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Efficient functional coupling of the human D3 dopamine receptor to G_o subtype of G proteins in SH-SY5Y cells

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1 The D3 dopamine receptor presumably activates G_i/G_o subtypes of G-proteins, like the structurally analogous D2 receptor, but its signalling targets have not been clearly established due to weak functional signals from cloned receptors as heterologously expressed in mostly non-neuronal cell lines.

2 In this study, recombinant human D3 receptors expressed in a human neuroblastoma cell line, SH-SY5Y, produced much greater signals than those expressed in a human embryonic kidney cell line, HEK293. Quinpirole, a prototypic agonist, markedly inhibited forskolin-stimulated cyclic AMP production and Ca^{2+}-channel (N-type) currents in SH-SY5Y cells, and enhanced GTPγ^{35}S binding in isolated membranes, nearly ten times greater than that observed in HEK293 cell membranes.

3 GTPγ^{35}S-bound Gα subunits from quinpirole-activated and solubilized membranes were monitored upon immobilization with various Gα-specific antibodies. Gα_o subunits (not Gα_i) were highly labelled with GTPγ^{35}S in SH-SY5Y, but not in HEK293 cell membranes, despite their abundance in the both cell types, as shown with reverse transcription-polymerase chain reaction and Western blots. N-type Ca^{2+} channels and adenylyl cyclase V (D3-specific effector), on the other hand, exist only in SH-SY5Y cells.

4 More efficient coupling of the D3 receptor to G_o subtypes in SH-SY5Y than HEK293 cells may be attributed, at least in part, to the two D3 neuronal effectors only present in SH-SY5Y cells (N-type Ca^{2+}-channels and adenylyl cyclase V). The abundance of G_o subtypes in the both cell lines seems to indicate their availability not a limiting factor.

Keywords: Human D3 dopamine receptor; G_o subtypes of G-proteins; SH-SY5Y cells; signalling of D3 dopamine receptor; GTP^{35}S bound Gα subunits

Abbreviations: AC, adenylyl cyclase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; CHO, Chinese hamster ovary; EC_{50}, a half maximal concentration; GPCR, G protein coupled receptor; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, a half maximal inhibitory concentration; IP_3, inositol 1,4,5-triphosphate; K_D, dissociation constant; K_I, inhibition constant; PCR, polymerase chain reaction; RT, reverse transcription; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid

Introduction

The D3 dopamine receptor, a G protein-coupled receptor (GPCR) with seven transmembrane segments, belongs to a family of D2-like dopamine receptors (D2–D4) (Kebabian & Calne, 1979; Civelli *et al*., 1993; Seeman & Van Tol, 1994; Missale *et al*., 1998), and has been the target of potential drugs for psychotic disorders and Parkinson's disease (Sokoloff *et al*., 1990; Sokoloff & Schwarz, 1995). Although the human D3 receptor has been heterologously expressed and examined in various mammalian cells, its signalling pathways have not been clearly established. The D3 receptor is known to inhibit adenylyl cyclases, but its signal was considerably weaker than that of the D2 and D4 receptor in Chinese hamster ovary (CHO) cells, a putatively responsive cell line, (Chio *et al*., 1994; Potenza *et al*., 1994; McAllister *et al*., 1995; Missale *et al*., 1998), or hardly detectable in many other cell lines, such as C6 glioma, LTK-, a rat fibroblast, SK-N-MC human epithelioma, CCL1.3 fibroblast, and GH4C1 pituitary cells (Sokoloff *et al*., 1990; Seabrook *et al*., 1992; Tang *et al*., 1994; Freedman *et al*., 1994; Pilon *et al*., 1994; MacKenzie *et al*., 1994; Cox *et al*., 1995). Also absent were other signals frequently observed with some GPCRs, like the release of arachidonate or inositol-1,4,5-trisphophate (IP_3), and the modulation of K^+- or Ca^{2+}-channels (Seabrook *et al*., 1992; Freedman *et al*, 1994). Even in the first step of G-protein activation, agonist-induced GTPγ^{35}S binding, the D3 response was much smaller than that of the D2 or D4 receptors in CHO cells (Gardner *et al*., 1996). Further understanding of D3 signalling pathways, therefore, seems to depend on the discovery of a cell line containing appropriate cellular machinery for D3 signals. In this study we report greater agonist-induced responses for the human D3 receptor heterologously expressed in human neuroblastoma cells (SH-SY5Y) as compared to those in human embryonic kidney cells (HEK293), and its efficient coupling to the G_o subtype of G proteins.

Methods

A [^{3}H]-IP_3 radioreceptor assay kit, a FlashPlate [^{125}I]-cyclic AMP assay kit, [^{3}H]-raclopride and GTPγ^{35}S were purchased from DuPont NEN. A PRISM Ready Reaction DyeDeoxy Terminal Cycle Sequencing Kit was purchased from Perkin-

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Elmer/Applied Biosystems Division, and a mammalian expression vector, PCI-Neo, from Promega. The antibodies selective for $G\alpha_i$-$G\alpha_o$, $G\alpha_s$, $G\alpha_{q/11}$ or $G\alpha_{13}$ were purchased from Calbiochem, and the antibodies selective for $G\alpha_{i3}$ or $G\alpha_o$ (K-20) from Santa Cruz Biotechnology. The mouse monoclonal antibody against bovine G_o protein was obtained from Chemicon. Hybond-P (polyvinylidene difluoride membrane) and ECL Plus Western blotting detection system were purchased from Amersham Pharmacia Biotech.

The human cDNA for the D3 dopamine receptor was cloned from a human cDNA library and inserted into a PCI-Neo mammalian expression vector. This was expressed in HEK293 or SH-SY5Y cells, using Ca^{2+} phosphate precipitation techniques, and subsequent G-418 selection. Membranes from transfected cells were prepared by standard procedures including cell homogenization and differential centrifugation as described elsewhere (Pregenzer *et al*., 1993). Binding of radioactive ligands was measured in membranes expressing the recombinant receptor, using filtration techniques as described elsewhere (Pregenzer *et al*., 1993). Briefly, [^{3}H]-raclopride binding was measured in medium containing (mM): NaCl 100, $MgCl_2$ 2, EDTA 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 (pH 7.4) and 5–15 μg membrane protein, using the radioactive ligand at varying concentrations (0.1–20 nM for typical binding profiles). Incubations were performed in a total volume of 500 μl at 23°C for 60 min. Reaction mixtures were filtered over Whatman GF/B filters under vacuum, and filters were washed three times with 4 ml of ice cold 50 mM Tris/HCl buffer (pH 7.4). Non-specific binding was estimated in the presence of excess unlabelled raclopride (10 μM). All the stock solutions for ligands were prepared in 0.1 % ascorbic acid (w v^{-1}). Displacements of [^{3}H]-raclopride by test compounds (competition assays) were carried out in the same assay buffer with the radioactive ligand at 1 nM.

GTPγ^{35}S binding was measured following the procedures reported earlier (Chabert *et al*, 1994; Pregenzer *et al*., 1997) in medium containing (mM) HEPES 25,: NaCl 100, EDTA 1, $MgCl_2$ 3, dithiothreitol, 0.003% digitonin (w v^{-1}) 0.5, GTPγ^{35}S ($5-3\times10^5$ cpm/assay) 2, and about 20 μg membrane protein in a volume of 120 μl. The use of digitonin at trace amounts here was solely to make the membranes permeable to GTPγ^{35}S. Test ligands were included at 10 μM, unless indicated otherwise. Membranes were preincubated with 100 μM 5′-adenylylimidodiphosphate for 30 min, and then with 10 μM GDP for 10 min on ice. The reaction was initiated by adding treated membranes to reaction mixtures and continued at 30°C for 30 min. Reaction mixtures were filtered over Whatman GF/B filters under vacuum, and filters were washed three times with 4 ml of an ice-cold buffer containing (mM): NaCl 100, Tris/HCl, pH 8.0 20, $MgCl_2$ 25. Agonist-induced GTPγ^{35}S binding was obtained by subtracting that observed without agonists. The binding data were analysed using a nonlinear regression method (Sigma Plot), and presented as mean ± s.e.mean from three or more experiments.

Agonist-induced IP_3 release in intact cells was measured using the [^{3}H]-IP_3 radioreceptor assay. Briefly the cells were seeded on a 24-well plate and grown to about 80% confluency. The cells were treated with 10 μM quinpirole or 1 mM carbachol at 37°C for 0, 30, 45 or 60 s, and the reaction was stopped with trichloroacetic acid (TCA, 20% final concentration). After removal of TCA with 1,1,2-trichloro-1,2,2-trifluoroethane and trioctyl amine, an aliquot was analysed for IP_3, using [^{3}H]-IP_3/IP_3 receptor preparations from calf cerebellum, following the protocols provided by the vendor (DuPont NEN). For each experiment, a dose-response profile for IP_3 was constructed by adding known amounts of exogenous IP_3 to TCA extracts of untreated cells.

Cellular changes in cyclic AMP were measured using a FlashPlate [^{125}I]-cyclic AMP assay. Briefly, cells were grown to about 80% confluency in a 96-well plate, and then treated with forskolin at a submaximal concentration (typically 10 μM) with or without quinpirole or test ligands for 30 min in the presence of a phosphodiesterase inhibitor. Cyclic AMP in cell lysates was measured, through competition between [^{125}I]-cyclic AMP and non-radioactive antigen for a fixed number of antibody binding sites in microplates coated with solid scintillant.

The whole-cell configuration of the patch clamp technique (Hamill *et al*., 1981) was used to record Ca^{2+}-channel currents, as described elsewhere (Ito *et al*., 1994). Briefly, currents were recorded by a current-voltage converter consisting of an Axopatch-1D amplifier and a CV-4 headstage (Axon Instrument Co., U.S.A.), and stored on diskettes in an IBM-PC compatible computer using the 'pClamp' acquisition software (Axon Instrument Co.). The Pipette solution contained (mM): N-methylglucamine 140, EGTA 10, glucose 5 and HEPES 10, pH 7.2. External solution contained (mM): $BaCl_2$ 50, CsCl 50, tetraethylammonium chloride 25, glucose 25, HEPES 10, (pH 7.3), GTP 0.3 and tetrodotoxin 0.5. All experiments were performed at room temperature (23°C).

GTPγ^{35}S-bound $G\alpha$ subunits of G proteins were identified following the method described elsewhere (Okamoto *et al*., 1992) with some modifications. The key change was that receptor-activation by agonists in the presence of GTPγ^{35}S preceded membrane solubilization with detergents while the sequence was reversed in the original procedure. This change is beneficial in that G proteins are activated prior to general structural disruptions of GPCRs, G proteins and surrounding membranes by detergents. Briefly, membranes (~50 μg or less membrane proteins) were incubated in the presence of GTPγ^{35}S (4 nM) and quinpirole (10 μM) under the conditions identical to those for GTPγ^{35}S binding as described above. Such treated membranes were solubilized with an equal volume of a buffer containing (mM): Tris/HCl, 100, pH 8.0, $MgCl_2$ 10, NaCl 100 and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS, w v^{-1}) 0.6% for 30 min on ice, and then diluted to a final CHAPS concentration to 0.125% (w v^{-1}). Aliquots of the mixtures (typically 300 μl) were transferred to wells which had been coated successively with goat anti-rabbit (or anti-mouse) antibodies (1 : 100 dilution), bovine serum albumin (5 mg ml^{-1}) and one of the affinity-purified antibodies specific for various $G\alpha$ subunits (1 : 200 dilution). Amounts of membranes were chosen to be in the range where quinpirole-induced GTPγ^{35}S association with the antibody for $G\alpha_i-G\alpha_o$ linearly increased. Here we used the antibody selective for $G\alpha_i-G\alpha_o$ (against the C-terminal sequence of $G\alpha_{i3}$, KNNLKECGLY (345–354) which shares the same last four residues (CGLY) with $G\alpha_o$), the antibodies selective for $G\alpha_s$ (against the C-terminal sequence, 385–394), $G\alpha_{q/11}$ (against the common C-terminal sequence, QLNLKEYNLV), or $G\alpha_{13}$ (against the sequence, 367-377) (Calbiochem). We also used the antibody cross-reacting with $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ due to their common residues (KNNLK), but not with $G\alpha_o$ (Santa Cruz), and the mouse monoclonal antibody raised against bovine G_o protein (Chemicon). The plates were incubated for 1 h at room temperature. Individual wells were washed free of unbound GTPγ^{35}S, and counted using a standard scintillation cocktail and a β-counter. Ligand specific-GTPγ^{35}S binding was computed by subtracting that observed without test ligands.

Transcripts for $G\alpha_o$ and adenylyl cyclase V were estimated using semi-quantitative reverse transcription with polymerase chain reaction (RT–PCR). RT reactions were carried out with an aliquot of RNA fractions (2 μg) extracted from SH-SY5Y or HEK293 cells, RNase inhibitor, oligo dT, random hexamers, dNTP (1.5 mM) and reverse transcriptase (Life Technologies) in a vendor-supplied buffer, for 1 h at 37°C. PCR was performed in a 50 μl reaction mixture containing 2 μl of RT reaction mixtures, 1 U Amplitaq DNA polymerase in the vendor supplied buffer, 200 μM each dNTP, 10 pmol primers. The cycle parameters were at 94° for 30 s, 54° for 30 s and 72° for 90 s with a final extension for 10 min after 28 cycles. For $G\alpha_o$, the primers were designed from the published sequence of the human $G\alpha_{o1}$ (bp 726–746 and bp 1029–1049) and $G\alpha_{o2}$ (bp 729–748 and bp 1025–1047) (Tsukamoto *et al*., 1991), and for adenylyl cyclase V (bp 1192–1212 and bp 1484–1502) from the partial sequence of human adenylyl cyclase V in Genbank (Accession number M83533). RT–PCR yielded fragments of the expected size. Additionally, all PCR products from the two cell lines were purified through agarose gel electrophoresis, and were directly sequenced using the PRISM Ready Reaction DyeDeoxy Terminal Cycle Sequencing Kit.

For Western blots, aliquots of cell lysates (20 μg protein) in the presence of protease inhibitors (leupeptin, 2 μg/ml and phenylmethylsulfonylfluoride, 1 mM) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), using reducing gels and transferred to Hybond-P™ (polyvinylidene difluoride membrane). Membranes were probed with the $G\alpha_o$-specific polyclonal rabbit antibody K-20 (1 : 500 dilution) (Santa Cruz), which was raised against a highly divergent $G\alpha_o$ domain, KMVCDVVSRMEDTEPFSAEL (105–124), and was not cross-reactive with other $G\alpha$ subunits. Detection was performed using the ECL Plus Western blotting detection system.

Results

We examined ligand binding properties of the human D3 dopamine receptor expressed in HEK293 or SH-SY5Y cells. Scatchard analysis of binding data for [^{3}H]-raclopride at various concentrations, fit to the binding equation with a single class of binding sites (linearity) and yielded dissociation constant (K_D) of 1.5 ± 0.1 and 2.1 ± 0.3 nM, and maximal binding of 1.9 ± 0.2 and 2.3 ± 0.2 pmoles/mg protein in membranes from HEK293 and SH-SY5Y cells, respectively. Competition binding experiments using [^{3}H]-raclopride, were carried out with standard dopaminergic ligands at various concentrations. The inhibition constants (K_I) for spiperone, sulpiride, raclopride, quinpirole, dopamine, pramipexole and terguride (Table 1), as obtained using Cheng and Prusoff equation (Cheng & Prusoff, 1973) were in good agreement between the two cell lines, and were also comparable with those reported in the literature (Sokoloff *et al*, 1990). For the D3 receptor, agonists are known to display small affinity differences (less than 10 fold) between their high and low affinity sites (G-protein coupled and uncoupled phenotype, respectively) (Sokoloff *et al*., 1990), which could be further obscured by high receptor densities in cloned cells (Boundy *et al*, 1996; Butkerait *et al*., 1995). Consistent with this expectation, data from the displacement of [^{3}H]-raclopride by quinpirole were fitted to the one-site binding model with its K_I of 27 ± 2 nM (Figure 1) and were not altered upon addition of GTPγS at 10 μM (data not shown).

High threshold-activated Ca^{2+} channel currents in SH-SY5Y cells are largely blocked by ω-conotoxin (Friederich *et al*., 1993; Reeve *et al*., 1995), as N-type channels. Here, currents (Ba^{2+} ions as charge carriers) were evoked by pulses of 25 mV and 400 ms-duration from a holding potential of -100 mV. Quinpirole concentration-dependently blocked Ca^{2+}-channel currents in SH-SY5Y cells heterologously expressing the human D3 receptor, with an IC_{50} value of 5.6 ± 1.8 nM and maximal inhibition of 55 ± 3 % (Figure 1). In untransfected cells, no effect on Ca^{2+} channel currents was observed with quinpirole at 1 μM. Certain GPCRs activate phospholipase C, *via* $G\alpha_{q/11}$ or $G\beta\gamma$ subunits, and increase intracellular IP_3. Quinpirole failed to enhance IP_3 release in SH-SY5Y cells expressing D3 while carbachol markedly increased IP_3 release in the same cell line by activation of endogenous m3 muscarinic receptors (Figure 1).

Agonist-bound GPCRs catalyze the exchange of GDP with GTP on G-protein α subunits as the first step of G-protein activation. This step was monitored with GTPγ^{35}S, a slowly hydrolyzing GTP analogue. Quinpirole concentration-dependently enhanced GTPγ^{35}S binding in membranes from the SH-SY5Y cells, with a half maximal concentration (EC_{50}) of 25 ± 4 nM, and maximal binding of 149 ± 21 fmoles/mg protein, but by only 14 ± 6 fmoles/mg protein in membranes from HEK293 cells (Figure 2). Quinpirole-induced GTPγ^{35}S binding in SH-SY5Y cell membranes was blocked by haloperidol (10 μM), an antagonist (Figure 2). Haloperidol alone reduced the basal GTPγ^{35}S binding by 17%, as normalized to that observed with quinpirole, probably due to some population of receptors in constitutively active states. Several known agonists for D3 were also examined for their effects on GTPγ^{35}S binding in SH-SY5Y cell membranes. Dopamine, pramipexole and terguride concentration-dependently enhanced GTPγ^{35}S binding with an EC_{50} value of 42 ± 4, 18 ± 2, and 1.4 ± 0.2 nM, respectively, and maximal stimulation of 96 ± 4, 97 ± 9 and 47 ± 5%, respectively, as normalized to that of quinpirole (Table 2).

We examined the effect of quinpirole on forskolin-stimulated cyclic AMP production in SH-SY5Y and HEK293 cells, where forskolin at 10 μM (a submaximal concentration)

Table 1 Comparison of binding properties of the human D3 dopamine receptor expressed in SH-SY5Y and HEK293 cells

Compounds	*SH-SY5Y* K_I, nM	*HEK293* K_I, nM
Spiperone	0.15 ± 0.03	0.28 ± 0.05
Sulpiride	6.4 ± 0.6	8.9 ± 0.6
Raclopride	2.1 ± 0.3	1.5 ± 0.1
Quinpirole	27 ± 2	23 ± 1
7-OH DPAT	0.8 ± 0.3	1 ± 0.1
Dopamine	15 ± 1.3	15 ± 2
Pramipexole	5.8 ± 0.9	8.5 ± 0.2
Terguride	0.8 ± 0.1	0.6 ± 0.2

The IC_{50} values were obtained from competition binding experiments using [^{3}H]-raclopride in the presence of test ligands at various concentrations. IC_{50} values from dose-response profiles were converted to K_I using the Cheng and Prusoff equation. Experiments were carried out at room temperature in membranes from SH-SY5Y or HEK293 cells expressing the human D3 dopamine receptor, as described in Methods. The data represent mean$\pm$s.e. from at least two full dose-response profiles (six concentrations) and one or more abbreviated ones (three concentrations). The maximal binding sites as obtained from [^{3}H]-raclopride binding were 2.3 ± 0.2 and 1.9 ± 0.2 pmol/mg protein for SH-SY5Y and HEK293 cells, respectively.

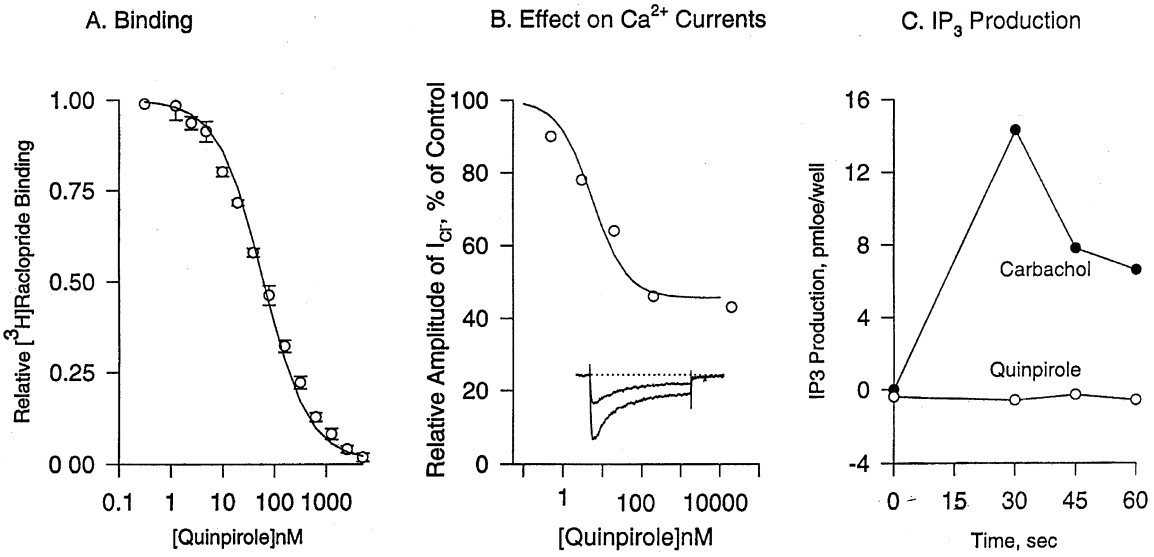

Figure 1 Plots showing the displacement of [^{3}H]-raclopride binding to D3 by quinpirole, effects of quinpirole on N-type Ca^{2+}-channel currents and on IP_3 production in SH-SY5Y cells expressing the human D3 dopamine receptor. (A) Binding of [^{3}H]-raclopride was measured in the presence of quinpirole at various concentrations in isolated membranes from SH-SY5Y cells. The solid line represents the data fitting with the binding equation for a single class of binding sites with the K_I of 27 ± 2 nM. (B) Quinpirole concentration-dependently blocked Ca^{2+}-channel currents (Ba^{2+} as the charge carrier) in SH-SY5Y cells expressing the human D3 dopamine receptor. The inset shows representative traces for Ca^{2+}-channel currents in the absence or presence of quinpirole at 1 μM, obtained using a pulse potential of 25 mV for 400 ms from a holding potential of -100 mV. The amplitude of the peak current without the drug was 120 pA. The dotted line represents the current baseline obtained upon application of La^{3+} (100 μM) at the end of the recording. (C) Quinpirole at 10 μM had no effect on intracellular level of IP_3 in SH-SY5Y cells while carbachol at 1 mM transiently enhanced IP_3 in the same cell line.

typically increased cyclic AMP by 4–5 pmoles per well (a 96-well plate). Quinpirole concentration-dependently reduced the cyclic AMP increase in SH-SY5Y cells, and its maximal inhibition amounted to $63 \pm 10\%$. Composite dose-response profiles (Figure 2), when normalized to maximal inhibition observed with individual experiments, showed an IC_{50} value of 0.95 ± 0.5 nM for quinpirole. Parallel assays in HEK293 cells showed the maximal inhibition of adenylyl cyclases by quinpirole amounting to $27 \pm 3\%$ of that observed in SH-SY5Y cells, and with an IC_{50} value of 1.1 ± 0.4 nM (Figure 2). We also examined the effects of several other agonists on adenylyl cyclases in SH-SY5Y cells. Dopamine, pramipexole and terguride concentration-dependently reduced forskolin-stimulated cyclic AMP with EC_{50} values of 0.8 ± 0.2, 0.5 ± 0.3 and 0.8 ± 0.3 nM, respectively, and maximal inhibition of 91 ± 4, 92 ± 5 and $96 \pm 6\%$, respectively, as normalized to that of quinpirole (Table 2). Haloperidol by itself had no appreciable effect on forskolin-stimulated cyclic AMP level, but blocked the dopamine action, as expected for an antagonist (Figure 2). Note that the agonist EC_{50} values in this cyclic AMP assay were 30–50-fold less than those in the GTPγ^{35}S assay, except for terguride with only a 2 fold difference. Moreover, terguride behaved like a full agonist with the cyclic AMP assay (96% of quinpirole), but like a partial agonist with the GTPγ^{35}S assay (45% of quinpirole), as noted above. It appears that the maximal inhibition of adenylyl cyclase by agonists may occur at a receptor occupancy considerably lower than that observed with GTPγ^{35}S binding. Moreover, terguride, a seemingly partial agonist in the GTPγ^{35}S assay, may achieve maximal inhibition of adenylyl cyclase like full agonists, at its receptor occupancy much greater than that for full agonists.

Taking advantage of robust agonist-induced GTPγ^{35}S binding in SH-SY5Y cell membranes, we attempted to trap quinpirole-dependent GTPγ^{35}S-labelled Gα subunits from solubilized membranes, with Gα-specific antibodies, following the procedure previously described (Okamoto *et al.*, 1992), with some modifications (see Methods). After receptor activation with quinpirole at 10 μM in the presence of GTPγ^{35}S (4 nM), G proteins were solubilized with 0.3% CHAPS (w v^{-1}), and trapped with antibodies specific for various Gα subunits; Gα_i, Gα_o, Gα_i–Gα_o (cross-reacting with both), Gα_s, G$\alpha_{q/11}$, G_{13}. The highest level of GTPγ^{35}S was observed with the antibody for Gα_i–Gα_o (Figure 3). Typically, GTPγ^{35}S associated with the antibody was about three times greater than that observed in the control well without quinpirole activation; e.g., 6182 ± 325 vs 1610 ± 240 c.p.m. per well. The next highest level of GTPγ^{35}S was associated with Gα_o, $75 \pm 3\%$ of that for Gα_i–Gα_o, followed by Gα_i, only $8 \pm 5\%$. Relative degrees of GTPγ^{35}S-association with the other antibodies, as normalized to that observed with the antibody against Gα_i–Gα_o in SH-SY5Y cell membranes, were 7 ± 3, -1 ± 2 and $-2 \pm 3\%$ for Gα_s, G$\alpha_{q/11}$, G_{13}, respectively (Figure 3). These results point to Gα_o subtypes of G proteins as the primary targets of D3 (Figure 3). Moreover, quinpirole (200 nM)-induced GTPγ^{35}S association with the Gα_i–Gα_o antibody was blocked by coapplication of haloperidol (10 μM) (data not shown). Similar experiments with membranes from HEK293 cells expressing D3 showed no marked association of GTPγ^{35}S with any antibodies we tested, including those specific for Gα_i–Gα_o or Gα_o (Figure 3). Two isotypes of Gα_o (Gα_{o1} and Gα_{o2}) are known (Tsukamoto *et al.*, 1991), and their presence in the two cell lines was examined, using RT–PCR with isotype-specific primers, in a semi-quantitative manner (see Methods). Gα_{o1} transcripts were well amplified in samples from HEK293 and SH-SY5Y cells, and Gα_{o2} transcripts were detectably amplified, but its product was somewhat less for HEK293 than SH-SY5Y cells (Figure 4). Direct sequencing of the PCR products from the both cell lines confirmed their identities as Gα_{o1} and Gα_{o2} (Tsukamoto *et al.*, 1991). The protein levels of Gα_o subtypes in the two cell lines were estimated with Western blots using the antibody specific for Gα_o, albeit not isotype-specific. The Western blot (Figure 4) displayed the primary band at the molecular weight of

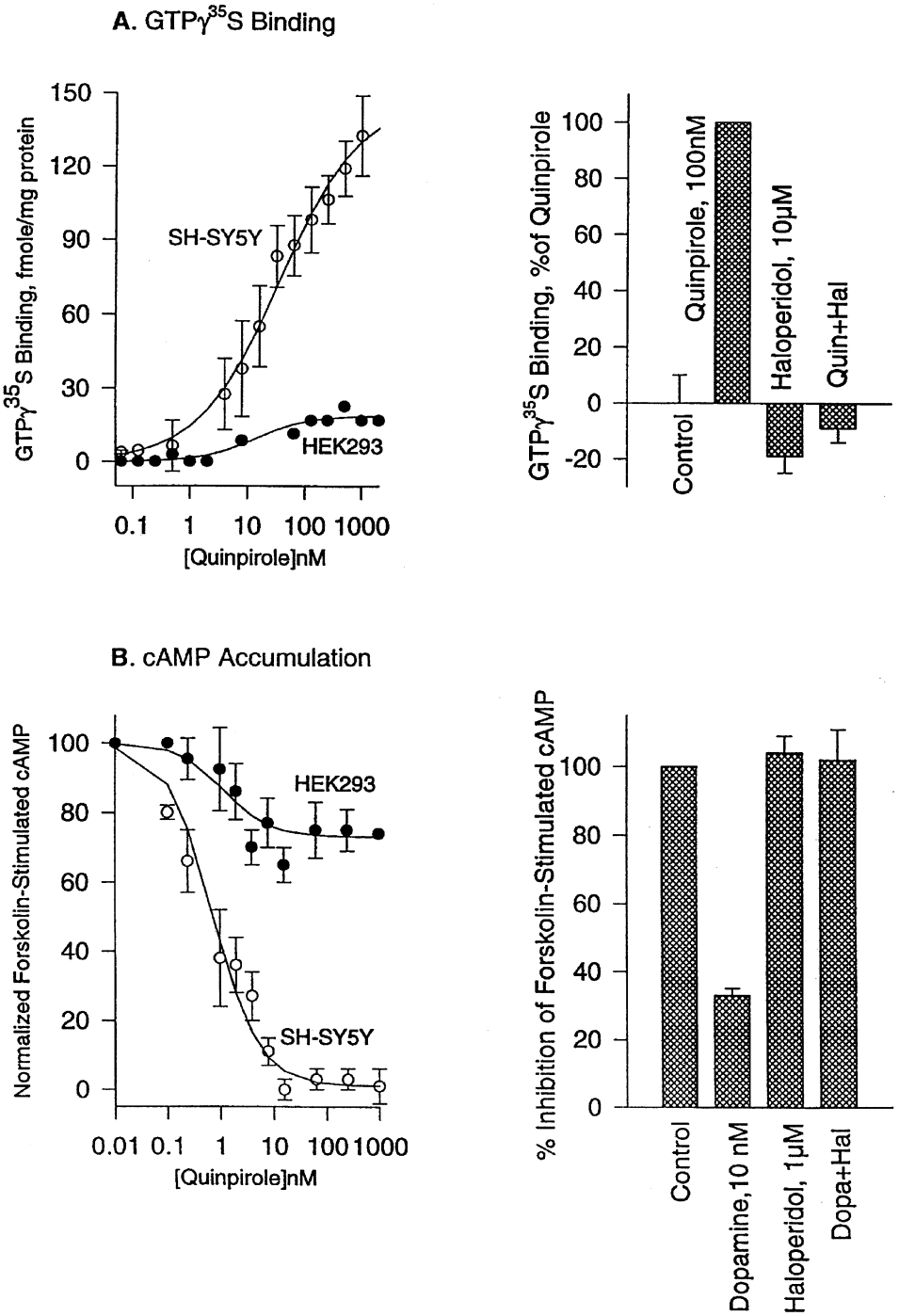

Figure 2 Comparison of quinpirole-induced GTPγ^{35}S binding in isolated membranes and inhibition of forskolin (10 μM)-stimulated cyclic AMP production in HEK293 cells and SH-SY5Y cells expressing the human D3 dopamine receptor. (A) Quinpirole dose-dependently increased GTPγ^{35}S binding in SH-SY5Y cell membranes, nearly 10 times more than that in HEK293 cell membranes. The quinpirole-induced GTPγ^{35}S binding was blocked by haloperidol, which by itself reduced the basal GTPγ^{35}S binding by 17%. (B) Quinpirole dose-dependently blocked forskolin-stimulated cyclic AMP production in SH-SY5Y cells much more robustly than in HEK293 cells. The degrees of inhibition at various concentrations were normalized to the maximal inhibition observed with quinpirole in SH-SY5Y cells in parallel assays. Dopamine similarly inhibited the cyclic AMP production in SH-SY5Y cells and its action was blocked by haloperidol.

39 k.d.a., which is identical to that of Gα_o subunits as resolved with SDS–PAGE. Intensities of the 39 k.d.a. band indicate comparable abundance of Gα_o subunits in the two cell lines. A minor band at the molecular weight of 31 k.d.a. was detected only in SH-SY5Y cells (Figure 4), probably partially digested Gα_o.

Among nine isotypes of adenylyl cyclases in mammalian cells, adenylyl cyclase V (ACV) has been recently proposed as a selective D3 target, because its heterologous coexpression with the D3 dopamine receptor in HEK293 cells resulted in robustly enhanced quinpirole-induced inhibition of forskolin-stimulated cyclic AMP production (Robinson & Caron, 1997). We examined here the presence of ACV transcripts in the two cell lines, using RT–PCR with ACV-specific primers (see Methods). Transcripts for ACV were well amplified in samples from SH-SY5Y cells, but were not detectably amplified in

Table 2 Intrinsic efficacy of standard agonists for the human D3 dopamine receptor as measured with GTPγ^{35}S binding and inhibition of cyclic AMP production

	GTPγS binding		*Inhibition of adenylyl cyclase*	
Compounds	*EC_{50}, nM*	*Maximum efficacy % of quinpirole*	*EC_{50}, nM*	*Maximum efficacy % of quinpirole*
Quinpirole	25±4	100	1.0±0.5	100
Dopamine	42±4	96±4	0.5±2	91±4
Pramipexole	18±2	97±9	0.9±0.3	88±5
Terguride	1.4±0.2	47±5	0.8±0.3	96±6

GTPγ^{35} binding was measured in SH-SY5Y cell membranes in presence of test ligands at various concentrations, and agonist-induced level was computed by subtracting that observed in the absence of test ligands, and normalized to that observed with quinpirole (10 μM) in the same experiments. Changes in forskolin-stimulated cyclic AMP production was measured in intact cells upon treatment with forskolin (10 μM) and test ligands at various concentrations, using Flash-Plate Kits (NEN). The dose-response profiles were fitted using the model with a single class of binding sites for test ligands. The data represent the mean±s.e.mean from three experiments.

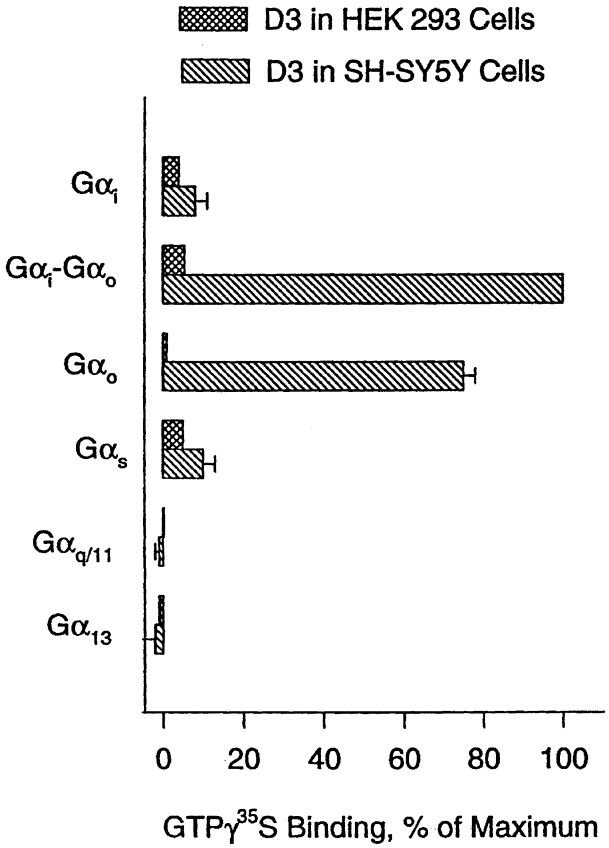

Figure 3 Plot showing relative levels of quinpirole-induced GTPγ^{35}S association with the antibodies specific for various Gα subunits. Membranes from SH-SY5Y and HEK293 cells expressing the human D3 dopamine receptor were treated with quinpirole at 10 μM in the presence of GTPγ^{35}S at 4 nM, and then solubilized with 0.3% CHAPS (w v^{-1}). The mixtures were diluted to final CHAPS concentration of 0.125% (w v^{-1}) and then added to the wells coated with indicated antibodies for Gα isotypes. After washing out unbound GTPγ^{35}S, the wells were counted for ^{35}S radioactivity. The GTPγ^{35}S binding level for each antibody was normalized to that observed with the Gα_i–Gα_o antibody. The data represent the mean±s.e.mean from four experiments.

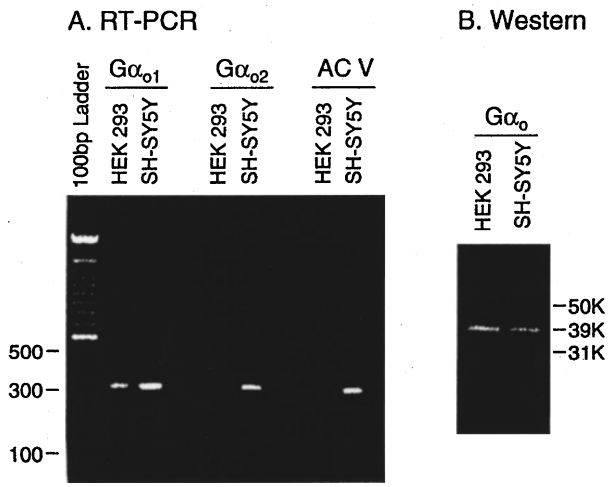

Figure 4 Detecting transcripts for Gα_{o1}, Gα_{o2} and adenylyl cyclase V (ACV) *via* RT–PCR, and expression levels of Gα_o subunits *via* Western blots with cell lysates from HEK293 and SH-SY5Y cells. (A) RT–PCR were carried with RNA extracts from HEK293 and SH-SY5Y cells and primers specific for Gα_{o1}, Gα_{o2} and ACV as described in detail in Methods. (B) Cell lysates from HEK293 and SH-SY5Y cells were resolved on reducing SDS–PAGE gels, transferred to PVDF membranes, and blotted with the antibody specific for Gα_o subunits.

those from HEK293 cells. The identity of the PCR product from SH-SY5Y cells was confirmed with direct sequencing.

Discussion

In this study we have shown that the human D3 dopamine receptor, when heterologously expressed in SH-SY5Y cells, produced robust functional signals, which included agonist-induced stimulation of GTPγ^{35}S binding, inhibition of adenylyl cyclases and reduction of N-type Ca^{2+}-channel currents. These responses were much greater than those obtained with HEK 293 cells, where the receptor was expressed as abundantly as in SH-SY5Y cells. Agonist-induced GTPγ^{35}S binding, representing the first step of G protein activation, was nearly 10 fold greater in SH-SY5Y than HEK293 cell membranes. Immobilization studies with antibodies specific for various Gα subunits revealed that quinpirole induced exclusive associations of GTPγ^{35}S with Gα_o subtypes of G-proteins in SH-SY5Y cells, but not with Gα_i. It should be pointed out that the specificity of the Gα_o-and Gα_i-selective antibodies has been well documented by their vendors (see Methods), and antibody efficiencies to bind target subunits appear to be comparable. For example, similar studies with 5-HT_{1B} receptors which are known to couple to G_i but not G_o subtypes showed the expected pattern, robust associations of GTPγ^{35}S with Gα_i and Gα_i–Gα_o, but not Gα_o (unpublished results). Immunoprecipitation studies with the antibodies specific for other α subunits, Gα_s–G$\alpha_{q/11}$ and Gα_{13}, revealed no appreciable association with GTPγ^{35}S in SH-SY5Y cell membranes.

From the above considerations, it is reasonable to propose that G_o subtypes are the major G proteins activated by D3 dopamine receptors in SH-SY5Y, but not in HEK293 cells. $G\alpha_o$ subunits are highly enriched in neurons, and also seem to be reasonably abundant in HEK293 cells, as shown in this study by RT–PCR with $G\alpha_o$ specific primers and by Western blots with the $G\alpha_o$-specific antibody (K-20 from Santa Cruz). The K-20 antibody which was raised against the internal sequence unique for $G\alpha_o$ (Residue 105–124) has been previously documented for its binding specificity and efficiency for $G\alpha_o$ in Western blots (Schandar *et al*., 1998). This apparent presence of $G\alpha_o$ in HEK293 cells raises the question of why D3 receptors failed to produce robust G protein couplings (e.g., as measured with agonist-induced GTPγ^{35}S binding) which seems to occur promiscuously between G proteins and various receptors in isolated/reconstituted states. One possibility has been recently proposed that such couplings would not occur in intact cells in the absence of target effectors. Such coupling specificities in cells may require formation of structurally restrained complexes among receptors, G-proteins and target effectors (Chidiac, 1998).

Numerous $G\alpha_o$-coupled effectors would be expected to exist in neuronal cell lines, and here we examined only two effectors, adenylyl cyclases and N-type Ca^{2+} channels. Adenylyl cyclases consist of nine isotypes including the most recently discovered type IX (Premont *et al*., 1996). Among the multiple isotypes, ACV has been reported to be a selective target for D3 (Robinson & Caron, 1997). Only when the D3 receptor and ACV were coexpressed in HEK293 cells, quinpirole robustly blocked forskolin-stimulated cyclic AMP, despite the presence of other endogenous adenylyl cyclases such as ACII, III, IV and VII (Hellevuo *et al*., 1993). Using RT–PCR, we have shown here the presence of ACV transcripts in SH-SY5Y cells, but not in HEK293 cells. In the brain, ACV is highly expressed in selective regions, such as the striatum and nucleus accumbens which are the important regions for both dopaminergic and serotonergic neurotransmission (Glatt & Snyder, 1993; Premont *et al*., 1996). ACV thus appears to be a physiologically relevant target for the D3 receptor. In addition to ACV, SH-SY5Y cells contain high threshold N-type Ca^{2+} channels, selectively inhibited by ω-conotoxin and also by G_o coupled receptors, such as neuropeptide Y receptors (Friederich *et al*., 1993; Reeve *et al*., 1995). Generally, N-type Ca^{2+}-channel currents are inhibited by various G_o coupled receptors, such as opiate, muscarinic, $GABA_B$ and neuropeptide Y receptors, but are much less so by G_i coupled receptors (Lledo *et al*., 1992). The potent inhibition of Ca^{2+}-channel currents in SH-SY5Y cells by quinpirole is consistent with the coupling of D3 to G_o subtypes of G proteins. As described above, $G\alpha_o$ subunits consists of the two splice variants, $G\alpha_{o1}$ and $G\alpha_{o2}$ (Tuskamoto *et al*, 1991), in HEK293 and SH-SY5Y cells, but their relative abundance will not be known, until the antibodies specific for the isotypes become available. So far, however, no isotype-specific functional differences have been established, e.g., both isotypes have been reported to be active for N-type Ca^{2+} channels (Lledo *et al*., 1992). $G\alpha_o$ subtypes seem not to be a limiting factor in D3 signallings in HEK293 cells.

In this study, we were able to point out two effectors, N-type Ca^{2+} channels and ACV which could contribute, at least in part, to the D3 signalling differences in HEK293 and SH-SY5Y cells, including robust agonist-induced GTPγ^{35}S binding. Yet, a number of questions remain to be resolved. (1) To what extent do these effectors contribute to the GTPγ^{35}S binding? (2) What other effectors are involved in D3 receptor signaling in SH-SY5Y cells? (3) Are there other molecular entities required for D3 signallings? Recently, caveolins, representing a family of scaffolding proteins for organizing preassembled signalling complexes at the plasma membranes, have been proposed to regulate the activation state of associated signalling molecules (Okamoto *et al*., 1998). Some of these questions could be resolved in the near future by removing ACV and/or N-type Ca^{2+} channels from SH-SY5Y cells, using various molecular biological techniques, and also by expressing these effectors in HEK293 cells.

In this study, intrinsic efficacy of several standard ligands was compared as measured with agonist-induced GTPγ^{35}S binding and inhibition of forskolin-stimulated cyclic AMP production in SH-SY5Y cells. One noteworthy point is that the agonist EC_{50} values for the cyclic AMP assay were 30–50-fold less than those for the GTPγ^{35}S assay (Table 2). This indicates that the receptor occupancy required for the maximal inhibition of adenylyl cyclases by agonists was considerably lower than that observed for the GTPγ^{35}S assay. Moreover, terguride behaved like a partial agonist in the GTPγ^{35}S assay (45% of quinpirole), but behaved like a full agonist in cyclic AMP assay (97% of quinpirole), when its receptor occupancy became much greater than that for full agonists. This points out difficulties in using the cyclic AMP assay or other low threshold assays for estimating intrinsic efficacy of test ligands, because of large receptor reserves. In this respect, the GTPγ^{35}S assay seems to be advantageous for intrinsic efficacy measurements because the assay, representing the first step of G-protein activation, seems to be solely dependent on the number of functionally activated receptor complexes without downstream amplification or threshold steps.

In summary, human D3 dopamine receptors appear to activate G_o subtypes of G proteins in SH-SY5Y cells, as evidenced by robust agonist-induced GTPγ^{35}S binding, but not in HEK293 cells, due to the presence of D3-specific target effectors (e.g., ACV and N-type Ca^{2+} channels) only in SH-SY5Y cells.

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