

Calcium Signaling by Dopamine D5 Receptor and D5-D2 Receptor Hetero-Oligomers Occurs by a Mechanism Distinct from That for Dopamine D1-D2 Receptor Hetero-Oligomers

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ABSTRACT

In this report, we investigated whether the D5 dopamine receptor, given its structural and sequence homology with the D1 receptor, could interact with the D2 receptor to mediate a calcium signal similar to the $G_{q/11}$ protein-linked phospholipase C-mediated calcium signal resulting from the coactivation of D1 and D2 dopamine receptors within D1-D2 receptor hetero-oligomers. Fluorescent resonance energy transfer experiments demonstrated close colocalization of cell surface D5 and D2 receptors (<100 Å), indicating hetero-oligomerization of D5 and D2 receptors in cells coexpressing both receptors. Coactivation of D5 and D2 receptors within the D5-D2 hetero-oligomers activated a calcium signal. However, unlike what is observed for D1 receptors, which activate extensive calcium mobilization

only within a complex with the D2 receptors, a robust calcium signal was triggered by D5 receptors expressed alone. Hetero-oligomerization with the D2 receptor attenuated the ability of the D5 receptor to trigger a calcium signal. The D5 and D5-D2-associated calcium signals were $G_{q/11}$ protein-linked and phospholipase C-mediated but were also critically dependent on the influx of extracellular calcium through store-operated calcium channels, unlike the calcium release triggered by D1-D2 hetero-oligomers. Collectively, these results demonstrate that calcium signaling through D5-D2 receptor hetero-oligomers occurred through a distinct mechanism to achieve an increase in intracellular calcium levels.

The neurotransmitter dopamine controls a variety of brain functions such as locomotion, cognition, and emotion. Dysfunction of this system has been linked to a number of pathological conditions (Pivonello et al., 2007). Dopamine function is mediated by five G protein-coupled receptors, which are classified into two different subtype families based on their homology and G protein coupling. The D1-like receptors, of

which the D1 and D5 receptors are members, couple to $G_{s/olf}$ proteins to increase cAMP production by adenylyl cyclase. The D2-like receptors, D2, D3, and D4, couple to $G_{i/o}$ proteins to decrease cAMP production by adenylyl cyclase. Despite the fact that D1 and D2 receptor subtypes have opposing effects on adenylyl cyclase function, these receptors, when coactivated, have demonstrated functional synergism in many studies (Svenningsson et al., 2000; Waszczak et al., 2002; Nolan et al., 2007), which may occur within neurons coexpressing D1 and D2 receptors (Bertorello et al., 1990; Surmeier et al., 1992; Aizman et al., 2000; Lee et al., 2004).

A potential mechanism by which this synergism may occur within these neurons is through the hetero-oligomerization of the coexpressed D1 and D2 receptors. D1 and D2 receptors

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ABBREVIATIONS: PLC, phospholipase C; HEK, human embryonic kidney; HA, hemagglutinin; U73122, 1-[6-[(17 β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; AM, acetoxymethyl ester; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; 2-APB, 2-aminoethyl diphenyl borate; SKF 81297, (\pm)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide; ADP β S, adenosine-5'-O-(2-thiodiphosphate) trilithium salt; FRET, fluorescence resonance energy transfer; trFRET, time-resolved fluorescence resonance energy transfer; APC, allophycocyanin; PBS, phosphate-buffered saline; SCH 23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; IP3, inositol triphosphate; SKF 96365, 1-(β -(4-methoxyphenyl)propoxy)-4-methoxyphenethyl-1H-imidazole; YM254890, (1*R*)-1-[(3*S*,6*S*,9*S*,12*S*,18*R*,21*S*,22*R*)-21-acetamido-18-benzyl-3-[(1*R*)-1-methoxyethyl]-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,20-hepta-oxo-1,19-dioxo-4,7,10,13,16-pentaazacyclodocosan-6-yl]-2-methylpropyl rel-(2*S*,3*R*)-2-acetamido-3-hydroxy-4-methylpentanoate.

form functional hetero-oligomeric complexes in cells and in vivo as we showed previously (Rashid et al., 2007). When D1 and D2 receptors were coexpressed in cells, they formed hetero-oligomers that activated a novel $G_{q/11}$ protein-linked, phospholipase C (PLC)-mediated calcium signal, completely independent of extracellular calcium influx, that was not activated by either homo-oligomer (Lee et al., 2004; Rashid et al., 2007).

Because of the inability of D1-like receptor agonists and antagonists to distinguish between the closely related D1 and D5 receptors, the synergism observed in behavioral and physiological studies that had been credited to interactions between coactivated D1 and D2 receptors could also be mediated by coactivated D5 and D2 receptors. Although the distribution of the D5 receptor in brain is quite limited in comparison to the expression of the D1 receptor, there are regions where D5 receptors could potentially be coexpressed with D2 receptors. Because D1 and D2 receptors interacted to form hetero-oligomers, we postulated that a similar effect may also occur between the D5 and D2 receptors, because the D5 receptor is very similar to the D1 receptor in amino acid sequence homology (O'Dowd, 1993). Therefore, we queried whether such an interaction between these two receptors could occur at the level of intracellular calcium mobilization. In this study, we report that a robust $G_{q/11}$ protein-linked, PLC-mediated calcium signal was activated by the D5 receptor expressed alone, and this signal was significantly modified by the formation of D5-D2 heteromers in cells coexpressing both receptors. Unlike D1-D2 signaling, D5 receptor-associated signals were critically dependent on the availability of calcium ions from both the intracellular and extracellular stores.

Materials and Methods

Cell Culture and Receptor Expression. All cell culture and transfection reagents were obtained from Invitrogen (Carlsbad, CA). HEK 293T cells were maintained as monolayer cultures at 37°C in minimal essential medium supplemented with 6% fetal bovine serum and antibiotics. For fluorescence resonance energy transfer experiments, HA and Myc epitope tags were introduced after the initiation methionines of the D5 and D2 receptors, respectively, by polymerase chain reaction, inserted into pcDNA3 vector (Invitrogen), and transiently transfected into HEK 293T cells using LipofectAMINE (Invitrogen, Carlsbad, CA). Stable cell lines coexpressing the N-terminal HA epitope-tagged human D5 receptor and N-terminal FLAG epitope-tagged human D2 receptor were created in HEK 293T cells using the bicistronic pBudCE 4.1 vector (Invitrogen). In brief, the D5 receptor cDNA was inserted into the EF1 α multicloning site and the D2 receptor cDNA into the cytomegalovirus site. Antibiotic-resistant clones (selected with 300 μ g/ml Zeocin) from each transfection were isolated and tested for expression of corresponding receptors using saturation binding analysis.

Measurement of Calcium Signal. Calcium mobilization assays were carried out using a FLEXstation multiwell plate fluorometer (Molecular Devices, Sunnyvale, CA). Stably transfected cells were seeded in black microtiter plates at a density of 100,000 cells/well and grown for 24 h. The cells were then loaded with 2 μ M Fluo-4/AM indicator dye (Invitrogen) in advanced minimum essential medium supplemented with 2.5 mM probenecid for 1 h and subsequently washed twice with Hanks' balanced salt solution, without sodium bicarbonate and phenol red, supplemented with 20 mM HEPES (Invitrogen). Baseline fluorescence values were measured for 15 s, and changes in fluorescence corresponding to alterations in intracellular calcium levels upon the addition of agonists thereafter were

recorded. Fluorescence values were collected at 3-s intervals for 100 s. For calculation of dose-response curves, the peak fluorescence values for each agonist concentration were determined and analyzed using Prism software (GraphPad Software Inc., San Diego, CA). In signaling pathway inhibition studies, cells were treated with 10 μ M U73122 (Sigma-Aldrich, Oakville, ON), 3 μ M thapsigargin (Calbiochem, San Diego, CA), or 10 μ M BAPTA-AM (Sigma) for 1 h, with 0.25 μ g/ml pertussis toxin (Sigma) for 18 h, with 10 μ M YM254890 (a kind gift from Astellas Pharma Inc., Tokyo, Japan), 500 μ M SQ22536 (Sigma), 2-APB (Cayman Chemicals, Ann Arbor, MI), 10 μ M dantrolene (Sigma), 250 μ M EGTA (Sigma), 1 μ M SKF 96365 (Sigma), and 1 μ M nifedipine (Sigma) for 5 min before calcium measurements. SKF 81297, quinpirole, and ADP β S were purchased from Sigma.

Fluorescence Immunohistochemistry of Brain Tissue. Brain tissue from male Sprague-Dawley rats (75 days old and ~350 g) prepared after intracardiac perfusion and fixation was examined. Immunocytochemical procedures followed the indirect (secondary antibody-labeled) fluorescent technique described previously using 16- μ m coronal sections (Lee et al., 2004). The primary antibody for the D5 receptor (Luedtke et al., 1999) was obtained from Millipore Bioscience Research Reagents (Temecula, CA), and the specific anti-D2 receptor antibody, previously confirmed for specificity (Lee et al., 2004), was acquired from Millipore Bioscience Research Reagents. The specificity of the D5 antibody was characterized by individually expressing each of the five dopamine receptors in HEK cells, fixing them, and screening the antibody for reactivity by immunocytochemistry. This strategy was used previously to demonstrate the specificity of D1 and D2 receptor antibodies (Lee et al., 2004). The antibody did not react with D1, D2, D3, or D4 dopamine receptors and only reacted with the D5 receptor (data not shown). Secondary antibodies to visualize D5 and D2 receptors were labeled with Alexa Fluor 350 (Invitrogen) or conjugated to Cy5 (Rockland Immunochemicals, Gilbertsville, PA), respectively. Negative controls were performed for each of the primary and secondary antibodies by ensuring that there was no signal in the absence of these individually as well as no bleed-through for the fluorophore emissions. Confocal laser microscopy was performed using a Zeiss LSM 510 system (Carl Zeiss Inc., Thornwood, NJ).

Sections of brain were obtained progressing caudally from bregma +1.5 to +0.9 cm. Nonadjacent 16- μ m sections were analyzed by two-dimensional stereology using systematic sampling of 12 to 16 fields of 750 μ m² size, viewed under 63 \times magnification using oil immersion, to avoid counting the same neuron more than once.

Time-Resolved Fluorescence Resonance Energy Transfer. This protocol was similar to that of McVey and colleagues (2001) with minor modifications. In brief, the donor and acceptor fluorophores were Europium chelate and allophycocyanin (APC), respectively (PerkinElmer Life and Analytical Sciences, Waltham, MA). Europium chelate was conjugated to the anti-FLAG antibody. APC was conjugated to the anti-HA antibody. Each antibody was diluted in a solution of 50% phosphate-buffered saline (PBS)/50% fetal bovine serum. Cells expressing HA-D5 and myc-D2 receptors together or separately and mixed were incubated with these antibodies for 3 h at 37°C on a rotating wheel. After incubation, samples were pelleted at 5000 rpm, washed twice with PBS, and then resuspended in a final volume of 300 μ l of PBS. A 100- μ l portion of each sample was then divided into aliquots on a 96-well plate in triplicate. FRET analysis was performed on a Victor² multilabel plate reader (PerkinElmer Life and Analytical Sciences) with excitation at 340 nm and emission measured, after a 400- μ s delay, at 615 nm (Eu³⁺ signal) and 665 nm [time-resolved fluorescence resonance energy transfer (trFRET) signal]. Energy transfer (E) was calculated as $E = (EAD_{665}/EAD_{615}) - (ED_{665}/ED_{615})$, where EAD₆₆₅ and EAD₆₁₅ represent emission at 665 and 615 nm, respectively, in the presence of both the donor and acceptor fluorophores, and ED₆₆₅ and ED₆₁₅ represent the emission at 665 and 615 nm, respectively, from samples containing the donor only.

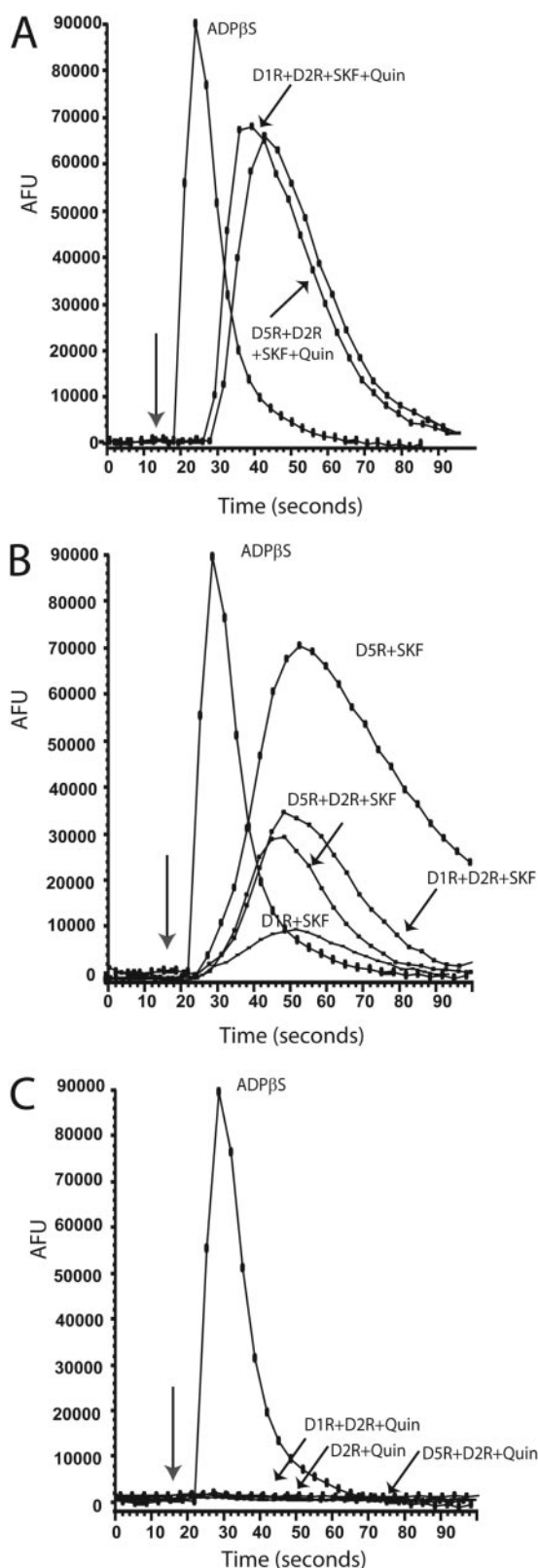


Fig. 1. Intracellular calcium release by activation of D1 or D5 receptors stably expressed alone and coexpressed with the D2 receptor in HEK cells. Calcium elevations are representative of $n = 4$ to 6 measurements. Data are expressed in absolute fluorescence units (AFU) and are shown alongside the calcium signal activated by P2Y1 receptor with 1 μ M ADP β S. A, calcium elevations from coactivation of receptors with 1 μ M SKF 81297 and 1 μ M quinpirole in cells coexpressing D5 and D2 receptors or coexpressing D1 and D2 receptors. B, calcium elevations from

Membrane Preparations and Radioligand Binding. Cells stably expressing dopamine receptors were washed with PBS, resuspended in hypotonic lysis buffer with protease inhibitors (5 mM Tris-HCl, 2 mM EDTA, 5 μ g/ml leupeptin, 10 μ g/ml benzamide, and 5 μ g/ml soybean trypsin inhibitor, pH 7.4), and homogenized with a Polytron apparatus (Kinematica, Littau-Lucerne, Switzerland). The homogenate was centrifuged to pellet unbroken cells and nuclei, and the supernatant was collected. The supernatant was centrifuged at 40,000g for 30 min to isolate a membrane fraction enriched in plasma membrane, and the resulting pellet (P2 membranes) was washed and resuspended in lysis buffer. Protein content was determined by the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Saturation binding experiments were performed with 15 to 30 μ g of membrane protein with increasing concentrations of [3 H]raclopride (final concentration, 250–10,000 pM) for estimation of D2 receptor density or [3 H]SCH 23390 (final concentration, 100–4000 pM) for estimation of D5 receptor density. Nonspecific binding was determined by 10 μ M (+)-butaclamol. Competition experiments were done in duplicate with increasing concentrations (10^{-11} to 10^{-3} M) of unlabeled agonists. The concentration of [3 H]SCH 23390 used in the competition assays for the D5 receptor was approximately equivalent to its K_d value (1 nM). The final concentration of [3 H]raclopride used in the competition assays for the D2 receptors was 2 nM. Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Montreal, QC, Canada) using Whatman GF/C filters (Whatman, Clifton, NJ).

Statistical Analysis. The data obtained from calcium signaling experiments were fitted, when appropriate, by least-squares nonlinear regression using the computer program Prism version 3.00 for Windows (GraphPad Software Inc.). Data from multiple experiments were averaged and expressed as the means \pm S.E.M. The results were considered significantly different when the probability of randomly obtaining a mean difference was <0.05 using the paired Student's t test, also performed using Prism. For competition binding experiments, the data were best-fitted by the program postulating binding to two sites.

Results

To explore the possibility of a D5 receptor-activated calcium signal, stable cell lines expressing the D5 receptor alone and coexpressing the D5 and D2 receptors were generated in HEK 293T cells (receptor density as follows: D5R = 529.2 ± 45.2 fmol/mg, D5R in D5-D2R = 994.0 ± 155.0 fmol/mg, D2R in D5-D2R = 692.0 ± 106.3 fmol/mg, $n = 4-6$). The ability of these receptors to activate a calcium signal by 1 μ M agonist addition was compared with that of D1 and D2 receptors (Fig. 1). When the D1/D5 receptor agonist SKF 81297 and the D2 receptor agonist quinpirole were coadministered, robust calcium signals were generated in D1-D2 and D5-D2 coexpressing cells. These signals peaked 15 to 20 s slower compared with the signal activated by the endogenously expressed purinergic P2Y1 receptor that was activated by the P2Y1 receptor agonist ADP β S (Fig. 1A). The addition of SKF 81297 alone resulted in a $\sim 50\%$ lower level of calcium signal activation in D1-D2 and D5-D2 coexpressing cells compared with the signals generated upon full coactivation of both dopamine receptors, with no alteration in the time course of the signal generation (Fig. 1B). There was a robust calcium signal as-

receptor activation with 1 μ M SKF 81297 in cells expressing the D5 receptor alone, coexpressing D5 and D2 receptors, the D1 receptor alone, or coexpressing D1 and D2 receptors. C, calcium elevations from receptor activation with 1 μ M quinpirole in cells expressing D2 receptor alone, or coexpressing D5 and D2 receptors or D1 and D2 receptors. Arrows, time of agonist addition.

sociated with D5 receptor activation in cells expressing this receptor alone. This signal peaked at a maximum similar to that of the calcium signal from D1-D2 and D5-D2 receptor coactivations but peaked several seconds slower (Fig. 1B). Activation of the D1 receptor with SKF 81297 in cells expressing the D1 receptor alone produced a weak calcium signal significantly lower in magnitude than that from the coactivation of both D1 and D2 receptors or activation of the D1 receptor by SKF 81297 in D1-D2 cells (Fig. 1B). Activation of the D2 receptor by quinpirole in cells expressing the D2 receptor alone or in D5-D2 cells did not activate a calcium signal (Fig. 1C).

To compare the mechanism of activation of the D5 receptor-associated calcium signals with that of the D1-D2 signal, inhibitors of signal transduction cascades were used to elucidate the pathways involved. Similar to the D1-D2 signal, the addition of the adenylyl cyclase inhibitor SQ22536 did not affect the peak heights of the agonist-induced calcium signal associated with activation of the D5 receptor ex-

pressed alone or coexpressed with the D2 receptor in D5-D2 cells (Fig. 2A). The $G_{i/o}$ protein inhibitor pertussis toxin attenuated the D5 and D5-D2 calcium signals and the D1-D2 calcium signal to a similar extent (Fig. 2B). This observation could represent an effect of overnight pertussis toxin treatment because the signal associated with the activation of the P2Y1 receptor, which is solely mediated through the activation of $G_{q/11}$ proteins, was also decreased to a similar degree. The D5 and D5-D2 receptor-associated signals were decreased by the phospholipase C inhibitor U73122 (Fig. 2C) and the G_q protein inhibitor YM254890 (Fig. 2D), just as for the D1-D2 calcium signal.

$G_{q/11}$ protein-coupled receptors are able to mobilize intracellular calcium by initiating both calcium release from intracellular stores and by influx of calcium ions from the extracellular space through calcium channels (Putney et al., 2001). We therefore determined the source of the D5 and D5-D2 receptor triggered increases in intracellular calcium. First, to explore the involvement of calcium release from

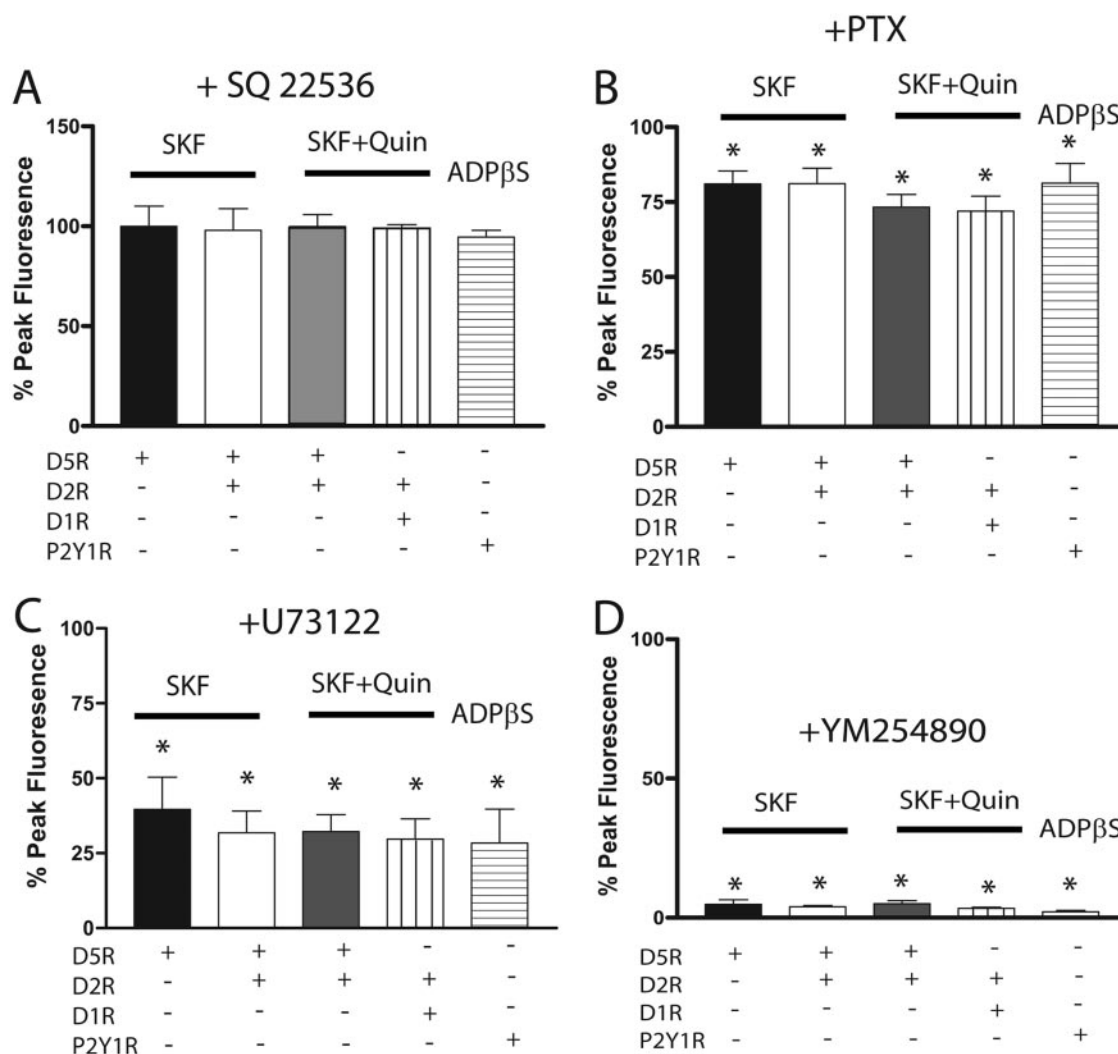


Fig. 2. The D5 receptor-activated calcium signal in the presence and absence of the D2 receptor used a $G_{q/11}$ protein-dependent signaling pathway. Data are represented as the percentage of peak fluorescence of the calcium signal from vehicle-treated cells (*, $p < 0.05$). Each bar represents the mean \pm S.E.M. of $n = 4$ to 6 experiments. The bars represent the effect of 1 μ M SKF 81297 on the D5 receptor expressed alone (bar 1) or together with the D2 receptor (bar 2), 1 μ M SKF 81297 and quinpirole on D5-D2 (bar 3) or D1-D2 receptors (bar 4), and 1 μ M ADP β S on P2Y1 receptors (bar 5). The calcium signal was measured in the presence of 500 μ M adenylyl cyclase inhibitor SQ22536 (A), after overnight treatment with 250 ng/ml pertussis toxin (B), pretreatment with 10 μ M PLC inhibitor U73122 for 1 h (C), or pretreatment with 1 μ M $G_{q/11}$ protein inhibitor YM254890 for 5 min (D).

these sources, intracellular stores were depleted by the sarcoplasmic endoplasmic reticulum calcium pump inhibitor thapsigargin (Fig. 3A), or intracellular calcium was chelated by BAPTA-AM (Fig. 3B). Depleting intracellular calcium by either means significantly decreased all of the receptor-activated calcium signals tested. Because calcium stores are gated by IP₃ receptors and ryanodine receptors, the IP₃ receptor antagonist 2-APB and the ryanodine receptor antagonist dantrolene were tested next. The addition of 2-APB significantly decreased all signals tested (Fig. 3C). These results were confirmed with another IP₃ receptor antagonist, Xestospongine C (data not shown). Treatment with dantrolene, which inhibits ryanodine receptors, did not decrease any of the calcium signals (Fig. 3D). These data indicate that intracellular calcium release gated by IP₃ receptors plays an important role in the generation of the D5, D5-D2, and the D1-D2 receptor-associated calcium signals.

To explore the involvement of extracellular calcium ions for signal generation, the calcium chelator EGTA and inhibitors of calcium influx, SKF 96365 and nifedipine, were

used. EGTA greatly attenuated D5 and D2-D5 receptor activated calcium signaling and slightly inhibited D1-D2 receptor-mediated signaling but did not affect P2Y₁ receptor signaling (Fig. 4A). This result suggested that D5 and D2-D5 receptor-activated signals were highly dependent on extracellular calcium ion concentrations, in sharp contrast to the D1-D2 signal. Receptor-operated calcium channels have been demonstrated to allow calcium ions to enter cells after receptor activation (Kawanabe and Nauli, 2005). To elucidate whether receptor-operated calcium channels played a part in signal generation, an inhibitor of the receptor activated, store-operated calcium channels, SKF 96365, was used and was observed to significantly attenuate the D5 and D5-D2 receptor-associated signals but not the D1-D2 or P2Y₁ receptor-activated signals (Fig. 4B). Because SKF 96365 may have some selectivity for voltage-dependent calcium channels, the L-type calcium channel inhibitor nifedipine was used. No significant effect of nifedipine treatment was apparent on the calcium signals tested (Fig. 4C). These data suggested that the calcium signals involving the D5 receptor or the D5-D2 heteromeric complex differed from that of

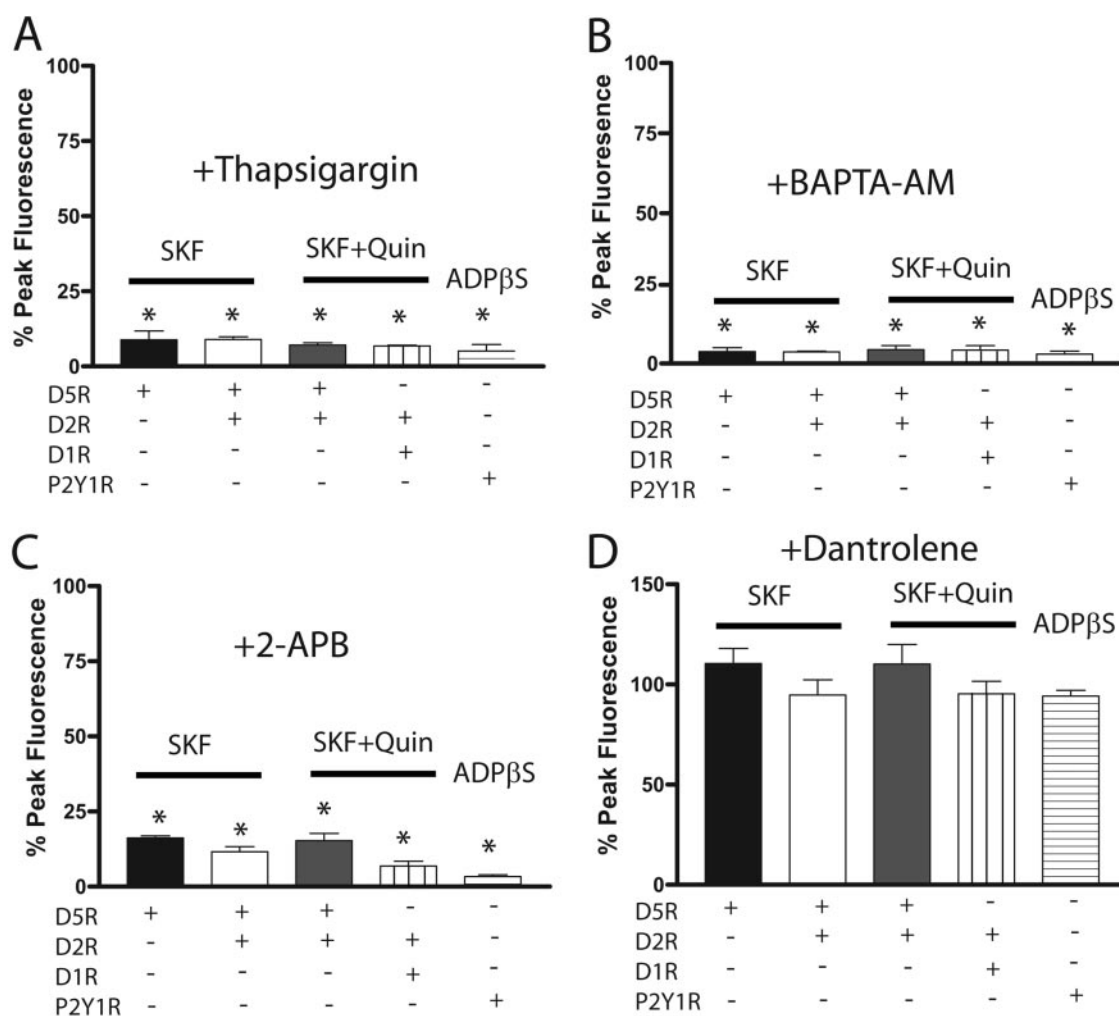


Fig. 3. The inhibition of mediators of intracellular calcium release abolished the increase in calcium associated with D5 receptor activation in cells expressing D5 receptors alone or coexpressed with D2 receptors ($n = 3-7$). Data were represented as the percentage of peak fluorescence of the calcium signal from vehicle-treated cells \pm S.E.M. Effect of 1 μ M SKF 81297 on the D5 receptor expressed alone (bar 1) or together with the D2 receptor (bar 2). Effect of 1 μ M SKF 81297 and quinpirole on D5-D2 (bar 3) or D1-D2 receptors (bar 4). Effect of 1 μ M ADP β S on P2Y₁ receptors (bar 5). Calcium signal after depletion of intracellular calcium in cells pretreated with sarcoplasmic endoplasmic Ca²⁺ ATPase pump inhibitor thapsigargin 3 μ M for 1 h (A), after treatment with 10 μ M BAPTA-AM for 1 h (B), in the presence of IP₃ antagonist 2-APB 10 μ M (C), or in the presence of ryanodine receptor antagonist dantrolene 10 μ M (D) (*, $p < 0.05$).

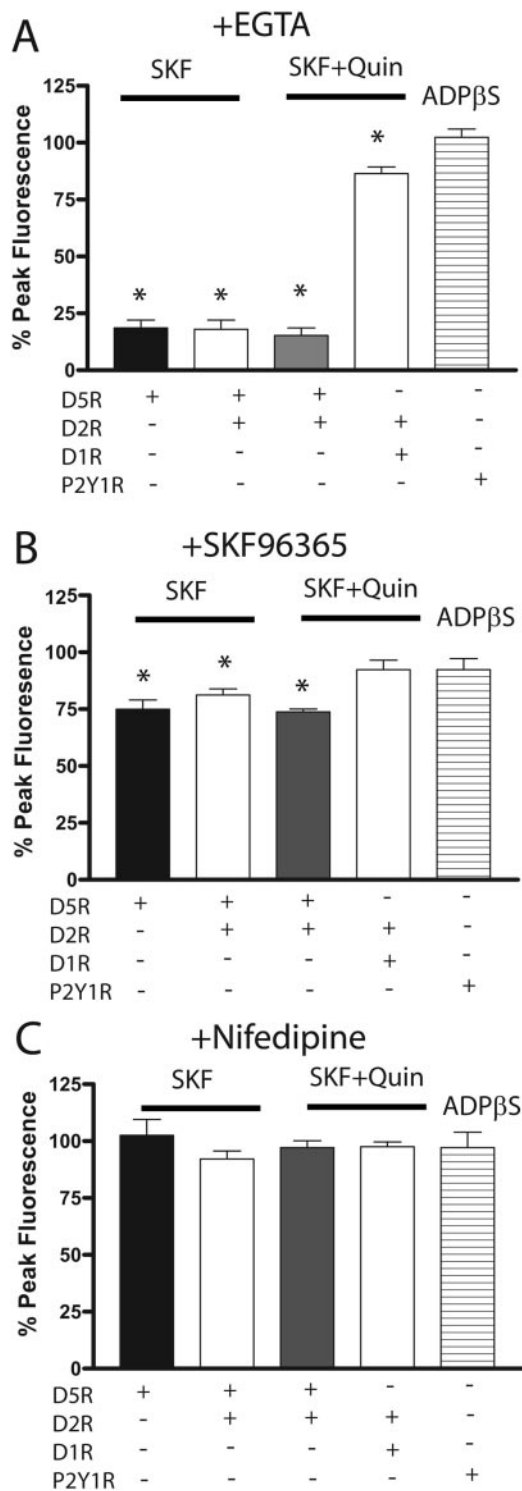


Fig. 4. Inhibition of extracellular calcium entry attenuated D5 receptor activation of the calcium signal when D5 receptors are expressed alone or coexpressed with D2 receptors in HEK 293T cells ($n = 3-5$). Data are represented as the percentage of peak fluorescence of the calcium signal from vehicle treated cells \pm S.E.M. Effect of 1 μ M SKF 81297 on the D5 receptor expressed alone (bar 1) or together with the D2 receptor (bar 2). Effect of 1 μ M SKF 81297 and quinpirole on D5-D2 (bar 3) or D1-D2 receptors (bar 4). Effect of 1 μ M ADPβS on P2Y1 receptors (bar 5). Calcium signal in the presence of extracellular calcium chelator EGTA 250 μ M (A), store-operated calcium channel inhibitor SKF 96365 10 μ M (B), or calcium-channel blocker nifedipine 1 μ M (C) (*, $p < 0.05$).

the D1-D2 signal in that they require the presence and influx of extracellular calcium for proper signal activation.

To explore the agonist concentration required to generate the calcium signal activated by D5 receptors expressed alone, increasing concentrations of SKF 81297 were used to activate receptors (Fig. 5). This resulted in a concentration-dependent calcium response with an EC_{50} value of 4.9 ± 1.4 nM ($n = 5$) (Fig. 5, A and B). This signal was significantly inhibited by the D1/D5 receptor antagonist SCH 23390 but not by the D2 receptor antagonist eticlopride (Fig. 5C).

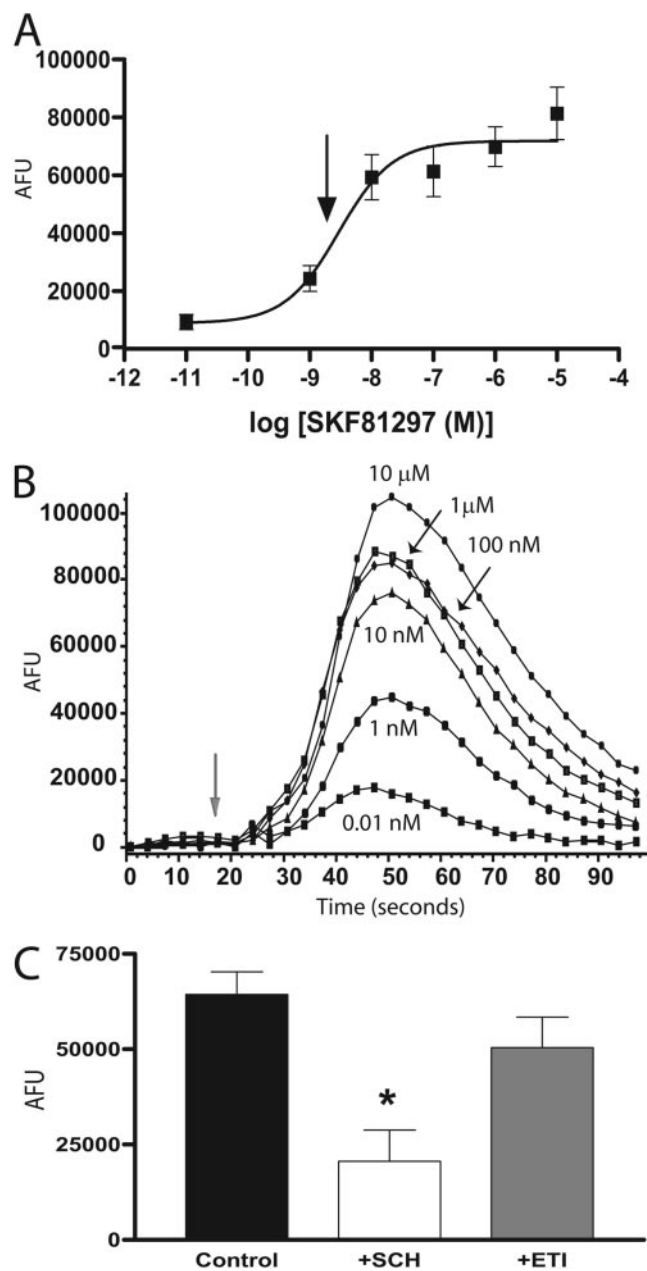


Fig. 5. SKF 81297 triggered the D5 receptor-activated calcium signal dose-dependently in HEK 293T cells ($n = 5-6$). Data are expressed in absolute fluorescence units (AFU). A, concentration-response of the calcium signal mediated by the activation of the D5 receptor with increasing concentrations of SKF 81297. Arrow, EC_{50} value. B, calcium mobilization tracings elicited by increasing concentrations of SKF 81297 in cells expressing the D5 receptor. C, calcium signal at 100 nM SKF 81297 (■) in the presence of 1 μ M D1/D5 receptor antagonist SCH 23390 (□) (*, $p < 0.05$), or 1 μ M D2 receptor antagonist eticlopride (▣).

To explore the concentration-dependent response of the calcium signal activated by D5 receptors in D5-D2 cells, D5 receptors were activated with increasing concentrations of SKF 81297 (Fig. 6A). The peak responses of the calcium signal generated by D5 receptor activation with SKF 81297 were significantly lower ($61.3 \pm 6.8\%$ of the signal generated by the D5 receptor alone, $n = 5$, $p < 0.05$) in D5-D2 compared with D5 cells (Fig. 6A). A significant increase in the EC_{50} value of the response of the D2-D5 cells compared with D5 cells was also observed for SKF 81297 (209.2 ± 81.2 nM, $n = 5$, $p < 0.05$ in D5-D2 cells compared with 4.9 ± 1.4 nM for D5 cells). Representative calcium tracings of this concentration-response effect in D5-D2 cells are shown in Fig. 6B. Activation of D2 receptors in D5-D2 cells did not significantly activate a calcium signal at any concentration tested (Fig. 6C). Cotreatment with either the D1/D5 receptor antagonist SCH 23390 or the D2 receptor antagonist eticlopride significantly decreased the D5 receptor-activated calcium signal mediated by SKF 81297 in D5-D2 cells (Fig. 6D).

When both D5 and D2 receptors in D5-D2 cells were coactivated with increasing concentrations of SKF 81297 and quinpirole, a concentration-dependent calcium response was observed. The EC_{50} value of this response was lower than that for the D5 receptor activated alone in D5-D2 cells but was significantly greater than that from D5 receptor activa-

tion in cells expressing the D5 receptor alone (19.8 ± 5.1 nM, $n = 4$, $p < 0.05$ compared with D5R + SKF and D5-D2R + SKF) (Fig. 7, A and B). When increasing the concentrations of SKF 81297 in the presence of a saturating concentration of quinpirole (10 nM), the EC_{50} value of this response was reduced greatly compared with the EC_{50} value in the absence of quinpirole (0.4 ± 0.05 nM, $n = 3$, $p < 0.05$ compared with D5-D2R + SKF) (Fig. 7, C and D). Either the D5 or D2 receptor antagonist was able to decrease this signal, indicating the necessity of the heteromeric complex for this effect (Fig. 7E). To test whether dopamine could activate calcium signals in cells expressing the D5 receptor or coexpressing D5-D2 receptors, increasing concentrations of dopamine were used to activate the calcium signal in these cells (Fig. 7F). Dopamine was able to activate a calcium signal in cells expressing the D5 receptor alone with an EC_{50} value similar to the signal activated by SKF 81297 (D5R + dopamine = 9.9 ± 3.6 nM, $n = 6$). Dopamine activated a calcium signal in cells coexpressing D5-D2 receptors with an EC_{50} value similar to when the signal was activated by coadministration of SKF 81297 and quinpirole (D5-D2R + dopamine = 34.1 ± 4.0 nM, $n = 6$, $p > 0.05$ compared with D5-D2R + SKF + quinpirole).

Because D1 and D2 receptors form hetero-oligomers and D1 and D5 receptors are significantly homologous in their protein sequences, the possibility that D5-D2 receptor het-

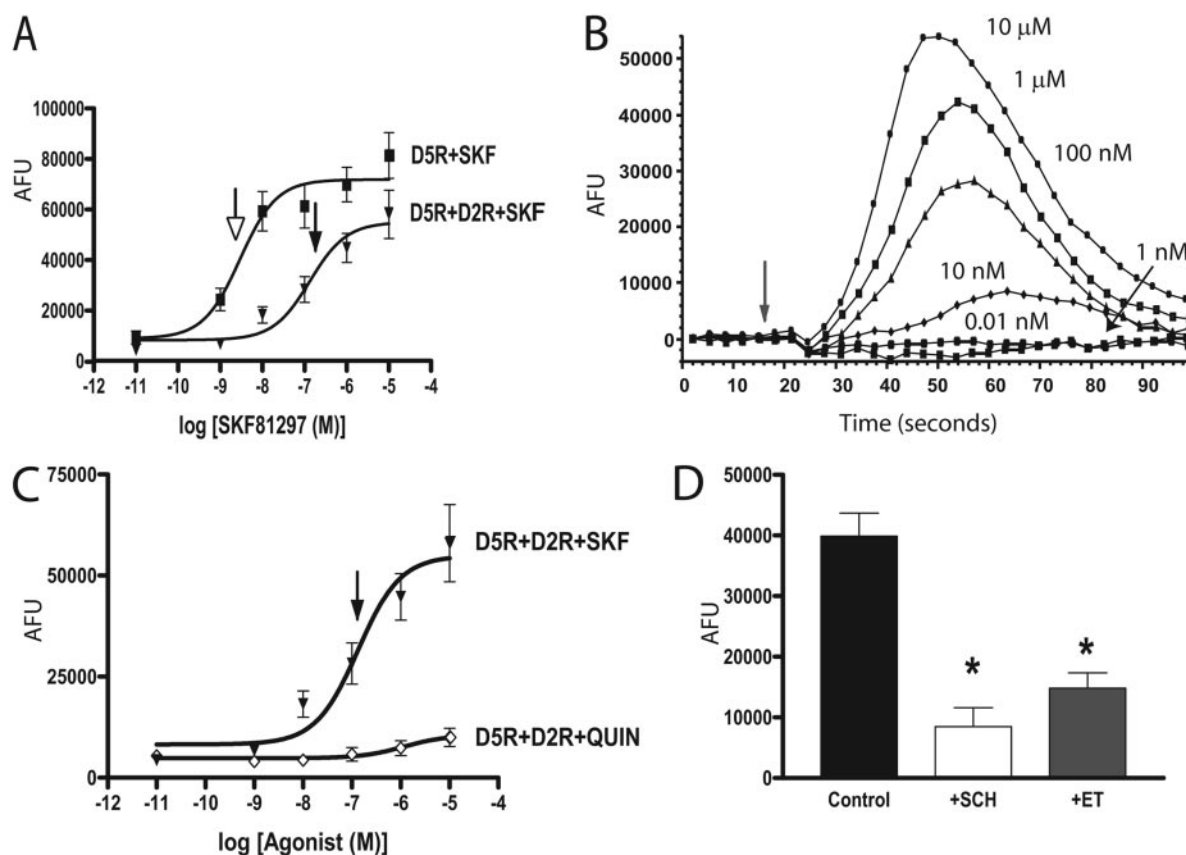


Fig. 6. Calcium signaling in cells coexpressing D5 and D2 receptors activated by SKF 81297 is decreased compared with that of D5 receptors expressed alone in HEK 293T cells ($n = 5-6$). Data are expressed in absolute fluorescence units (AFU). A, concentration-response of the calcium signal mediated by activation of the D5 receptor in cells expressing the receptor alone compared with the activation of the D5 receptor in D5-D2 cells with increasing concentrations of SKF 81297. Arrows, EC_{50} values. B, calcium mobilization tracing elicited by increasing concentrations of SKF81297 in cells coexpressing D5 and D2 receptors. C, concentration-response of the calcium signal mediated by activation of D2 receptors in D5-D2 cells with increasing concentrations of quinpirole shown compared with that elicited by SKF 81297. D, the calcium signal at 100 nM SKF 81297 in cells coexpressing D5 and D2 receptors (■) in the presence of 1 μ M D1/D5 receptor antagonist SCH 23390 (□) or 1 μ M D2 receptor antagonist eticlopride (■) (*, $p < 0.05$).

ero-oligomers form in cells coexpressing D5 and D2 receptors was explored using trFRET assays. Cell surface trFRET was observed in cells coexpressing D5-D2 receptors but not when receptors were transfected individually and the cells were then mixed (Fig. 8A). Because FRET demonstrated that coexpressed D5 and D2 receptors existed in close proximity on the cell surface to likely form hetero-oligomers, we tested

whether the affinity of the agonists used herein was affected by hetero-oligomer formation. Competition of [3 H]SCH 23390 binding to the D5 receptor with dopamine (Fig. 8B and Table 1) and SKF 81297 (Fig. 8C and Table 1) in cells expressing the D5 receptor alone or coexpressing D5-D2 receptors demonstrated no change in the affinity of these agonists for D5 receptors when coexpressed with D2 receptors ($p > 0.05$).

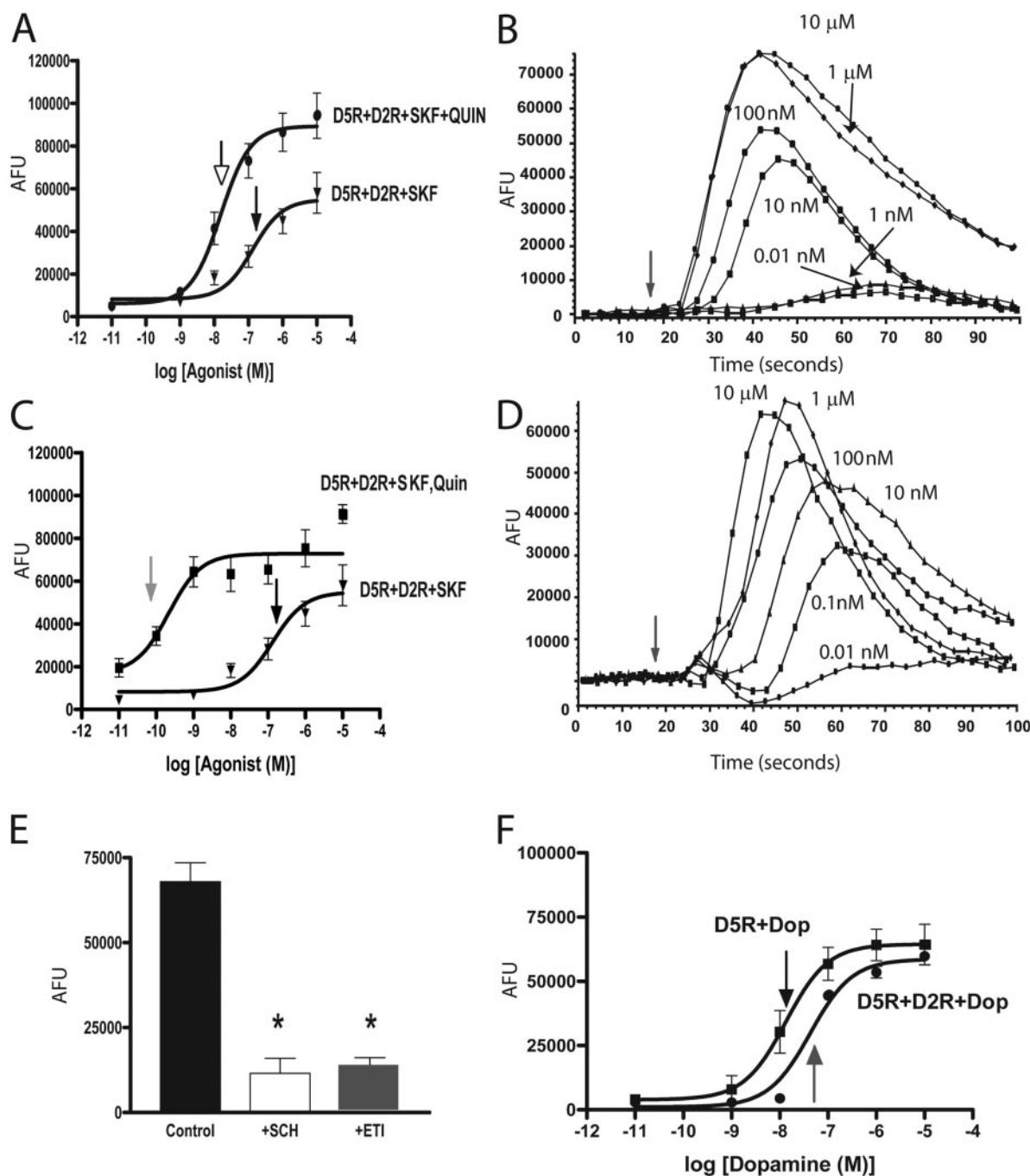


Fig. 7. Full calcium signal generation was elicited by SKF 81297 and quinpirole for coexpressed D5 and D2 receptors in HEK 293T cells ($n = 5-7$). Data are expressed in absolute fluorescence units (AFU). **A**, concentration-response of the calcium signal from coactivation of both receptors with increasing concentrations of SKF 81297 and quinpirole compared with that elicited by SKF 81297 alone. **B**, representative calcium mobilization tracing elicited by increasing concentrations of SKF 81297 and quinpirole in cells coexpressing D5 and D2 receptors. **C**, concentration-response of the calcium signal from activation of D5 receptors with increasing concentrations of SKF 81297 in the presence of 10 nM quinpirole compared with that elicited by SKF 81297 alone. **D**, representative calcium mobilization tracing elicited by increasing concentrations of SKF 81297 in the presence of 10 nM quinpirole in cells coexpressing D5 and D2 receptors. **E**, the calcium signal at 100 nM SKF 81297 and quinpirole (■) in the presence of the 1 μ M D1/D5 receptor antagonist SCH 23390 (□) or the 1 μ M D2 receptor antagonist eticlopride (●) (*, $p < 0.05$). **F**, calcium signaling activated with increasing concentrations of dopamine in cells expressing the D5 receptor alone or coexpressing D5 and D2 receptors. Arrows, EC₅₀ values.

Competition of [^3H]raclopride binding to the D2 receptor with dopamine (Fig. 8D and Table 2) and quinpirole (Fig. 8E and Table 2) in cells expressing D2 receptors alone or coexpressing D5-D2 receptors demonstrated no change in the affinity of dopamine or quinpirole for D2 receptors when coexpressed with D5 receptors ($p > 0.05$).

To determine whether there was a potential physiological basis for the novel D5-D2 receptor synergism observed in this

report, the coexistence of D5 and D2 receptors in individual neurons was examined using selective antibodies. Using confocal microscopy and receptor-specific antibodies, colocalization of D5 and D2 receptors was observed in neurons within the rat cerebral cortex (Fig. 9A), ventral pallidum (Fig. 9B) and caudate nucleus (Fig. 9C). Note that in the cortex, there are D5-positive neurons that do not express D2 receptor, whereas in caudate nucleus, there are many D2-positive neu-

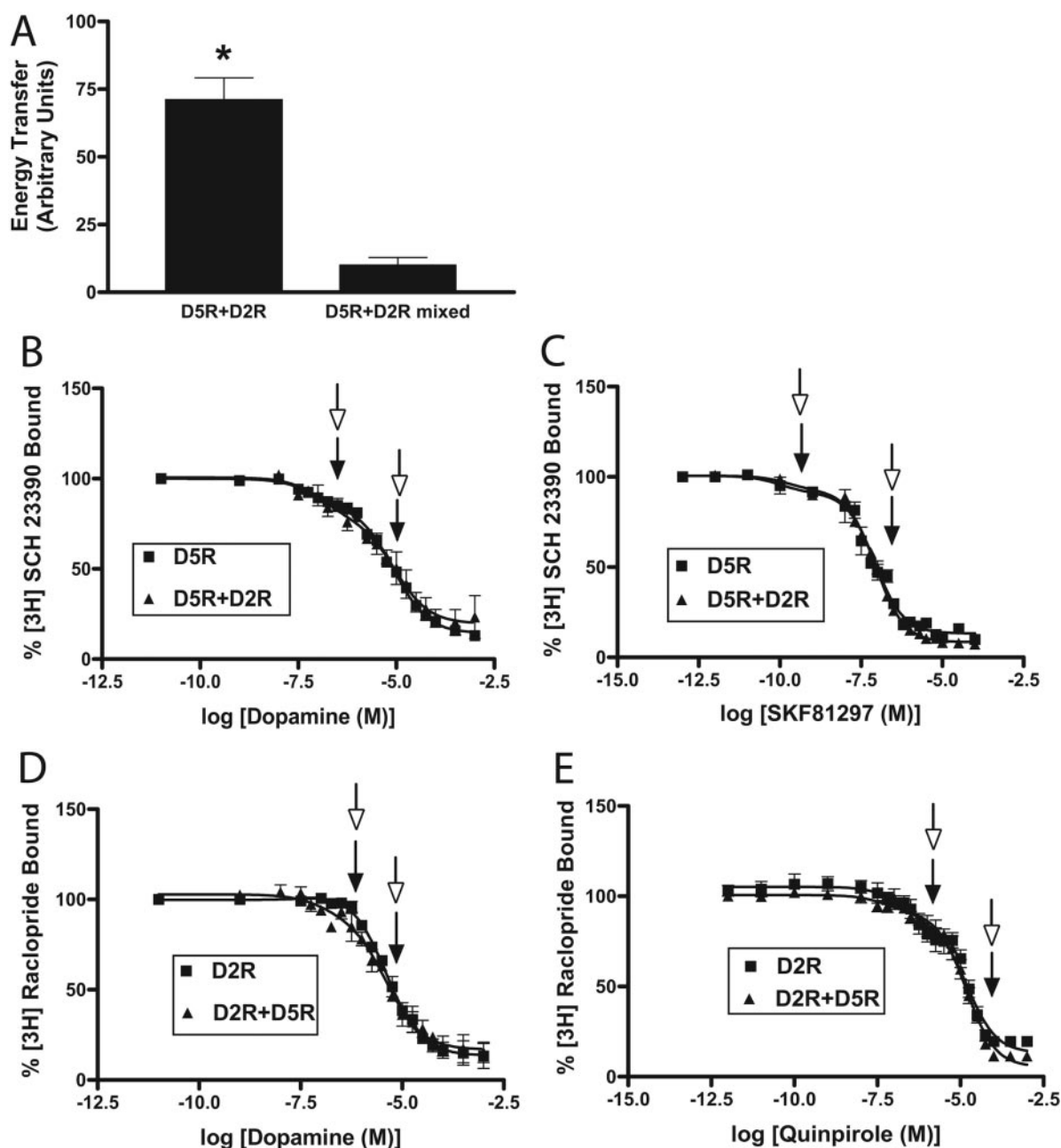


Fig. 8. Dopamine D5 and D2 receptors form hetero-oligomers, which do not display altered affinity profiles to selected agonists. A, a close proximity of coexpressed D5 and D2 receptors on the cell surface was detected using time-resolved FRET with Europium chelate as the donor and APC as the acceptor ($n = 5$ independent cotransfections). Energy transfer on the cell surface was detected in cells cotransfected with the amino-terminally tagged HA-D5 receptors and amino-terminally tagged myc-D2 receptors (■) but not when these receptors were transfected separately and cells mixed (□) (*, $p < 0.05$). B-E) Coexpression of D5 and D2 receptors does not alter agonist binding affinities and proportion of agonist-detected high- and low-affinity states ($n = 6$). B, competition of [^3H]SCH 23390 binding by dopamine in membranes prepared from cells expressing the D5 or coexpressing D5 and D2 receptors. C, competition of [^3H]SCH 23390 binding by the D1/D5-specific agonist SKF 81297 in cell membranes prepared from cells expressing the D5 or coexpressing D5 and D2 receptors. D, competition of [^3H]raclopride binding by dopamine in membranes prepared from cells expressing the D2 or coexpressing D5 and D2 receptors. E, competition of [^3H]raclopride binding by the D2 selective agonist quinpirole in membranes prepared from cells expressing the D2 or coexpressing D5 and D2 receptors. Black arrows, high- and low-affinity sites for receptors expressed alone; white arrows, high- and low-affinity sites for receptors coexpressed.

rons that do not express the D5 receptor. D2 receptor-labeled neurons (100–200) were assessed in 750- μm^2 two-dimensional planes from each of several sections from $n = 3$ rats, for a total of 12 to 16 fields at 63 \times magnification under oil immersion. Of the D2 receptor-positive neurons in caudate putamen, there was coincident labeling for the D5 receptor in 10% of neurons, whereas in cortex and ventral pallidum, there was D5 labeling in 20 and 25% of D2 receptor-containing neurons, respectively. Collectively, these results set the stage for the D5-D2 receptor interactions observed in this report to occur in vivo.

Discussion

In this report, coactivation of dopamine D5 and D2 receptors within D5-D2 hetero-oligomers resulted in the generation of a calcium signal. The intracellular calcium release associated with D5-D2 receptor activation was sensitive to chelation of extracellular calcium ions and inhibition of receptor-dependent, store-operated calcium channels and to inhibition of $G_{q/11}$ and PLC. However, unlike the D1 receptor, the D5 receptor expressed alone triggered a robust calcium signal upon activation. The calcium signal from D5 receptor activation was attenuated when D5 receptors hetero-oligomerized with coexpressed D2 receptors. This effect was disinhibited when both receptors were activated. This is unlike what was observed with D1-D2 hetero-oligomers, in which coactivation of both D1 and D2 receptors were necessary to generate a robust calcium signal that was not observed when the D1 receptor was expressed alone. The calcium signals associated with the activation of the D5 receptor, expressed either alone or within the D5-D2 receptor complex, required $G_{q/11}$ and PLC activity, similar to the D1-D2 and the purinergic P2Y1 receptor-mobilized calcium signals but were distinct in having a critical dependence on the influx of extracellular calcium concomitantly.

In this report, the D5 receptor was demonstrated to activate a robust calcium signal when expressed alone. There has been some previous evidence to suggest that D5 action triggered a calcium signal. Increases or decreases in IP3

levels have been demonstrated to be controlled by D5 activity depending on the cell lines in which the D5 receptors were expressed (Sidhu et al., 1998). This activity may be associated with the abilities of the D5 receptors to couple to G proteins in specific cell lines. For instance, D5 receptors have been reported to interact with calcium signal-simulating G_{12} and G_{13} proteins in the kidney (Zheng et al., 2003). This ability of D5 receptors to mobilize calcium may also occur in vivo, because colocalization of D5 receptors with IP3 receptors was observed in neurons of the prefrontal cortex (Paspalas and Goldman-Rakic, 2004), suggesting that the D5 receptors modify IP3 receptor functions in the brain. Furthermore, activation of the D5 receptor and not the D1 receptor potentiated L-type channel-controlled burst firing in the subthalamic nucleus of the basal ganglia (Baufreton et al., 2003), suggesting that D5 receptors can modify calcium fluxes by modifying channel activities. This greater ability of the D5 receptor, but not the D1 receptor, to couple to calcium-signaling pathways is not predictable considering the similar overall amino acid homology between D1 and D5 receptors. However, because the greatest differences between the D1 and D5 receptors are in the intracellular domains, which mediate G protein interactions, it is entirely possible that the disparity in these regions can result in differences in G protein coupling, selectivity, and specificity. Because the differences in these domains mediate the significantly higher agonist-independent constitutive adenylyl cyclase activity and the higher affinity for dopamine the D5 receptor displays compared with the D1 receptor (Demchyshyn et al., 2000), it is also possible that these regions mediate the differences in G protein coupling.

These calcium signals generated by the D5 receptor, either alone or within a complex with the D2 receptor, required the presence of extracellular calcium, unlike the D1-D2-generated calcium signal. There are a number of possibilities as to why the signaling of the D5 receptor-containing complexes was sensitive to changes in the calcium ion concentrations in the extracellular media. First, D5 receptor-associated calcium signals may be tied closely to the activation of receptor-

TABLE 1

Agonist binding affinities of the dopamine D5 receptor expressed alone or coexpressed with the dopamine D2 receptor

Competition binding experiments using [^3H]SCH 23390 were performed in membranes from cells stably transfected with the D5 receptor alone or cotransfected with the D2 receptor. The data represent the means \pm S.E.M of six experiments, each performed in duplicate.

	Fraction	D5R		Fraction	D5R + D2R	
		KH	KL		KH	KL
		<i>nM</i>	<i>nM</i>		<i>nM</i>	<i>nM</i>
Dopamine	0.317 \pm 0.072	132.4 \pm 72.0	3936 \pm 997.2	0.338 \pm 0.048	171.9 \pm 69.1	6407 \pm 1396
SKF 81297	0.217 \pm 0.041	8.1 \pm 4.1	33.9 \pm 8.5	0.198 \pm 0.033	8.2 \pm 2.9	20.4 \pm 3.5

KH, high-affinity constant; KL, low-affinity constant.

TABLE 2

Agonist binding affinities of the dopamine D2 receptor expressed alone or coexpressed with the dopamine D5 receptor

Competition binding experiments using [^3H]raclopride were performed in membranes from cells stably transfected with the D2 receptor alone or cotransfected with the D5 receptor. The data represent the means \pm S.E.M of six experiments, each performed in duplicate.

	Fraction	D2R		Fraction	D2R + D5R	
		KH	KL		KH	KL
		<i>nM</i>	<i>nM</i>		<i>nM</i>	<i>nM</i>
Dopamine	0.291 \pm 0.084	256.6 \pm 105.3	4836 \pm 1612	0.271 \pm 0.073	144.0 \pm 84.4	4030 \pm 1113
Quinpirole	0.293 \pm 0.047	13.8 \pm 8.7	1196 \pm 366	0.253 \pm 0.034	25.3 \pm 3.4	875.5 \pm 270.1

KH, high-affinity constant; KL, low-affinity constant

operated channels, such as the transient receptor potential channels (Clapham et al., 2001), and therefore the function of these channels could have facilitated downstream PLC-linked signals (Kawanabe and Nauli, 2005). Second, D5 receptor activation of PLC β may be highly contingent on the process of calcium-induced calcium release, in which calcium influx through receptor or store-operated calcium channels is initiated by prior PLC-mediated calcium release from intracellular stores. PLC β and IP3 receptor activity is then stimulated in a positive feedback mechanism (Thore et al., 2005). A third possibility is the activation by D5 receptors of the δ isoform of PLC, of which little is known except that it is very sensitive to influx of extracellular calcium, which could activate the enzyme (Yagisawa et al., 1999).

Hetero-oligomerization with the D2 receptor resulted in different effects on the signaling functions of D5 and D1 receptors. Hetero-oligomerization of the D1 and D2 receptors, on one hand, produced a functional unit for calcium generation, which was not observed for either the D1 or D2 receptor homo-oligomers. On the other hand, oligomerization between D5 and D2 receptors, which formed cell surface hetero-oligomers as demonstrated by FRET, negatively modified a previously functional unit of calcium signaling, D5 homo-oligomers. D5-D2 hetero-oligomers could elicit maximal calcium release associated with D5 receptor homo-oligomers only if both receptors within the signaling complex were activated. This observation supports the model suggesting that, for maximal signal activation, both receptors within a dimer needed to be activated for full activation to occur (Baneres and Parelo, 2003; Kniazeff et al., 2004). Although this was described for homodimeric G protein-coupled recep-

tors, we show that it is applicable for both constituents of a receptor hetero-oligomer as well. The smaller calcium signal associated with SKF 81297 in D5-D2-expressing cells also points to this same mechanism. Primary activation of a single receptor type by SKF 81297 within the D5-D2 complex only generated a partial increase in calcium. Another interpretation of the effect of SKF 81297 administered alone, which is still supportive of the dimer model of signal activation, is that the smaller signal activated is the result of the full activation of the D5 receptor and partial activation of the D2 receptor by this agonist. This could be true, because we have shown that SKF 81297 has affinity for the D2 receptor within the D1-D2 complex as a partial agonist (Rashid et al., 2007). Also in support of this premise, D1 or D2 antagonists, used singly, efficaciously abolished this signal elicited by SKF 81297 alone in D5-D2-expressing cells.

Differences between D1 and D5 receptors pertaining to interactions with D2 receptors in coexpressing cells have also been observed in another study at the level of cAMP amplification. Coactivation of D5 and D2 receptors in cells coexpressing both receptors resulted in increased cAMP levels, whereas coactivation of D1 and D2 receptors attenuated D1 receptor-mediated cAMP production (Lee et al., 2000). These data may suggest another functional difference between D1 and D5 receptors, within complexes with D2 receptors, in a different signaling pathway. D5-D2 interactions have also been revealed in D5-null mice, in which it regulates exploration and motor coordination (O'Sullivan et al., 2005).

In this report, we demonstrate the differential abilities of the D1-like receptors, D5 and D1, to interact with the D2 receptor to control intracellular calcium release. The D5 receptor and the D5-D2 hetero-oligomeric complex both are able to robustly stimulate intracellular calcium release but in a manner distinct from the D1-D2 receptor complex. This may translate into the functional differences noted, in that D5 and D2 receptors have been shown to cooperate functionally to facilitate motor activity and striatal long-term depression, whereas D1 and D2 receptors are mainly involved in striatal long-term potentiation and motor inhibition (Centonze et al., 2003). In addition, because there exists anatomic specificity of D5 and D1 receptor localization (Bergson et al., 1995) and thus different localization of the D5-D2 and D1-D2 receptor complexes, these data also suggest differential mechanisms by which intracellular calcium can be regulated by dopamine in different regions of the brain and in the periphery. This complements and advances our understanding of the complex interplay among the dopamine receptor subtypes in integrating and mediating critical brain functions. The effects of the heteromeric complexes composed of dopamine receptors may have far-reaching effects on dopaminergic neurotransmission and provide distinct mechanisms by which this neurotransmitter can regulate rapid-onset calcium-signaling systems in the brain.

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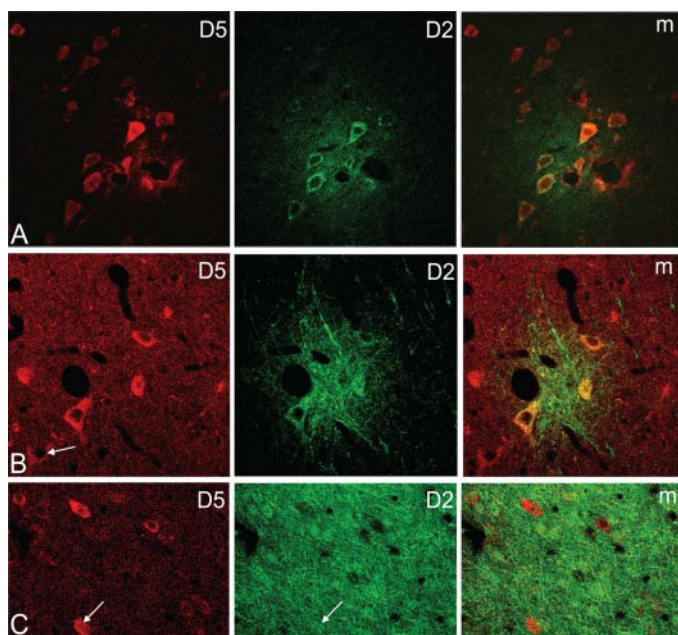


Fig. 9. Confocal microscopy of the immunocytochemical visualization of D5 and D2 receptors in rat cortex (A), ventral pallidum (B), and caudate putamen (C). B, the arrow indicates neuron expressing the D5 receptor but no D2 receptor. C, the arrows indicate a neuron expressing both D5 and D2 receptors, whereas there are multiple neurons present expressing either D5 or D2 receptors. The D5 antibody labeling was visualized by secondary antibody conjugated to Alexa fluor 350 (red), and the D2 antibody labeling was visualized by secondary antibody conjugated to Cy5 (green). Each part shows the labeling of D5 or D2 receptors and the merged picture (m). The sections were visualized at 60 \times magnification.

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