Fluorescence Studies Reveal Heterodimerization of Dopamine D_1 and D_2 Receptors in the Plasma Membrane[†]

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ABSTRACT: Evidence for hetero-oligomerization has recently been provided for various G protein-coupled receptors. In this paper, we have studied the possibility that dopamine D_1 and D_2 receptors physically interact with each other. Human dopamine D_1 and D_2 receptors were fluorescently tagged with derivatives of green fluorescence protein and transiently coexpressed in the membrane of human embryonic kidney 293 cells. Using qualitative fluorescence spectroscopy, as well as quantitative Förster resonance energy transfer (FRET) analysis, performed in a single cell by confocal microscopy and fluorescence lifetime microscopy, we show that dopamine D_1 and D_2 receptors can form hetero-oligomers in the plasma membrane. The degree of receptor protein−protein interaction is significantly enhanced by concomitant addition of D_1 and D_2 receptor subtype-specific agonists. Our investigations extend biochemical and electrophysiological studies and give insights into the regulation and synergistic mode of operation of dopamine receptors.

It has become widely accepted that plasma membrane heptaspanning G protein-coupled receptors (GPCRs)[1] can exist as dimeric or oligomeric complexes (*1*, *2*). The dimerization of membrane receptors (which was first demonstrated for the tyrosine kinase receptor superfamily) can be essentially relevant to receptor function (*3*−*5*) and signal transduction.

Dopamine D_1−D_2 receptor interaction plays an important role for these receptors in the functioning of the human brain and processes underlying reward and cognition. Abnormalities of dopaminergic neurotransmission are implicated in the etiology of several major neurological or psychiatric disorders, including Parkinson's disease, schizophrenia, Huntington's chorea, attention deficit hyperactivity disorder, and drug abuse. The dopamine receptors are targets of several drugs used to treat some of these disorders. Moreover, drugs of abuse like cocaine or amphetamine are dopaminergic stimulants (*6*).

The biological effects of dopamine are mediated by two families of receptors, originally distinguished on the basis of ligand binding and second-messenger coupling (*7*). Molecular cloning of dopamine receptors has enabled further distinctions in two dopamine receptor classes. D_1 and D_5 receptors belong to the D1-like class, whereas D_2−D_4 receptors fall within the D2-like group (*8*). Of all these, D_1 and D_2 are the most widely expressed receptors in the central nervous system.

Evidence that dopamine D_1, D_2 and D_3 receptors can form homodimers in transfected cell lines has been provided (*9*−*11*), and D_2 receptors have been shown to exist as dimers in human and rat brain tissues (*12*). Scarselli et al. (*13*) have shown that D_2 and D_3 receptors can interact with each other to form a functional heterodimer that exhibits unique functional properties. The physical interactions of dopamine receptors with other GPCR (*14*−*18*) and ligand-gated ion channels (*19*−*21*) are also well-documented.

An important feature of normal dopaminergic transmission is that for many behavioral, electrophysiological, and gene-activating effects of dopamine, the concomitant stimulation of D_1 and D_2 receptors is required (*22*−*29*), a phenomenon often termed requisite synergism (*30*). LaHoste et al. (*31*) have provided further evidence that it is the D_2 receptor itself, not other members of the D_2-like receptor family (namely, D_3 or D_4), which synergizes with the dopamine D_1 receptor.

The molecular mechanism underlying the above-mentioned D_1−D_2 receptor synergism has not been fully elucidated, since the cellular localization of D_1 and D_2 receptors is still a matter of controversy. Generally, it has been well documented that these receptors are localized in two independent sets of GABA-ergic projection neurons (*32*). Thus, striatonigral neurons have been found to express D_1 mRNA, whereas striatopallidal neurons have been found to express D_2 receptor mRNA. However, more recently, using immunohistochemistry at the light microscope level (*33*), in situ

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[1] Abbreviations: GPCR, G protein-coupled receptor; FRET, Förster resonance energy transfer; CFP, cyan fluorescence protein; YFP, yellow fluorescence protein; (±)-chloro-APB HBr, (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; HEK, human embryonic kidney.

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hybridization of adjacent brain sections (*34*), single-cell reverse-transcription PCR of dissociated striatal neurons in vitro (*35*), and, finally, confocal microscopy (*36*), evidence for at least some cellular colocalization of D_1 and D_2 mRNA and protein has been provided. As demonstrated by patch−clamp studies in acutely dissociated striatal cultures (*37*), approximately one-half of all medium spiny neurons coexpress functional D_1 and D_2 class receptors. The extent of D_1 and D_2 receptor colocalization appears to correlate with the neuropeptide profile of individual neurons, with low-level colocalization (15−20%) in enkephalin or substance P single positive cells as compared to 70% colocalization in the smaller enkephalin/SP double-positive population.

Therefore, it has been hypothesized that dopamine D_1−D_2 receptor synergistic actions, well-documented in the literature, may result from physical interaction, i.e., oligomerization, of these receptors in the plasma membrane of neurons, on which these two subtypes of dopamine receptors reside. Recently, it has been shown that concurrent coactivation of D_1 and D_2 receptors within the same cells results in the activation of a novel phospholipase C-dependent calcium signaling pathway (*38*). Additionally, it has been demonstrated that the receptors could be co-immunoprecipitated from cells and from brain (*38*), which indicated that D_1−D_2 receptor synergism may occur directly at the receptor protein level. However, it has been frequently emphasized (*1*) that in studies in which dimerization or oligomerization is assessed by the ability of receptors tagged with different epitopes to be co-immunoprecipitated, some artifacts can often arise. The hydrophobicity of the transmembrane domains of receptors raised concerns that apparent co-immunoprecipitation might reflect little more than nonspecific aggregation following detergent extraction of proteins from cell membranes; another key issue is whether the samples are fully solubilized prior to the immunoprecipitation step. On the other hand, failures to detect associated receptors might be due to the harsh and stringent solubilization conditions that might prevent the detection of constitutive dimers by promoting their partial or total dissociation.

Because of these uncertainties in biochemical approaches, we have employed more sensitive biophysical techniques to study the significance of D_1 and D_2 receptor interaction in the plasma membrane. We show here that hetero-oligomerization indeed occurs and that it can be enhanced in the presence of specific receptor agonists.

MATERIALS AND METHODS

Materials. The pcDNA3.1 plasmid, encoding human D_1 and D_2 receptors, was from UMRcDNA Resource Center (Univeristy of Missouri, Rolla, MO). The pECFP-N1 and pEYFP-N1 vectors were from BD Biosciences, Clontech (Palo Alto, CA). All molecular biology reagents were from Fermentas (Vilnius, Lithuania). All cell culture reagents were from Gibco and Sigma (St. Louis, MO). (±)-Chloro-APB HBr (±SKF82958 HBr), a dopamine D_1 receptor agonist, was obtained from Sigma RBI and quinpirole (dopamine D_2 receptor agonist) from Tocris. Human embryonic kidney 293 (HEK 293) cells were obtained from American Type Culture Collection (Manassas, VA).

Construction of Fusion Proteins. The cDNA encoding human D_1 and D_2 receptors was PCR-amplified using a reverse primer without a stop codon and carrying a unique XmaI restriction site and as a forward primer the universal primer for pcDNA3.1. The entire coding sequence was inserted into the pECFP-N1 and pEYFP-N1 vectors using NheI and XmaI restriction enzymes. The pECFP-YFP construct was obtained by CFP PCR amplification using the reverse primer without the stop codon, carrying a unique EcoRI restriction site, the forward primer typical for pECFP-N1, and then the entire coding sequence was inserted into the pEYFP-N1 plasmid, using NheI and EcoRI restriction enzymes.

The dopamine D_2 receptor was tagged with CFP protein and was used as the fluorescence donor, and the D_1 receptor, with YFP, was used as a fluorescence acceptor.

Cell Culture and Transfection. Human embryonic kidney 293 (HEK 293) cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified essential medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen Life Technologies) and 1% L-glutamine. Cells were seeded into 100 mm dishes (2.5×10^6 cells/dish) and transiently transfected with 12 μg of the indicated DNA/dish (donor:acceptor DNA ratio of 1:1), using a calcium phosphate precipitation procedure (*39*). The cells were harvested 48 h after transfection.

For fluorescence lifetime measurements, and confocal imaging, the cells were seeded on glass cover slips in 30 mm dishes (1×10^6 cells/dish).

Membrane Preparation and Radioligand Binding Assay. For binding experiments, the transfected HEK 293 cells were washed with phosphate-buffered saline (PBS), scraped from the dish in PBS, and centrifuged at 1000 rpm for 5 min. The pellet was frozen at −30 °C until it was used.

Frozen pellets were resuspended in the binding buffers [50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, and 1 mM EDTA for D_1 dopamine receptor binding and in 5 mM phosphate buffer (pH 7.4) for D_2 dopamine receptor binding], using an Ultra Turrax homogenizer. The homogenates were centrifuged at 30000*g* for 10 min. That step was repeated twice. [^{3}H]SCH23390 (specific activity of 86 Ci/mmol, NEN, Boston, MA) was used as the dopamine D_1 receptor-specific radioligand and [^{3}H]spiperone (specific activity of 15.7 Ci/mmol, NEN) as the dopamine D_2 receptor-specific radioligand.

Binding assays were performed in a total volume of 500 μL. Saturation studies were carried out on a fresh membrane preparation (final protein concentration of 20 or 40 μg/tube for the D_1 and D_2 dopamine receptor, respectively) using concentrations of [^{3}H]SCH23390 ranging from 0.06 to 6 nM (nonspecific binding was assessed by addition of 10 μM *cis*-(*Z*)-flupentixol, Lundbeck) or concentrations of [^{3}H]spiperone ranging from 0.01 to 4 nM [nonspecific binding was assessed by addition of 50 μM butaclamol, Research Biochemicals Inc. (Natick, MA)].

Tubes were incubated for 90 min at room temperature ([^{3}H]SCH23390) or for 30 min at 37 °C ([^{3}H]spiperone), and then binding was terminated with rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed four times with 5 mL of ice-cold washing buffer [50 mM Tris-HCl (pH 7.4) and 120 mM NaCl for the D_1 receptor and 5 mM phosphate buffer (pH 7.4) for the D_2 receptor], and bound radioactivity was determined by liquid scintillation counting (Beckman LS 650).

Estimation of the radioligand binding parameters, K_d and B_{max}, was carried out using the GraphPad Prism 2.0 curve fitting program (GraphPad Software, San Diego, CA).

Fluorescence Spectroscopy Measurements. Fluorescence readings were performed 48 h after transfection. The cell culture from a single 100 mm dish was washed and detached from a plate using PBS, and then the cells were resuspended in 1 mL of isotonic buffer [137.5 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 6 mM KCl, 5.6 mM glucose, 10 mM HEPES, and 0.4 mM NaH_2PO_4 (pH 7.4)]. Spectrofluorimetric measurements of the cell suspension were recorded on a Fluorolog 3 instrument (Jobin Yvon). The CFP derivative was excited at 433 nm (excitation slits of 2 nm), and YFP derivatives were excited at 475 nm (excitation slits of 2 nm); their fluorescence emission was detected at 450−550 nm through a double monochromator. The spectral contributions arising from light scattering and the nonspecific fluorescence of the cells were eliminated by subtracting the emission spectra of mock-transfected cells from the fluorescence spectra of cells expressing the D_2 receptor−CFP and D_1 receptor−YFP constructs. To determine the effect of dopamine D_1 and D_2 receptor subtype-specific agonists on FRET efficiency, the cell suspension was incubated at a saturating concentration of (±)-chloro-APB (10^{-7} M) or quinpirole (10^{-7} M) at 37 °C for 30 min (or 2 h). All fluorescence emission spectra were recorded in a 10 mm quartz cuvette (Hellma) with continuous stirring at 37 °C. We performed all measurements by normalizing for the cell density.

Confocal Microscopy Analysis of FRET. HEK 293 cells grown on cover slips were transiently transfected with the cDNA encoding the indicated proteins and were incubated with 10^{-7} M receptor agonists [chloro-ABP (D_1), quinpirole (D_2), or both] for 30 min (or 2 h). For FRET experiments, cells were fixed with a 3.5% paraformaldehyde solution in PBS for 15 min at room temperature, before being washed in PBS and mounted onto slides. Confocal laser scanning microscopy was performed using a Leica TCS SP2 microscope (Leica Microsystem, Mannheim, Germany) equipped with an acousto-optical beam splitter and a 100 mW argon laser for excitation at 458 and 514 nm. CFP was excited with a 458 nm laser, and YFP was excited with a 514 nm laser; images were acquired in the following sequence. A pre-photobleach YFP (acceptor) image was acquired by scanning while exciting with the 514 nm laser line. A pre-photobleach CFP (donor) image was acquired by scanning while exciting with the 458 nm laser line. A region of interest was selected, and the acceptor (YFP) was subsequently photobleached by scanning repeatedly with the 514 nm laser line until the fluorescence signals were at 50% levels. A post-photobleach image for CFP was acquired by scanning with the 458 nm laser line, and a second post-photobleach image for YFP was acquired by scanning with the 514 nm laser. The FRET efficiency was calculated for a region of interest according to eq 1.

$$\mathrm{FRET}_{\mathrm{Eff}} = \frac{D_{\mathrm{post}} - D_{\mathrm{pre}}}{D_{\mathrm{post}}} \quad (1)$$

The statistical significance was assessed using a one-way ANOVA, followed by the Dunnett's test for post-hoc comparison.

Measurements of FRET by Fluorescence Lifetime Microscopy. Time-correlated single-photon-counting (TCSPC) measurements were performed using picosecond diode pulse laser as a light source coupled to the Olympus platform IX 71 inverted fluorescence microscope. The excitation diode laser (Jobin Yvon, IBH) was at 434 nm with 1 MHz repetition. The diode laser power was adjusted to the level which allows pulse pile-up to be avoided. A picosecond single-photon detection TBX-04 module (Jobin Yvon, IBH), mounted into the microscope optical port, was used as a detector. This TBX-04 module was integrated with the fast photomultiplier, a high-voltage power supply, a gigahertz preamplifier, and a picosecond timing discriminator. For data acquisition, a Jobin Yvon, IBH data station has been used, and for the data decay analysis, DAS 6 software provided by the manufacturer has been used.

The fluorescence decay from the single HEK 293 cell, transfected with the dopamine D_2 receptor−CFP construct, were observed using a 40× objective and an Olympus fluor cube with a dichroic beam splitter at 455 nm combined with an interference filter (CFP emission filter, Semrock, Rochester, NY, centered at 480/20 nm), and a >475 nm cutoff filter (Endower Corp.). Approximately 15 s was needed to achieve appropriate photon statistics for the determination of the fluorescence lifetime of the receptor−CFP fluorescence donor. All measurements were performed at 37 °C, and each recorded lifetime was averaged from ∼15 independent decay measurements.

Intensity data were analyzed using the following multiexponential decay law:

$$I_t = \sum_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where α_i and τ_i are the pre-exponential factor and decay time of component i, respectively. The fractional fluorescence intensity of each component is defined as

$$f_i = \frac{\alpha_i \tau_i}{\sum \alpha_i \tau_i} \quad (3)$$

The data were analyzed with the software from Jobin Yvon, IBH. Best fit parameters were obtained by minimization of the reduced χ^2 value as well as residual distribution of experimental data.

The average efficiency of energy transfer, $\langle E \rangle$, was calculated from the average donor lifetime in the presence $\langle \tau_{da} \rangle$ or absence $\langle \tau_d \rangle$ of the acceptor:

$$\langle E \rangle = 1 - \frac{\langle \tau_{da} \rangle}{\langle \tau_d \rangle} \quad (4)$$

The average lifetime was calculated according to the following equation:

$$\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad (5)$$

RESULTS

Characterization of Dopamine D_1 and D_2 Receptors Expressed in HEK 293 Cells. The HEK 293 cell line,

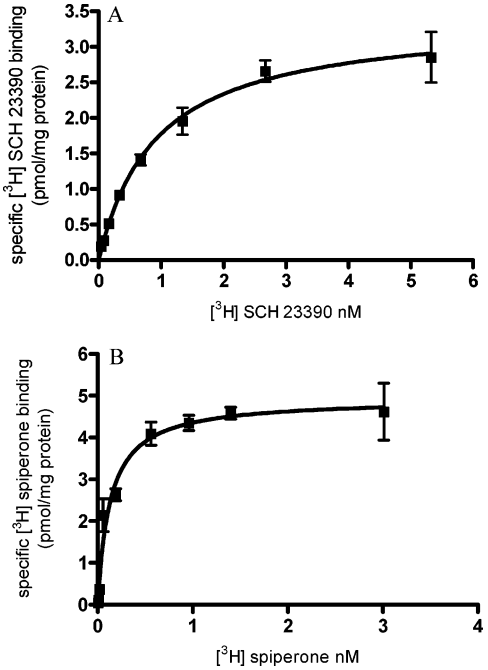

FIGURE 1: Saturation binding of [^{3}H]SCH 23390 (A) and [^{3}H]-spiperone (B) to human D_1 and D_2 dopamine receptors, respectively, coexpressed in HEK 293 cells. Data are from a single experiment performed in triplicate and are representative of at least three independent experiments each.

transiently transfected with the cDNA3.1 plasmid with an insert encoding D_1 or D_2 dopamine receptors, was established for the binding studies as described in Materials and Methods. The saturation analysis, performed with [^{3}H]SCH 23390 or [^{3}H]spiperone for the D_1 or D_2 receptor, respectively, has shown that transfected HEK 293 cells can specifically bind these ligands and revealed K_d values equal to 0.92 nM for the D_1 receptor and 0.1 nM for the D_2 receptor (Figure 1A,B). The observed values are close to the values shown for these receptors in the brain (*8*). The density of the receptors (B_{max}) expressed in HEK 293 cells is equal to 3.25 pmol/mg of protein for the D_1 receptor and 5.0 pmol/mg of protein for the D_2 receptor. Our saturation analysis (data not shown) of the D_1 and D_2 receptors coupled with the YFP and CFP fluorescence proteins, respectively, indicates that fluorescently tagged dopamine receptors exhibit the same K_d values as wild-type receptors. Coupling of the CFP and YFP fluorescence proteins to dopamine receptors did not change their expression in the HEK 293 cell line, as can be judged by the B_{max} value for the D_1-YFP and D_2-CFP species.

The binding parameters (K_d and B_{max} values) obtained from separately transfected HEK 293 cells with D_1 or D_2 receptors are in agreement with the values that were detected, while HEK 293 cells are transiently cotransfected with both receptors. These results clearly indicate that the fusion of the dopamine receptors to derivatives of green fluorescence protein did not change their functional ligand binding properties.

Fluorescence Spectroscopy Measurements of D_1-D_2 Receptor Interaction. Figure 2A shows the representative spectra of the mixture of HEK 293 cells, one transfected with only the D_2-CFP fusion receptor and the second one with the D_1-YFP receptor. In the mixed cell population, the FRET phenomenon was not observed, when they were

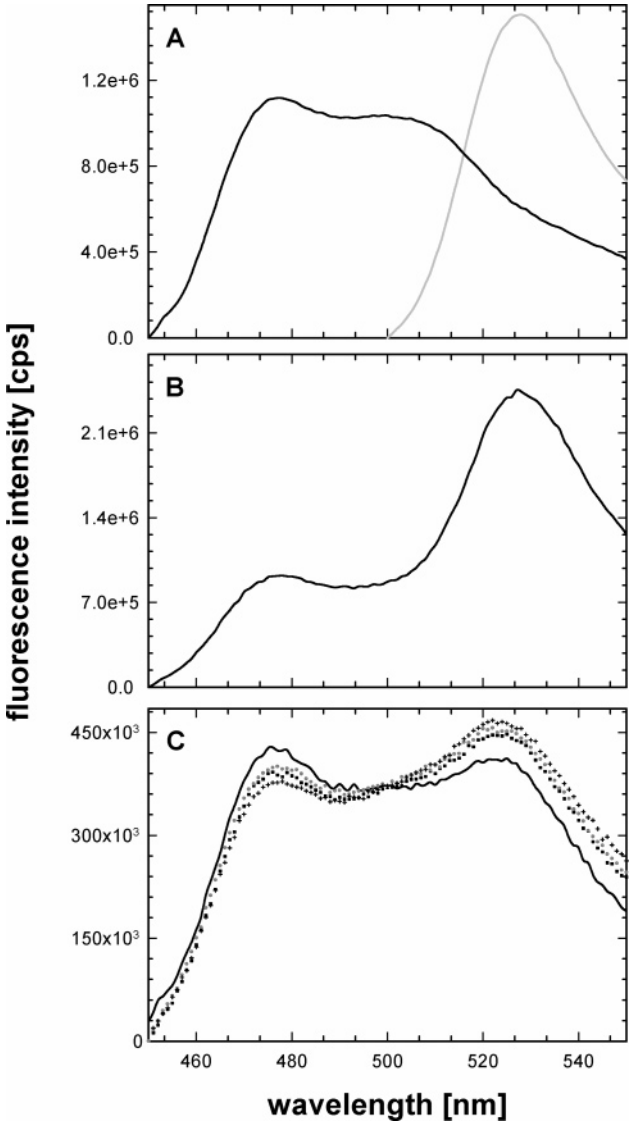

FIGURE 2: Fluorescence emission spectra of HEK 293 cells expressing the CFP and YFP proteins coupled to dopamine D_2 and D_1 receptors, respectively. (A) Negative FRET control: (black) spectrum from cells expressing the D_2-CFP receptor, when excited at 434 nm, and (gray) spectrum from cells expressing the D_1-YFP receptor, when excited at 475 nm. (B) Positive FRET control. Spectrum from cells expressing CFP coupled directly through a 15-amino acid linker to YFP, when excited at 434 nm. (C) Spectrum from cells coexpressing the D_2-CFP and D_1-YFP dopamine receptors, when excited at 434 nm: (—) in the absence of receptor agonists, (■) in the presence of 0.1 μM chloro-APB, the D_1 receptor agonist, (●) in the presence of 0.1 μM quinpirole, the D_2 receptor agonist, and (+) in the presence of both receptor agonists, chloro-APB and quinpirole, for 30 min.

excited at a donor D_2-CFP absorption of 434 nm, and the recorded spectra exhibit the same shape as in the case of HEK 293 cells transfected separately with a plasmid containing D_2-CFP or D_1-YFP fusion proteins. When the HEK 293 cells were transfected with the plasmid encoding the CFP−YFP hybrid mutant, in which both cyan (CFP) and yellow (YFP) were linked together by a short 15-amino acid chain, the efficient FRET phenomenon was observed (Figure 2B) following an excitation at 434 nm. One can expect that for this CFP−YFP mutant protein the efficiency of resonance energy transfer should be the highest, in comparison to those of other derivatives of fluorescence proteins such as D_2-CFP and D_1-YFP fusion receptors. However, since FRET

efficiencies depend on many factors such as dipole orientation factor, there is no a priori knowledge of how efficient they will be. Therefore, the CFP−YFP fusion can be considered only as a positive control. The fluorescence intensity ratio, measured at maximal fluorescence emissions of ~475 and ~528 nm for CFP- and YFP-tagged proteins, respectively, is equal to ca. 0.38. FRET experiments in HEK 293 cells cotransfected with D_2−CFP and D_1−YFP constructs clearly indicate (Figure 2C) that the dopamine D_1 receptor can form oligomers with D_2 receptors. The fluorescence intensity ratio, measured at maximal fluorescence emissions of 475 and 528 nm for D_2−CFP and D_1−YFP constructs, respectively, is equal to 1.18 when they are excited at 434 nm. The ratio of fluorescence intensity of the fluorescence donor, D_2−CFP (excited at 434 nm), measured at 475 nm, to the fluorescence intensity measured at 528 nm (Figure 2A) is equal to 1.85.

Figure 2C also demonstrates that in the presence of a dopamine D_1 receptor agonist, chloro-APB, as well as in the presence of a dopamine D_2 receptor agonist, quinpirole, the efficiency of the FRET phenomenon increases, as can be judged by an increase in the fluorescence intensity of the D_1−YFP acceptor, accompanied with the decrease of the D_2−CFP donor fluorescence intensity. However, the most pronounced hetero-oligomerization of the dopamine D_1 and D_2 receptors can be observed in the presence of both D_1 and D_2 receptor agonists, chloro-APB and quinpirole.

Confocal Microscopy Studies of Dopamine D_1−D_2 Receptor Interaction. To validate the qualitative results from FRET experiments, performed in the HEK 293 cell suspension by fluorescence spectroscopy, we aimed to investigate the D_1−D_2 receptor oligomerization using confocal microscopy. As can be seen in Figure 3, both studied receptors are expressed at the cell membrane. An analysis of FRET using confocal microscopy supports the observations made with the use of fluorescence spectroscopy. Cotransfection of HEK 293 cells with both D_1−YFP and D_2−CFP receptor proteins resulted in constitutive resonance energy transfer between the fluorescence donor and acceptor (Figure 4). Such a phenomenon was not observed when HEK 293 cells were cotransfected with plasmids encoding CFP and YFP (data not shown).

The efficiency of FRET was slightly enhanced by the incubation of the transfected cells with subtype-specific agonists (chloro-APB or quinpirole) and was significantly enhanced when both agonists were added together to the incubation medium for 30 min (Figure 4). The same results were obtained when cells were grown in the presence of both agonists for 2 h (data not shown).

Fluorescence Lifetime Microscopy Studies of D_1−D_2 Receptor Interaction. We have used fluorescence lifetime imaging microscopy to observe FRET phenomena in living cells. Since fluorescence lifetime measurements are independent of any change in fluorophore concentration or excitation intensity, this kind of measurement can provide quantitative information about the interactions between the labeled proteins of interest. As can be seen in Figure 5, the HEK 293 cells, fluorescently transfected with the construct encoding only the CFP, exhibited a double-exponential decay, when excited at 434 nm, characterized by an average fluorescence lifetime, τ, equal to 2.61 ns. On the other hand, when HEK 293 cells were transfected with the construct encoding the CFP donor protein connected by a short 15-amino acid linker to the YFP acceptor protein, the average fluorescence lifetime of the CFP donor was reduced to the value of 1.68 ns, due to the FRET phenomenon. The average FRET efficiency calculated according to eq 2 resulted in a value of ~0.35. Figure 6 shows that similar results were obtained in the HEK 293 cells transfected with the constructs encoding the D_2−CFP receptor and the D_1−YFP receptor in the presence of chloro-ABP or quinpirole. In each case, lifetime measurements of CFP alone as well as when it was

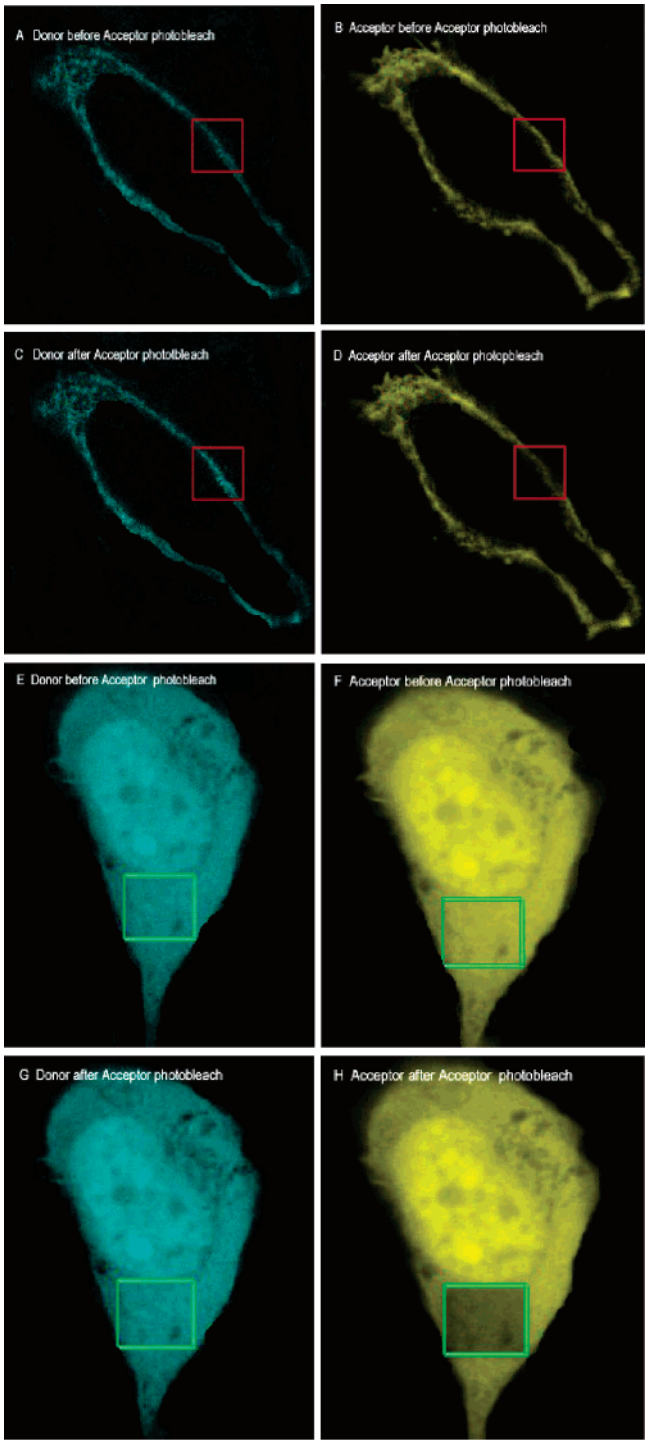

FIGURE 3: Colocalization of fluorescently tagged dopamine D_1 and D_2 receptors on the membrane surface of transiently transfected HEK 293 cells (A−D), contrasted to the colocalization of CFP and YFP (E−H). The proteins were subjected to FRET measurements with the use of acceptor photobleaching, as described in Materials and Methods.

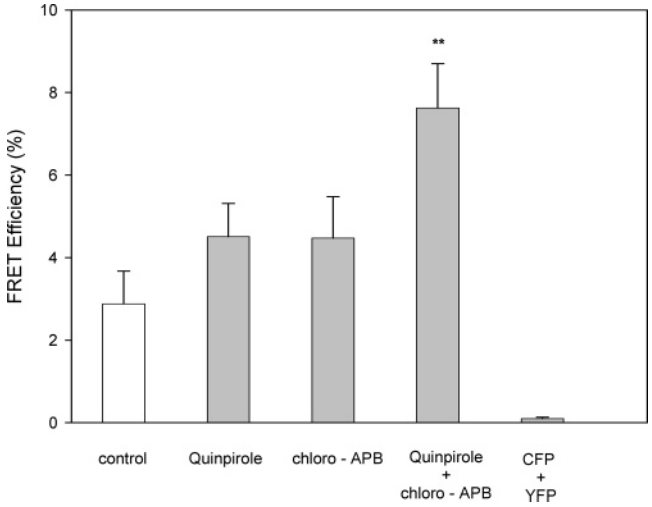

FIGURE 4: FRET efficiency of the D_2−CFP (fluorescence donor) and D_1−YFP (fluorescence acceptor) pairs by acceptor photobleaching. Data are means ± the standard error of the mean of four independent experiments performed in duplicate. Receptor agonists (0.1 μM) were present in the incubation medium for 30 min prior to cell fixation. The statistical significance was evaluated using a one-way ANOVA, followed by a Dunnett's test for post hoc comparisons. Asterisks indicate a p of <0.01.

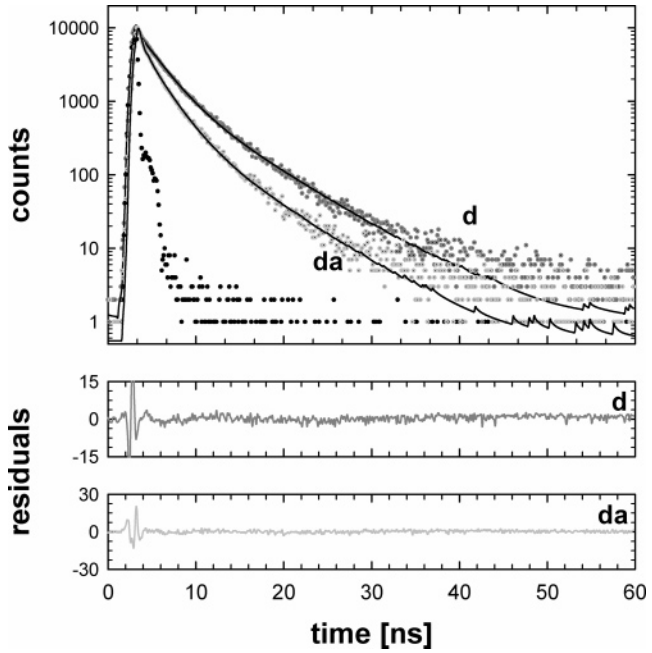

FIGURE 5: Fluorescence lifetime microscopy measurements of the FRET positive control of HEK 293 cells expressing the donor CFP (d) and acceptor protein YFP, coupled to a donor, CFP (da), through a 15-amino acid linker. The dark gray dotted curves show the intensity decay of the donor (d) alone or linked to the acceptor (da). The black solid lines and weighted residuals (bottom panels) are for the triple-exponential fits. The excitation pulse diode laser profile, set up at 434 nm, is shown as a black dotted curve. Experiments were performed as described in Materials and Methods.

bound to the dopamine D_2 receptor, measured several times for each specimen, had to be fitted to a multiexponential decay. However, the double-exponential decay was characterized by lower values of reduced χ^2 and better residual distributions. In some cases, the triple-exponential decays were recorded with the third lifetime values higher than the value recorded for CFP alone, and with pre-exponential factor α_3 close to zero. Therefore, this component of the decay was

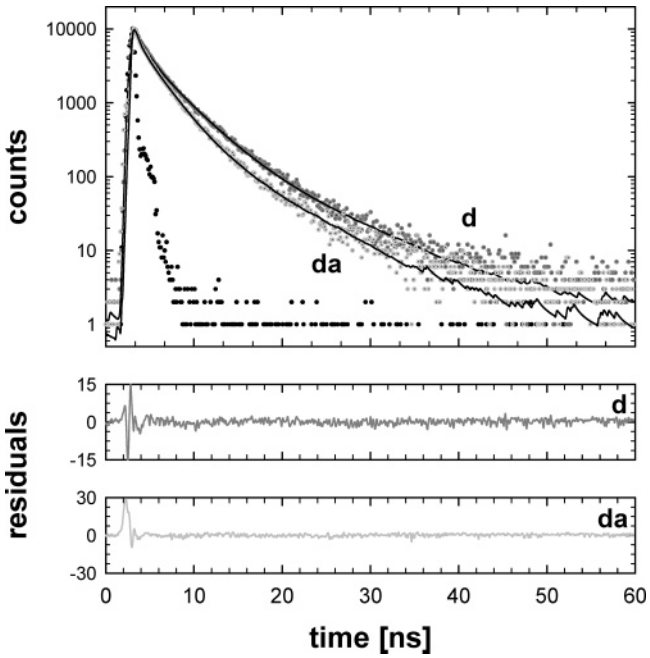

FIGURE 6: Fluorescence lifetime microscopy measurements of HEK 293 cells, expressing dopamine D_1 and D_2 receptors. The dark gray dotted curve (d) shows the intensity decay of the D_2−CFP dopamine receptor expressed in HEK 293 cells, and the gray dotted curve (da) shows the intensity decay of the HEK 293 cells coexpressing the donor, the D_2−CFP receptor, and the acceptor, the D_1−YFP receptor, in the presence of 0.1 μM dopamine D_2 and D_1 receptor agonists, quinpirole and chloro-APB, respectively. The black solid lines and weighted residuals (bottom panels) are for the triple-exponential fits. The excitation pulse diode laser profile, set up at 434 nm, is shown as a black dotted curve. Experiments were performed as described in Materials and Methods.

not included in the calculated average values of fluorescence lifetimes $\langle\tau_d\rangle$ and $\langle\tau_{da}\rangle$.

The values for average fluorescence lifetimes of CFP as well as of the dopamine D_2−CFP construct in the absence and presence of acceptor YFP and the dopamine D_1−YFP construct are presented in Table 1. Additionally, the values of average efficiency of energy transfer are shown. The transfer efficiency, E, varies from a maximal value of ∼35% in the case of the CFP−YFP construct to a value of ∼3.2% in the case of cotransfection with D_2−CFP and D_1−YFP constructs. The subtype-specific agonists of each dopamine receptor, i.e., chloro-APB (D_1) and quinpirole (D_2), added to the cell suspension, caused a significant increase in the FRET efficiency, especially when they were added concomitantly (Table 1).

DISCUSSION

The dopamine D_1 and D_2 receptors, tagged with fluorescence proteins, YFP and CFP, respectively, expressed in HEK 293 cells are located in the plasma membrane, as judged both by laser confocal microscopy and by saturation binding experiments. Qualitative fluorescence spectroscopy, as well as quantitative FRET analysis, performed in a single cell by confocal microscopy and fluorescence lifetime microscopy indicate that dopamine D_1 and D_2 receptors can form hetero-oligomers. The degree of the protein−protein interaction is enhanced by concomitant addition of dopamine receptor subtype-specific agonists. This is the first demonstration by three independent biophysical techniques that

Table 1: Summary of Energy Transfer Measurements by Fluorescence Lifetime Microscopy in HEK 293 Cells[a]

species	$\langle t_d \rangle$ (ns)	$\langle t_{da} \rangle$ (ns)	transfer efficiency $\langle E \rangle$ (%)
CFP[b]	2.61 ± 0.05	–	–
CFP−YFP[c]	–	1.68 ± 0.06	35.0
D_2−CFP[d]	2.48 ± 0.05	–	–
D_2−CFP and D_1−YFP[e]	–	2.40 ± 0.04	3.2
D_2−CFP, D_1−YFP, and APB[f]	–	2.36 ± 0.06	4.0
D_2−CFP, D_1−YFP, and quinpirole[g]	–	2.34 ± 0.05	5.6
D_2−CFP, D_1−YFP, APB, and quinpirole[h]	–	2.27 ± 0.04	8.3

[a] Excitation was set up at 434 nm, and emission was observed through appropriate interference filters, as described in Materials and Methods. The standard errors of means are given. [b] Measured in cells expressing the CFP protein. [c] Measured in cells expressing CFP coupled directly to YFP through a 15-amino acid linker (CFP−YFP). [d] Measured in cells expressing the D_2 receptor coupled to CFP (D_2−CFP). [e] Measured in cells coexpressing the D_2−CFP construct and D_1 receptor coupled to YFP (D_1−YFP). [f] Measured in cells coexpressing the D_2−CFP and D_1−YFP constructs in the presence of 0.1 μM D_1 receptor agonist, chloro-APB. [g] Measured in cells coexpressing the D_2−CFP and D_1−YFP constructs in the presence of 0.1 μM D_2 receptor agonist, quinpirole. [h] Measured in cells coexpressing D_2−CFP and D_1−YFP constructs in the presence of 0.1 μM dopamine D_2 and D_1 receptor agonists, quinpirole and chloro-APB, respectively.

dopamine D_1 and D_2 receptors, coexpressed in the HEK 293 cells, can physically interact as hetero-oligomers.

Oligomerization of GPCRs is difficult to analyze in native cells; therefore, a human embryonic kidney cell line has been widely used in resonance energy transfer studies of membrane receptors, since these cells provide an accepted model in which fluorescently tagged receptor proteins can be efficiently expressed. As reported by Mercier et al. (*40*), the extent of dimerization of β_2-adrenergic receptors (shown by BRET) was unchanged over a 20-fold range of expression levels (from 1.4 to 26.3 pmol/mg of protein). While studying the homodimerization of neuropeptide Y receptors, Dinger et al. (*41*) have also demonstrated that the FRET effect was independent of the amount of receptor expression. Similarly, the BRET signal was stable for receptor expression levels ranging from 0.58 to 6.5 pmol/mg of protein for β_2/β_3-adrenergic receptor hetero-oligomers (*42*). The studies presented here were performed with reasonable D_1 and D_2 receptor levels, ranging from ca. 3 to 5 pmol/mg of protein. These findings imply that examples of GPCR dimerization are not merely artifacts derived from the high levels of expression that are often achieved in heterologous cell systems.

The findings obtained in this study place dopamine D_1 and D_2 receptors within the large group of GPCRs, which have been shown to form homo- and/or hetero-oligomers. Moreover, the ability of dopamine D_1 and D_2 receptors to physically interact with each other, especially upon agonist activation, may be a crucial molecular mechanism underlying the well-known synergism of these dopamine receptors. In recent years, the GPCR oligomerization has been intensively investigated, using FRET and BRET methodology, but to our knowledge, fluorescence lifetime imaging microscopy has been used for the first time in such studies. Since the fluorescence lifetime measurements of the CFP donor do not depend on the protein concentration and excitation intensity, the recorded values of FRET parameters can provide more reliable information about protein−protein interaction in the living cell. In these studies, only the donor (CFP, coupled with the dopamine D_2 receptor) fluorescence lifetime was measured, so the photobleaching of the acceptor (D_1−YFP) was not necessary. Nevertheless, the FRET efficiency values, determined by lifetime imaging microscopy, are in excellent agreement with the FRET efficiency values determined by confocal microscopy, both in the absence and in the presence of dopamine D_1 and D_2 subtype-specific agonists. This additionally validates the acceptor photobleaching methodology used herein, although one has to be aware that this approach might sometimes be subject to various errors, as described recently (*43*). These complementary observations further support the main finding of our study, indicating that dopamine D_1 and D_2 receptors can form hetero-oligomers in the cell membrane.

In this FRET study, using three independent approaches, we show that D_1 and D_2 receptors form hetero-oligomers with a transfer efficiency of 3.8%, which is increased to ca. 8.3% upon stimulation of both receptors with their subtype-specific agonists. Since the basal level of constitutive heterodimerization was observed in this study, the possibility that ligands promote the changes in distance or orientation between preassembled receptors cannot be ruled out, as suggested for melatonin receptors (*44*). The observed FRET efficiency for D_1-D_2 receptor hetero-oligomerization is comparable to the value of ca. 5−10%, observed for oligomerization of other GPCRs, such as α_{1A}- and α_{1B}-adrenergic receptor interaction (*45*). This rather low level of FRET efficiency can be actually expected, since we have observed the highest level of FRET efficiency, ca. 35%, in the case of a CFP−YFP construct expressed in HEK 293 cells.

More recently, O'Dowd et al. (*46*), using a nuclear localization signal (NLS) sequence linked to the dopamine D_1 receptor, visualized ensembles of dopamine receptor hetero-oligomers in living cells. Their elegant set of data demonstrated the effect of dopamine antagonists on translocation of receptor complexes to nucleus; however, the influence of agonists has not been investigated.

Here, we are able to show that the extent of D_1-D_2 receptor oligomerization is significantly enhanced in the presence of subtype-specific agonists of both dopamine receptors. This remains in line with the data provided by Lee et al. (*38*), who have shown that agonist stimulation of coexpressed D_1 and D_2 receptors resulted in an increase in intracellular calcium levels via a signaling pathway neither activated by the receptor alone nor when only one of the coexpressed receptors was activated by a selective agonist. These authors have also shown, by co-immunoprecipitation from rat brain and from cells coexpressing the receptors, that D_1 and D_2 receptors are part of the same heteromeric protein complex. In further work, they have demonstrated the novel pattern of agonist-induced D_2 receptor internalization, when the D_2 receptor was coexpressed with the D_1 receptor in the HEK 293 cells (*47*). However, it should be emphasized that the extent of internalization of these two receptors upon the presence of their agonists at a micromolar concentration for 30 min was not very high and ranged from 10 to 15%. The studies presented here extend these findings by demonstrating

that indeed D_1 and D_2 receptors physically interact with each other in the cell membrane, and this physical interaction is significantly enhanced in the presence of agonists of both dopamine receptors [which is not always a case for other GPCR pairs for which heterodimerization has been proved (*48*)]. We strongly hypothesize that such an interaction, enhanced over a basal level by concomitant activation of both receptors by their agonists, represents a molecular mechanism underlying the synergistic link between these two receptors, widely reported in the literature, which indicates that for many effects of dopamine, the concomitant stimulation of both D_1 and D_2 receptors is required. Therefore, in addition to stimulation (D_1) and inhibition (D_2) of adenylate cyclase, the D_1-D_2 receptor complex, the formation of which is significantly enhanced in the presence of both receptor agonists, might be responsible for the activation of a different signaling pathway, for example, the generation of the calcium signal, as shown by Lee et al. (*38*).

Given the important role of central dopamine receptors in the regulation of behavior and in various neurological and psychiatric disorders, one can imagine that the degree of D_1-D_2 receptor oligomerization may be altered under certain pathological conditions. Therefore, the ligands able to selectively target heterodimer pairs need to be identified and their function needs to be analyzed in physiological settings, for example, in dopamine D_3-D_2 heterodimers, for which antiparkinsonian agents have a higher affinity than they do for the corresponding homodimers (*49*).

In conclusion, our work, in which three independent biophysical methods have been used to demonstrate physical interaction of D_1 and D_2 dopamine receptors, which was significantly enhanced upon the concurrent presence of both receptor agonists, extends and confirms the results obtained in other laboratories, using a biochemical methodology. Therefore, our results validate various biochemical approaches applied in the studies of GPCR oligomerization.

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