A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D₁ Receptors

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ABSTRACT

The indoloquinolizidine-peptide 28 [(3S,12bR)-N-((S)-1-((S)-2-carbamoylpyrrolidin-1-yl)-3-(4-fluorophenyl)-1-oxopropan-2-ylamino)-4-cyclohexyl-1-oxobutan-2-yl]-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizine-3-carboxamide], a trans-indoloquinolizidine-peptide hybrid obtained by a combinatorial approach, behaved as an orthosteric ligand of all dopamine D₂-like receptors (D₂, D₃, and D₄) and dopamine D₁ receptors, but as a negative allosteric modulator of agonist and antagonist binding to striatal dopamine D₁ receptors. Indoloquinolizidine-peptide 28 induced a concentration-dependent hyperbolic increase in the antagonist apparent equilibrium dissociation constant values and altered the dissociation kinetics of dopamine D₁ receptor antagonists. The negative allosteric modulation was also found when agonist binding to D₁ receptors was assayed. Indoloquinolizidine-peptide 28 was a weak ago-allosteric modulator but markedly led to a decreased potency without decreasing the maximum partial/full agonist-mediated effect on CAMP levels. Compounds able to decrease the potency while preserving the efficacy of D₁ receptor agonists are promising for exploration in psychotic pathologies.

G-protein-coupled receptors (GPCRs) represent a high percentage of the current market for therapeutic agents and remain a primary focus of many biomedical research and pharmaceutical drug discovery programs. Much attention is focused on the identification and study of molecules that act as orthosteric ligands at a given GPCR to elicit a pharmacological effect. These compounds compete with the endogenous ligand(s) and thus preclude simultaneous occupation of the receptor by the two molecules. In addition to orthosteric sites, many GPCRs have been found to possess allosteric binding sites that are structurally distinct from the orthosteric sites (Christopoulos, 2002; May et al., 2007; Bridges and Lindsley, 2008). Allosteric sites may be less conserved across subtypes than orthosteric sites, providing a means for true selectivity (Bridges and Lindsley, 2008). One characteristic feature of

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; IP28, indoloquinolizidine-peptide 28, (3S,12bR)-N-((S)-1-((S)-2-carbamoylpyrrolidin-1-yl)-3-(4-fluorophenyl)-1-oxopropan-2-ylamino)-4-cyclohexyl-1-oxobutan-2-yl]-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizine-3-carboxamide (C₃₀H₄₁F₂N₆O₄); SCH 23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SKF 38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine hydrobromide; SKF 83566, 8-bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benza zepin-7-ol hydrobromide; YM-09151-2, cis-5-chloro-2-methoxy-4(methylamine)-N-[2-methyl-1(phenylmethyl)-3-pyridinyl]-benzamide; HBSS, Hank’s balanced salt solution; FRT, Flp recombination target; HEK, human embryonic kidney.
the allosteric interaction is that the receptor is able to simultaneously bind an orthosteric and an allosteric ligand, introducing complexity into pharmacological responses by modifying the affinity or the signal imparted by orthosteric ligand (May et al., 2007). The ability of allosteric modulators to fine tune pharmacological responses has sparked interest in their potential applications in both clinical and basic science settings (Bridges and Lindsley, 2008; Conn et al., 2009). This interest is more relevant in the case of neurotransmitter receptor targets, where synaptic neurotransmission occurs in extremely complex circuits implicated in many neurological functions. An allosteric modulator will preserve the physiological relevance of receptor signaling while modulating the potency of the endogenous neurotransmitter (Conn et al., 2009).

Allosteric sites have been described for dopamine receptors (Schatz, 2005). Dopamine receptors are grouped into two classes: D₁-like receptors, which include D₁ and D₅ receptors, and D₂-like receptors, which include D₂, D₃, and D₄ receptors (Neve et al., 2004). As for many GPCRs (see Ferré et al., 2009 for review), there is evidence that dimerization/oligomerization of D₁ receptors (Ng et al., 1994; George et al., 1998) is important for membrane expression (Kong et al., 2006). In addition, dopamine D₂ receptors form higher-order oligomers at physiological expression levels with dimers, the minimal repetitive structural unit (Guo et al., 2008; Han et al., 2009). An allosteric site associated with the D₂ dopamine receptor is recognized byamiloride and analogs of this diuretic drug, such asbenzamil and methylisobutylamiloride (Hoare et al., 2000). These compounds also decrease antagonist binding to D₁, D₅, and D₄ dopamine receptors (Hoare et al., 2000). L-Proline-L-leucine-L-glycine, an endogenous hypothalamic factor that inhibits the release of melanocyte-stimulating hormone from the anterior pituitary, is another dopamine receptor allosteric modulator enhancing agonist binding to D₂ and D₄ receptors (Verma et al., 2005). The allosteric modulation of dopamine receptors by ions has been described extensively (Schatz, 2005). At millimolar concentrations, sodium decreases agonist binding but increases antagonist binding to D₂ dopamine receptors (Ericksen et al., 2009). Zinc ion allosterically modulates dopamine receptors (Schatz and Sibley, 2001), and it may be therapeutically relevant in antipsychotic drug treatments (Schatz, 2005).

Because dopamine is an important neurotransmitter involved in the regulation of several biological functions, including locomotor activity, emotion, cognition, and neuroendocrine secretion, and because striatum receives the densest dopamine innervations and contains the highest density of dopamine receptors in the brain (Gerfen, 2004), dopamine receptors are targets in the pathophysiology of Parkinson’s disease and schizophrenia (Andersen and Nielsen, 1991; Wu et al., 2005). We have recently developed a library of indoloquinolizidine-peptide hybrids as multiple ligands for different dopamine receptor subtypes by a combinatorial approach that combines the solution-phase synthesis of two indolo[2,3-a]quinolizidine scaffolds with solid-phase peptide chemistry (Vendrell et al., 2009). Some trans-indoloquinolizidine-peptide hybrids were selected showing an affinity (Kᵢ) in the low-micromolar range for both families of dopamine receptors (Vendrell et al., 2009). Here, the functional characterization of one of these compounds, the indoloquinolizidine-peptide 28 (IP28), on binding to dopamine D₁-like and D₂-like receptors is described. By means of kinetic assays and competition experiments in radioligand binding, it has been demonstrated that the IP28 behaved as an orthosteric ligand of dopamine D₂, D₃, D₄, and D₅ receptors but as an allosteric modulator of the D₁ dopamine receptors. IP28 decreased the affinity of both agonist and antagonist binding to the receptor and, at the same time, behaved as a D₁ receptor partial agonist. IP28, which was then the first described ago-allosteric modulator of D₁ dopamine receptors, decreased receptor potency, whereas it preserved agonist-induced maximal cAMP production. This type of compound may be relevant to treat some psychotic pathologies.

Materials and Methods

Cell Transfection and Generation of an Inducible Cell Line Expressing the Human Dopamine D₁ Receptor. Human embryonic kidney 293 (HEK-293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 2 mM L-glutamine, and 100 μg ml⁻¹ sodium pyruvate (all from Invitrogen, Paisley, UK), at 37°C in a humidified atmosphere of 5% CO₂. HEK-293 cells were grown to 60% confluence and transfected by the polyethylenimine (Sigma-Aldrich Chemie, Steinheim, Germany) method with 5 μg of cDNA corresponding to human dopamine D₁, D₄, and D₅ receptors. Cells were incubated (4 h) with the corresponding cDNA together with polyethyleneiminine (5.47 mM in nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in Hank’s balanced salt solution (HBSS) with 10 mM glucose, detached, and resuspended in the same buffer containing 1 mM EDTA. Human dopamine receptor expression was tested by binding to membranes from these cells.

The Flp-In T-REx System (Invitrogen) was used to generate a stable mammalian cell line exhibiting tetracycline-inducible expression of human dopamine D₁ receptor from a specific genomic cDNA location. The Flp-In T-REx-293 cells used in this system contain a single integrated Flp recombination target (FRT) site, stably express the Tet repressor, and allow research to proceed directly to the generation of a stable cell line. The human dopamine D₁ receptor cDNA was amplified by use of sense and antisense primers and the iProof kit (Bio-Rad, Hercules, CA) as indicated by the manufacturer. To the amplified fragment, an adenine nucleotide was added in its 3’ end, with use of the iTaq kit (Bio-Rad), to be cloned into pcDNA5/FRT/TO-TOPO-D₁R construct. Flp-In T-REx-293 cells were cotransfected with the pcDNAs/FRT/TO-TOPO-D₁R construct and the pOG44 plasmid (Invitrogen) to allow the integration of the expression vector pcDNA5/FRT/TO-TOPO-D₁R under the control of a tetracycline-inducible promoter into the genome via Flp recombinase-mediated DNA recombination at the FRT site. A polyclonal selection of isogenic cell lines was performed by use of 15 μg/ml blasticidin and 200 μg/ml hygromycin B, as recommended by the manufacturer. Flp-In T-REx-293-D₂, D₃, and D₄ cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 4.5 mg/ml glucose and 0.11 mg/ml sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 15 μg/ml blasticidin, and 200 μg/ml hygromycin B (all supplements were from Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. Expression of human dopamine D₂ receptor was induced by overnight incubation of Flp-In T-REx-293-D₂, D₃, and D₄ cells with different concentrations of tetracycline (Invitrogen) and tested by binding experiments.

Membrane Preparation and Protein Determination. Membrane suspensions from sheep brain striatum or from cells expressing particular subtypes of dopamine receptors were processed as
described previously (Casado et al., 1990; Sarrió et al., 2000). Tissue or cells were disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for three 5-s periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing a proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris was removed by centrifugation at 1500g for 10 min at 4°C, and membranes were obtained by centrifugation at 105,000g (40 min, 4°C). Membranes were resuspended and re centrifuged under the same conditions. The pellet was stored at −80°C, washed once more as described earlier, and resuspended in 50 mM Tris-HCl buffer for immediate use. Protein was quantified by the biocinchoninic acid method (Pierce Chemical Co., Rockford, IL) by use of bovine serum albumin dilutions as standard.

Radioilgand Binding Experiments. For competition experiments, membrane suspensions (0.5 mg of protein/ml) were incubated for 2 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ with the indicated free concentration of the dopamine D₁-like receptor antagonist [³H]SCH 23390 or [³H]YM-09151-2 (PerkinElmer Life and Analytical Sciences, Wellesley, MA) or the D₂-like receptor antagonist [³H]YM-09151-2 (PerkinElmer Life and Analytical Sciences) and increasing concentrations of IP28 (11 different concentrations from 0.1 nM to 50 μM; Tocris, Arounmouth, UK), increasing concentrations of SCH 23390 (13 different concentrations from 0.01 nM to 10 μM; Tocris, Arborntown, UK), increasing concentrations of YM-09151-2 (10 different concentrations from 0.01 nM to 10 μM; Tocris, Aronmouth, UK), increasing concentrations of SCH 23390 (13 different concentrations from 0.1 nM to 50 μM; Sigma-Aldrich) in the absence or in the presence of the indicated concentration of IP28 (preincubated 60 min with membranes) or increasing concentrations of dopamine D₁-like receptor agonist SKF 38393 (13 different concentrations from 0.1 nM to 50 μM; Tocris) in the absence or in the presence of the indicated concentration of IP28 (preincubated 60 min with membranes).

Non specific binding was determined in the presence of 50 μM SKF 83566 (the same values were obtained in the presence of 50 μM SCH 23390 or SKF 38393) or 50 μM YM-09151-2 (the same nonspecific binding was obtained in the presence of 50 μM quinpirole). Free and membrane-bound ligand were separated by rapid filtration of 500-μl aliquots in a cell harvester (Brandel, Gaithersburg, MD) through Whatman GF/C filters embedded in 0.3% polyethyleneimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA) overnight at room temperature, and radioactivity counts were determined by use of a Tri-Carb 1600 scintillation counter (PerkinElmer H scintillation cocktail (National Diagnostics, Atlanta, GA) through Whatman GF/C filters embedded in 0.3% polyethyleneimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA) overnight at room temperature, and radioactivity counts were determined by use of a Tri-Carb 1600 scintillation counter (PerkinElmer Life and Analytical Sciences) with an efficiency of 62% (Sarrió et al., 2000).

For dissociation kinetic assays, sheep brain striatum membranes (0.5 mg of protein/ml) or membranes from receptor-expressing cells (0.2 mg of protein/ml) were incubated 60 min at 25°C with medium, the indicated concentrations of SKF 83566 (Tocris) or IP28 in Tris-HCl buffer (50 mM, pH 7.4) containing 10 mM MgCl₂, before adding the indicated concentrations of [³H]SCH 23390 or [³H]YM-09151-2. After 2 h the dissociation was initiated by the addition of 10 μM SCH 23390 or YM-09151-2. At the indicated time interval total binding was measured by rapid filtration and determination of radioactivity counts as indicated above. Nonspecific binding was measured after a 90-min incubation in the presence of 50 μM SKF 83566 or 50 μM quinpirole.

All displacers were dissolved in dimethyl sulfoxide and diluted in the binding medium. The dimethyl sulfoxide concentration in the binding incubates was less than 0.5% and, at this concentration, it did not affect agonist or antagonist affinity for dopamine receptors.

Binding Data Analysis. Because dopamine D₁ and D₂ receptors are expressed as dimers or higher-order oligomers (see the introduction), radioligand competition curves were analyzed by nonlinear regression with use of the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the specific binding data to the mechanistic two-state dimer receptor model (Franco et al., 2005, 2006). This model considers a homodimer as the minimal structural unit of the receptor. To calculate the macroscopic equilibrium dissociation constants, the following equation for a competition binding experiment deduced by Casado et al. (2007) was considered:

\[ A_{\text{total}} = (K_{D2A}A + 2A^2 + K_{D2A}A B/K_{DAB}R_A) \left( K_{D1A}K_{D2A} + K_{D1A}A + K_{D1A}K_{D2A}B/K_{DB1} \right) + K_{D1A}K_{D2A}B^2/K_{DB1}^2 + A_{\text{nonspecific}} \]

where A represents free radioligand (the D₁-like receptor antagonist [³H]SCH 23390 or the D₂-like receptor antagonist [³H]YM-09151-2) concentration, \( R_A \) is the total amount of receptor dimers, and \( K_{D1A} \) and \( K_{D2A} \) are the macroscopic equilibrium dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed competing compound (the dopamine D₁-like receptor antagonist SCH 23390, the dopamine D₂-like receptor agonist SKF 38393, or the dopamine D₂-like receptor antagonist YM-09151-2) concentration, and \( K_{DB1} \) and \( K_{DB2} \) are, respectively, the macroscopic equilibrium dissociation constants of the first and second binding of B; \( K_{DAB} \) is the hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semioccupied by A.

Because the radioligand A (being the antagonist [³H]SCH 23390 or the agonist [³H]YM-09151-2) showed noncooperative behavior (Franco et al., 2006; Casado et al., 2007), eq. 1 was simplified to eq. 2 because \( K_{D2A} = 4K_{D1A} \) (Casado et al., 2007):

\[ A_{\text{total}} = (4K_{D1A}A + 2A^2 + 4K_{D1A}A B/K_{DAB}R_A) \left( 4K_{D1A}^2 + 4K_{D1A}A + 4K_{D1A}A B/K_{DB1} \right) + 4K_{D1A}^2 B^2/K_{DB1}^2 + A_{\text{nonspecific}} \]

The dimer cooperativity index for the competing ligand B (the dopamine D₁ receptor agonist SKF 38393) was calculated as (Casado et al., 2007):

\[ D_{CB} = \log(4K_{DB1}/K_{DB2}) \]

When \( K_{DAB} = 2K_{DB1} \), the binding of the radioligand to one protomer in the dimer does not modify the binding of the competing ligand to the other empty protomer in the dimer. In contrast, values of \( K_{DAB} < 2K_{DB1} \) or \( K_{DAB} > 2K_{DB1} \) indicate, respectively, a positive or negative effect (see radioligand/competitor modulation in Supplemental Data). According to this, a new parameter, “the dimer radioligand/competitor modulation index” (\( D_{AB} \)), ("SCH 23390/SKF 38393 modulation index" for the D₁ receptor ligands reported here) is introduced, which is defined as \( D_{AB} = \log(2K_{DAB}/K_{DAB}) \). The index is defined in such a way that its value is “0” when the presence of radioligand does not affect the competitor binding to the empty protomer in the dimer. Positive or negative values of \( D_{AB} \) indicate that the presence of radioligand increases or decreases, respectively, the competitor affinity for binding to the empty protomer in the dimer.

A direct calculation of the concentration of B providing half-saturation (\( B_{50} \)) was obtained according to (Casado et al., 2007):

\[ B_{50} = (K_{DB1}K_{DB2})^{1/2} \]

In the experimental conditions, when both radioligand A (the antagonist [³H]SCH 23390) and competitor B (the dopamine D₁ receptor agonist SKF 38393) show noncooperativity, \( K_{D2A} = 4K_{D1A} \) and \( K_{DB2} = 4K_{DB1} \), and eq. 1 is simplified to:

\[ A_{\text{total}} = (4K_{D1A}A + 2A^2 + 4K_{D1A}A B/K_{DAB}R_A) \left( 4K_{D1A}^2 + 4K_{D1A}A + 4K_{D1A}^2 B^2/K_{DB1}^2 \right) + 4K_{D1A}^2 B^2/K_{DB1}^2 + A_{\text{nonspecific}} \]

When both radioligand A (being the antagonist [³H]SCH 23390 or the antagonist [³H]YM-09151-2) and competitor B (SCH 23390 or
For biphasic curves (or complex dissociation kinetics), after 5 min, cells were placed on ice and centrifuged at 2500 rpm for 10 min at 37°C and treated or not with the indicated ligands.

Bated with 50 μM glucose and resuspended with 200 μl of HBSS containing 10 mM glucose, the pellet was washed twice in HBSS containing 10 mM glucose, and resuspended.

Dissociation kinetic data were fitted to the following empirical equation:

\[
A_{\text{total bound}} = A_{\text{total bound}}^i = \sum_{i=1}^{n} A_i e^{-kt} + A_{\text{nonselective bound}}
\]

where \(A_i\) represents the initial radioligand (the D1 receptor antagonist \([3H]SCH 23390\) or the D2 receptor antagonist \([3H]YM-09151-2\)) bound for each molecular specie \(i\), \(t\) is time, and \(k_i\) is the dissociation rate constants for the \(n\) different molecular species. For monophasic curves (or simple dissociation kinetics), \(n = 1\), and for biphasic curves (or complex dissociation kinetics), \(n = 2\).

Goodness of fit was tested according to reduced \(\chi^2\) value given by the nonlinear regression program. The test of significance for two different population variances was based on the Student’s \(t\) distribution (see Casado et al., 1990 for details). By use of this \(F\) test, a probability greater than 95% (\(p < 0.05\)) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70% (\(p > 0.30\)) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values ± S.E.M. of three to four independent experiments.

cAMP Determination. The Flp-In T-REx-293-D1R cells were grown in six-well plates to 80% confluence and incubated with 15 ng/ml tetracycline for 16 h in serum-free medium before the experiment [in these conditions, the expression level of dopamine D1 receptors was similar to the expression in brain striatum (0.4–0.5 pmol/mg protein)]. Cells (equivalent to 0.4 mg of protein) were harvested, washed twice in HBSS containing 10 mM glucose, and suspended in HBSS supplemented with 10 mM glucose and 10 mM MgCl\(_2\) to a final volume of 1 ml in plastic tubes. Cells were preincubated with 50 μM zardaverine (Tocris) as phosphodiesterase inhibitor for 10 min at 37°C and treated or not with the indicated ligands. After 5 min, cells were placed on ice and centrifuged at 2500g for 5 min at 4°C. The pellet was washed with ice-cold HBSS containing 10 mM glucose and resuspended with 200 μl of ice-cold HClO\(_4\) (4%) for 30 min; 1.5 M KOH was added to reach neutral pH. Samples were centrifuged at 15,000g for 30 min at 4°C, and the supernatant was frozen at −20°C. The accumulation of cAMP in the samples was measured by a \([3H]cAMP\) assay system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as instructed by the manufacturer. Student’s \(t\) test for unpaired samples was used for statistics.

Results

The IP28 Binds to Dopamine D1 Receptors but Is Unable to Completely Displace Agonist Binding. The IP28 (Fig. 1a) is a \(\alpha\)-indoloquinolizidine-peptide hybrid obtained as described previously by applying a combinatorial approach that combines the solution-phase synthesis of an indolo[2,3-\(\alpha\)]quinolizidine scaffold with solid-phase peptide chemistry (Vendrell et al., 2009). As described in Vendrell et al. (2009), IP28 binds to dopamine D1 and D2 receptors. We have tested whether IP28 has some degree of selectivity for dopamine receptors because its affinity constants for other GPCRs, namely adenosine A\(_2\) or histamine H\(_3\) receptors, were 70 or 50 μM, respectively (results not shown). These values are more than 30-fold higher than the affinity for the D\(_2\) receptors (1.5 μM; Vendrell et al., 2009). To characterize the IP28 binding to dopamine D1 receptors, IP28 was compared with the D\(_1\) dopamine receptors antagonist SCH 23390 as displacer of \([3H]SCH 23390\) binding. Competition experiments were performed with a constant concentration of \([3H]SCH 23390\) (2.5 nM) and increasing concentrations of unlabeled SCH 23390 (0.01 nM to 50 μM) or IP28 (10 nM to 50 μM) by using brain striatal membranes as described under Materials and Methods. As expected (see Fig. 1b), SCH 23390 fully displaced the specific binding of the \([3H]SCH 23390\), whereas IP28 decreased the radioligand binding with an IC\(_{50}\) of 2.5 ± 0.2 μM, but it was unable to completely inhibit the specific binding of \([3H]SCH 23390\) (Fig. 1b). Very high IP28 concentrations led to 78% reduction of radioligand binding, and this cannot be explained by binding to the orthosteric site. Therefore, the results suggested that IP28 might behave as an allosteric effector of D1 receptors.

IP28 Is a Negative Allosteric Modulator of Antagonist SCH 23390 Binding to Dopamine D1 Receptors. To test whether the IP28 was an allosteric modulator of the antagonist binding to dopamine D1 receptors, its effect on the antagonist SCH 23390 binding affinity was carefully evaluated. Competition experiments were performed with a constant amount of \([3H]SCH 23390\) (2.8 nM) and increasing concentrations of unlabeled antagonist (0.01 nM to 50 μM) in

![Fig. 1. Competition curves of dopamine D1 receptor antagonist \([3H]SCH 23390\) binding versus increasing concentrations of \(SCH 23390\) or IP28. a, structure of IP28, Smiles formula: O=C(N[C@@H](CC1=C(C/F=C(C1)C([N2CC(C)]C[=C(])C)=O)C@H(CCC3CCCC3)N(C@H(C4/C@[N@@H](CC6)=C6)C76)=O)C76)=O. b, competition experiments of the antagonist \([3H]SCH 23390\) (2.5 nM) versus increasing concentrations of \(SCH 23390\) (0.01 nM to 50 μM) (●) or IP28 (10 nM to 50 μM) (○) were performed as indicated under Materials and Methods by use of brain striatal membranes. Data are mean ± S.E.M. from a representative experiment (\(n = 3\)) performed in triplicate. For some data points, error is smaller than symbol.](image-url)
the absence or presence of different IP28 concentrations (1–50 μM) using brain striatal membranes. From Fig. 2a, two related effects can be seen. One is that total amount of [3H]SCH 23390 bound is decreased by increasing IP28 concentration in agreement with the results shown in Fig. 1b. The other is that IP28 concentration-dependently increased the SCH 23390 concentration needed to displace 50% of the radioligand binding, indicating that antagonist affinity decreases in the presence of IP28. Because it has been described that D1 receptors are dimers or higher-order oligomers (Ng et al., 1994; George et al., 1998; Kong et al., 2006), binding data were fitted to a dimeric receptor model as described under Materials and Methods, i.e., to eq. 6, and the equilibrium dissociation constant KDA1 values, which are a measure of affinity for the antagonist, were calculated by nonlinear regression (KDA2 was calculated as 4KDA1 for this noncooperative antagonist; see Materials and Methods). IP28 concentration-dependently increased the apparent antagonist equilibrium dissociation constant (KDA1 values until a seemingly constant KDA1 value was reached (Fig. 2b). To better understand the results, an analysis of the variation of dissociation constants assuming the binding of IP28 to the orthosteric site was performed (see Supplemental Data). The results of this analysis (dotted line in Fig. 2b) indicate that, in such circumstances, when the [3H]SCH 23390 binding is noncooperative and there is not radioligand-competitor modulation (KDB = 2KDA1; see Supplemental Data), a linear relationship between apparent KDA1 and the IP28 concentration would happen. Therefore, the apparently hyperbolic relationship depicted in Fig. 2b (solid line connecting real data points) indicates that IP28 is not an orthosteric ligand and suggest that it is a negative allosteric modulator of the antagonist binding to dopamine D1 receptors.

To further prove the allosteric behavior of IP28 on dopamine D1 receptors binding, dissociation kinetic experiments were performed. First, 2.5 nM [3H]SCH 23390 was incubated with brain striatal membranes in the absence or in the presence of 3 nM dopamine D1 receptor antagonist SKF 83566 or 3 μM IP28, as indicated under Materials and Methods; dissociation was then initiated by the addition of 10 μM SCH 23390. The time course of the different dissociations is displayed in Fig. 3. In the absence of SKF 83566 or IP28, the dissociation of [3H]SCH 23390 is biphasic, and dissociation rate constants are listed in Table 1. As expected, the presence of the orthosteric competitor, SKF 83566, did not affect the values of the dissociation rate constants for [3H]SCH 23390 (Table 1). In contrast, IP28 led to a different dissociation curve in such a way that the presence of the indoloquinolizidine-peptide led to simple [3H]SCH 23390 dissociation kinetics and to the disappearance of the slow radioligand dissociation rate constant (Fig. 3, Table 1). This suggests that IP28 modifies the receptor conformation increasing orthosteric ligand dissociation. This finding is consistent with a negative allosteric modulation exerted by IP28 on the antagonist SCH 23390 binding to dopamine D1 receptors. Thus, IP28 binds to an allosteric site on D1 receptors.

![Fig. 2. Competition curves of dopamine D1 receptor antagonist [3H]SCH 23390 binding versus increasing concentrations of SCH 23390 in the presence or in the absence of IP28. a, competition experiments of the antagonist [3H]SCH 23390 (2.8 nM) versus increasing concentrations of SCH 23390 were performed with brain striatal membranes as indicated under Materials and Methods in the presence of increasing concentrations of IP28 (top to bottom: 0 (○), 1 (○), 3 (■), 10 (□), 20 (▲), 30 (△), 40 (▼), 50 (▲) μM). Binding data were fitted to eq. 6 (see Materials and Methods) to obtain the equilibrium binding parameters. b, the calculated values for the apparent equilibrium dissociation constant (apparent KDA1) versus the IP28 concentrations are plotted. The dotted straight line represents the plot of the apparent equilibrium dissociation constants, assuming that IP28 binds to the orthosteric site and competes for the [3H]SCH 23390 binding (see Supplemental Data). Data are mean ± S.E.M. from a representative experiment (n = 3) performed in triplicate. In a, for some data points, error is smaller than symbol.](image)

![Fig. 3. Dissociation kinetic curves for [3H]SCH 23390 binding to dopamine D1 receptors in the absence or in the presence of the agonist SKF 83566 or IP28. Brain striatal membranes were incubated with 2.5 nM [3H]SCH 23390 in the absence (○), or in the presence of 3 nM SKF 83566 (△), or in the presence of 3 μM IP28 (■). In all cases, the dissociation was initiated by the addition of 10 μM unlabeled SCH 23390 (see Materials and Methods). Data are mean ± S.E.M. from a representative experiment (n = 3) performed in triplicate. For some data points, error is smaller than symbol.](image)
Because the D<sub>1</sub> receptor expression in striatal membranes is very high with respect to D<sub>2</sub> receptor expression (Araki et al., 2007), the above-described results are representative of the IP28 binding to D<sub>1</sub> receptors. There is no selective radiolabeled ligand available for the different subtypes of D<sub>1</sub>-like receptors (D<sub>4</sub> and D<sub>5</sub> receptors); therefore, the IP28 binding to D<sub>5</sub> receptors was analyzed in membranes from HEK-293 cells expressing D<sub>5</sub> receptors. Cells were generated, and membranes were obtained as described under Materials and Methods. Dissociation kinetic experiments were performed by incubating cell membranes (0.2 mg of protein/ml) with 2.5 nM [3H]SCH23390 in the absence or in the presence of 3 μM IP28, and dissociation was then initiated by the addition of 10 μM SCH23390. In the presence of IP28, the [3H]SCH23390 binding decreased by 40%. In the absence of IP28, the dissociation of [3H]SCH23390 was monophasic, and the dissociation rate constant was 0.12 ± 0.02 min<sup>-1</sup>. It is noteworthy that the presence of IP28 did not change the [3H]SCH23390 dissociation rate for D<sub>5</sub> receptor, which was 0.11 ± 0.02 min<sup>-1</sup> for the calculated constant. Thus, IP28 is not an allosteric modulator of dopamine D<sub>5</sub> receptors, which suggests that IP28 behaves as an orthosteric ligand.

**IP28 Is a Negative Allosteric Modulator of Agonist SKF 38393 Binding to Dopamine D<sub>1</sub> Receptors.** To test whether IP28 was an allosteric modulator of agonist binding to dopamine D<sub>1</sub> receptors, competition experiments were performed with a constant amount of [3H]SCH23390 (2.3 nM) and increasing concentrations of the D<sub>1</sub> receptor agonist SKF 38393 (0.1 nM to 50 μM), in the absence or presence of different IP28 concentrations (1 μM to 30 μM) using brain striatal membranes. Binding data (Fig. 4) were fitted assuming that D<sub>1</sub> receptors are dimers and statistically (F test) testing whether the agonist SKF 38393 binding was cooperative (fitting to eq. 2) or noncooperative (fitting to eq. 5). In the absence of IP28 or at low IP28 concentrations (1 and 3 μM), binding data were well fitted to eq. 2. In contrast, at high concentrations (10–30 μM), binding data fitted to eq. 5. Therefore, the presence of IP28 led to a progressive disappearance of agonist binding cooperativity (see Table 2). One advantage provided by the two-state dimer receptor model is the quantification of cooperativity by calculation of a cooperativity index (D<sub>CB</sub>; Table 2). D<sub>CB</sub> measures the affinity modifications occurring when a protomer senses the binding of the same ligand molecule to the partner protomer in a dimer (see Materials and Methods). The D<sub>CB</sub> value of −0.7 indicates negative cooperativity for SKF 38393 binding. The D<sub>CB</sub> values (Table 2) changed in the presence of IP28 until cooperativity completely disappeared (D<sub>CB</sub> = 0), which indicates that IP28 binding to its allosteric site prevents the homotropic cooperativity in agonist binding. Furthermore, apparent dissociation constants for the agonist/competitor (K<sub>DB1</sub> and K<sub>DB2</sub>) increased (p < 0.01) in the presence of increasing IP28 concentrations until a constant value was attained. These results indicate that IP28 is also a negative allosteric modulator of the agonist binding.

The two-state dimer receptor model can even provide an hybrid equilibrium dissociation constant (K<sub>DAB</sub>; Table 2) that corresponds to the equilibrium dissociation constant of the agonist SKF 38393 binding to a receptor dimer semioccupied by another compound, such as an antagonist (SCH 23390; see Casado et al., 2009a,b). Accordingly, a “dimer radioligand/competitor modulation index” (D<sub>AB</sub>) can be calculated as indicated under Materials and Methods. D<sub>AB</sub> is a measure of competitor affinity modifications occurring when a protomer senses the binding of another molecule to the partner protomer (in a dimer). As shown in Table 2, in the absence of IP28, the antagonist [3H]SCH23390 binding to an empty receptor dimer positively modulates the agonist SKF 38393 binding to the other subunit in the dimer (D<sub>AB</sub> = 0.43), whereas the modulation is completely disrupted by IP28 (D<sub>AB</sub> = 0).

**IP28 Is a Weak Ago-Allosteric Effector but a Strong Negative Modulator of D<sub>1</sub> Receptor Potency.** Dopamine D<sub>1</sub> receptors couple to Gs/olf proteins and its main signaling pathway is the stimulation of the adenyl cyclase-protein kinase A cascade (Neve et al., 2004). Thus, to investigate the IP28-mediated consequences on dopamine D<sub>1</sub> receptor signaling, cAMP assays were performed. Flp-In T-REx-293-D<sub>R</sub> cells were treated with increasing concentrations of IP28 or with the dopamine D<sub>1</sub> receptor partial or full agonists in the absence or the presence of 10 μM IP28. IP28 was able by itself to increase concentration-dependently the cAMP concentration (Fig. 5) in cells induced with tetracycline, but not in noninduced cells, which lack D<sub>1</sub> receptor expression. The maximum effect was approximately 22% of that exerted by the partial agonist (Fig. 6a) and approximately
Parameter values obtained by fitting data of antagonist [3H]SCH 23390 binding to dopamine D1 receptors in competition experiments with the agonist SKF 38393 and different concentrations of IP28.

Data points in Fig. 4 were fitted assuming radioligand binding to D1 receptor dimers. Values were calculated by fitting data to eq. 2 or 5, introducing the K_{D_{1_{H}}} value for [3H]SCH 23390 binding to D1 receptor obtained from Fig. 2 to calculate the macroscopic dissociation constants in the absence or in the presence of IP28. Data are mean ± S.E.M. values of three experiments.

$$\begin{array}{cccccc}
\text{Parameters}\textsuperscript{a} & 0 & 1 & 3 & 10 & 20 & 30 \\
\text{IP28} & \text{μM} \\
\text{Apparent } K_{D_{1_{H}}}\text{, μM} & 0.020 ± 0.002 & 0.35 ± 0.04 & 1.2 ± 0.2 & 1.7 ± 0.3 & 3 ± 1 & 2.2 ± 0.5 \\
\text{Apparent } K_{D_{2_{H}}}\text{, μM} & 0.4 ± 0.1 & 7 ± 1 & 9 ± 1 & 7 ± 1 & 12 ± 4 & 9 ± 2 \\
\text{Apparent } K_{D_{A_{B}}}\text{, μM} & 0.015 ± 0.009 & 0.70 ± 0.08 & 2.4 ± 0.4 & 3.4 ± 0.6 & 6 ± 3 & 4 ± 1 \\
D_{C_{B}} & -0.7 ± 0.1 & -0.7 ± 0.1 & -0.3 ± 0.1 & 0 & 0 & 0 \\
D_{A_{B}} & 0.5 ± 0.3 & 0 & 0 & 0 & 0 & 0 \\
B_{50}\text{, μM} & 0.09 ± 0.01 & 1.5 ± 0.2 & 3.3 ± 0.4 & 3.4 ± 0.6 & 6 ± 2 & 4.4 ± 0.9 \\
\end{array}$$

\textsuperscript{a} K_{D_{1_{H}}} and K_{D_{2_{H}}} respectively, are the equilibrium dissociation constants of the first and second binding of B (SKF 38393) to the dimer. K_{D_{A_{B}}} is the hybrid equilibrium dissociation constant of B binding to a receptor dimer semioccupied by A ([3H]SCH 23390). D_{C_{B}} is the dimer cooperativity index for the binding of ligand B. D_{A_{B}} is the dimer radioligand/competitor modulation index (SCH 23390/SKF 38393 modulation index). B_{50} is the concentration providing half-saturation for B.

17% of that exerted by the full agonist (Fig. 6b); therefore, IP28 behaved as a weak ago-allosteric agonist. It is noteworthy that the presence of IP28 led to a marked shift to the right of the dose–response curves of any orthosteric agonists (Fig. 6). The EC_{50} values for the partial agonist SKF 38393 or the full agonist SKF 81297 in the absence of IP28 (116 ± 15 nM or 41 ± 8 nM, respectively) were lower \((p < 0.001)\) than the values obtained in the presence of IP28 (4.4 ± 0.5 μM or 1.2 ± 0.2 μM, respectively), indicating that IP28 strongly decreased the receptor potency. IP28 did not decrease the efficacy of full/partial agonists (Fig. 6). All of these results indicate that IP28 is an ago-allosteric modulator of dopamine D1 receptors that decreases the agonist potency while preserving the receptor efficacy.

**IP28 Is Not an Allosteric Modulator of Dopamine D2 Receptors.** IP28 not only binds to dopamine D1 receptors, but also to dopamine D2 receptors (Vendrell et al., 2009). To characterize IP28 binding to dopamine D2 receptors, IP28 was compared with the D2 dopamine receptors antagonist YM-09151-2 as displacer of [3H]YM-09151-2 binding. Competition experiments were performed with a constant concentration of [3H]YM-09151-2 (1 nM) and increasing concentrations of unlabeled YM-09151-2 (0.01 nM to 50 μM) or IP28 (10 nM to 100 μM) using brain striatal membranes as described under Materials and Methods. Both YM-09151-2 and IP28 fully displaced the specific binding of [3H]YM-09151-2 (Fig. 7a). For IP28 a EC_{50} of 2.6 ± 0.4 μM was calculated. These results are compatible with IP28 binding to the orthosteric site of D2 receptors. To further prove this hypothesis, dissociation kinetic experiments were performed. First of all, 0.5 nM [3H]YM-09151-2 was incubated with brain striatal membranes in the absence or in the presence of 3 μM IP28 as indicated under Materials and Methods and dissociation was then initiated by the addition of 10 μM YM-09151-2.
Materials and Methods. Dissociation kinetic experiments were performed by incubating cell membranes (0.2 mg of protein/ml) from HEK-293 expressing D_2 or D_4 receptors with 0.5 nM [^{3}H]YM-09151-2 in the absence or in the presence of 3 μM IP28, as indicated under Materials and Methods, and dissociation was then initiated by the addition of 10 μM YM-09151-2. In both cases, the [^{3}H]YM-09151-2 binding decreased in the presence of IP28 (33% for the D_2 receptor and 36% for the D_4 receptor), and the dissociation of [^{3}H]YM-09151-2 was biphasic. It is noteworthy that the presence of IP28 did not affect the values of the dissociation rate constants (Table 3). This indicates that IP28 did not modify the orthosteric ligand dissociation from D_3 or D_4 receptors. This finding is consistent with orthosteric binding of IP28 to all dopamine D_2-like receptors.

Discussion

The indoloquinolizidine scaffold constitutes a novel and synthetically accessible structure, which can potentially interact at transmembrane binding sites and exhibits good solubility in ethanol-aqueous media. The combinatorial exploration of indoloquinolizidine-peptide hybrids and their behavior at dopamine receptors has been studied recently (Vendrell et al., 2009). Here, we reported that one of the indoloquinolizidine peptide hybrids, IP28, is able to decrease the antagonist affinity for D_3 dopamine receptors. The increase in the K_D values for the antagonist in the presence of increasing concentrations of IP28 was nonlinear, thus indicating that IP28 binds to an allosteric site. To detect and quantify such allosteric interaction, the allosteric modulator effect on the rates of dissociation of orthosteric ligands has been one of the methods of election (May et al., 2007). Affinity modulators induce a conformational change that may alter one or both association or dissociation rates of binding to the orthosteric site. The most common method is to assay dissociation kinetics, because the only way dissociation of a pre-bound GPCR orthosteric ligand complex can be modified is by the concomitant binding of a modulator to a topographically distinct site (May et al., 2007). For this purpose, dissociation experiments (of a radiolabeled orthosteric ligand) can be performed in the presence of the allosteric modulator. These assays demonstrated that IP28 is an allosteric modulator that increased the dissociation rate of the radiolabeled neutral antagonist bound to D_1 dopamine receptors. This effect is specific for D_1 dopamine receptors because the dissociation rate of the radiolabeled antagonist bound to membranes from cells expressing D_3 dopamine receptors did not change in the presence of IP28. In addition, IP28 did not change the dissociation rate of the radiolabeled antagonist bound to membranes from cells expressing D_4 dopamine receptors determined in the absence or in the presence of IP28 (3 μM).

Because the D_2 receptor expression in striatal membranes is very high with respect to the expression of other D_2-like receptors, namely D_3 and D_4 (Lidow et al., 1998; Araki et al., 2007), the above-described results are representative of the IP28 binding to D_2 receptors. Because there are no selective radiolabeled ligands for the different subtypes of D_2-like receptors, the IP28 binding to D_2 and D_4 receptors was analyzed in cells expressing D_3 or D_4 receptors. Cells were generated and membranes were obtained as described under


TABLE 3

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Experimental Conditions</th>
<th>k_{i,slow}</th>
<th>k_{i,fast}</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_3</td>
<td>Control</td>
<td>0.002 ± 0.001</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>D_3</td>
<td>IP28</td>
<td>0.003 ± 0.002</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>D_4</td>
<td>Control</td>
<td>0.0033 ± 0.0003</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>D_4</td>
<td>IP28</td>
<td>0.004 ± 0.001</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

Dissociation rate constants for [^{3}H]YM-09151-2 binding to dopamine D_3 and D_4 receptors determined in the absence or in the presence of IP28 (3 μM)

Binding data from dissociation kinetic experiments were fitted assuming monophasic (n = 1 in eq. 7) and biphasic (n = 2 in eq. 7) dissociation curves. By the F test, a significant better fit was obtained by considering n = 2 (see Materials and Methods). Parameter values represent the mean ± S.E.M. of three experiments.
D4 dopamine receptors, indicating that IP28 is not an allosteric modulator of D2-like receptors and suggesting that it behaves as an orthosteric ligand of D2-like dopamine receptors. Although IP28 binds to all subtypes of dopamine receptors, it is a specific allosteric modulator for dopamine D1 receptors. This is interesting from a pharmacological point of view, because the study of IP28 could open new avenues for the design of more affinity and selective drugs acting as allosteric modulators.

An allosteric effect results in a positive modulation if the modulator facilitates an interaction, or in a negative modulation if it inhibits the interaction of the ligand with the orthosteric binding site (May et al., 2007; Schwartz and Holst, 2007; Conn et al., 2009). According to these concepts, IP28 is an allosteric ligand of dopamine D1 receptors that negatively modulates the agonist and antagonist binding to the orthosteric site of the receptor. Moreover, IP28 was able to disrupt the homotropic cooperativity in agonist binding to dopamine D1 receptors and also the positive cross talk observed between the antagonist and the agonist binding in competition experiments. In summary, the binding of IP28 to its allosteric site reduces the orthosteric-mediated ligand-induced molecular cross-talk between the two protomers in the D1 receptor dimer. In terms of signaling, it is interesting that IP28 was able to induce cAMP increases in cells expressing D1 dopamine receptors (and not in parental cells). This result suggested that IP28 would be an ago-allosteric modulator, which recently has been defined as a ligand that functions both as an agonist on its own and as an allosteric modulator of the effect of the agonists (Schwartz and Holst, 2006, 2007; Bridges and Lindsley, 2008). This distinguishes ago-allosteric from allosteric modulators, i.e., modulators that, as defined by International Union of Pharmacology (IUPHAR: http://www.iuphar.org/) (Neubig et al., 2003), enhance or inhibit the affinity and/or the effect of the orthosteric agonist but have no effect on their own. The effect of the ago-allosteric modulator can be positive with regard to both efficacy and potency but might also be negative or inhibitory in terms of, for example, potency while being positive in terms of efficacy (Schwartz and Holst, 2007). As demonstrated in this article, IP28 increased the EC90 values obtained from curves of cAMP response versus increasing D1 dopamine receptor agonist concentrations. This indicates that IP28 is an ago-allosteric negative modulator of agonist potency without decreasing the agonist-mediated maximum effect, i.e., without affecting efficacy of the full/partial agonists used.

Diverse evidence suggests that D1 dopamine antagonists may have neuroleptic properties (Andersen and Nielsen, 1991; Wu et al., 2005). An important role for D1 dopamine receptors was in the pathophysiology of schizophrenia has been described (Goldman-Rakic, 1999; Sedvall et al., 1995). It was demonstrated that selective D1 antagonists had antipsychotic activity in preclinical studies, but a clinical trial of selective D1 antagonists demonstrated no antipsychotic activity, and instead may have aggravated psychoses in some patients (Miyamoto et al., 2005). In contrast to the inef-fic-tiveness of D1 antagonists in the treatment of schizophrenia, low doses of selective full D1 receptor agonists, such as dihy-drexidine, A77636 and SKF81297, have been reported to have cognitive-enhancing actions in non-human primates (Cai and Arnsten, 1997). Cognitive impairment has been found across all subtypes of schizophrenia. It has been postulated that either insufficient or excessive D1 receptor stimulation is deleterious to cognitive function of the prefrontal cortex; thus, an “optimal” level of D1 receptor activation is necessary for normal cognitive function (Goldman-Rakic et al., 2000), and overactivation of D1 dopamine receptors may exacerbate psychotic symptoms in patients with schizophrenia (Bubenikova-Valesova et al., 2009). It may be speculated that compounds able to decrease the potency while preserving the efficacy of D1 receptor agonists are promising targets of exploration as modulators of dopamine compounds in psychotic pathologies. Because IP28 binding to an allosteric site on D1 dopamine receptors decreases the ligand affinity and the receptor potency while preserving the receptor efficacy, a D1-like receptor agonist stimulation in the presence of this type of compound, showing weak agonist properties by its own, is promising in terms of therapeutic potential. Mapping the interacting zone in the D1 receptor combined with strategies to improve selectivity and the affinity of the interaction between the allosteric compound and the receptor are required to meet this desirable objective.

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References
Cai JX and Arnsten AF (1997) Dose-dependent effects of the dopamine D1 receptor antagonists A77636 or SKF81297 on spatial working memory in aged monkeys. J Pharmacol Exp Ther 283:183–189.


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