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Pharmacological Characterization of (*E*)-*N*-(3-iodoprop-2-enyl)-2β-Carbomethoxy-3β-(4′-methylphenyl)nortropane as a Selective and Potent Inhibitor of the Neuronal Dopamine Transporter[1]

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ABSTRACT

The pharmacological properties of the iodinated derivative of cocaine (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane (PE2I) were evaluated in vitro in the rat. Binding experiments on rat striatal membranes showed that PE2I selectively recognized the dopamine transporter (DAT) according to a single binding site model with high affinity (K_d = 4 nM, B_{max} = 12 pmol/mg protein). In the cortical membranes, the binding of PE2I was also selectively associated with the DAT (IC_{50} for GBR 12909 = 6 nM versus more than 1000 nM for paroxetine), with similar affinity to that of the striatum. Autoradiographic experiments on rat brain sections with [^{125}I]PE2I were in agreement with the localization of the DAT. In addition, PE2I was shown to be a potent inhibitor of dopamine uptake, with IC_{50} values similar to those for GBR 12909 and 2β-carbomethoxy-3β-(4′-iodophenyl)-tropane (β-CIT) (2–6 nM). All of these findings, combined with previously published data, support the use of PE2I as a selective and potent tool to study the DAT both in vivo and in vitro.

The membrane dopamine (DA) transporter (DAT) has major physiological roles in regulating neurotransmission processes through the rapid removal of DA from the synaptic cleft back into the presynaptic nerve endings. It also mediates the pharmacological effects of drugs such as cocaine and amphetamine (Giros et al., 1996) and the entry of neurotoxins such as 1,2,3,6-tetrahydro-1-methyl-4-phenylpyridine into DA neurons (Gainetdinov et al., 1997). Moreover, this transporter is very involved in a variety of disease processes such as the physiopathological mechanisms of Parkinson's disease (Uhl et al., 1994). The DAT appears, therefore, to be an essential neurochemical factor, and its exploration is highly valuable for the understanding of the mechanisms of action of several drugs, as well as for the diagnosis and follow-up of various cerebral diseases.

One reliable approach to such study involves the use of radioactive probes in vitro or, as more recently described, in vivo for scintigraphic investigations. The development of such radioligands is generally based on compounds known to bind to the DAT. A large panel of drugs interacts with this transporter, the molecular structure of which has been elucidated (Giros et al., 1991). However, the strong structural similarities among the three membrane monoamine transporters [DA, 5-hydroxytryptamine (5-HT; serotonin), and norepinephrine (NE)] contribute to the lack of selectivity of many radioligands (Amara and Kuhar, 1993). For example, the pharmacological effects of cocaine are essentially due to an inhibitory action of this drug at the DAT (Ritz et al., 1990a; Giros et al., 1996), but it also binds to the 5-HT and NE transporters (Ritz et al., 1990a). More potent ligands of the DAT have been developed, in particular, the cocaine derivative WIN 35,428 (Boja et al., 1990) and its closely related iodinated derivative 2β-carbomethoxy-3β-(4′-iodophenyl)-tropane (β-CIT) (Boja et al., 1991). The compound β-CIT is currently used for in vitro study of the DAT when labeled with ^{125}I (Boja et al., 1992a) and in vivo when labeled with ^{11}C for positron emission tomography (Farde et al., 1994) or with ^{123}I for single-photon emission computed tomography (SPECT; Laruelle et al., 1994a). β-CIT has, however, two major disadvantages: it binds to both the DA and 5-HT transporters (Boja et al., 1992a; Rothman et al., 1994),

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ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; PE2I, (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane; 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; SERT, serotonin transporter; SPECT, single-photon emission computed tomography; β-CIT, 2β-carbomethoxy-3β-(4′-iodophenyl)-tropane; IPT, (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-chlorophenyl)nortropane.

and it has in vivo kinetics that require at least 20 h to obtain maximal specific binding to the DAT (Brücke et al., 1993). This delay is not suitable for [^{11}C]β-CIT (half-life = 20 min) and is not fully appropriate for [^{123}I]β-CIT (half-life = 13 h).

To obtain a radioiodinated tracer possessing both high selectivity for the DAT and suitable kinetics for short half-life isotopes, we recently developed several new tropane derivatives (Emond et al., 1997). One of these compounds, (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl) nortropane (PE2I), is 300 times more selective than β-CIT for the DAT compared with the 5-HT transporter (Emond et al., 1997). PE2I also specifically binds in vivo to the DAT in the rat and in the primate, where it reaches maximum specific binding to the DAT at 1 h after injection (Guilloteau et al., 1998). In a rat model of Parkinson's disease, PE2I is able to detect a 10% decrease in striatal DAT as early as 24 h after 6-hydroxydopamine lesion of the nigrostriatal pathway, appearing therefore to be a reliable index of DAT density (Chalon et al., 1999). In addition, preliminary in vitro postmortem autoradiographic studies on human brains (Hall et al., 1999) and in vivo SPECT studies in healthy subjects (Kuikka et al., 1998) show that this compound is a good candidate for exploration of the DAT in humans.

All of these findings support the future use of PE2I for quantification of DAT in clinical applications; however, it is essential to have a thorough knowledge of this tool for optimal interpretation of such explorations. We therefore present a pharmacological characterization of PE2I with the rat model and demonstrate that this compound is a potent and highly selective inhibitor of the DAT, suitable for both in vivo and in vitro investigations.

Materials and Methods

Animals and Drugs

All experiments were conducted on male Wistar rats weighing 250 to 300 g (Center d'Elevage R. Janvier, Le Genest St. Isle, France) in accordance with French law on animal experimentation.

Stable PE2I and β-CIT were synthesized as previously described (Emond et al., 1997). Natural cocaine was obtained from Coopération Pharmaceutique Française (Melun, France). GBR 12909, nisoxetine, haloperidol, sulpiride, and SCH 23390 were obtained from RBI Bioblock (Illkirch, France). Paroxetine was obtained from SmithKline Beecham (Nanterre, France). [^{3}H]DA (specific activity, 21.5 Ci/mmol) was purchased from NEN (Boston, MA).

The radiolabeling of [^{125}I]PE2I was performed from the stannyl precursor according to a previously described method (Guilloteau et al., 1998). After purification, [^{125}I]PE2I was obtained in a no-carrier-added form with a specific activity of 2000 Ci/mmol. It was kept in ethanol at −20°C; it is stable for 1 month under these storage conditions.

Binding studies

In Vitro Binding Assays on Striatal Membranes. *Tissue preparation.* Male rats were sacrificed by decapitation on the day of the assay, and both striata of each animal were removed on ice and weighed (two rats were used for each experiment). The tissue was homogenized in 10 volumes of 0.32 M sucrose with an Ultraturrax T25. After 1000*g* centrifugation at 4°C for 10 min, the supernatant was kept, and the pellet was treated as described above. Both supernatants were then pooled and centrifuged at 17,500*g* for 30 min at 4°C; 20 volumes of the incubation buffer was added to the pellet, and the mixture was homogenized and centrifuged at 50,000*g* for 10 min at 4°C. The final pellet was suspended in a minimum volume of the assay buffer, and the protein concentration was measured according to Bradford (1976) with BSA as standard.

Saturation studies. Several experimental procedures were used. In all experiments, [^{125}I]PE2I was used at a tracer dose of 20 pM together with 0.3 to 20 nM stable PE2I in 200 μl of buffer. Nonspecific binding was always determined in the presence of 30 μM cocaine. The mixture of [^{125}I]PE2I and PE2I was incubated with 30 μg of protein in a total volume of 1 ml in a Tris · HCl, pH 7.4, buffer (50 mM Tris · HCl, 120 mM NaCl, 5 mM KCl) for 30, 60, or 90 min at 22°C. Samples were then rapidly filtered through Whatman GF/B fiber filters soaked with 0.1% polyethylenimine (Sigma, St. Quentin-Fallavier, France). The filters were washed twice with 4 ml of cold buffer, and the residual radioactivity was measured in a gamma counter (Cobra 5010; Packard).

Influence of Na^+ was tested with Tris · HCl buffer with different concentrations of NaCl (30, 120, or 300 mM).

The influence of the buffer was tested with phosphate instead of Tris · HCl buffer as the incubation medium (10.14 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4). Data were analyzed with the use of RADLIG-EBDA analysis software (Biosoft).

Competition studies. For these studies, a mixture of [^{125}I]PE2I (20 pM) and PE2I (4 nM) was incubated with 30 μg of protein in a total volume of 1 ml in the Tris · HCl, pH 7.4, buffer (50 mM Tris · HCl, 120 mM NaCl, 5 mM KCl) for 90 min at 22°C in the presence of different drugs (GBR 12909, cocaine, β-CIT, paroxetine, nisoxetine, haloperidol, sulpiride, SCH 23390) at concentrations of 10^{-6} to 10^{-10} M. Samples were then treated as described above. Total binding was determined in the absence of any drug, and nonspecific binding was measured in the presence of 30 μM cocaine. The IC_{50} values were determined graphically for each compound, and the K_i values were calculated according to Cheng and Prussoff (1973), as fully competitive inhibition was the assumed mechanism.

In Vitro Binding Assays on Cortical Membranes. *Tissue preparation.* Male rats were sacrificed by decapitation on the day of the assay, and the prefrontal cortex of each animal was removed on ice and weighed (two rats were used for each experiment). The tissue was homogenized in 10 volumes of 0.32 M sucrose with an Ultraturrax T25. Cortical membranes were then prepared as described above for the striatum.

Saturation studies. These studies were conducted as described above for striatal membranes, except for the protein concentration and the volume of incubation (180 μg protein/assay in a total volume of 500 μl).

Competition studies. They were conducted as described above for striatal membranes, except for the protein concentration and the volume of incubation (180 μg protein/assay in a total volume of 500 μl) in the presence of GBR 12909 or paroxetine at concentrations of 10^{-5} to 10^{-10} M.

In Vitro Autoradiographic Studies

Male Wistar rats weighing 200 to 250 g were sacrificed by decapitation, and their brains were removed on ice and then rapidly frozen at −35°C. Then, 20-μm coronal sections were cut with a cryostat microtome (Reichert-Jung Cryocut 1800; Leica, Rueil-Malmaison, France), thaw mounted on gelatin microscope slides, and kept at −80°C until use. Sections were incubated for 90 min with 100 pM [^{125}I]PE2I in 100 μl of a pH 7.4 phosphate buffer (10.14 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4). Adjacent sections were incubated in the presence of 100 μM cocaine, 1 μM GBR 12909, 20 nM paroxetine, or 20 nM nisoxetine. Slices were then washed twice for 20 min in the phosphate buffer at 4°C and rinsed for 1 sec in distilled water. After drying, slices were exposed to sensitive film (Hyperfilm β-max; Amersham International, Buckinghamshire, UK) in X-ray cassettes for 3 days together with standards (^{125}I-microscales; Amersham). Regional optical densities were measured with an image analyzer (Biocom, Les Ulis, France) after identification of anatomical regions according to the atlas of Paxinos and Watson (1986).

Inhibitory Properties of [^{3}H]DA Uptake

Uptake experiments were performed on a synaptosomal fraction obtained from rat striata. After dissection on ice and weighing, the tissue was homogenized in 10 volumes of 0.32 M sucrose with a Potter homogenizer (Potter S Braun; Roucaire, Les Ulis, France). After 1000*g* centrifugation at 4°C for 10 min, the supernatant was kept and pellet was treated as described above. Both supernatants were then mixed and centrifuged at 17,500*g* for 30 min at 4°C. The final pellet was suspended in a minimum volume of the assay buffer (Krebs-Ringer medium, pH 7.6, according to Amejdki-Chab et al., 1992, with minor modifications) consisting of 109 mM NaCl, 3.55 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5.5 mM glucose. The protein concentration was measured according to Bradford (1976) with BSA as standard.

The assay buffer containing 0.1 mM pargyline was gassed with 95% O_2/5% CO_2 at 37°C for 30 min. Aliquots (50 μl corresponding to 100 μg of protein) of the synaptosomal fraction were then preincubated for 5 min at 37°C with 50 μl of the assay buffer with or without various concentrations (10^{-5} to 10^{-10} M) of competitor (cocaine, GBR 12909, β-CIT, or PE2I) and 850 μl of the assay buffer. Incubation was continued for 3 min with the addition of 15 nM/50 μl of [^{3}H]DA (specific activity, 21.5 Ci/mmol); the reaction was then stopped with 5 ml of ice-cold medium. The mixture was immediately filtered through Whatman GF/B fiber filters. The filters were washed twice with 5 ml of ice-cold medium, and the residual radioactivity was measured in a beta counter (LKB Rack Beta 1215). Specific uptake of [^{3}H]DA was defined as the difference between the total uptake and the uptake at 37°C in the presence of 10^{-6} M GBR 12909. The IC_{50} values (concentrations inhibiting 50% of control uptake) were determined graphically for each competitor.

Results

Binding Studies

In Vitro Binding on Striatal Membranes. *Saturation studies.* The affinity and density of specific [^{125}I]PE2I binding sites were measured using a constant concentration of [^{125}I]PE2I (20 pM) and increasing concentrations of unlabeled PE2I (0.3–20 nM). Preliminary experiments with different incubation times (30, 60, and 90 min) showed that binding equilibrium was reached between 60 and 90 min. We then chose to incubate for 90 min for the following experiments. With Tris · HCl buffer containing 120 mM NaCl, the Scatchard transformation of the resulting data (Fig. 1) revealed a linear curve suggesting a one-site model (n_H = 1) with a K_d value of 3.9 ± 0.8 nM (mean ± S.D. of 7 independent determinations, each performed in triplicate) and a B_{max} value of 11.8 ± 2.6 pmol/mg protein (mean ± S.D.).

Similar results were obtained when NaCl was present in the buffer at a concentration of 300 mM, whereas significant increases in K_d and B_{max} values were observed with only 30 mM NaCl (Table 1).

A series of saturation experiments was also performed in the presence of phosphate instead of Tris · HCl buffer as incubation medium, and these yielded a K_d value of 4.9 ± 0.7 nM and B_{max} value of 12.3 ± 3.8 pmol/mg protein (mean ± S.D. of 3 independent determinations, each performed in triplicate).

Competition studies. The pharmacological profile of specific [^{125}I]PE2I binding in the striatum was studied using drugs known to bind to the DA, 5-HT, and NA transporters and to various DA receptors. This profile was consistent with the binding of [^{125}I]PE2I to the DAT (Fig. 2), as the rank order of potency of these drugs was GBR 12909 = β-CIT > cocaine = paroxetine = nisoxetine > haloperidol = sulpiride = SCH 23390 (Table 2).

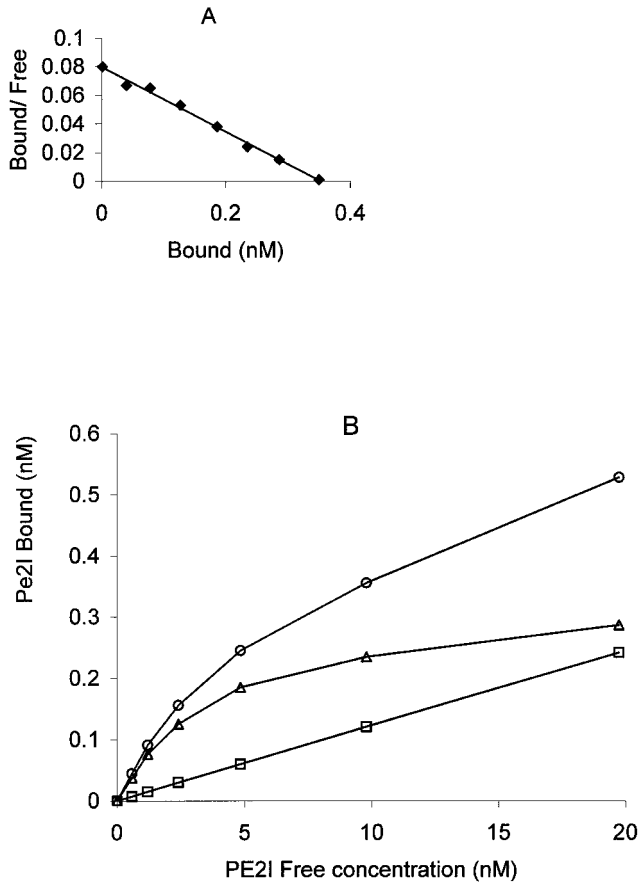

Fig. 1. Saturation experiments of [^{125}I]PE2I binding in the rat striatum. Striatal membranes (30 μg protein/assay) were incubated with a constant concentration of [^{125}I]PE2I (20 pM) and increasing concentrations of unlabeled PE2I (0.3–20 nM) in Tris · HCl buffer, pH 7.4, for 90 min at 22°C. Nonspecific binding was determined in the presence of 30 μM cocaine. A, Scatchard transformation of the data. B, one representative saturation curve. ○, total; □, nonspecific; △, specific.

TABLE 1

Effect of concentration in Na^+ on the binding of PE2I to rat striatal membranes

A mixture of [^{125}I]PE2I (20 pM) and unlabeled PE2I (0.3–20 nM) was incubated with 30 μg of protein for 90 min at 22°C in Tris · HCl buffer, pH 7.4, containing different concentrations of Na^+ as NaCl. The nonspecific binding was determined in the presence of 30 μM cocaine. K_d and B_{max} values were calculated after Scatchard analysis.

[NaCl]	K_d	B_{max}
mM	*nM*	*pmol/mg protein*
30	13.3 ± 5.7	30.6 ± 7.9
120	3.9 ± 0.8	11.8 ± 2.6
300	4.4 ± 0.8	11.2 ± 3.5

Values are mean ± S.D. of 3 independent experiments, each performed in triplicate.

In Vitro Binding on Cortical Membranes. *Saturation studies.* The affinity and density of specific [^{125}I]PE2I binding sites were measured using a constant concentration of [^{125}I]PE2I (20 pM) and increasing concentrations of unlabeled PE2I (0.3–20 nM) in the presence of 180 μg of proteins from the membrane preparation. With Tris · HCl buffer containing 120 mM NaCl, the Scatchard transformation of the resulting data (Fig. 3) revealed a linear curve that suggested a one-site model (n_H = 1) with a K_d value of 5.7 ± 1.2 nM

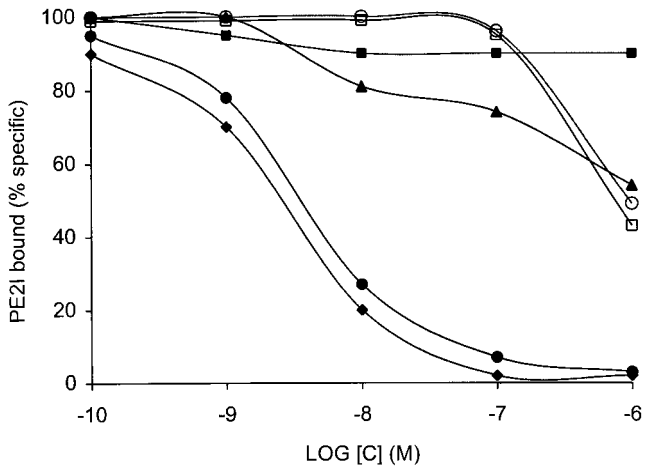

Fig. 2. Displacement of [^{125}I]PE2I binding in rat striatum by various drugs. Striatal membranes were incubated with 20 pM [^{125}I]PE2I with 4 nM PE2I and increasing quantities of drugs (10^{-10} to 10^{-6} M). Each curve is representative of 3 independent experiments, each performed in triplicate. The inhibitory constants of the drugs are given in Table 2. ◆, GBR 12909; ●, β-CIT; ▲, cocaine; □, paroxetine; ○, nisoxetine; ■, sulpiride, SCH 23390, haloperidol.

(mean ± S.D. of 7 independent determinations, each performed in triplicate) and a B_{max} value of 1.0 ± 0.3 pmol/mg protein (mean ± S.D.).

Competition studies. Competition studies on cortical membranes were performed with GBR 12909 and paroxetine to determine whether the specific [^{125}I]PE2I binding was related to the DA or 5-HT transporters. These experiments revealed a pharmacological profile consistent with that of binding to the DAT, with an IC_{50} value of 6.4 ± 2.5 nM (mean ± S.D.) for GBR 12909 and more than 1000 nM for paroxetine (n = 5 independent experiments for each value).

In Vitro Autoradiographic Studies

Semiquantitative data were obtained by the determination of regional optical densities. As shown on Table 3, the highest binding of [^{125}I]PE2I (total binding) was observed in the striatum, with a higher level in the lateral part than in the medial. [^{125}I]PE2I also bound to the nucleus accumbens and olfactory tubercle, whereas a low level was found in the frontal cortex. In DAT-rich regions (striatum, nucleus accumbens, olfactory tubercle), cocaine and GBR 12909 considerably decreased (−75 to −90%) the binding of [^{125}I]PE2I, whereas paroxetine and nisoxetine had no significant effect. In the cortex, a small reduction in [^{125}I]PE2I binding was observed with cocaine, GBR 12909, and paroxetine, whereas no effect was observed with nisoxetine.

Inhibitory Properties of [^{3}H]DA Uptake

The ability of PE2I to inhibit [^{3}H]DA uptake on rat striatal synaptosomes was close to that of GBR 12909 and β-CIT, whereas cocaine showed the weakest potency (Table 4 and Fig. 4).

Discussion

To obtain a specific and reliable DAT ligand, we recently developed an iodinated congener of cocaine, PE2I (Emond et al., 1997). Precise understanding of the pharmacological profile of such a probe is essential for reliable interpretation of data obtained with the use of it. The first aim of this study was therefore to characterize the binding properties of PE2I to the DAT on rat striatal membranes. We chose to perform all experiments on fresh tissue because the use of frozen tissue with derivatives of cocaine as in vitro tracer could introduce artifacts (Kirifides et al., 1992). Under these experimental conditions, PE2I bound to the DAT according to a one-site model in two different buffer systems (Tris and phosphate), with a K_d value of ~4 nM. In addition, such binding was shown to be Na^+ dependent, as already observed for inhibitors of DA uptake such as GBR (Janowsky et al., 1986; Bonnet et al., 1988) and β-CIT (Wall et al., 1993). It must be

TABLE 2

Inhibition of specific [^{125}I]PE2I binding in rat striatal membranes

A mixture of 20 pM [^{125}I]PE2I and 4 nM unlabeled PE2I was incubated with 30 μg of protein in Tris · HCl buffer, pH 7.4, for 90 min at 22°C in the presence of different drugs at concentrations of 10^{-5} to 10^{-10} M. Total binding was determined in the absence of drugs, and nonspecific binding was determined in the presence of 30 μM cocaine. IC_{50} values were determined graphically, and the K_i values were calculated according to the method of Cheng and Prussoff (1973): $K_i = 1/[1 + (1/K_d)]$.

Drug	K_i
	nM
PE2I	6.8 ± 0.4
GBR 12909	1.3 ± 0.3
β-CIT	1.4 ± 0.8
Cocaine	480 ± 28
Paroxetine	416 ± 76
Nisoxetine	450 ± 50
Haloperidol	>1000
Sulpiride	>1000
SCH 23390	>1000

Values are mean ± S.D. of 3 independent experiments, each performed in triplicate.

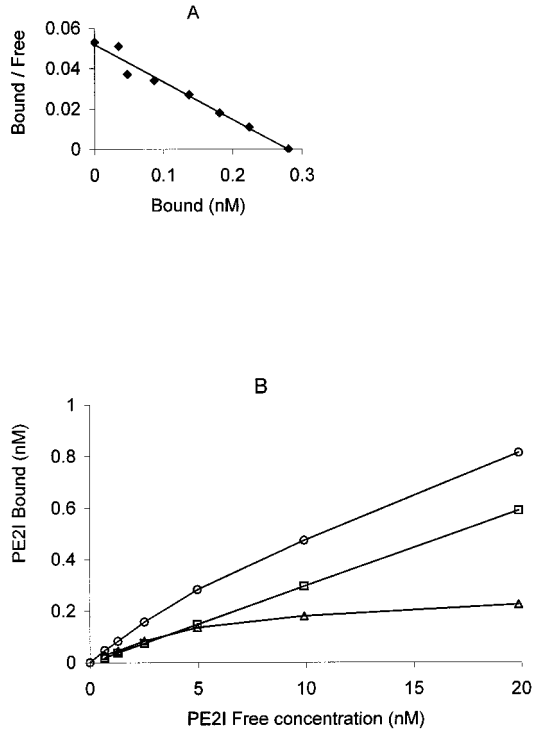

Fig. 3. Saturation experiments of [^{125}I]PE2I binding in the rat frontal cortex. Cortical membranes (180 μg protein/assay) were incubated with a constant concentration of [^{125}I]PE2I (20 pM) and increasing concentrations of unlabeled PE2I (0.3–20 nM) in Tris · HCl, pH 7.4, buffer for 90 min at 22°C. Nonspecific binding was determined in the presence of 30 μM cocaine. A, Scatchard transformation of the data. B, one representative saturation curve. ○, total; □, nonspecific; △, specific.

TABLE 3

Autoradiographic analysis of [^{125}I]PE2I binding to several areas of coronal rat brain sections

Sections were incubated with 100 pM [^{125}I]PE2I either alone or in the presence of 100 μM cocaine, 1 μM GBR 12909, 20 nM paroxetine, or 20 nM nisoxetine. Exposure was carried out for 3 days, and regional optical densities were measured on identified anatomical regions according to the atlas of Paxinos and Watson (1986).

Cerebral Area	Total Binding	Cocaine (100 μM)	GBR 12909 (1 μM)	Paroxetine (20 nM)	Nisoxetine (20 nM)
Frontal cortex	2.2 ± 0.3	1.5 ± 0.3*	1.8 ± 0.1*	1.4 ± 0.2*	2.6 ± 0.5
Medial striatum	31.4 ± 2.6	6.4 ± 0.8**	8.3 ± 0.9**	26.3 ± 1.4	25.7 ± 2
Lateral striatum	37.8 ± 1.6	7.1 ± 1.0**	11.1 ± 0.9**	33.5 ± 1.6	34.2 ± 2.2
Accumbens nucleus	29.9 ± 2.6	5.2 ± 0.1**	8.7 ± 1.0**	26.6 ± 2.0	27.3 ± 2.2
Olfactory tubercle	20.2 ± 3.3	2.2 ± 0.2**	4.3 ± 1.0**	16.2 ± 2.2	16.6 ± 1.0

Values are the mean ± S.D. for 6 rats.
* $P < .05$, ** $P < .001$, t test for unpaired values.

TABLE 4

Inhibitory potency on [^{3}H]dopamine uptake in rat striatal synaptosomes

Potencies of drugs in inhibiting the specific uptake of 15 nM of [^{3}H]DA were determined on rat striatal synaptosomes. The total uptake of [^{3}H]DA was defined in the absence of drug and the nonspecific uptake in the presence of 10^{-6} M GBR 12909. IC_{50} values were determined graphically.

Drug	IC_{50}
	nM
PE2I	6.0 ± 1.4
GBR 12909	3.2 ± 0.3
β-CIT	2.1 ± 0.9
Cocaine	466 ± 57

Values are mean ± S.D. of 3 independent experiments, each performed in triplicate.

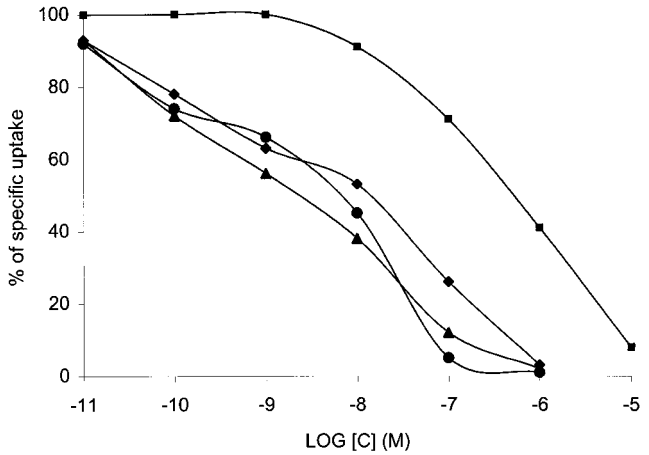

Fig. 4. Inhibition of [^{3}H]DA uptake in rat striatal synaptosomes. The total uptake of [^{3}H]DA was defined in the absence of drugs and the nonspecific uptake in the presence of 10^{-6} M GBR 12909. ◆, PE21; ●, GBR 12909; ▲, β-CIT; ■, cocaine.

noted that PE2I and β-CIT differed in their in vitro binding on rat striatal membranes, as β-CIT followed a two-site model, with high-affinity sites ($K_d \sim 0.1$–1 nM) and low-affinity sites (K_d = 3–40 nM; Boja et al., 1991, 1992a; Laruelle et al., 1994b; Rothman et al., 1994). A distinction can therefore be made between cocaine derivatives developed as ligands of the DAT possessing either single or two binding sites in vitro. In particular, two binding sites have been described for β-CIT, WIN 35,065-2 (Ritz et al., 1990b), and RTI-121 (Boja et al., 1995). By contrast, a single binding site has been determined for (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-chlorophenyl)nortropane (IPT) (Kung et al., 1995), *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(3′,4′-dichlorophenyl)nortropane (Garreau et al., 1997), and altropane (Madras et al., 1998). In view of the chemical structure of all these tropane derivatives, it should be noted that IPT, *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(3′,4′-dichlorophenyl)nortropane, and altropane, in contrast with β-CIT, WIN 35,065-2, and RTI-121, all possess an iodopropenyl group on the nitrogen of the tropane structure. It can therefore be hypothesized that the conformational changes provided by the presence of this iodopropenyl group might prevent the binding of such compounds to the low-affinity binding site, resulting in the labeling of only the high-affinity site. However, because PE2I and its close chemical structure congeners possess B_{max} values in the same order of magnitude as β-CIT and WIN 35,065-2, it cannot be excluded that these compounds did not bind to a single site but to two sites with equal affinity. In the case of the recognition of a single binding site in the striatum, this would be an advantage for quantifying the density of DAT in vitro and in vivo. As it has been suggested that only the functional state of the DAT seems to be associated with the high-affinity binding site (Pristupa et al., 1993), a further value of a compound such as PE2I could be to provide exclusive evidence of this state.

Competition studies on rat striatal membranes showed that ligands of the DA D_1 and D_2 receptors, which are present in high amounts in the striatum, had no effect on PE2I binding. By contrast, binding was inhibited by several drugs, with an efficiency corresponding closely to the pharmacological profile of a DAT ligand: β-CIT and GBR 12909 highly inhibited the binding of PE2I, whereas inhibition by paroxetine, nisoxetine, and cocaine was poor. Poor inhibitory potency of cocaine has always been observed in vitro on various ligands of the DAT possessing the tropane structure such as WIN 35,428 (Boja et al., 1990), β-CIT (Boja et al., 1992a; Rothman et al., 1994), RTI-121 (Boja et al., 1995), IPT (Kung et al., 1995), and altropane (Madras et al., 1998), in agreement with the poor affinity of cocaine for the DAT (Madras et al., 1989). PE2I therefore binds selectively to the DAT in the striatum, as demonstrated by competition experiments on membrane preparations as well as by autoradiographic studies. This is an advantage compared with β-CIT, which also binds to the 5-HT transporter (SERT) in this cerebral region (Rothman et al., 1994). With in vitro experiments on striatal membranes for binding to the DAT and cortical membranes for binding to the SERT, previous findings already demonstrated that PE2I was 29 times more potent on the DAT than on the SERT (Emond et al., 1997). This high selectivity is close to that of altropane (SERT/DAT selectivity ratio = 28; Madras et al., 1998), whereas lower ratios have been found for IPT and RTI-121 (SERT/DAT = 4.5 for both compounds; Boja et al., 1992b; Goodman et al., 1994), compared with the inverted ratio observed for β-CIT (SERT/DAT = 0.89; Boja et al., 1992b).

The high selectivity of PE2I for the DAT led us to use it to study the transporter in the frontal cortex where the concentration is low. Indeed, the mesocortical dopaminergic system has major regulatory roles, but its study is limited, partly due to the lack of a highly selective ligand for the DAT. In the frontal cortex, the DAT has been demonstrated with immunochemical methods (Ciliax et al., 1995), but few attempts have been performed with radioactive markers. In our experiments on cortical membranes, the identification of the DAT with PE2I required 12 times greater tissue concentration than in the striatum, thus reflecting much lower concentrations of these sites in this cerebral region. We obtained data corresponding to a single binding site model, with a K_d value close to that obtained in the striatum (~5 nM) and a B_{max} value 10 times lower. Moreover, competition studies showed that the binding of PE2I was essentially related to the DAT and not to the SERT. Comparative experiments using 30 times greater tissue concentrations than for the striatum showed that RTI-121 was able to label the DAT in a two-site model, with similar K_d values for high and low binding sites as in the striatum and B_{max} values 45 and 9 times lower, respectively (Boja et al., 1998). However, it was shown that the cortical binding of RTI-121 was probably not related exclusively to the DAT but also to the 5-HT and NE transporters. No data are to date available concerning the binding of altropane, the pharmacological properties of which seem to be close to those of PE2I, in the frontal cortex. From our binding experiments on membrane preparation, it could therefore be assumed that PE2I selectively recognizes the DAT in the cortex. However, these findings were not confirmed in our autoradiographic experiments where a slight competitive effect of paroxetine was observed in the cortex. A minor binding of PE2I to the SERT can therefore not be excluded in this cerebral area.

With the aim of characterizing the properties of a probe such as PE2I, autoradiographic experiments allow visualization of the distribution of the marker at a given cerebral level. These studies confirmed that the localization of PE2I in the rat brain is consistent with that of the DAT, as already observed using an ex vivo biodistribution method (Guilloteau et al., 1998). The highest binding of PE2I was observed in the striatum, accumbens nucleus, and olfactory tubercle, a distribution similar to that observed for the DAT characterized with an immunochemical method (Ciliax et al., 1995). The intensity of labeling in the striatum followed a lateromedial gradient, as already found with β-CIT (Fujita et al., 1994; Coulter et al., 1995). The selectivity of PE2I labeling in the striatum, accumbens nucleus, and olfactory tubercle was assessed with competition studies. A moderate (1 μM) dose of GBR 12909 had a strong displacing effect, whereas a similar effect was obtained with cocaine at a 100-fold concentration. By contrast, paroxetine and nisoxetine had no significant effect in these cerebral regions. These findings, together with autoradiographic studies on postmortem human brain sections (Hall et al., 1999), still showed the selective binding of PE2I to the DAT. All the binding studies therefore demonstrated that PE2I is a potent and selective ligand for the DAT.

In addition, competition experiments on the uptake of DA clearly showed that PE2I is a potent inhibitor of this process, as it had the same inhibitory effect as β-CIT and GBR 12909 in our experimental conditions. The inhibitory potency of these three compounds was ~100 times greater than that of cocaine, the K_i value of which was similar to previously published data (Boja et al., 1992b; Pristupa et al., 1993).

We therefore demonstrated that PE2I is a DAT inhibitor that binds this transporter with high affinity and selectivity. These findings, associated with previously published data, support the use of PE2I as a potent tool to study DAT both in vivo and in vitro.

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