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A D1/D2 chimeric dopamine receptor mediates a D1 response to a D2-selective agonist

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D1 and D2 dopamine receptors are G-protein coupled receptors and have seven transmembrane spanning regions (TM) typical of this receptor superfamily. Although dopamine binds equally to D1 and D2 receptors, many compounds are highly selective. To probe the receptors for regions that determine subtype specificity, plasmid constructs coding for the D1 or a D1/D2 chimeric receptor were made and transfected into cells to study the binding and agonist properties of non-selective or subtype-selective compounds The results suggest that the D2-selective agonist, quinpirole, gains much of its selectivity by binding to within TM VI and VII of the D2 receptor.

D1 dopamine receptor; D2 dopamine receptor; Chimeric dopamine receptor

1. INTRODUCTION

Dopamine receptors have been classified into D1 and D2 subtypes [1] and this classification has been extended to newly discovered dopamine receptors (D3, D4, D5) which have been categorized as either 'D1-like' or 'D2-like' [2]. The D1–D2 classifications are largely based on binding affinities as well as signalling properties of the receptors. Briefly, D1 receptors bind the benzazapine SCH 23390 with low nanomolar affinity and D1 agonists stimulate adenylyl cyclase whereas D2 receptors bind the butyrophenone spiperone with picomolar affinity and D2 agonists inhibit adenylyl cyclase [3]. In the present study, we explored the receptor basis of subtype-selective dopaminergic ligands by making a D1/D2 receptor chimera.

Previous studies suggested that the transducing domain of G-protein coupled β_2-adrenergic receptors resided in the third cytoplasmic loop [4,5]. Moreover, additional studies employing chimeric receptors and site-directed mutagenesis indicated that the ligand subtype selectivity for adrenergic receptors might derive from sequences in TM VI and VII [5,6]. To see if these results would extend to another set of catecholaminergic receptors, namely dopamine receptors, we made a D1/D2 receptor chimera containing D1 sequences from the N-terminal through to the C-terminal end of the third cytoplasmic loop and D2 sequences from the beginning of TM VI to the C-terminal of the receptor. We predicted that the chimeric receptor would differ from the wild-type D1 receptor by exhibiting (1) decreased affinity for D1-selective ligands, (2) increased affinity for a D2 ligand, and (3) activation of a D1 response by a D2 ligand.

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2. MATERIALS AND METHODS

2.1. *Wild-type human D1 and chimeric D1/D2 receptor constructs*

Standard procedures were used for DNA manipulations [7]. The polymerase chain reaction (PCR) was used to construct the chimeric D1/D2 receptor such that the junction between D1 and D2 segments was the beginning of TM VI (Fig. 1). To obtain the segment of the human D1 receptor including up to TM VI, one oligonucleotide was made, tatgcggccgcagagcccctgatgtgctt, that spanned the initiator methionine and another oligonucleotide, tgcgctagcgtcttcaggactttagtttc, was made that spanned the junction of the third cytoplasmic loop and TM VI of the human D1 receptor. To obtain TM VI and VII of the rat D2 receptor, one oligonucleotide was made that spanned the third cytoplasmic loop and TM VI, cctgctagccattgttctcggtgtgttca, and another oligonucleotide was made, atcgggcccatgaggtctggcctgcata, that spanned the termination codon of the rat D2 receptor. These pairs of oligonucleotides were used in separate PCR reactions containing plasmid DNA encoding either the human D1 receptor [8] or the rat D2 receptor [9] using a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) according to the supplier's protocol. Rat, as opposed to human, D2 sequences were used because we had the rat cDNA on hand and the amino acid sequences of these species from the beginning of TM VI to the C-terminal differ by only one conservative substitution (rat Leu-438 → human Met-438) located in the cytoplasmic tail of the receptor [10]. The D1 product was cut with *Not*I and *Nhe*I restriction enzymes and the D2 product was cut with *Nhe*I and *Apa*I restriction enzymes. Following gel purification, the products were subcloned into *Not*I–*Apa*I sites of the eukaryotic expression plasmid Rc/CMV (Invitrogen, San Diego, CA). The resulting plasmid was completely se-

quenced on both strands to be sure errors were not introduced during the PCR or subcloning. The wild-type human D1 receptor plasmid has previously been described [11]. All plasmid DNAs used for transfections were purified twice using cesium chloride gradients.

2.2. *Expression of wild-type and mutant receptors in COS-7 and HEK-293 cells*

COS-7 cells (ATCC# CRL1651) were maintained and transfected for binding assays using the DEAE-dextran method as previously described [11] HEK-293 cells (ATCC# CRL1573) were grown and maintained in minimal essential medium (HEPES-buffered) containing 10% fetal bovine serum and antibiotic/antimycotic solution HEK-293 cells were transfected for functional assays with DNA (20 μg/ 3.5×10^6 cells) using a modified calcium phosphate procedure [12]. Cells were used in either binding or functional assays 72 h post-transfection.

2.3. *Receptor binding assays*

Transiently transfected COS-7 cells were harvested 72 h post-transfection and the membranes of untransfected cells and cells transfected with the D1 or D1/D2 chimeric constructs were assayed by saturation binding analysis for D1 binding sites using the D1-specific radioligand [^{3}H]SCH 23390. The concentrations of [^{3}H]SCH 23390 ranged from 0.1–12 nM for the wild-type saturation curves and from 1.25–60 nM for the analyses of the membranes derived from the untransfected and D1/D2 transfected cells. For competition assays, membranes were incubated with [^{3}H]SCH 23390 and increasing concentrations of competitor in the presence or absence of 10 μM SCH 23390 to determine non-specific binding. The concentration of radioligand used for wild-type D1 or chimeric D1/D2 competition assays was approximately equal to the K_D obtained from the saturation assays. All binding experiments were performed three or more times. All binding parameters were determined using non-linear least-squares regression analysis as provided by the program Inplot (GraphPad, San Diego, CA). A two-site curve was chosen for the competition data if the residual sum of squares associated with the two-site fit was significantly ($P < 0.05$) lower than that associated with a one-site fit according to the manual for Inplot (GraphPad, San Diego, CA). All other aspects of the binding assays were as previously described [11].

2.4. *cAMP accumulation assays*

The cAMP accumulation assays were performed as previously described [11]. All assays were performed in triplicate The EC_{50} determinations were performed using the non-linear least squares regression analysis program of Inplot (GraphPad, San Diego, CA).

2.5 *Compounds*

All unlabelled ligand compounds were obtained from Research Biochemicals Inc. (Natick, MA). The radioligand, [^{3}H]SCH 23390 (75 Ci/mmol), was obtained from Amersham (Arlington Heights, IL).

3. RESULTS AND DISCUSSION

Saturation binding analysis of untransfected COS-7 cells revealed a low affinity site ($K_D > 100$ μM, data not shown) of greatly variable capacity. This site did not interfere with the saturation curves of the D1 or D1/D2 receptors since these curves modelled to single sites of much higher affinities.

Transient expression in COS-7 cells was high for both constructs (Fig. 2). Results from saturation binding analyses showed that the affinity of the chimeric D1/D2 receptor for the labelled D1-selective antagonist, [^{3}H]SCH 23390, was decreased nearly 20-fold relative to that of the wild-type D1 receptor (Fig. 2). Given the K_Ds

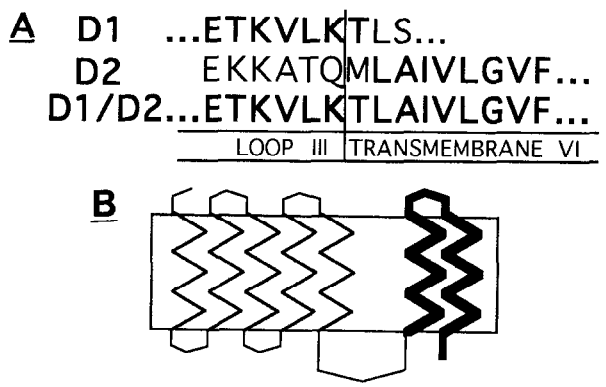

Fig. 1. (A) Amino acid sequences of the junction site of the human D1 and rat D2 receptors to make the human D1/rat D2 chimeric receptor. (B) Scheme of the D1/D2 chimeric receptor.

of the two receptors for the radioligand, we were then able to derive the K_is of other compounds (Table I) by the Cheng-Prusoff equation [13] after determining their IC_{50}s in competitive binding assays (Fig. 3). The data

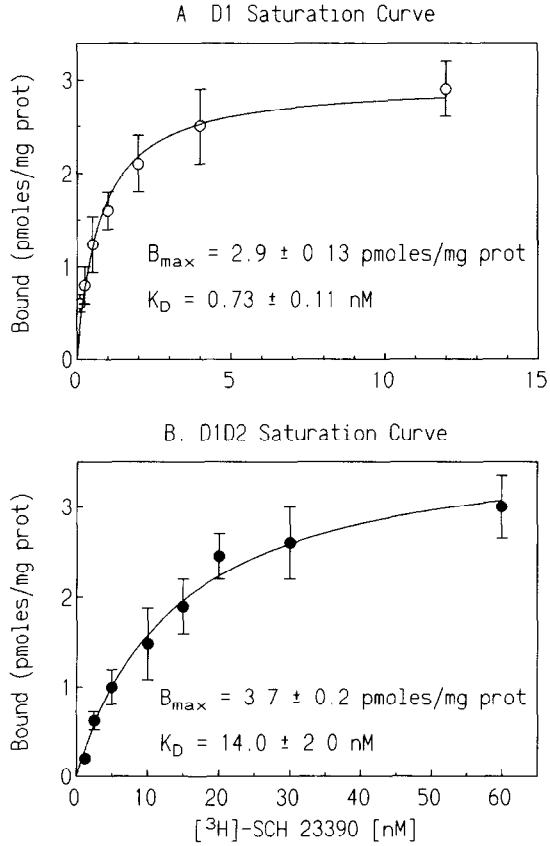

Fig. 2. Saturation curves using the labelled D1 receptor antagonist [^{3}H]SCH 23390 as the radioligand. Binding assays were performed on membranes of COS-7 cells transfected with constructs coding for either the human D1 receptor (A) or the human D1/rat D2 chimeric receptor (B).

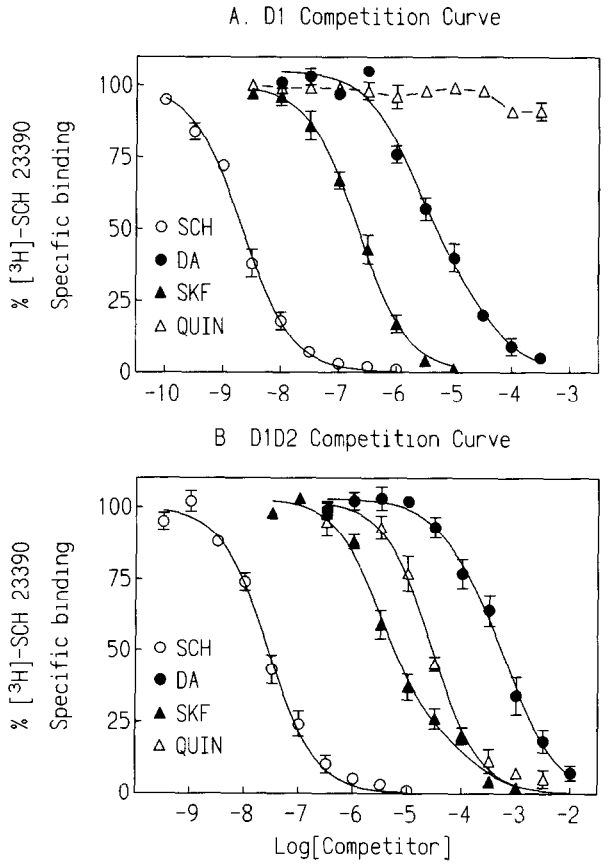

Fig. 3. Competition curves of various dopaminergic compounds using membranes from COS-7 cells transfected with constructs coding for the human D1 receptor (A) or the human D1/rat D2 chimeric receptor (B). The concentration of the radiolabel [^{3}H]SCH 23390 was approximately 0.8 nM in (A) and approximately 12 nM in (B). K_i values for these curves appear in Table I.

from Table I show that, relative to the wild-type D1 receptor, the chimeric D1/D2 receptor lost affinity for the D1-specific compounds, SCH 23390 and SKF 38393 and gained affinity for the D2-specific ligand, quinpirole. An unexpected result was that the chimeric receptor also lost affinity for the non-selective ligand, dopamine. Indeed, the drop in affinity for dopamine was greater than that for the D1-selective agents.

Results from the agonist-stimulated cAMP assays (Fig. 4, Table II) mirrored the pattern of results obtained in the binding experiments. Again, relative to the wild-type human D1 receptor, both the D1- and non-selective compounds, SKF 38393 and dopamine, respectively, lost potency. The loss of potency by dopamine was greater and this was accompanied by a diminished maximal responsiveness to dopamine (Fig. 4). In contrast, the potency of the D2-selective agonist, quinpirole, was greatly enhanced.

The loss of dopamine binding and potency by the chimeric receptor was not predicted since it is thought that catecholamines bind to residues contained within

Table I

K_is (mean ± S.E.M., μM) of compounds determined from competition curves

Compound	Receptor	
	D1	D1/D2
SCH 23390	0.001 ± 0.0004	0.013 ± 0.001
Dopamine	H 0.986 ± 0.44 (67%)* L 15.7 ± 1.8 (33%)*	H 42.8 ± 5.1 (27%)* L 421.6 ± 21 (63%)*
SKF 38393	0.107 ± 0.003	H 1.6 ± 0.03 (79%)* L 64 2 ± 7.71 (21%)*
Quinpirole	> 1,000	13.8 ± 0.17

*% of receptor population in high (H) or low (L) affinity when the competition curve is best fit by a two-site model as described in section 2.3. See Fig. 3 for curves.

the first five TMs [11,14,15] and these are intact in the chimeric receptor. Moreover, the chimeric receptor is composed of two receptor subtypes, both with equal affinity for dopamine. Nonetheless, it is possible that both dopamine and the benzazapines interact with subtype selective sites in TM VI and VII which prevent the formation of a normal binding pocket from a D1/D2 chimeric receptor. Unfortunately, it is also possible that substitution of the rat D2 sequences produced a global effect on the molecule to affect interactions between sites of the preceding D1 sequences. Therefore, in light of the negative impact of the chimeric receptor on dopamine interactions, the decreased binding of the D1 selective compounds for the chimera cannot be interpreted as evidence for the presence of D1 selective determinants in TM VI and VII.

The fact that the binding of the D2-selective quinpirole was greatly enhanced in the chimera strongly points to sites in TM VI and/or VII which contribute to quinpirole binding. Moreover, the striking ability of this D2 agonist, typically associated with D2-mediated inhibition of adenylyl cyclase, to stimulate cyclase in cells transfected with the D1/D2 receptor indicates that the D2 binding sites provided by TM VI and VII also pro-

Table II

EC_{50}s (mean ± S.E.M., μM) determined from agonist-stimulated cAMP accumulation dose-response curves

Compound	Receptor	
	D1	D1/D2
Dopamine	0.37 ± 0.01	4.06 ± 0.07
SKF 38393	0.11 ± 0.01	0.57 ± 0.06
Quinpirole	566.60 ± 51.60	0.46 ± 0.01

See Fig. 4 for curves.

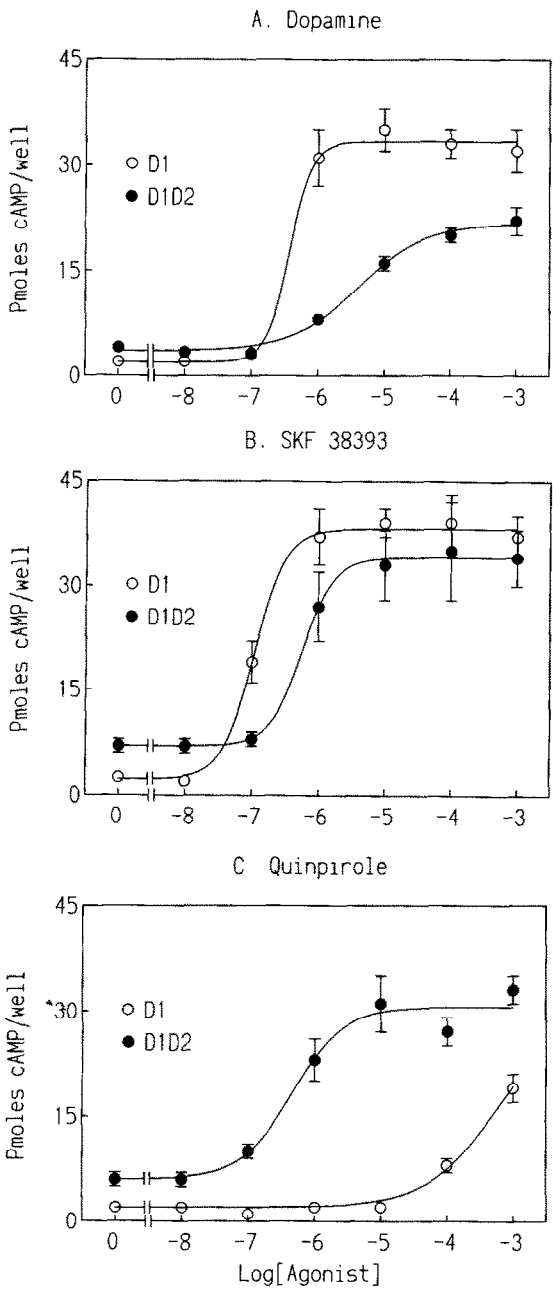

Fig. 4. Dose–response curves of agonist-stimulated cAMP accumulation in HEK-293 cells transfected with constructs coding for the human D1 receptor or the human D1/rat D2 chimeric receptor. The EC_{50}s for these curves appear in Table II. Basal cAMP accumulation values (pmols/well) were: D1-transfected cells = 3 ± 0.2 and D1/D2-transfected cells = 6 ± 0.6. Forskolin (10 μM)-stimulated cAMP accumulation values (pmol/well) were: D1-transfected cells = 56 ± 8 and D1/D2-transfected cells = 79 ± 4. There was no cAMP response to any of the dopaminergic agonists when tested in untransfected HEK-293 cells.

mote receptor activation by the agonist. Although the affinity of quinpirole for the chimera is greatly improved relative to the D1 receptor, it is still well below the affinity for the D2 receptor (typically found to be approximately 1 μM, [16]), suggesting that quinpirole might interact with other aspects of the D2 receptor beyond TM VI and VII. Further studies are necessary to determine whether all D2 selective compounds gain D2 selectivity by interacting at TM VI and VII determinants and also which residues within TM VI and VII mediate the binding of quinpirole.

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