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The preferential dopamine D_3 receptor agonist *cis*-8-OH-PBZI induces limbic *Fos* expression in rat brain

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Abstract

The affinity, selectivity and agonistic properties of a constrained dopaminergic compound, the benz[e]indole *cis*-8-hydroxy-3-(*n*-propyl)1,2,3a,4,5,9b-hexahydro-1H-benz[e]indole (*cis*-8-OH-PBZI), for the dopamine D_3 receptor were evaluated in competition binding experiments with cloned human dopamine receptor subtypes and, to further extend its profile, in in vitro radioligand binding assays. The K_i value measured for competition binding of this compound to the dopamine D_3 receptor was 27.4 ± 3.1 nM; this was 775-fold, 550-fold, 90-fold and 10-fold higher affinity than that measured at dopamine D_{1A}, D_5, D_{2s} and D_4 receptors, respectively. Evidence of dopamine receptor activation by *cis*-8-OH-PBZI was obtained by measuring dose-dependent increases in extracellular acidification rates and decreases in cAMP synthesis. In vivo, *cis*-8-OH-PBZI potently induced *Fos* protein immunoreactivity in the rat medial prefrontal cortex and shell region of the nucleus accumbens, but only marginally in the motor dorsolateral striatum, indicating a selective limbic site of action. In conclusion, the present data identify *cis*-8-OH-PBZI as having preference for the dopamine D_3 receptor in vitro, and as having dopamine agonist activity and limbic sites of action in vivo. © 1997 Elsevier Science B.V.

Keywords: Dopamine D_3 receptor; Receptor binding; G protein; Acidification rate; cAMP accumulation; *Fos* protein immunoreactivity

1. Introduction

The molecular cloning in recent years of a number of human dopamine receptors has suggested the possibility of designing ligands which are selective for receptor subtypes localized in limbic brain regions thought to be involved in schizophrenic symptomatology. A further goal has been to focus effort on those receptor subtypes that are not expressed in motor areas involved in the extrapyramidal side effects common to many neuroleptic drugs currently in use. Taken together, this pattern of receptor localization should be in agreement with an anatomical profile of activity considered desirable for therapeutic intervention (evidence recently reviewed by Andreasen (1996)).

At present, the known subtypes of the dopamine D_2 receptor family include short and long forms of the human dopamine D_2 receptor (Bunzow et al., 1988; Dal Toso et al., 1989; Grandy et al., 1989; Monsma et al., 1989) and dopamine D_3 receptor (Giros et al., 1990; Sokoloff et al., 1990; Fishburn et al., 1993) and variable-length forms of the dopamine D_4 receptor (Van Tol et al., 1992). Human subtypes of the dopamine D_1 family now include the dopamine D_{1A} (Dearry et al., 1990; Sunahara et al., 1990; Zhou et al., 1990) and dopamine D_5 (Sunahara et al., 1991) receptors. Localization evidence published in these studies indicates that the human dopamine D_3 receptor subtype is preferentially localized in limbic areas.

A number of dopaminergic ligands have now been characterized which possess differing degrees of selectivity towards the dopamine D_3 receptor. This effort has yielded a better understanding of cellular functions likely to be mediated by this receptor subtype. The dopamine D_3 receptor has been noted as having high affinity for dopamine and other agonists (Robinson et al., 1994), and most ligands which are selective for this receptor have been

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found to possess agonist activity. Agonists such as dopamine, quinpirole, 7-hydroxy-2-(di-*n*-propylamino)tetralin (7-OH-DPAT) and PD 128,907 each increase mitogenesis in NG108-15 cells expressing the human dopamine D_3 receptor (Griffon et al., 1995; Sautel et al., 1995), an effect which is blocked by the preferential dopamine D_3 antagonist nafadotride. The ability of the dopamine agonists to mediate changes in transcriptional activation in NG108-15 cells via the dopamine D_3 receptor, measured as changes in *c-fos* expression, has also been described (Pilon et al., 1994).

As a means of further identifying ligands selective for dopamine receptor subtypes we screened compound libraries, using in vitro assays selective for each of the known dopamine receptors. In pursuing this strategy we identified the novel benz[e]indole *cis*-8-OH-PBZI, a compound previously characterized as having only weak affinity in in vitro tests of dopamine D_1 and D_2 receptor binding (Cruse et al., 1993), as having high selectivity for the dopamine D_3 receptor. We now provide a detailed description of the receptor binding and signal transduction capabilities of *cis*-8-OH-PBZI. These results demonstrate its dopamine D_3 receptor selectivity when compared to that of other ligands with preference for this receptor and dopamine agonist activity. In addition, measurements of *Fos* protein immunoreactivity were used to assess the activity of this compound in different brain regions. These data further allowed us to establish an anatomical pattern of activity for *cis*-8-OH-PBZI in concordance with that expected for a compound having a limbic site of action.

2. Materials and methods

2.1. Drugs, reagents

The hydrochloride salt of *cis*-8-hydroxy-3-(*n*-propyl)1,2,3a,4,5,9b-hexahydro-1H-benz[e]indole (*cis*-8-OH-PBZI) was synthesized as previously described by Cruse et al. (1993). The synthesis of 7-OH-DPAT was performed as previously described by Wikström et al. (1985). Haloperidol and (−)-apomorphine were purchased from Sigma Chemicals (St. Louis, MO). Haloperidol was subsequently converted to a hydrochloride salt at Novo Nordisk. Dopamine, (−)-quinpirole, PD 128,907, SCH-23390, (+)-butaclamol and (−)-raclopride were purchased from Research Biochemicals (Natick, MA). The adenylyl cyclase activating agent 7-β-deacetyl-7-β-(γ-*N*-methylpiperazino)-butyryl-forskolin was purchased from Calbiochem–Novabiochem (La Jolla, CA). Clozapine was a gift of Sandoz (Basel). *Cis*-flupenthixol was a gift of H. Lundbeck (Denmark). The radioligands [^{3}H]SCH-23390 (81.4 Ci/mmol) and [^{3}H]Spiperone (15 Ci/mmol) were purchased from Dupont NEN Research Products (Boston, MA). Both [^{3}H]*R*(+)-7-OH-DPAT (138 Ci/mmol) and [^{125}I]cAMP (2000 Ci/mmol) were purchased from Amersham (Buckinghamshire). Rabbit anti-*Fos* antiserum was purchased from Oncogene Science (Uniondale, NY). Biotinylated swine anti-rabbit IgG was purchased from DAKO (Dacopatts). Steptavidin-biotinylated horseradish-peroxidase was purchased from Vector ImmunoChemicals (Burlingame, CA).

2.2. Dopamine receptor-expressing cell lines

The human dopamine D_3 receptor was recloned by reverse transcriptase-polymerase chain reaction (PCR) and stably expressed as follows: total RNA was isolated from the human embryonal kidney cell line HEK-293 (ATCC#CRL1573) by the acid–guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987) [1]. First strand cDNA was prepared by reverse transcription of 10 μg of total RNA, using an oligo-dT primer and SuperScript RNaseH reverse transcriptase (GIBCO-BRL). PCR was then performed for 40 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, using AmpliTaq DNA polymerase (Perkin Elmer–Cetus), first strand cDNA (prepared from 2.5 μg total RNA) as template and the oligonucleotides 5′-ATGGCATCTCT-GAGTCAGCTGAGTAGC and 5′-TCAGCAAGACAG-GATCTTGAGGAAGGC (Giros et al., 1990) as sequence-specific primers. Restriction sites were added to the primary PCR product by using the oligonucleotides 5′-CAAGGTACCTGGACCATGGCATCTCTGAGT and 5′-GAGTGTTCCCTCTAGAGCTCCCTCAGCAAG as primers in a subsequent PCR reaction. The resulting 1.2-kb PCR product was digested with *Asp*718I and *Xba*I restriction endonucleases and inserted into the cloning vector pBlueScript IISK + (Stratagene) for sequence verification. The resulting full length human dopamine D_3 receptor cDNA was further subcloned into the mammalian expression vector Zem219B. The baby hamster kidney cell line BHK21tk$^-$ (ATCC#CRL1632) was then transfected with 10 μg of this expression plasmid, using the lipofectin method (GIBCO-BRL). Subclones resistant to 1 μM methotrexate were isolated and receptor expression was determined by radioligand binding.

The human dopamine D_{1A} receptor was stably expressed in BHK21tk$^-$ cells and the human dopamine D_5 receptor was stably expressed in CHO cells, as previously described by Pedersen et al. (1994). The human dopamine D_{2s} receptor was stably expressed in Ltk$^-$ cells, as described by Bunzow et al. (1988). Cell membranes prepared from CHO cells expressing the $D_{4.2}$ variant of the human dopamine D_4 receptor (described by Van Tol et al. (1992)) were purchased from Receptor Biology (Baltimore, MD). Cell lines were propagated in Dulbecco's modified Eagle's media (DMEM) supplemented with fetal calf serum (10%, v/v), penicillin/streptomycin (20 μg/ml) and either 0.5 mg/ml of G-418 (dopamine D_{1A}, D_{2s}) or 5 μM

[1] The possible presence of the dopamine D_3 receptor in kidney was previously noted by Sokoloff et al. (1990).

methotrexate (dopamine D_5, D_3). The cell membranes used in radioligand binding experiments were prepared from clonal cell lines expressing individual receptor subtypes by hypotonic lysis, essentially as described previously by Scheideler and Zukin (1990). Membrane protein concentrations were estimated by using a modified Lowry method (Peterson, 1977).

2.3. Specific radioligand binding to dopamine receptor subtypes

Cell membranes (3–40 μg) were resuspended in assay buffer (20 mM Hepes, pH 7.4, containing 2 mM $MgCl_2$) and incubated with 0.5 nM [^{3}H]*R*(+)-7-OH-DPAT (dopamine D_3), 0.1 nM [^{3}H]SCH 23390 (dopamine D_{1A}, D_5) or 0.1 nM [^{3}H]Spiperone (dopamine D_{2s}, D_4) for 45 min at 25°C. Free and bound ligand were separated by rapid filtration through Whatman GF/B filters, the filters were then washed with 8 ml of assay buffer containing 100 mM NaCl and the filter-bound radioactivity was determined by liquid scintillation counting. Non-specific binding was assessed in the presence of 5 μM of (−)-quinpirole (dopamine D_3), 2 μM *cis*-flupenthixol (dopamine D_{1A}, D_5) or 3 μM (+)-butaclamol (dopamine D_{2s}, D_4).

2.4. In vitro selectivity for receptor, ion channel and drug uptake sites

Various radioligand binding assays specific for neurotransmitter receptors, ion channels and uptake sites were used to further evaluate the selectivity of *cis*-8-OH-PBZI for the dopamine D_3 receptor. These assays included competitive ligand binding to adrenoceptors (α_1, α_2, β_1, β_2), acetylcholine (M_1), adenosine (A_1, A_2), serotonin (5-HT_{1A}, 5-HT_2, 5-HT_3), histamine (H_1, H_2, H_3) and opiate (μ, κ) receptors, glycine (A, B), glutamate (kainate, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA)) and gamma-aminobutyric acid (GABA) ligand-gated channel sites, voltage-gated sodium and calcium channel sites and uptake sites for GABA, dopamine, norepinephrine and serotonin. The assay conditions and radioligands used have previously been described by Andersen (1989) and Suzdak et al. (1992).

2.5. Measurement of extracellular acidification rates

The ability of dopamine and *cis*-8-OH-PBZI to alter the basal acidification rate of BHK21tk$^-$ cells expressing the dopamine D_3 receptor was analyzed using a Cytostar microphysiometer (Molecular Devices, Menlo Park, CA). The instrument uses a silicon sensor to detect small changes in pH in the media bathing cells that have been placed in a microvolume sensor chamber (described by McConnell et al. (1992)). Cells were seeded into cytosensor microphysiometer cups (2×10^5 cells/cup) 18–24 h before the experiments were started. Flow chambers containing the cells were perfused at a rate of 100 μl/min with a running buffer composed of bicarbonate-free DMEM (pH 7.4) containing 44 mM NaCl. A 2-min pump cycle was used throughout each experiment, as follows: 80 s at a flow rate of 100 μl/min, and 40 s with the flow stopped. The extracellular pH was measured for 30 s during each cycle, while the flow was stopped. Test compounds were diluted into the running buffer and perfused through a second fluid path. The second fluid path was opened for 180 s for two pump cycles in order to perfuse the cell cups with test compound. A 25-min recovery period was allowed between drug exposures to allow for the stabilization of acidification rates.

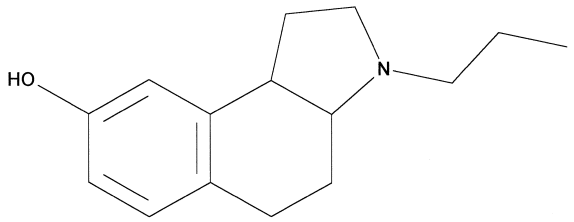


Fig. 1. Structure of *cis*-8-OH-PBZI (*cis*-1,2,3a,4,5,9b-hexahydro-1H-benz[e]indole).

2.6. Determination of cellular cAMP synthesis

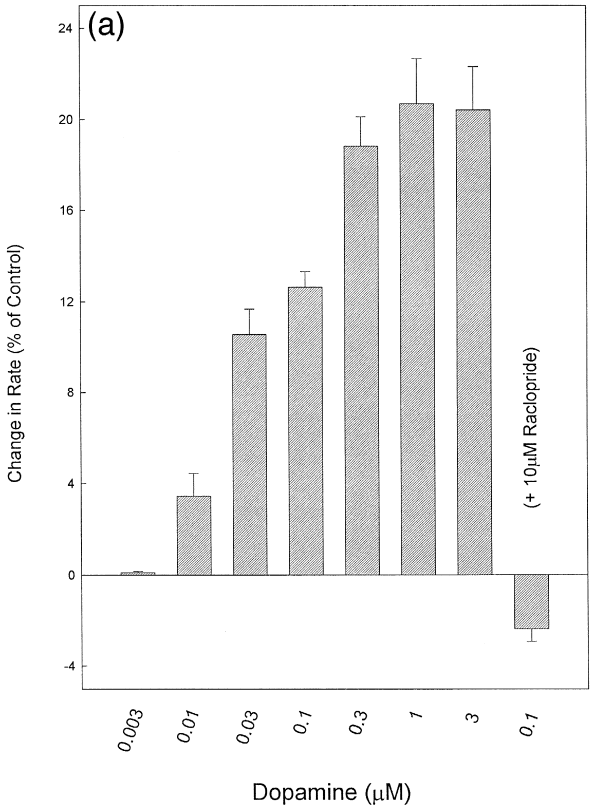
Ltk$^-$ cells expressing the dopamine D_{2s} receptor were grown to confluency on 60-mm plates and treated for 45 min with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) before experiments were started. IBMX was first dissolved in a minimum volume of 1 N NaOH, further diluted in DMEM to a concentration of 5 mM and adjusted to pH 7.2 prior to use. To start experiments test drugs (serially diluted in dH_2O) were added to cell cultures to yield the indicated concentrations. After 10 min 7β-deacetyl-7-β-(γ-*N*-methylpiperazino)–butyryl–forskolin, dissolved in

Table 1
K_i values (nM) for the displacement of radioligand binding to human dopamine D_{2s}, D_3 and D_4 receptor subtypes

	D_{2s}	D_3	D_4
cis-8-OH-PBZI	1770 ± 760	27 ± 8.2	276 ± 80
(−)-Quinpirole	80 ± 42	1.2 ± 0.8	14 ± 11
PD 128,907	202 ± 123	0.4 ± 0.3	114 ± 23
Dopamine	6.2 ± 0.3	1.8 ± 1.0	18 ± 3.5
(−)-Apomorphine	41 ± 7.1	9.5 ± 6.4	6.5 ± 0.7
Clozapine	50 ± 5.7	110 ± 2.1	11 ± 2.8
(−)-Raclopride	7.9 ± 7.3	19 ± 2.1	2100 ± 181

K_i values were calculated from the Cheng–Prusoff equation (Cheng and Prusoff, 1973), using IC_{50} values obtained from competitive displacement studies in which at least 5 different ligand concentrations were tested. The results shown represent the mean S.D. of 2–8 separate experiments. The K_d values used in these calculations were established in saturation binding experiments performed for each dopamine receptor subtype.

dH_2O, was added to yield a final concentration of 150 μM. After a further 15 min the media was aspirated, the monolayer of cells was washed once with phosphate-buffered saline (PBS), pH 7.2, and the cells were removed from the plates in 3 ml of 60% ethanol (v/v). After sonication, the cell membranes were pelleted in a microcentrifuge and the supernatants were dried under nitrogen. The cAMP content of each sample was estimated by using the Biotrak cAMP scintillation proximity assay system (Amersham, Buckinghamshire). Antibody-bound radioactivity was determined using a Packard TopCount β-Scintillation Counter. Results were normalized to the protein content of each plate of cells; this was estimated by a modified Lowry method (Peterson, 1977) after each cell membrane pellet was dissolved in 0.1 N NaOH by heating at 70°C.



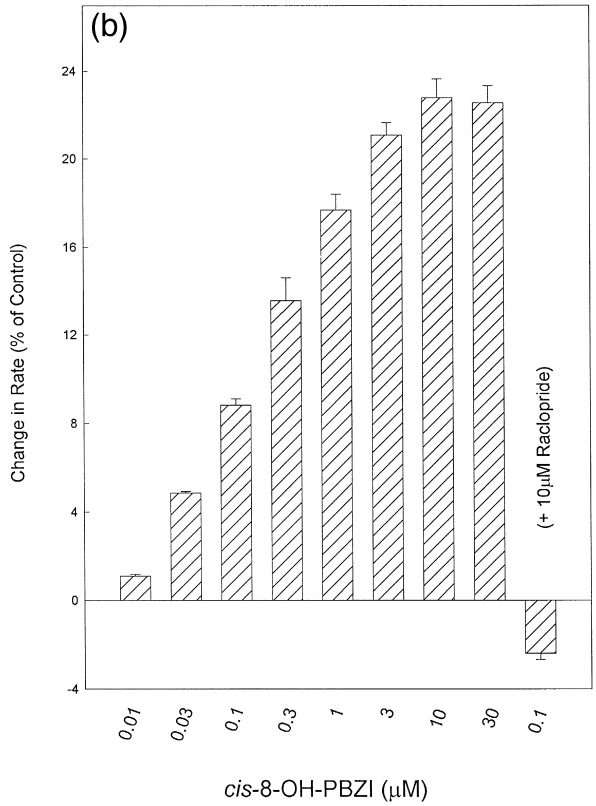


Fig. 2. Dopamine D_3 receptor-mediated changes in extracellular acidification rates. The dose-dependent effects of dopamine (A) and *cis*-8-OH-PBZI (B) on D_3-BHK21tk$^-$ cell acidification rates are shown. The final lane in each bar graph shows results obtained following the addition to the cells of both agonist and the dopamine receptor antagonist raclopride. The data shown represent peak acidification rates. These values were calculated as the change in basal rates measured in the absence of drug, using the highest rate measured after drug perfusion. The results shown represent the mean ± S.E.M. of 4 determinations, and are representative of 3 independent experiments.

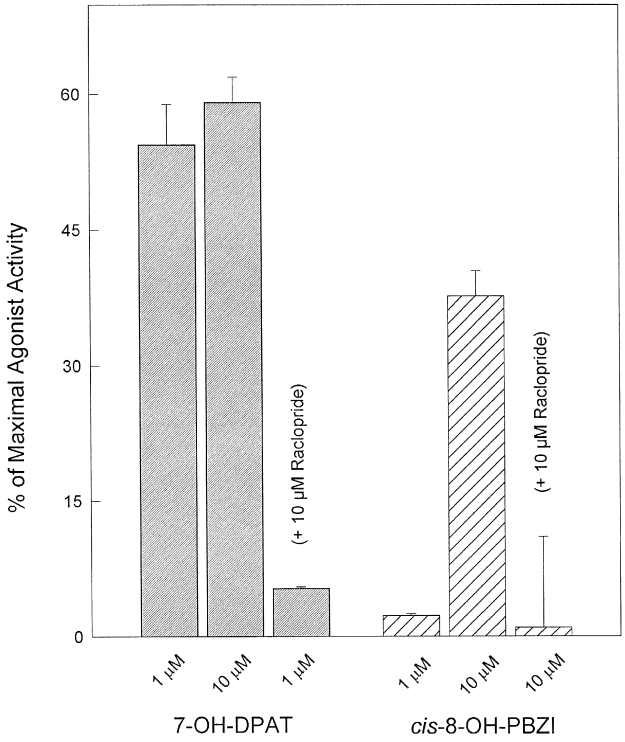


Fig. 3. Dopamine D_{2s} receptor-mediated regulation of cAMP synthesis. The ability of 7-OH-DPAT or *cis*-8-OH-PBZI to block forskolin-stimulated cAMP synthesis in D_{2s}-Ltk$^-$ cells is shown as a percentage of the maximal inhibition measured in the presence of 10 μM dopamine. Each drug was also tested in the presence of the dopamine receptor antagonist (−)-raclopride, as indicated. The results shown are for a representative experiment from 3 independent studies as the mean ± S.E.M. of triplicate determinations.

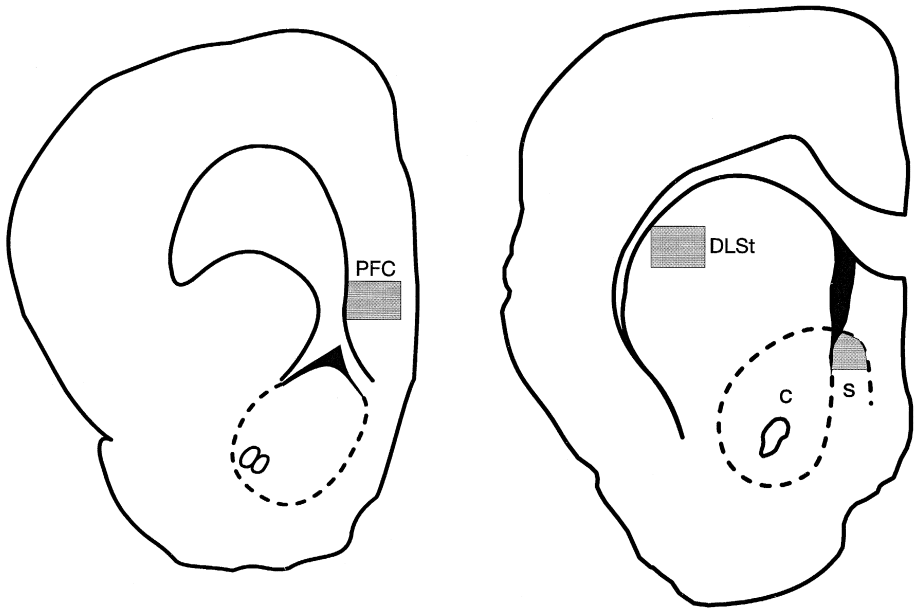


Fig. 4. *Fos* counting areas in rat brain. Camera lucida drawings of coronal rat brain sections depict the areas used for counting *Fos*-positive nuclei (in gray shaded boxes). The counting areas shown are located in the dorsolateral striatum (DLSt), medial prefrontal cortex (PFC) and shell region of the nucleus accumbens (S). Brain sections used in studies of the medial prefrontal cortex were located 2.7 mm anterior to the Bregma. Sections used in studies of the dorsolateral striatum and shell region of the nucleus accumbens were located 1.6 mm anterior to the Bregma.

2.7. Animals / in vivo drug administration

Male Sprague–Dawley rats weighing 250 ± 20 g were used (Moellegaards Breeding Labs, Skensved). The animals were housed in temperature-controlled rooms (20–22°C) with a 12-h light/dark cycle (light from 06.00–18.00) and access to food and water ad libitum. The experimental protocols used were in accordance with the internationally accepted principles for the care and use of laboratory animals and have been approved by the Danish Committee for Animal Research. The *cis*-8-OH-PBZI used in in vivo studies was dissolved in physiologic saline, adjusted to a pH between 6.5 and 7.0 with 1 N NaOH. (−)-Apomorphine was dissolved in distilled water containing 0.2 mg/ml ascorbic acid. All drug solutions used in these studies were prepared immediately before use.

2.8. Determination of Fos protein immunoreactivity in rat brain

Animals (five rats per group) were injected subcutaneously (s.c.) with saline, (−)-apomorphine (0.3, 3.0 mg/kg) or *cis*-8-OH-PBZI (0.1, 1.0, 10.0 mg/kg). Two hours later [2] the rats were anesthetized, perfused transcardially (essentially as described by Kjær et al. (1994)) and killed. Their brains were then rapidly removed, fixed for 90 min, and cryprotected in PBS containing 30% sucrose for two days. They were then frozen and cut on a cryostat (Leica, Jung CM3000) at −23°C in 40 μm thick sections. These sections were then divided into 5 fractions and added to vials containing PBS. Two of these fractions were randomly chosen for assessment of background immunoreactivity. The remaining fractions were used to determine *Fos* protein immunoreactivity on free-floating sections by a modification of the avidin–biotin complex method described by Larsen (1992) and Kjær et al. (1994). Briefly, after endogenous peroxidase activity was blocked, sections were incubated in PBS containing non-immune 5% (v/v) swine serum, 1% (w/v) bovine serum albumin and 0.3% (w/v) Triton X-100 (1×20 min) and then incubated overnight at 4°C in rabbit anti-*Fos* antiserum (diluted 1:200). Binding of the primary antibody was visualized with biotinylated swine anti-rabbit IgG (diluted 1:400, v/v) and steptavidin-biotinylated horseradish-peroxidase (diluted 1:500, v/v). The peroxidase activity was visualized with 0.025% (w/v) 3,3-diaminobenzidine, tetra-hydrochloride, and $NiSO_4$ was used to enhance the signal. The sections were then mounted on chrom–alum–gelatine-coated slides, dried for 48 h, degreased for 4 h in a chloroform–ethanol bath, and after rehydration counterstained with thionin to visualize the Nissl substance. Primary or secondary antisera, or the tertiary complex, were omitted from control sections. Counting areas were identified and *Fos*-immunoreactive cells were counted as earlier described by Fink-Jensen et al. (1995). The results from drug-treated and control animals were compared by use of a one-way analysis of variance followed by Student–Newman–Keuls or Dunnett's posthoc test.

[2] Pretreatment interval established in experiments where 0.1 mg/kg of *cis*-8-OH-PBZI was administered (s.c.) for 0.5, 1, 2 and 4 h.

3. Results

3.1. Interaction with dopamine receptors subtypes

The direct interaction of *cis*-8-OH-PBZI (structure shown in Fig. 1) with the human dopamine D_{1A}, D_5, D_{2s}, D_3 and D_4 receptor binding sites was studied in detail by radioligand binding, using membrane preparations from clonal cells expressing each receptor. These results were then compared with receptor binding values obtained for selected dopaminergic compounds with varying selectivities for these receptor subtypes.

The data from the competition binding studies revealed that *cis*-8-OH-PBZI has high affinity and selectivity for the dopamine D_3 receptor in these in vitro assays (Table 1). Within the dopamine D_2 receptor family, *cis*-8-OH-PBZI exhibited 90-fold selectivity for the dopamine D_3 receptor when its binding was compared with that measured at the dopamine D_{2s} receptor, and 10-fold selectivity for dopamine D_3 versus D_4 receptor binding. Data obtained in these assays for the dopamine agonists (−)-quinpirole and PD 128,907 also revealed a dopamine D_3 receptor selectivity, as expected. However, both *cis*-8-OH-PBZI and (−)-quinpirole displayed a lower selectivity for dopamine D_3 and D_4 receptor binding than was seen for PD 128,907. Both dopamine and the agonist (−)-apomorphine proved to be non-selective compounds which interacted with high affinity at each of the receptor subtypes. Thus, the observed selectivity of the compounds for the dopamine D_3 receptor in these assays was not attributable to the preferential binding of agonists at the dopamine D_3 receptor. The dopamine receptor antagonist clozapine had a modest preference for the dopamine D_4 receptor, as expected, whereas (−)-raclopride exhibited preferential binding to the dopamine D_{2s} and D_3 receptors.

3.2. Regulation of receptor-mediated signaling

Changes in extracellular acidification rates were measured in BHK21th$^-$ cells expressing the dopamine D_3 receptor as a means of determining the potential agonist activity of *cis*-8-OH-PBZI, since earlier experiments had shown that dopamine was unable to regulate cAMP synthesis in these cells. Both dopamine and *cis*-8-OH-PBZI dose dependently increased the extracellular acidification rate recorded for these cells (Fig. 2), showing that both compounds have agonist activity at the dopamine D_3 receptor. EC_{50} values [3] measured in these experiments for dopamine were 24.3 ± 5.6 nM and 117.3 ± 4.4 nM for *cis*-8-OH-PBZI. Further, the peak signal amplitude elicited by 3 μM *cis*-8-OH-PBZI matched that determined for a maximally effective (1 μM) concentration of dopamine, showing that *cis*-8-OH-PBZI is also a full agonist at the dopamine D_3 receptor. Significantly increased extracellular acidification rates were already observed after the cells were treated with low drug concentrations which approximated the K_i values measured in in vitro receptor binding assays (binding data summarized in Table 1). Addition of dopamine to a final concentration of 3 nM produced a signaling response which was 9.2% that of the maximum measured rate (Fig. 2A), whereas the response elicited by 30 nM *cis*-8-OH-PBZI was 20.7% of this maximum rate (Fig. 2B). Cotreatment of the cells with 10 μM of the antagonist (−)-raclopride blocked the signal generated by either *cis*-8-OH-PBZI or dopamine. A transient decrease in the basal acidification rate was consistently observed following the addition of both test compound and (−)-

[3] Mean ± the standard error of the mean for 3 independent experiments.

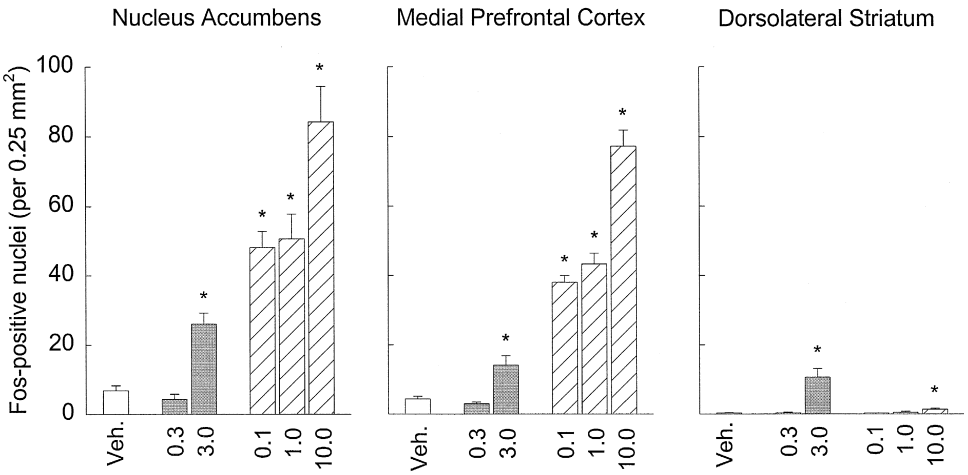


Fig. 5. In vivo induction of *Fos* protein immunoreactivity. The number of *Fos*-positive nuclei were counted following the administration of vehicle (open bars), (−)-apomorphine (gray bars) or *cis*-8-OH-PBZI (hatched bars) to the following brain areas: (A) nucleus accumbens; (B) medial prefrontal cortex; (C) dorsolateral striatum. Results are presented as the average number of *Fos*-immunoreactive nuclei in 4–6 counting areas (mean ± S.E.M.). The number of *Fos*-positive cells is expressed per 0.25 mm^2. A star shown above a bar indicates that the data are significantly different statistically from those shown for vehicle.

raclopride. These decreases did not appear to be due to the antagonism of a constitutive receptor-mediated activity, as treatment of the cells with (−)-raclopride alone did not alter extracellular acidification rates. As expected, exposure to either dopamine or *cis*-8-OH-PBZI failed to elicit a signal in BHK21tk$^-$ cells, which do not express the dopamine D_3 receptor.

Dopamine strongly inhibited cAMP synthesis in Ltk$^-$ cells expressing the dopamine D_{2s} receptor, which made it possible for us to use this assay in order to see if cis-8-OH-PBZI possessed significant dopamine agonist activity at other receptors of the dopamine D_2 family Fig. 3. As shown in Fig. 4, 1 μM 7-OH-DPAT effectively blocked cAMP synthesis in these cells. By comparison, 1 μM *cis*-8-OH-PBZI was ineffective whereas a concentration of 10 μM was 38% as effective as 10 μM dopamine in reducing cAMP synthesis. The dopamine D_2 receptor antagonist (−)-raclopride blocked the agonist effect of each compound tested.

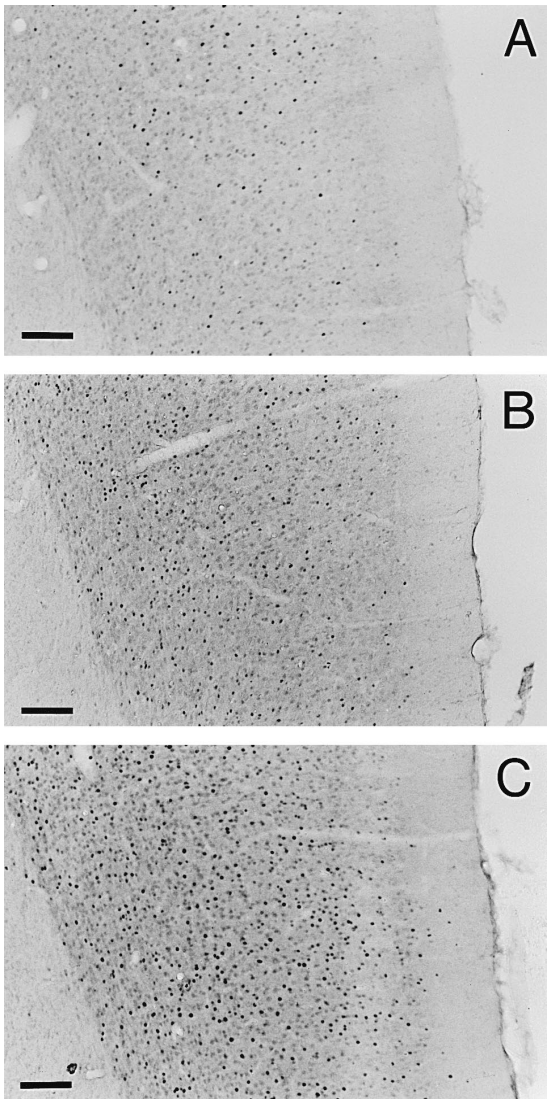


Fig. 6. Staining of *Fos* positive nuclei in the prefrontal cortex. Effects of saline (A), 3 mg/kg (−)-apomorphine (B) and 10 mg/kg *cis*-8-OH-PBZI (C) on immunohistochemical labeling of *Fos* protein in the rat medial prefrontal cortex 2 h following subcutaneous administration.

3.3. In vitro receptor binding profile

An extended characterization of *cis*-8-OH-PBZI was undertaken in order to investigate its in vitro selectivity for the dopamine D_3 receptor. Within the dopamine D_1 receptor family *cis*-8-OH-PBZI had negligible affinity for the dopamine D_{1A} and D_5 subtypes (775 and 550-fold lower affinity compared to dopamine D_3 receptor binding, respectively). Further investigation of *cis*-8-OH-PBZI in radioligand binding assays selective for neurotransmitter receptors, ion channels and uptake sites showed that the compound was inactive at most sites ($IC_{50} > 5$ μM). *Cis*-8-OH-PBZI had a weak interaction with muscarinic M_1 and serotonin 5-HT_{1A} receptors, α_2-adrenoceptors and serotonin uptake sites. However, the IC_{50} values measured in these assays were above 1 μM.

3.4. In vivo Fos protein immunoreactivity

In order to establish whether *cis*-8-OH-PBZI acts selectively in limbic regions of rat brain, we measured the expression of *Fos* protein in the shell region of the nucleus accumbens, medial prefrontal cortex and dorsolateral striatum. A camera lucida drawing of the sampling areas used for counting *Fos*-positive nuclei is shown in Fig. 4. The results of counting *Fos*-positive cell nuclei are summarized in Fig. 5. *Cis*-8-OH-PBZI induced a strong and selective *Fos* protein immunoreactivity response in both the shell region of the nucleus accumbens ($P < 0.001$) and in the medial prefrontal cortex ($P < 0.001$) (Fig. 5A, B). A weak increase was also observed in the dorsolateral striatum, as shown in Fig. 5C. However, this response corresponded to only 1.37 positive cells per 0.25 mm^2 at the highest dose tested (10 mg/kg) versus 0.25 positive cells per 0.25 mm^2 in the vehicle group ($P = 0.003$). The highest dose of (−)-apomorphine tested (3 mg/kg) increased *Fos* immunoreactivity in the shell region of the nucleus accumbens ($P < 0.001$), medial prefrontal cortex ($P = 0.002$) and dorsolateral striatum ($P < 0.001$). However, the (−)-apomorphine response in both the shell region of the nucleus accumbens and the medial prefrontal cortex was considerably weaker than that observed for *cis*-8-OH-PBZI. Photographs of representative sections from the medial prefrontal cortex showing comparative *Fos* protein staining after subcutaneous administration of saline or drug (Fig. 6) demonstrate the relatively intense *Fos* protein immunoreactivity induced by *cis*-8-OH-PBZI, in comparison to that seen following treatment with either saline or (−)-apomorphine.

4. Discussion

The results of radioligand binding experiments with cloned dopamine receptor subtypes establish the benz[e]indole *cis*-8-OH-PBZI as having high affinity and selectivity for the dopamine D_3 receptor. A further comparison of these data with affinity values measured for (−)-quinpirole and PD 128,907, compounds previously reported to be preferential dopamine D_3 receptor agonists (Lévesque et al., 1992; Sokoloff et al., 1992; Pugsley et al., 1995), showed that *cis*-8-OH-PBZI has a similar preference for the dopamine D_3 receptor. A significant difference was noted, however, in the higher affinity of *cis*-8-OH-PBZI for the dopamine D_4 receptor relative to that of PD 128,907. The binding preferences measured for several dopaminergic compounds also tested in these in vitro assays corresponded to their earlier reported selectivities for dopamine receptor subtypes. The agonists dopamine and (−)-apomorphine lacked selectivity for the different receptor subtypes, as described previously by Freedman et al. (1994) and Van Tol et al. (1991). The dopamine antagonist (−)-raclopride was shown to have high affinity for the dopamine D_{2s} and D_3 receptor subtypes and relatively weak affinity for dopamine D_4 receptors, as earlier reported by Lahti et al. (1993) and Malmberg et al. (1993). Clozapine showed high affinity and modest selectivity for the dopamine D_4 receptor, as originally reported by Van Tol et al. (1992).

In vitro signal transduction measurements for the dopamine D_3 and D_{2s} receptor subtypes allowed us to determine whether *cis*-8-OH-PBZI acts as an agonist or antagonist in assays specific for each receptor subtype. We recorded changes in extracellular acidification rates in $BHK21tk^-$ cells expressing the dopamine D_3 receptor as a means of assessing agonist activity, as Lajiness et al. (1995) had previously shown that increases in cellular hydrogen efflux occur in response to dopamine D_3 receptor activation. Dopamine and *cis*-8-OH-PBZI were equally effective at inducing a signaling response in these cells. Thus, *cis*-8-OH-PBZI possesses full agonist activity at the dopamine D_3 receptor. Notably, the concentrations of *cis*-8-OH-PBZI which altered cellular acidification rates were similar to those that yielded an effective inhibition of radioligand binding at the dopamine D_3 receptor in in vitro assays. In order to see if this agonist activity extended to other members of the dopamine D_2 receptor family, we also measured dopamine D_{2s} receptor-mediated changes in cellular cAMP synthesis. These studies were performed as described by Bunzow et al. (1988), using clonal Ltk^- cells expressing the dopamine D_{2s} receptor. *Cis*-8-OH-PBZI partially inhibited cAMP synthesis, but only when added to cells at the high concentrations shown in in vitro radioligand binding assays to be necessary for establishing occupation of this receptor site. These data demonstrate that *cis*-8-OH-PBZI is capable of activating both the dopamine D_3 and D_{2s} receptor subtypes, but that large differences exist in the effective drug concentrations and maximal signaling efficacy at each receptor.

Immunohistochemical studies provided convincing evidence of an anatomical selectivity in the in vivo action of *cis*-8-OH-PBZI. Even at very low doses (0.1 mg/kg) the compound induced a strong *Fos* protein response, reflecting changes in *c-fos* expression, in the limbic medial prefrontal cortex and shell region of the nucleus accumbens. By contrast, the *Fos* response to *cis*-8-OH-PBZI in the non-limbic dorsolateral striatum was extremely weak and is probably without functional importance. Notably, the nonselective dopamine agonist (−)-apomorphine induced *Fos* protein immunoreactivity in all three brain areas. Its effect was weaker in the medial prefrontal cortex and shell region of the nucleus accumbens, but stronger in the dorsolateral striatum relative to that of *cis*-8-OH-PBZI. Our results with *cis*-8-OH-PBZI are consistent with earlier reports that dopamine D_2/D_3 agonists *induce Fos* protein immunoreactivity in NG108-15 neuroblastoma–glioma hybrid clonal cells expressing the dopamine D_3 receptor (Pilon et al., 1994). The selective induction of in vivo *Fos* expression that we observed also agrees with the reported localization of the dopamine D_3 receptor in brain areas including the shell region of the nucleus accumbens and medial prefrontal cortex, but not in the dorsolateral striatum (Sokoloff et al., 1990). The ability of *cis*-8-OH-PBZI to induce *Fos* protein expression in the medial prefrontal cortex was interesting, as its action was comparable to the previously observed effect of the atypical neuroleptic clozapine in this brain area (Robertson and Fibinger, 1992; Fink-Jensen and Kristensen, 1994; Fink-Jensen et al., 1995). However, because 7-OH-DPAT cannot modify this clozapine-mediated activity in the medial prefrontal cortex (Guo et al., 1995) it is unclear whether the dopamine D_3 receptor was the direct target of clozapine in these studies. In this respect, the high affinity of clozapine for the dopamine D_4 receptor (Van Tol et al., 1991) and relatively low selectivity of *cis*-8-OH-PBZI towards this receptor is noteworthy. Extended in vitro investigation of *cis*-8-OH-PBZI failed to reveal any additional possible ligand binding site interactions. Only a weak possible interaction was observed at pharmacophore recognition sites for several neurotransmitter receptors (serotonin 5-HT_{1A}, muscarinic M_1, α_2-adrenoceptor) and at serotonin uptake sites. Thus, it appears likely that the in vivo activity of *cis*-8-OH-PBZI is limited to members of the dopamine receptor family.

In conclusion, *cis*-8-OH-PBZI has been shown to have in vitro selectivity for the dopamine D_3 receptor and dopamine agonist activity. The ability of *cis*-8-OH-PBZI at low doses to induce strong *Fos* protein immunoreactivity in the medial prefrontal cortex and shell region of the nucleus accumbens, but only a very weak response in the motor dorsolateral striatum, is consistent with a selective limbic action of this novel compound.

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