caspase-3 activity. Cells were exposed to 200 µM H₂O₂ and fixed by incubating in 4 % formaldehyde for 30 min. The cells were then permeabilized in PBS/0.2 % Triton X-100 for 10 min. The cells were incubated with blocking buffer (PBS/0.1 % Tween 20/5 % BSA) for 2 h at room temperature. Anti-active caspase-3 antibody (diluted 1 : 250) was added and incubated overnight at 4 °C. After washing, cells were incubated with donkey anti-rabbit Cy3-conjugate antibody (diluted 1 : 500)

for 2 h at room temperature. The cells were washed twice in PBS and the nuclei were stained with 1 µg/ml of the fluorescent DNA dye DAPI (in PBS) for 10 min and then washed with PBS. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) mounting medium. For caspase-3 activity, 0.5 × 10⁶ cells/100-mm-tissue-culture plates were grown for 24 h and treated with H₂O₂ as indicated. Active caspase-3 was measured using the Caspase-3/CPP32 fluorimetric assay kit (Biovision, Palo Alto, CA, U.S.A.). Enzymic activity was determined spectrofluorimetrically (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA, U.S.A.) by measuring the kinetics of fluorescence increase at excitation/emission wavelengths of 400/505 nm.

Plasmid transfection

To develop the PC12-D₂R cell line, the D_{2L} cDNA [19] was subcloned into pRC/RSV, transfected using lipofectAMINE into the PC12 cells and G418-resistant clones were isolated and screened for [³H]spiperone ligand binding.

[³H]Spiperone and [³⁵S]GTP[S] binding

[³H]Spiperone and [³⁵S]GTP[S] binding assays were carried out essentially as described in [20]. Membranes were incubated with [³⁵S]GTP[S] (0.5 nM), GDP (5 µM) and increasing concentrations of DA agonists at 37 °C for 20 min. Data were analysed by non-linear regression analysis using the Inplot curve fitting program (GraphPad v3.0).

Immunoblotting

PC12-D₂R cells (3 × 10⁶ cells/100-mm plate) were grown for 24 h and following respective treatments, the cells were washed twice with ice-cold PBS and lysed in buffer 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % Igepal C630, 1 mM PMSF, 1 mM sodium orthovanadate, 5 µg/ml aprotinin and a cocktail of protease inhibitors (Roche Diagnostics 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/PAGE. The resolved proteins were electrotransferred to nitrocellulose membrane and incubated with phosphorylated ERK1/2 or p38 kinase antibodies, and then detected with peroxidase-conjugated secondary antibodies and chemiluminescent ECL reagent. The blots were then stripped in stripping buffer containing 62.5 mM Tris/HCl, pH 6.7, 2 % SDS and 100 mM β-mercaptoethanol and probed for total ERK or p38 kinase protein.

Statistical analysis

Data were analysed by either two-tailed Student's *t* test or ANOVA followed by Tukey's test for multiple comparisons.

RESULTS

Oxidative-stress-induced apoptosis in PC12 cells

We used oxidative stress or withdrawal of trophic support to induce apoptosis in PC12 cells as a model system for studying the putative neuroprotective property of D₂-receptor agonists. Cells were incubated for 24 h with varying concentrations of H₂O₂, and cell viability was assayed using the MTT metabolism assay.

Figure 1 Concentration-dependent cell death induced by H₂O₂ in undifferentiated and differentiated PC12 cells

Cells were exposed to various concentrations of H₂O₂ for 24 h and cell viability was assessed using the MTT assay ($n = 8$).

Both undifferentiated and post-mitotic PC12 cells that had been differentiated into a neuronal phenotype by NGF exposure were studied. H₂O₂ reduced cell survival in a concentration-dependent manner (Figure 1). NGF-differentiated cells were more sensitive than undifferentiated cells to low H₂O₂ concentrations and were similar in sensitivity at higher H₂O₂ concentrations. In comparison with untreated controls, cell survival observed in differentiated cells exposed to 50 μM H₂O₂ was $72.1 \pm 4.8\%$ compared with $99.9 \pm 2.4\%$ in non-differentiated cells ($P < 0.001$). Cell survival observed with 200 μM H₂O₂ was 52.8 ± 2.2 and $51.5 \pm 2.1\%$ in differentiated and undifferentiated cells respectively.

Apoptotic degeneration is associated with activation of caspase-3-like proteases in *in vitro* models of PD [6,21,22]. To test whether the oxidative-stress-initiated cell death was associated with apoptosis, we evaluated the effects of H₂O₂ on caspase-3 activation (Figure 2). Results obtained with enzymic caspase-3 assay show that 100 μM H₂O₂ activates caspase-3 as early as 2 h and that activity continued to increase until 16 h (Figure 2C). With 200 μM H₂O₂ treatment, maximal caspase-3 activation was observed at 6 h (Figure 2C). These patterns of active caspase-3 were consistent with the greater degree of cell loss observed with increasing concentrations of H₂O₂ (Figure 1). To confirm that caspase-3 activation in these cells was associated with morphological features of apoptosis, differentiated cells were labelled for caspase-3 activation and stained with the DNA dye DAPI. H₂O₂ at 200 μM induced the characteristic pattern of chromatin condensation in cells that also stained positive for caspase-3 activation (Figure 2A). Apoptotic changes were evident 8 h after the addition of H₂O₂, and were noted in $42.2 \pm 6.9\%$ of nuclei after 16 h (Figure 2B).

Differentiated PC12 cells undergo apoptosis in the absence of NGF and serum [23]. After 24 h of trophic withdrawal, cell viability of differentiated PC12 cells was reduced by $48.5 \pm 3.0\%$ compared with control cells ($P < 0.01$). The trophic-withdrawn cells also stained positive for active caspase-3 and showed nuclear fragmentation (results not shown). Collectively, these data indicate that H₂O₂ or trophic withdrawal causes cell death in PC12 cells by inducing apoptosis.

Figure 2 Oxidative-stress-induced apoptosis in PC12 cells (A) and caspase-3 activation induced by H₂O₂ in differentiated PC12 cells was associated with nuclear condensation

(A) The nuclei were stained blue (DAPI) and active caspase-3, red. Left-hand panel, control; right-hand panel, PC12 cells 16 h following 200 μM H₂O₂ exposure. Note the association of caspase-3 activation with chromatin condensation. (B) Percentage of apoptotic cells with condensed nuclei and active caspase-3 determined by counting random fields ($n = 6$) at 8 and 16 h following H₂O₂ exposure, which showed a significant difference ($P < 0.01$) compared with the control. (C) H₂O₂-induced caspase-3 enzymic activity in undifferentiated PC12 cells was dependent on H₂O₂ concentration and incubation period ($n = 4$).

D₂-receptor activation is required for the prevention of apoptosis in PC12 cells

We studied the potential of DA agonists to protect against apoptosis induced by oxidative stress or by serum and NGF withdrawal in PC12 cells that lacked D₂ receptors. The absence of D₂ receptors in these cells was demonstrated by the lack of specific

Figure 3 Saturation analysis for [^3H]spiperone binding in membranes of PC12-D₂R cells

D₂L DA receptors were expressed in PC12 cells and saturation binding analysis using [^3H]spiperone was performed to determine levels of D₂ receptors. Binding parameters (K_d and B_{max}) were derived from the data, and values are expressed as the mean $\pm$ S.D. values from one experiment performed in triplicate, representative of three independent experiments.

binding using the D₂-receptor ligand [^3H]spiperone as well as by the inability to detect D₂-receptor mRNA by PCR (results not shown). The DA agonists tested, bromocriptine and pergolide, provided no protection against apoptosis in either differentiated or undifferentiated cells exposed to H₂O₂ or removed from NGF and serum. These results indicate that these DA agonists were not neuroprotective in the absence of functional D₂ receptors in this model system.

To study the possibility that the DA D₂ receptor might mediate neuroprotection, PC12 cells were stably transfected with the human DA D₂ receptor (PC12-D₂R). PC12-D₂R membranes showed specific [^3H]spiperone binding with a B_{max} of 545 ± 14 fmol/mg of protein, a K_d of 0.54 ± 0.07 nM (Figure 3) and competitive binding K_i values for various D₂-receptor agonists and antagonists consistent with reported values for the human D₂ receptor [19]. In PC12-D₂R cells, oxidative stress or withdrawal of trophic support induced cell loss and the stigmata of apoptosis similar to what was observed in PC12 cells that lacked D₂ receptors.

Undifferentiated PC12-D₂R cells were exposed to 200 μM H₂O₂ for 24 h in the presence or absence of various concentrations of the DA agonist bromocriptine, and cell viability was measured. In contrast to the lack of agonist-mediated neuroprotection observed in the parent cell line, bromocriptine dramatically protected undifferentiated PC12-D₂R cells from H₂O₂-induced apoptosis in a robust and concentration-dependent manner (Figure 4A). The protective activity of DA agonists was inhibited by the D₂-receptor antagonists haloperidol and butaclamol, further confirming that the neuroprotection was mediated by activation of the D₂ receptor (Figure 4B). In NGF-differentiated cells, 100 nM bromocriptine and pergolide similarly inhibited apoptosis induced by H₂O₂ and trophic-factor withdrawal (Figure 5).

To explore whether the D₂-receptor activation protects dopaminergic neurons, we investigated the neuroprotection by bromocriptine in the mouse immortalized nigral DA cell line SN4741, which expresses tyrosine hydroxylase, the DA transporter and the D₂ auto-receptors [16]. It has been reported that treatment with 1-methyl-4-phenylpyridinium (MPP⁺), neurotoxins and H₂O₂ equally induces oxidative-stress-dependent apoptotic cell death in SN4741 cells [16,24]. The DA agonist

Figure 4 D₂-receptor activation protects PC12 cells from oxidative-stress-induced apoptosis

(A) Bromocriptine increased cell survival of undifferentiated PC12-D₂R cells exposed to H₂O₂. Cells were exposed to 200 μM H₂O₂ for 24 h in the presence of increasing concentrations of bromocriptine and cell viability assessed by MTT assay ($n = 8$). * $P < 0.01$ compared with H₂O₂ alone. (B) DA agonist neuroprotection depends on interaction with the D₂ receptor. The protective effect of bromocriptine against H₂O₂-induced apoptosis was eliminated in the presence of the D₂-receptor antagonists haloperidol or butaclamol (1 μM ; $n = 8$). * $P < 0.01$ compared with bromocriptine + H₂O₂.

Figure 5 DA agonists protect against apoptosis in differentiated PC12-D₂R cells

Differentiated PC12-D₂R cells were exposed to 200 μM H₂O₂ or trophic withdrawal (TW) in the presence or absence of 100 nM bromocriptine (brom) and pergolide (perg; $n = 8$). * $P < 0.05$ compared with H₂O₂ or trophic withdrawal.

bromocriptine showed significant concentration-dependent increased cell survival against H₂O₂ (50 μM)-induced cell death in SN4741 cells (Figure 6). These results suggest that activation of an endogenous D₂ receptor can be neuroprotective.

Figure 6 Bromocriptine protects nigral DA cell line SN4741 from oxidative-stress-induced cell death

Cells were exposed to 50 μ M H₂O₂ for 18 h in the presence of increasing concentrations of bromocriptine and cell viability was assessed by MTT. Data are mean $\pm$ S.E.M. ($n = 16$). # $P < 0.001$ compared with control. * $P < 0.001$ compared with H₂O₂ alone.

Differential neuroprotective activity of DA agonists does not correlate with GTP[S] binding

DA agonists varied considerably in their ability to stimulate neuroprotection (Figure 7A). Bromocriptine provided the greatest neuroprotection in this assay, 7-OH-DPAT had low activity and pramipexole was nearly devoid of neuroprotective activity (Figure 7A). The major signalling pathway associated with activation of the D₂ receptor involves the heterotrimeric G-proteins, principally G_i. The capacity of agonists to stimulate G-protein activation was determined by [³⁵S]GTP[S] binding in membranes from PC12-D₂R cells (Figure 7B). Whereas bromocriptine and pramipexole showed marked differences in neuroprotective activity, they demonstrated similar agonist-stimulated [³⁵S]GTP[S] binding and they both exceeded the [³⁵S]GTP[S]-binding activity of pergolide and 7-OH-DPAT. These results indicate that DA agonists differ in their capacity to induce neuroprotection and further suggest that there is a weak correlation between activation of neuroprotective and G-protein signalling pathways.

Neuroprotection by D₂-receptor agonists involves the PI 3-kinase signalling cascade

To characterize the molecular mechanisms responsible for the D₂-receptor-mediated neuroprotection, we tested the effectiveness of several protein kinase inhibitors against bromocriptine-mediated cell survival in oxidative-stress-induced apoptosis. Inhibition of ERK by PD98059 (50 μ M) or p38 kinase by SB203580 (20 μ M) had no effect on the increased cell survival caused by D₂R activation (Figure 8 and results not shown). In contrast, the D₂ receptor-stimulated increase in cell survival required PI 3-kinase activation. The PI 3-kinase inhibitors wortmannin (100 nM) and LY294002 (10 μ M) completely abolished the capacity of bromocriptine to protect against oxidative-stress-induced cell death (Figure 8). Control toxicity studies indicated that, at the concentrations used, the inhibitors had no effect on cell survival, as determined by MTT assays (results not shown). However, the inhibitors of ERK and p38 kinase completely inhibited the activation of ERK by epidermal growth factor and p38 kinase by NaCl, respectively (Figure 9). These data suggest that activation of the PI 3-kinase pathway through the

Figure 7 Differential neuroprotective efficacy of DA agonists

(A) Comparison of DA agonists in protection of PC12-D₂R cells against oxidative stress. Cells were exposed to 200 μ M H₂O₂ for 24 h in the presence or absence of various concentrations of five DA agonists and the effects on cell survival determined by MTT assay. The rank order of protection observed was consistent in three independent experiments. (B) Concentration-response curve of the stimulation of [³⁵S]GTP[S]-specific binding to PC12-D₂R membrane by DA agonists. Data are means $\pm$ S.E.M. from one experiment performed in triplicate, representative of three independent experiments.

D₂ receptor contributes to the protection against oxidative-stress-induced apoptosis in PC12-D₂R cells.

DISCUSSION

Our results demonstrate that DA agonists can protect PC12 cells that express the human D₂ receptor and a nigral DA cell line, SN4741, from apoptosis induced by oxidative stress. Similar protective effects could not be obtained in PC12 cells that lacked D₂ receptors. These observations suggest that the D₂ receptor plays a critical role in the neuroprotective effects conferred by DA agonists in this model system. Furthermore, we find that specific DA agonists vary in their capacity to provide anti-apoptotic effects and that neuroprotective effects do not correlate closely with the capacity of the agonist to activate classical D₂-receptor-coupled G-protein signalling pathways. Furthermore,

Figure 8 Elimination of DA-agonist-mediated neuroprotection by inhibition of PI 3-kinase

Low concentrations of the PI 3-kinase inhibitor wortmannin (100 nM) or LY294002 (10 μ M) eliminate the protective action of bromocriptine on H₂O₂-induced apoptosis. Data plotted are from one experiment (means $\pm$ S.E.M., $n=8$), representative of four independent experiments. # $P < 0.001$ compared with control (no treatment). * $P < 0.001$ compared with H₂O₂ alone. #* $P < 0.001$ compared with bromocriptine + H₂O₂.

Figure 9 Representative Western immunoblots showing the effect of mitogen-activated protein kinase inhibitors on the phosphorylation of ERK and p38 kinase

(A) PC12-D₂R cells untreated (control) or pretreated with 50 μ M PD98059 (PD) for 1 h were stimulated with epidermal growth factor (EGF) for 10 min. Western blot analysis was performed using antibodies specific for phosphorylated and total ERK1/2. (B) Cells were untreated (control) or pretreated with 20 μ M SB203580 (SB) for 1 h and either untreated or treated with NaCl (0.4 M) for 1 h. Western blot analysis was performed using antibodies specific for phosphorylated and total p38 kinase.

we find that activation of the PI 3-kinase signalling pathway is required for anti-apoptotic activity of DA agonists in PC12-D₂R cells.

In our experiments in PC12 cells, we find no evidence for receptor-independent cytoprotective activities of DA agonists. Rather, we find that the activation of DA D₂ receptors is required for the prevention of apoptosis induced by H₂O₂ in undifferentiated PC12 cells and for protection against either H₂O₂ or trophic withdrawal in NGF-differentiated PC12 cells. This conclusion is based on the following observations: (i) no protection was observed in PC12 cells that do not express the D₂ receptor; (ii) the agonists bromocriptine, pergolide and quinpirole all protect PC12 cells from apoptosis in a concentration-dependent manner; (iii) this concentration-dependent protective effect is reversed by D₂ antagonists; and (iv) the promotion of cell survival is agonist-specific and independent of their capacity to activate G-protein coupled signalling pathway. Several previous studies have implicated activation of DA receptors in the neuroprotective effects observed with DA agonists [13,25,26]. For example, the capacity of bromocriptine to protect a mouse HT22 cell line against oxidative stress [26] and rodent dopaminergic neurons from levodopa-induced toxicity [27] is dependent on its ability to stimulate D₄ and D₂ DA receptors respectively. Thus several receptors may activate anti-apoptotic signalling.

Various studies have found that DA agonists can promote cell survival independently of receptor activation in several experimental paradigms [28–30]. Not all reports of DA agonist-mediated protection appear to rely on activation of DA receptors and other mechanisms may be applicable in other model systems. *In vitro* and *in vivo* studies have shown that DA agonists are capable of scavenging superoxide or hydroxyl radicals [30,31]. Pramipexole has been shown to up-regulate Bcl2 expression, which could provide an anti-apoptotic effect [32,33]. In addition, studies in isolated mitochondria have shown that the agonist can protect against membrane swelling induced by calcium or MPP⁺, which could not be accounted for by direct receptor activation [34]. In addition, blockade of DA receptors in dopaminergic cell cultures in some studies does not prevent the protective properties of DA agonists [32,35]. Further, the enantiomers of apomorphine and pramipexole, which do not bind to DA receptors, have been reported to protect dopaminergic neurons from MPP⁺, H₂O₂ or 6-hydroxydopamine toxicity [36]. These observations indicate that agonists can induce protective effects in some models independent of DA receptors [29,32,35]. These differences with our findings, in which the anti-apoptotic activity of DA agonists in PC12-D₂R cells is predominantly mediated by activation of the D₂ receptors, may result from differences in the experimental systems utilized and/or the DA agonist tested.

DA receptors belong to the rhodopsin family of heptahelical G-protein-coupled receptors. We find that the D₂-receptor-mediated increased cell survival is apparently independent of its activation of G_i/G_o heterotrimeric G-proteins. Recent studies have revealed that, in addition to heterotrimeric G-proteins, these receptors may interact with and activate a variety of signal mediators, including small G-proteins [37], Na⁺/H⁺-exchange factor [38], c-Src [39] and cGMP-operated Ca²⁺ channels [26]. The differences in their relative neuroprotective efficacy and their activation of G-proteins support the 'agonist signal trafficking' hypothesis that different agonists acting at the same receptor subtype can stabilize distinct receptor conformations and thereby preferentially activate subsets of the signalling pathways coupled to that receptor [40]. A recent study on D₂ receptor–G-protein interactions reported that specific agonists differed in their relative activity at promoting receptor complexing with either G_{i2} or G_o G-proteins, also supporting the formulation that specific agonists can stabilize the D₂ receptor in different conformation [20]. Our data show that specific agonists select between activation of only G_i/G_o G-protein pathways and the additional activation of a G-protein-independent

neuroprotective pathway. This distinction may be important in designing studies to determine the neuroprotective activity of DA agonists *in vivo*.

The present study demonstrates that PI 3-kinase inhibitors block the protective effect of D₂-receptor stimulation. Therefore, signal transduction via PI 3-kinase activation is necessary for this protective effect. Although PI 3-kinase is clearly important for growth-factor-mediated neuronal survival in many cell types and conditions, in other neuronal cell types and under different conditions, growth-factor-mediated activation of the ERK-signalling pathway appears to mediate survival effects [41]. We find no evidence for the involvement of ERK in D₂-receptor-mediated survival of PC12-D₂R cells from our studies using specific inhibitors. It was reported recently that D₂-receptor activation protects cortical neurons from glutamate-induced cytotoxicity by up-regulation of Bcl-2 protein expression via the PI 3-kinase cascade [42]. Therefore the effects of D₂-receptor activation on PI 3-kinase and cell survival appear to apply to a variety of cellular insults.

We find that a remarkably large effect on survival can be obtained with activation of the D₂ receptor by certain agonists. Furthermore, this improvement in survival is quite different with different agonists and does not correlate with the capacity of these agonists to activate classical G-protein signalling. These results suggest that D₂ receptors activate a trophic factor linked pro-survival signalling pathway in an agonist-specific manner. Thus it is likely that intracellular signalling by D₂-receptor stimulation can be manipulated for the development of more effective neuroprotective therapies.

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