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Lack of Discrimination by Agonists for D_2 and D_3 Dopamine Receptors

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The affinities of D_3 dopamine receptors for antagonists are similar to those of D_2 receptors. D_3 receptors have been reported, however, to have affinities nearly 100-fold higher than those of D_2 receptors for some agonists, including (±)-7-hydroxy-n,n-dipropyl-aminotetralin (7-OH-DPAT) and quinpirole. This has led to the use of these agonists to try to identify functional responses mediated by D_3 receptors in vivo. However, D_2 receptors exist in multiple states having high and low affinities for agonists. The G protein-coupled state of D_2 receptors is believed to be the functional state of these receptors. When receptors were labeled with the D_2 receptor antagonist [^{125}I]-(S)-3-iodo-N-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethoxysalicylamide ([^{125}I]-NCQ-298) under conditions that promote uncoupling of receptors from G proteins, the affinities of D_3 receptors were approximately 130-fold higher than those of D_2 receptors for 7-OH-DPAT and quinpirole. When receptors were labeled with the D_2 receptor agonist [^{125}I]-(R)trans-7-hydroxy-2-[N-propyl-N-(3'-iodo-2'-propenyl)-amino]tetralin ([^{125}I]-7-OH-PIPAT) under conditions that favor interactions of receptors with G proteins, the affinities of D_3 receptors were less than sevenfold higher than the affinities of D_2 receptors for the same drugs. Similarly, small differences in the affinities of D_2 and D_3 receptors for other agonists were seen when receptors were labeled with [^{125}I]-7-OH-PIPAT. These data demonstrate that putative D_3 receptor-selective agonists also interact with a high-affinity, G protein-coupled state of D_2 receptors. The similarities in affinities of the agonist-preferring state of D_2 and D_3 receptors means that currently available agonists cannot be used to discriminate between behavioral effects mediated by D_2 and D_3 receptors.
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KEY WORDS: *Dopamine D_2 receptor; Dopamine D_3 receptor; Dopamine; Agonist; (±)-7-hydroxy-n,n-dipropyl-aminotetralin (7-OH-DPAT)*

The D_2 subtype of dopamine receptor is thought to be an important site of action for antipsychotic drugs (Creese et al. 1976; Seeman et al. 1976). Recently, two new D_2-like receptor subtypes, termed D_3 and D_4 receptors, have been identified (Sokoloff et al. 1990; Van Tol et al. 1991). Based on their reported high affinity for many antipsychotic drugs and a relatively restricted distribution in limbic areas of the brain, D_3 receptors have been proposed as a possible target site for antipsychotic drug development (Schwartz et al. 1992). Currently, little is known about the physiological role of D_3 receptors. Studies of the properties and function of D_3 receptors in the brain have been hindered by a lack of selective drugs.

D_3 receptors are both structurally and pharmacologically similar to D_2 receptors. Both have seven putative membrane-spanning regions characteristic of neurotransmitter receptors that couple to guanine nucleotide-binding regulatory proteins (G proteins). D_3 receptors share approximately 75% amino acid homology with D_2 receptors within the membrane-spanning regions and 50% amino acid homology overall (Sokoloff

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"Agonist" and "antagonist" refer to the reported functions of these drugs at D2 receptors.

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et al. 1990). The affinities of D_3 receptors for many antagonists, including those used therapeutically in the treatment of psychoses, are comparable to the affinities of D_2 receptors for the same drugs (Sokoloff et al. 1990). However, in studies using transfected cells, D_3 receptors have been reported to have affinities nearly 100-fold higher than those of D_2 receptors for some agonists, including quinpirole and the putative dopamine autoreceptor agonist (±)-7-hydroxy-n,n-dipropylaminotetralin (7-OH-DPAT) (Lévesque et al. 1992).

The identification of 7-OH-DPAT as a D_3 receptor-selective drug has stimulated the use of this agent in laboratory animals to identify functional responses mediated by D_3 receptors (Caine and Koob 1993; Daly and Waddington 1993; Damsma et al. 1993). However, the validity of using 7-OH-DPAT—or other agonists—to discriminate between effects mediated by D_2 and D_3 receptors is problematic. Multiple signaling pathways including inhibition of adenylyl cyclase activity, inhibition of K^+ currents, Ca^{2+} mobilization, an increased rate of extracellular acidification, and potentiation of arachidonic acid release have been linked to activation of D_2 receptors (Neve et al. 1989; Vallar and Meldolesi 1989; Kanterman et al. 1991; Lledo et al. 1992). Although reports of functional effects of activation of D_3 receptors have appeared (Chio et al. 1994; Pilon et al. 1994; Potenza et al. 1994; Seabrook et al. 1994; Tang et al. 1994a), no functional responses have been consistently linked to the activation of D_3 receptors. Therefore, receptor selectivity has been defined using affinity values determined by competition of radiolabeled antagonist binding to receptors expressed in transfected cells.

D_2 receptors exist in multiple states having high and low affinities for agonists (Zahniser and Molinoff 1978; DeLean et al. 1982; Sibley et al. 1982; McDonald et al. 1984; George et al. 1985; Grigoriadis and Seeman 1985; Lahti et al. 1992). The agonist-preferring high-affinity state is believed to involve the formation of a ternary complex composed of agonist, the D_2 receptor, and a G protein (Wregget and DeLean 1984). To fully characterize the selectivity of a receptor for an agonist it is necessary to determine the affinities of multiple states of D_2 receptors for the agonist. Both G protein-coupled and uncoupled states of D_2 receptors are believed to have high affinity for radiolabeled antagonists. Computer-assisted analysis of data from the competition for radiolabeled antagonist binding has been used to determine the affinities of drugs for multiple populations of noninteracting receptor subtypes coexisting within the same tissue (Molinoff et al. 1981). Although these analytical methods are not directly transferrable to the study of interconverting affinity states of a single receptor subtype, similar techniques have been used to investigate the properties of multiple-affinity states of D_2 receptors (Sibley et al. 1982; McDonald et al. 1984; George et al. 1985; Chio et al. 1994). The inhibition of radiolabeled antagonist binding in the presence of guanine nucleotides provides a good estimate of the affinity of the uncoupled state of receptors for the agonist. An accurate determination of the affinity of an agonist for the G protein-coupled state of receptors cannot be obtained using an antagonist radioligand. An accurate estimate can be obtained in assays with radiolabeled agonists.

Based on the reported selectivity of D_3 receptors for 7-OH-DPAT, an iodinated derivative, [^{125}I](R)*trans*-7-hydroxy-2-[*N*-propyl-*N*-(3'-iodo-2'-propenyl)-amino]-tetralin ([^{125}I]-7-OH-PIPAT), was recently developed (Chumpradit et al. 1993; Foulon et al. 1993). In addition to D_3 receptors, a guanine nucleotide-sensitive state of D_2 receptors was found to bind [^{125}I]-7-OH-PIPAT with high affinity under conditions that promote the interaction of receptors with G proteins (Burris et al. 1994). The availability of [^{125}I]-7-OH-PIPAT, therefore, provides a radioligand with which the G protein-coupled state of D_2 receptors can be directly labeled. In the present study the affinities of the G protein-coupled state of D_2 receptors for drugs were determined by competition for [^{125}I]-7-OH-PIPAT binding under conditions ($MgCl_2$ present) that favor receptor interactions with G proteins. In addition, the affinities of the uncoupled state of D_2 receptors for drugs were determined by competition for the binding of [^{125}I]-NCQ-298, a D_2 receptor antagonist that binds with high affinity to D_2 and D_3 receptors (Hall et al. 1990; Filtz et al. 1993; Boundy et al. 1993a, 1993b) under conditions (guanine nucleotide present) that promote the uncoupling of receptors from G proteins. The affinity of D_2 receptors for [^{125}I]-NCQ-298 is increased when assays are carried out in the presence of sodium (Hall et al. 1990); therefore, NaCl (50 mM) was also included in assays with [^{125}I]-NCQ-298. For purposes of comparison, the affinities of D_3 receptors for the drugs were also determined under both conditions.

The affinities of D_2 receptors for agonists were much lower than those of D_3 receptors for the same drugs when assays were carried out under conditions that promote uncoupling of receptors from G proteins. However, the affinities of D_2 receptors for agonists were similar to the affinities of D_3 receptors for the same drugs under conditions that favor coupling of receptors to G proteins. Given the range of selectivity obtained in vitro with agonists, it is not possible to use differences in the in vivo potency of agonists, such as 7-OH-DPAT, to ascribe particular behavioral responses to activation of D_3 receptors.

MATERIALS AND METHODS

Materials and Drugs

Tissue culture dishes (100 × 20 mm) were purchased from Corning (Corning, NY). Eagle's minimum essen-

tial medium (EMEM), L-glutamine, and G418 sulfate were purchased from Gibco Laboratories (Grand Island, NY). Fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Tris[hydroxymethyl]aminomethane base (Tris), ethylenediamine tetraacetic acid disodium (Na_2EDTA), L-ascorbic acid, forskolin, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Guanylyl-imidodiphosphate tetralithium [Gpp(NH)p] and adenyl-yl-imidodiphosphate tetralithium [App(NH)p] were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). (+)Butaclamol hydrochloride, (−)quinpirole hydrochloride, dopamine hydrochloride, (−)apomorphine hydrochloride, (±)-7-hydroxy-n,n-dipropyl-aminotetralin hydrogen bromide, and S(−)sulpiride were purchased from Research Biochemicals Int. (Natick, MA). [^{3}H]-Adenine (26.9 Ci/mmol) was purchased from DuPont NEN (Wilmington, DE). [^{125}I]-NCQ-298 (2,200 Ci/mmol), [^{125}I]-7-OH-PIPAT (2,200 Ci/mmol), and (R,S)-7-OH-PIPAT were synthesized as previously described (Chumpradit et al. 1993; Foulon et al. 1993). In later experiments [^{125}I]-NCQ-298 (2,200 Ci/mmol) was provided by DuPont NEN.

Tissue Culture

HEK-293 cells, a human embryonic kidney cell line (Graham et al. 1977), were transfected with cDNA encoding the human D_{2L} (D_2 long isoform) and the rat D_3 receptor as previously described (Boundy et al., 1993b; Burris et al. 1994). Cells were grown in monolayer culture at 37°C in 5% CO_2 and fed with EMEM supplemented with 10% fetal bovine serum and 6418 (0.5 mg/ml). Cells were fed every fourth day and subcultured or harvested on day 7.

Preparation of Membranes

Cells were washed with ice-cold phosphate-buffered saline (138 mM NaCl, 4.1 mM KCl, 5.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 11.1 mM glucose, pH 7.4) and then incubated for 5 to 10 minutes with ice-cold 10 mM Tris buffer containing 5 mM Na_2EDTA. Cells were removed from plates, homogenized with a Brinkmann polytron, and centrifuged at 32,000/g for 20 minutes. The resulting supernatant was discarded, and the pellet resuspended by homogenization in 50 mM Tris buffer (pH 7.7 at 25°C) containing 1 mM Na_2EDTA and frozen at −70°C. On the day of an experiment membranes were thawed, incubated for 20 minutes at 37°C, and centrifuged for 20 minutes at 32,000/g. Supernatants were discarded and pellets resuspended by homogenization in 50 mM Tris buffer (pH 7.7 at 25°C).

Radioligand Binding

Binding assays were carried out as previously described (Burris et al. 1994). Crude membranes (0.5–5 µg of protein per assay tube) were incubated for 60 minutes at 37°C with [^{125}I]-7-OH-PIPAT or [^{125}I]-NCQ-298 and drugs as indicated in a total volume of 100 µl. When using [^{125}I]-7-OH-PIPAT the assay buffer consisted of 50 mM Tris (pH 7.7 at 25°C) containing 2 mM $MgCl_2$ and 0.1% BSA. For [^{125}I]-NCQ-298 the assay buffer consisted of 50 mM Tris (pH 7.7 at 25°C) containing 50 mM NaCl, 100 µM Gpp(NH)p, and 0.1% BSA. Assays were terminated by the addition of 5 ml of ice-cold wash buffer (20 mM Tris for experiments involving [^{125}I]-7-OH-PIPAT or 10 mM Tris containing 154 mM NaCl for experiments involving [^{125}I]-NCQ-298). Filtration was carried out using a Brandel cell harvester with glass-fiber filters (Schleicher and Schuell #32, previously soaked in 0.3% polyethylenimine) followed by washing with 15 ml of ice-cold wash buffer. Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

Maximum binding and K_d values were determined by Scatchard (1949) transformation of saturation binding data using unweighted linear regression analysis. Competition curves were analyzed by nonlinear regression for a one-site fit using an iterative curve-fitting program.

Accumulation of cAMP

Accumulation of cAMP was determined by a modification of the method of Jones et al. (1987) as described by Filtz et al. (1993). Cells were grown to confluency in 100-mm culture plates and incubated for 18 hour in EMEM supplemented with [^{3}H]-adenine (3 µCi/ml). Cells were rinsed twice with PBS at 37°C, then scraped from the plate and centrifuged for 5 minutes at 500/g. The resulting supernatant was discarded and the pellet resuspended in EMEM at 37°C. Cells were incubated in glass tubes for 10 minutes at 37°C in EMEM containing 1.4 mM IBMX and 13 mM HEPES, pH 7.4 (assay buffer). Drugs were added in assay buffer containing forskolin (final concentration of forskolin = 10 µM). After 7 minutes, reactions were stopped by the addition of 1 ml of 7.5% trichloroacetic acid at 4°C. [^{3}H]-cAMP was separated from [^{3}H]-ATP and [^{3}H]-ADP by sequential chromatography over Dowex and alumina columns.

RESULTS

Comparison of the Binding of [^{125}I]-7-OH-PIPAT to D_2 and D_3 Receptors

We have previously reported that [^{125}I]-7-OH-PIPAT binds with high affinity to both D_2 and D_3 receptors

(Burris et al. 1994). The binding of [^{125}I]-7-OH-PIPAT to D_2, but not to D_3, receptors is reduced in the presence of Gpp(NH)p. In the presence of $MgCl_2$ (2 mM), [^{125}I]-7-OH-PIPAT bound with a K_D value of 0.4 nM (B_{max} = 660 fmol/mg of protein) to receptors on membranes prepared from HEK-D_2 cells (Figure 1A). Gpp(NH)p decreased specific binding of [^{125}I]-7-OH-PIPAT in a concentration-dependent manner (Figure 1A, *inset*). The effect was selective for guanine nucleotides since Gpp(NH)p was at least two orders of magnitude more potent than App(NH)p. The maximum concentration of Gpp(NH)p tested (100 μM) decreased specific binding by 80% compared to control.

In membranes prepared from HEK-293 cells expressing transfected D_3 receptors (HEK-D_3 cells) [^{125}I]-7-OH-PIPAT bound with a K_D value of 0.1 nM and a density of approximately 180 fmol/mg of protein (Figure 1B). In contrast to the effect seen with cells expressing D_2 receptors, 100 μM Gpp(NH)p had no effect on the binding of [^{125}I]-7-OH-PIPAT to D_3 receptors (Figure 1B, *inset*).

Stimulation of D_2 Receptors by 7-OH-PIPAT and 7-OH-DPAT

Guanine nucleotide-sensitive binding of [^{125}I]-7-OH-PIPAT to D_2 receptors suggests that [^{125}I]-7-OH-PIPAT is an agonist at D_2 receptors. The ability of racemic 7-OH-PIPAT to stimulate D_2 receptors was examined directly in HEK-D_2 cells. Forskolin (10 μM) increased cAMP accumulation two- to fourfold over basal levels. Increasing concentrations of 7-OH-PIPAT potently (IC_{50} = 2.6 nM) inhibited the increase in cAMP accumulation stimulated by forskolin (Figure 2). Butaclamol (1 μM), a D_2 receptor antagonist, completely blocked the inhibition of cAMP accumulation by 100 nM 7-OH-PIPAT (Figure 2, *inset*).

7-OH-DPAT has been reported to inhibit release of

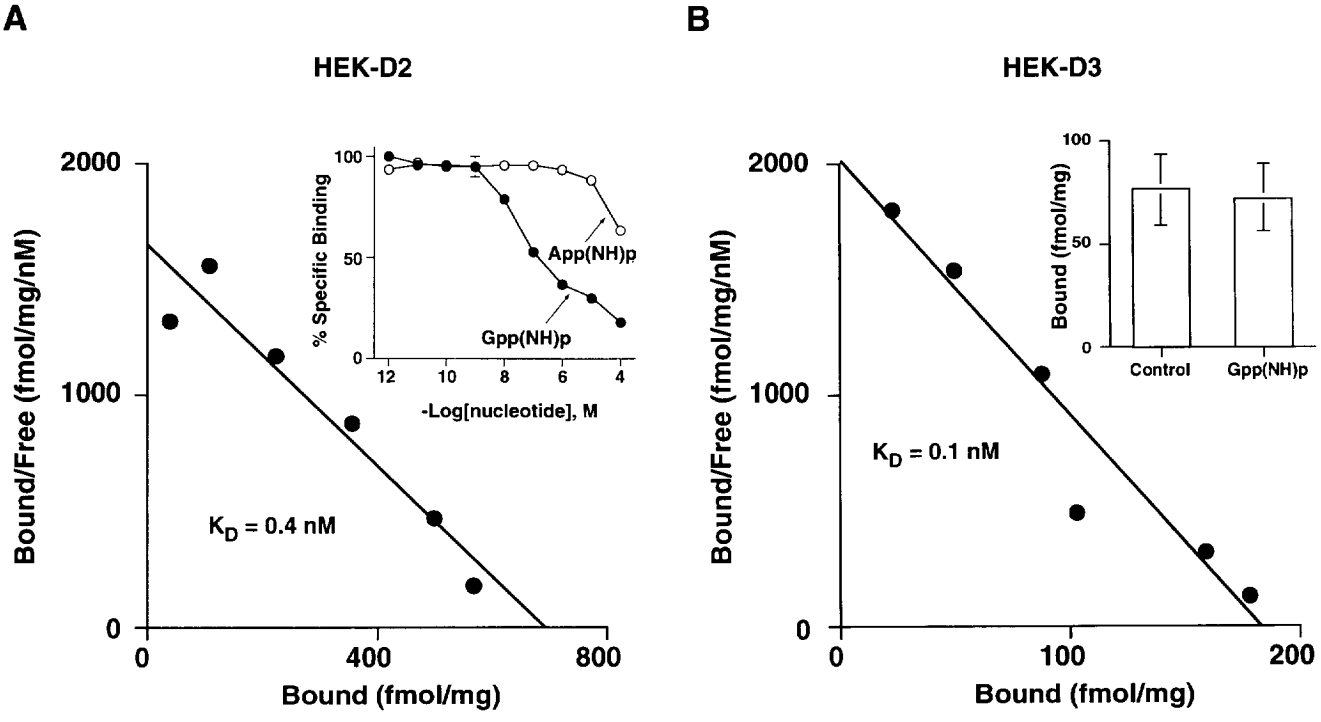

Figure 1. Binding of [^{125}I]-7-OH-PIPAT to D_2 and D_3 receptors. (**A**) Membranes prepared from HEK-D_2 cells were incubated with increasing concentrations of [^{125}I]-7-OH-PIPAT (0.03–3.17 nM) in the presence of 2 mM $MgCl_2$. Data shown are the means of specific binding for two separate tissue preparations assayed in triplicate. The inset shows the effect of Gpp(NH)p and App(NH)p on the binding of [^{125}I]-7-OH-PIPAT. Membranes were incubated with 0.54 nM [^{125}I]-7-OH-PIPAT and increasing concentrations of Gpp(NH)p or App(NH)p in the presence of 2 mM $MgCl_2$. Data shown are the means of specific binding for three separate tissue preparations assayed in triplicate. Nonspecific binding was determined with 2 μM butaclamol. (**B**) Membranes prepared from HEK-D_3 cells were incubated with increasing concentrations of [^{125}I]-7-OH-PIPAT (0.01–1.33 nM) in the presence of 2 mM $MgCl_2$. Data shown are the means of specific binding for three separate tissue preparations assayed in triplicate. The inset shows the effect of Gpp(NH)p on the binding of [^{125}I]-7-OH-PIPAT. Membranes were incubated with 0.15 nM [^{125}I]-7-OH-PIPAT in the absence or presence of 100 μM Gpp(NH)p. Data shown are the means of specific binding for three separate tissue preparations assayed in duplicate. Nonspecific binding was determined with 2 μM butaclamol.

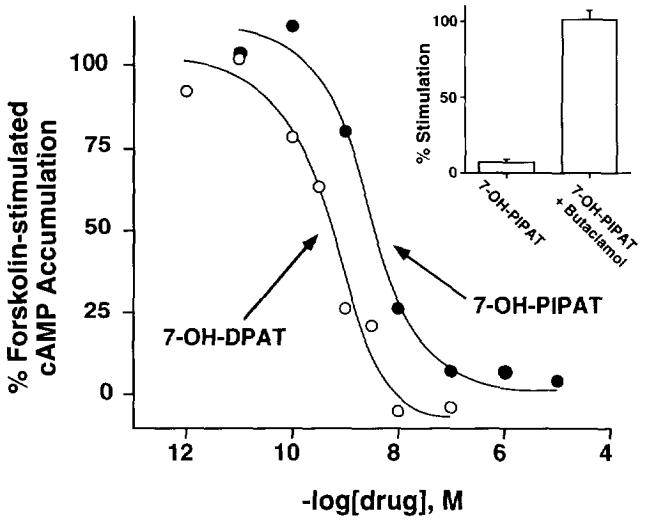

Figure 2. Inhibition of forskolin-stimulated accumulation of [^{3}H]-cAMP in HEK-D_2 cells. Cells were incubated with 10 μM forskolin and 1.4 mM IBMX in the absence or presence of increasing concentrations of 7-OH-DPAT or 7-OH-PIPAT. Data are expressed as a percentage of the stimulation of cAMP accumulation elicited by 10 μM forskolin. Accumulation of cAMP was measured as a percentage of [^{3}H]-cAMP to [^{3}H]-ATP and [^{3}H]-ADP. Data shown are the means of two experiments (7-OH-PIPAT) or three experiments (7-OH-DPAT). The inset shows the effect of 100 nM 7-OH-PIPAT alone and in the presence of 1 μM (+)butaclamol on [^{3}H]cAMP accumulation elicited by 10 μM forskolin.

dopamine in rat striatum through stimulation of D_2-like receptors (Mulder et al. 1987). Therefore, it was of interest to examine the ability of 7-OH-DPAT to inhibit forskolin-stimulated cAMP accumulation in HEK-D_2 cells. Increasing concentrations of 7-OH-DPAT potently (IC_{50} = 0.6 nM) inhibited forskolin-stimulated cAMP accumulation in HEK-D_2 cells (Figure 2). In contrast to their actions at D_2 receptors, neither 7-OH-PIPAT nor 7-OH-DPAT (at concentrations up to 100 nM) inhibited forskolin-stimulated cAMP accumulation in HEK-D_3 cells (data not shown).

Binding of Agonists and Antagonists to D_2 and D_3 Receptors

D_2 receptors exist in multiple states having low and high affinities for agonists (DeLean et al. 1982; Sibley et al. 1982; McDonald et al. 1984; George et al. 1985; Grigoriadis and Seeman 1985; Lahti et al. 1992). The affinities of D_2 receptors for agonists and antagonists were compared for receptors labeled with [^{125}I]-7-OH-PIPAT and with [^{125}I]-NCQ-298 under conditions that promote, respectively, coupling or uncoupling of receptors to G proteins. To facilitate comparison, identical assay conditions were used in studies of the properties of D_3 receptors.

When receptors were labeled with [^{125}I]-NCQ-298, D_2 and D_3 receptors displayed similar potencies for sulpiride, a D_2 receptor antagonist (Figure 3A, Table 1). When receptors were labeled with [^{125}I]-7-OH-PIPAT, the affinity of D_3 receptors for sulpiride was slightly less than the affinity of D_2 receptors for sulpiride (Figure 3B, Table 1). D_2 receptors displayed a 16-fold higher affinity for butaclamol than did D_3 receptors when receptors were labeled with [^{125}I]-NCQ-298 (Table 1). The limited selectivity of D_2 receptors for butaclamol was also seen when receptors were labeled with [^{125}I]-7-OH-PIPAT (Table 1).

D_3 receptors have been reported to have higher affinities for agonists than do D_2 receptors (Lévesque et al. 1992). When receptors were labeled with [^{125}I]-NCQ-298 in the presence of Gpp(NH)p and NaCl, D_3 receptors displayed, as expected, much higher affinities for most agonists than did D_2 receptors (Table 1). However, in the absence of Gpp(NH)p and NaCl, when

Table 1. Affinities of Agonists and Antagonists for D_2 and D_3 Receptors Labeled with [^{125}I]-NCQ-298 and [^{125}I]-7-OH-PIPAT

	[^{125}I]-NCQ-298			[^{125}I]-7-OH-PIPAT		
	K_i (*nM*)		D_2/D_3 Selectivity Index	K_i (*nM*)		D_2/D_3 Selectivity Index
	HEK-D_2	HEK-D_3		HEK-D_2	HEK-D_3	
Agonist						
7-OH-DPAT	165 ± 10 (3)	1.2 ± 0.1 (3)	138	2.6 ± 0.4 (6)	0.4 ± 0.1 (3)	7
Quinpirole	1065 ± 95 (3)	8.0 ± 0.4 (3)	133	6.4 ± 1.3 (5)	1.7 ± 0.2 (2)	4
Dopamine	1908 ± 652 (3)	44 ± 5 (3)	43	5.8 ± 0.4 (3)	5.6 ± 0.5 (3)	1
Apomorphine	168 ± 14 (3)	31 ± 3 (6)	5	4.9 ± 0.2 (4)	14 ± 2 (6)	0.4
Antagonist						
Sulpiride	12 ± 4 (3)	14 ± 1 (6)	0.9	60 ± 6 (2)	142 ± 17 (2)	0.4
Butaclamol	0.20 ± 0.03 (3)	3.2 ± 0.2 (3)	0.06	0.69 ± 0.05 (3)	5.0 ± 0.4 (3)	0.1

Competition experiments were carried out as described in Figure 3. K_i values were determined by the method of Cheng and Prusoff (1973). Data are the mean ± SEM, $n \geq 3$ experiments, or mean ± range, n = 2 experiments. The selectivity index is defined as the ratio of the K_i for D_2 compared to D_3 receptors. A high-selectivity index implies higher affinity for D_3 than for D_2 receptors.

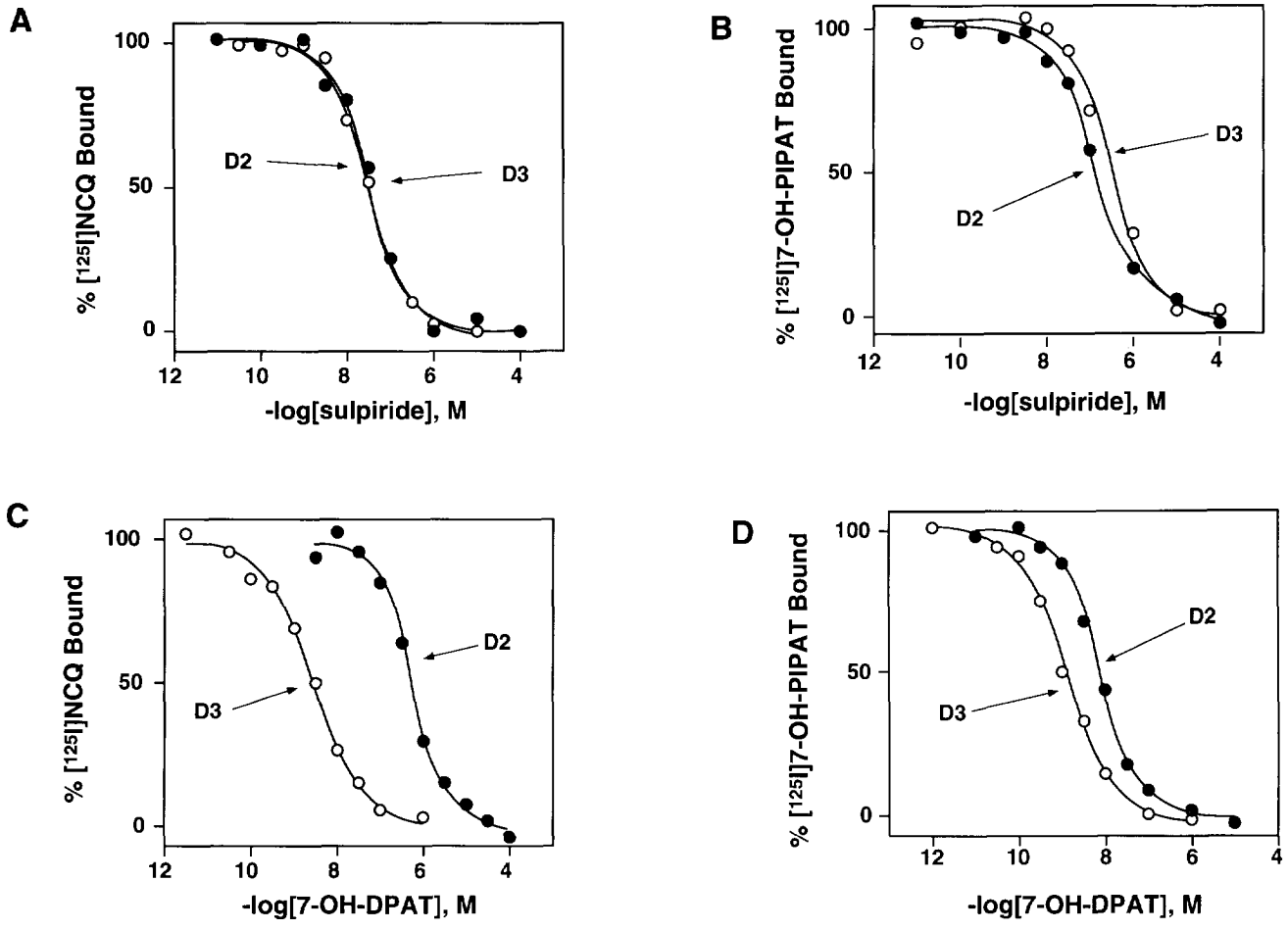

Figure 3. Inhibition of the binding of [^{125}I]-7-OH-PIPAT and [^{125}I]-NCQ-298 to D_2 and D_3 receptors expressed in HEK-293 cells. (**A**) Membranes were added to 0.15 nM [^{125}I]-NCQ-298 in the presence of 50 mM NaCl, 100 μM Gpp(NH)p, and increasing concentrations of sulpiride. Data shown are the means of specific binding for three separate tissue preparations assayed in triplicate. (**B**) Membranes were added to 0.62 nM (D_2) or 0.14 nM (D_3) [^{125}I]-7-OH-PIPAT in the presence of 2 mM $MgCl_2$ and increasing concentrations of sulpiride. Data shown are the means of specific binding for two separate tissue preparations assayed in triplicate. (**C**) Membranes were added to 0.15 nM [^{125}I]-NCQ-298 in the presence of 50 mM NaCl, 100 μM Gpp(NH)p, and increasing concentrations of 7-OH-DPAT. Data shown are the means of specific binding for three separate tissue preparations assayed in triplicate. (**D**) Membranes were added to 0.72 nM (D_2) or 0.16 nM (D_3) [^{125}I]-7-OH-PIPAT in the presence of 2 mM $MgCl_2$ and increasing concentrations of 7-OH-DPAT. Data shown are the means of specific binding for three separate tissue preparations assayed in triplicate. Nonspecific binding was determined with 2 μM butaclamol.

[^{125}I]-7-OH-PIPAT was used to label receptors, the affinities of D_3 receptors for agonists were similar to the affinities of D_2 receptors for the same agonists (Table 1). In particular, under conditions that promote uncoupling of G proteins from receptors, D_3 receptors displayed an affinity for 7-OH-DPAT nearly 140-fold higher than that of D_2 receptors (Figure 3C, Table 1). However, the affinity of D_3 receptors for 7-OH-DPAT was only slightly higher than that of D_2 receptors when the receptors were examined under conditions that favor receptor coupling to G proteins (Figure 3D, Table 1). Similarly, the affinities of D_3 receptors for quinpirole and dopamine were much higher than the affinities of D_2 receptors for the agonists in the presence of Gpp(NH)p and NaCl when [^{125}I]-NCQ-298 was used to label receptors; however, when Gpp(NH)p and NaCl were not present, and when [^{125}I]-7-OH-PIPAT was used, receptors bound quinpirole and dopamine with nearly equal affinities (Table 1).

Despite the different assay conditions, D_3 receptors labeled with [^{125}I]-7-OH-PIPAT and [^{125}I]-NCQ-298 displayed comparable affinities for agonists. In contrast, the G protein-coupled state of D_2 receptors (i.e., receptors labeled with [^{125}I]-7-OH-PIPAT) had 30- to

330-fold higher affinity for agonists than did the uncoupled state of D_2 receptors [i.e., [^{125}I]-NCQ-298-labeled receptors assayed in the presence of Gpp(NH)p]. 7-OH-DPAT, quinpirole, and dopamine displayed marked selectivity for D_3 receptors compared with D_2 receptors when Gpp(NH)p and NaCl were present in the assay and receptors were labeled with [^{125}I]-NCQ-298 (Table 1). However, this selectivity was reduced or absent when assays were carried out in the absence of Gpp(NH)p and NaCl and when receptors were labeled with [^{125}I]-7-OH-PIPAT (Table 1). Unlike the other agonists, apomorphine was only slightly D_3 receptor-selective when receptors were labeled with [^{125}I]-NCQ-298 (Table 1). However, when receptors were labeled with [^{125}I]-7-OH-PIPAT apomorphine displayed an approximately threefold selectivity for D_2 receptors (Table 1).

D_3 receptors labeled with [^{125}I]-7-OH-PIPAT bound agonists with two- to eightfold higher affinities than did receptors labeled with [^{125}I]-NCQ-298 (Table 1). Guanine nucleotides and NaCl have been shown to decrease the affinity of D_2 receptors for agonists (Grigoriadis and Seeman 1985; Neve et al. 1991). The presence of Gpp(NH)p and/or NaCl in the assay with [^{125}I]-NCQ-298 might also play a role in the decrease in the affinity of D_3 receptors for agonists. Therefore, the effects of Gpp(NH)p and NaCl on the affinity of D_3 receptors for 7-OH-DPAT were investigated. D_3 receptors bound 7-OH-DPAT with equally high affinity in the absence and presence of 100 μM Gpp(NH)p (Table 2). However, when 50 mM NaCl was added, the affinity of [^{125}I]-7-OH-PIPAT-labeled D_3 receptors for 7-OH-DPAT was reduced to a value approaching that obtained for [^{125}I]-NCQ-298-labeled receptors (Table 2). The affinity of D_3 receptors for the antagonist sulpiride also appeared to be a function of assay conditions. D_3 receptors labeled with [^{125}I]-NCQ-298 had a 10-fold higher affinity for sulpiride than did D_3 receptors labeled with [^{125}I]-7-OH-PIPAT (Table 1). D_3 receptors labeled with [^{125}I]-7-OH-PIPAT bound sulpiride with nearly equal affinity in the absence or presence of 100 μM Gpp(NH)p (Table 2). However, in the presence of 50 mM NaCl, the affinity of [^{125}I]-7-OH-PIPAT-labeled D_3 receptors for sulpiride was increased to a value similar to that seen for receptors labeled with [^{125}I]-NCQ-298 (Table 2).

DISCUSSION

Putative D_3 dopamine receptor-selective agonists have been used recently in studies of the biochemical properties and physiological roles of D_3 receptors (Caine and Koob 1993; Daly and Waddington 1993; Damsma et al. 1993; Meller et al. 1993). D_3 receptors and D_2 receptors have similar structural and pharmacological properties (Sokoloff et al. 1990). High densities of D_2 receptors exist in many of the same brain regions (e.g., nucleus accumbens and olfactory tubercle) in which D_3 receptors are found (Lévesque et al. 1992; Levant et al. 1993). Furthermore, the density of D_2 receptors is at least an order of magnitude higher than that of D_3 receptors (Boundy et al. 1993b). Therefore, agonists must have a high degree of selectivity if they are to effectively discriminate between actions at D_3 and D_2 receptors. D_3 receptors have been reported to have 100-fold higher affinity than D_2 receptors for 7-OH-DPAT based on affinity values determined by competition for binding of radiolabeled antagonists to receptors expressed in transfected cells (Lévesque et al. 1992). However, D_2 receptors exist in multiple states with varying affinities for agonists. High-affinity binding of agonists to D_2 receptors is dependent, in part, on coupling of the receptors to G proteins. In the original report in which the properties of D_2 and D_3 receptors were compared, assays were carried out under conditions (no $MgCl_2$ and 50 mM NaCl) that minimize high-affinity binding of agonists to D_2 receptors. In the present study, affinities of D_2 and D_3 receptors for 7-OH-DPAT and other agonists were determined directly by competition for the binding of a radiolabeled agonist under conditions that promote coupling of receptors to G proteins.

Table 2. Effect of Gpp(NH)p and NaCl on Binding of 7-OH-DPAT and Sulpiride to D_3 Receptors Labeled with [^{125}I]-7-OH-PIPAT or [^{125}I]-NCQ-298

	[^{125}I]-7-OH-PIPAT			[^{125}I]-NCQ-298
	Control	Gpp(NH)p	Gpp(NH)p + NaCl	Gpp(NH)p + NaCl
7-OH-DPAT	0.18 ± 0.01	0.20 ± 0.02	0.56 ± 0.11[a]	0.73 ± 0.13[b]
Sulpiride	165 ± 13	151 ± 38	19 ± 5[c]	14 ± 1[c]

K_i values were determined by the method of Cheng and Prusoff (1973) using IC_{50} values obtained in competition experiments as described in Figure 3 except that $MgCl_2$ was not included in the [^{125}I]-7-OH-PIPAT binding assay. The data are the mean ± SEM for three separate tissue preparations assayed in duplicate or triplicate.

[a] $p < .05$.

[b] $p < .025$.

[c] $p < .001$ for assay containing Gpp(NH)p and NaCl compared with control.

[^{125}I]-7-OH-PIPAT has been shown to label a guanine nucleotide-sensitive state of D_2 receptors (Burris et al. 1994 and present study). Furthermore, racemic 7-OH-PIPAT potently activated D_2 receptors coupled to the inhibition of cAMP accumulation. These data suggest that [^{125}I]-7-OH-PIPAT labeled a functional G protein-coupled state of D_2 receptors. When receptors expressed in HEK-293 cells were labeled with the antagonist [^{125}I]-NCQ-298 under conditions that inhibit interactions of receptors with G proteins, D_3 receptors displayed greater than a 100-fold higher affinity for 7-OH-DPAT than did D_2 receptors. This is consistent with the affinity of D_3 receptors for 7-OH-DPAT determined by inhibition of the binding of the antagonist [^{125}I]-iodosulpiride to receptors expressed in CHO cells (Lévesque et al. 1992). However, under conditions that favor coupling of receptors with G proteins, the affinity of D_3 receptors was only sevenfold higher than the affinity of D_2 receptors for 7-OH-DPAT. Similarly, D_3 receptors had much higher affinities than did D_2 receptors for the agonists dopamine and quinpirole under conditions that promote the uncoupled state of receptors, whereas the selectivity of D_3 receptors over D_2 receptors was diminished when the G protein-coupled state of the receptors was examined. The reported selectivity of agonists for D_3 receptors is, therefore, dependent on assay conditions.

The existence of multiple states of D_2 receptors having high and low affinities for agonists is well established. In membranes prepared from rat and bovine striatum and pituitary, which express high densities of D_2 receptors, high- and low-affinity components have been identified in studies of the inhibition of radiolabeled antagonist binding (George et al. 1985; McDonald et al. 1984; Sibley et al. 1982). Labeled agonists such as [^{3}H]-apomorphine, [^{3}H]-*N*-*n*-propyl-norapomorphine, and [^{3}H]-quinpirole have been used to directly label a guanine nucleotide-sensitive state of D_2 receptors having high affinity for agonists (Creese et al. 1979; Levant et al. 1992; Seeman and Schaus 1991; Sibley et al. 1982; Titeler and Seeman 1979). However, ^{3}H-labeled agonists often display a high degree of nonspecific binding. Furthermore, interpretation of results of studies with brain tissue are complicated by the existence of multiple D_2-like receptor subtypes. Clonal cell lines expressing transfected receptor cDNA allow the study of single subtypes of dopamine receptor. The D_2 agonist [^{3}H]-U-86170 binds to a guanine nucleotide-sensitive state of D_2 receptors transfected in CHO cells (Lahti et al. 1992). The affinities of [^{3}H]-U-86170-labeled D_2 receptors for agonists were similar to affinities determined for D_2 receptors labeled with [^{125}I]-7-OH-PIPAT, which is consistent with the finding that both radioligands label a G protein-coupled agonist-preferring state of the receptors.

The agonist-preferring high-affinity state is thought to be the functional state of D_2 receptors. In cells from the anterior pituitary, activation of D_2 receptors by agonists results in inhibition of prolactin release (George et al. 1985). This effect appears to be mediated through inhibition of adenylyl cyclase activity. The potency of agonists, as measured in studies of the inhibition of adenylyl cyclase activity and prolactin release, correlates with the affinity of the same agonists for the high-affinity state of D_2 receptors (McDonald et al. 1984; George et al. 1985). Whereas high-affinity binding of agonists to D_2 receptors is seen in membrane preparations, studies using radioligand binding assays with intact cells have not revealed the existence of D_2 receptors with a high affinity for agonists (Sibley et al. 1983). High-affinity binding of agonists to β receptors on intact cells has been seen under preequilibrium conditions (Toews et al. 1983), which is consistent with the hypothesis that agonists rapidly convert receptors from a state having high affinity for agonists to a state having low affinity for agonists (Pittman and Molinoff 1980). Nevertheless, functional studies using intact cells have demonstrated that agonists potently activate D_2 receptors (George et al. 1985; Neve et al. 1989; Lahti et al. 1992; Chio et al. 1994) suggesting that in vivo a high-affinity interaction between receptors and agonists occurs. However, the high-affinity state cannot be readily measured using radioligand binding assays in intact cells due to rapid conversion of receptors to a low-affinity state. In the present study, the potency of 7-OH-DPAT for inhibiting forskolin-stimulated accumulation of cAMP measured in intact cells was similar to the affinity of 7-OH-DPAT for D_2 receptors labeled with [^{125}I]-7-OH-PIPAT. Furthermore, we have previously determined that quinpirole and dopamine potently (IC_{50} values of 1 and 16 nM, respectively) inhibit cAMP accumulation in HEK-D_2 cells (Filtz et al. 1993). These values are similar to affinities determined in membrane preparations for the G protein-coupled state of D_2 receptors labeled with [^{125}I]-7-OH-PIPAT, but not for the state of D_2 receptors labeled with [^{125}I]-NCQ-298. This suggests that the affinities of the G protein-coupled state of D_2 receptors for agonists determined in membrane preparations reflect the affinities of receptors for the agonists in vivo.

In contrast to the wide range of affinities for agonists at G protein-coupled and uncoupled states of D_2 receptors, D_3 receptors displayed less than a 10-fold higher affinity for agonists at receptors labeled with [^{125}I]-7-OH-PIPAT than receptors labeled with [^{125}I]-NCQ-298. This small difference in affinity appears to be a consequence of the presence of NaCl in the assay with [^{125}I]-NCQ-298. Binding of [^{125}I]-7-OH-PIPAT to D_3 receptors was not decreased by adding Gpp(NH)p, suggesting that high-affinity binding of agonists to a G protein-coupled state of D_3 receptors in HEK-293 cells is insensitive to Gpp(NH)p or that D_3 receptors

bind agonists with high affinity in the absence of an interaction with G proteins. In addition, Gpp(NH)p did not decrease the affinity of D_3 receptors for 7-OH-DPAT. Similarly, D_3 receptors expressed in a variety of cell types have been reported to exist in a high-affinity state for agonists that is resistant to the effects of guanine nucleotides (Sokoloff et al. 1990, 1992; McAllister et al. 1993; Freedman et al. 1994; Tang et al. 1994b). Studies with CHO cells have in some instances revealed slight decreases in the affinities of D_3 receptors for agonists in the presence of guanine nucleotides (Sokoloff et al. 1992; Castro and Strange 1993; Chio et al. 1994; MacKenzie et al. 1994). The lack of large shifts in receptor affinity may be due to the absence of appropriate G proteins with which D_3 receptors can couple. Alternatively, it has not been determined with which signal transduction pathway D_3 receptors are coupled. Therefore, agonists at D_3 receptors have not been unambiguously defined. Chio et al. (1994) have recently reported functional effects of agonists on D_3 receptors transfected into CHO cells. Similar to D_2 receptors, D_3 receptors inhibit forskolin-stimulated cAMP accumulation, increase extracellular acidification, and stimulate mitogenesis, although the responses are considerably weaker than those seen at D_2 receptors. Furthermore, coupling of D_3 receptors to the inhibition of dopamine release and the regulation of Ca^{2+} conductance has been reported (Seabrook et al. 1994; Tang et al. 1994a).

In HEK-D_3 cells neither sensitivity to guanine nucleotides nor activation of D_3 receptors coupled to inhibition of cAMP accumulation was detected. However, binding of agonists to D_3 receptors, like that of D_2 receptors (Grigoriadis and Seeman 1985; Neve et al. 1991), was sensitive to NaCl. A decrease in the affinity of D_3 receptors for agonists upon addition of NaCl has been reported in studies with CHO cells expressing transfected D_3 receptors (MacKenzie et al. 1994). The ability of sodium to reduce the affinity of D_2 receptors for agonists is believed to involve an aspartate (Asp. 80) within the second membrane-spanning region of the receptor (Neve et al. 1991). A corresponding aspartate residue is present in the D_3 receptor, which may confer sodium sensitivity (Sokoloff et al. 1990; Schwartz et al. 1992).

With regard to antagonists, D_2 receptors displayed limited selectivity for butaclamol compared to D_3 receptors when receptors were labeled with [^{125}I]-NCQ-298. The affinities of D_3 receptors for butaclamol were similar whether receptors were labeled with [^{125}I]-NCQ-298 or [^{125}I]-7-OH-PIPAT. In contrast, the D_2 receptor antagonist sulpiride bound with 10-fold higher affinity to D_3 receptors labeled with [^{125}I]-NCQ-298 than to receptors labeled with [^{125}I]-7-OH-PIPAT. The presence of NaCl in the [^{125}I]-NCQ-298 assay, but not in the [^{125}I]-7-OH-PIPAT assay, appeared to mediate this difference in affinity. Sulpiride is a benzamide that requires sodium for high-affinity binding to D_2 receptors (Neve et al. 1991). Furthermore, the binding of [^{125}I]-iodosulpiride to D_3 receptors is increased in the presence of NaCl (Sokoloff et al. 1992). Similarly, the affinity of [^{125}I]-7-OH-PIPAT-labeled D_3 receptors for sulpiride was increased in the presence of NaCl, which is consistent with the presence of a sodium-sensitive site on the D_3 receptor.

The present results add to the emerging evidence that D_2 and D_3 receptors have similar pharmacological and biochemical properties. Nevertheless, the reports that 7-OH-DPAT and other D_2 agonists are selective for D_3 receptors have stimulated the use of these compounds in laboratory animals to identify functional responses mediated by D_3 receptors (Caine and Koob 1993; Daly and Waddington 1993; Damsma et al. 1993). For example, it was recently reported that 7-OH-DPAT, quinpirole, and apomorphine were effective in reducing cocaine self-administration in rats (Caine and Koob 1993). 7-OH-DPAT and quinpirole were more potent than apomorphine, a pattern suggesting the involvement of D_3 receptors rather than D_2 receptors in the reinforcing effects of cocaine. Central to the conclusion that these agonists elicit their effects on cocaine self-administration through interaction at D_3 receptors were reports that D_3 receptors have a high affinity for all three drugs, whereas D_2 receptors have a high affinity for apomorphine, but a low affinity for 7-OH-DPAT and quinpirole. However, as seen in the present study, D_2 receptors have either high or low affinities for 7-OH-DPAT, quinpirole, and apomorphine, depending on assay conditions. The G protein-coupled state of D_2 receptors had a high affinity for all three drugs. Furthermore, all three drugs are potent agonists at D_2 receptors (Chio et al. 1994; Lahti et al. 1992; Neve et al. 1989; and present results). In HEK-D_2 cells the potency of 7-OH-DPAT for activating D_2 receptors and the affinity of the G protein-coupled state of the D_2 receptor for 7-OH-DPAT were similar to the affinity of D_3 receptors for 7-OH-DPAT. Similar potencies of 7-OH-DPAT for activating D_2 and D_3 receptors have recently been reported (Chio et al. 1994; Liu et al. 1993; Potenza et al. 1994). These results suggest that 7-OH-DPAT and other agonists do not have sufficient selectivity to distinguish the subtype of receptor through which behavioral effects of agonists such as 7-OH-DPAT are mediated. Therefore, the elucidation of functional responses mediated by activation of D_3 receptors may require the development of subtype-selective dopamine receptor antagonists.

In summary, [^{125}I]-7-OH-PIPAT has been shown to interact with a functional G protein-coupled state of D_2 receptors that has a high affinity for agonists. Comparison of the affinities of D_2 and D_3 receptors for agonists defines the range of selectivity obtained with currently available agonists. When the agonist-labeled

G protein-coupled state of D_2 receptors is considered, D_3 receptor selectivity ascribed to agonists is diminished. The use of drugs to determine behavioral effects mediated by activation of D_3 receptors will require identifying compounds that exhibit a high degree of selectivity under a broad range of conditions.

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