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RTI-4793-14, a New Ligand With High Affinity and Selectivity for the (+)-MK801-Insensitive [^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine Binding Site (PCP Site 2) of Guinea Pig Brain

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KEY WORDS Phencyclidine receptor, RTI-4793-14, PCP, Biogenic amine reuptake carrier, (+)-MK801, Guinea pig brain

ABSTRACT [^{3}H]TCP, an analog of the dissociative anesthetic phencyclidine (PCP), binds with high affinity to two sites in guinea pig brain membranes, one that is MK-801 sensitive and one that is not. The MK-801-sensitive site (PCP site 1) is associated with NMDA receptors, whereas the MK-801-insensitive site (PCP site 2) may be associated with biogenic amine transporters (BAT). Although several "BAT ligands" are known that bind selectively to PCP site 2 and not to PCP site 1 (such as indatraline), these compounds have low affinity for site 2 (K_i values > 1 μM). Here we demonstrate that the novel pyrrole RTI-4793-14 is a selective, high affinity ligand for PCP site 2. We determined the IC_{50} values of RTI-4793-14 and several reference compounds [PCP, (+)-MK801 and indatraline] for PCP site 1 (assayed with [^{3}H](+)-MK801), PCP site 2 (assayed with [^{3}H]TCP in the presence of 500 nM (+)-MK801) and a variety of BAT-related measures ([^{3}H]CFT binding to the DA transporter, [^{3}H]nisoxetine binding to the norepinephrine transporter, [^{3}H]dopamine uptake, [^{3}H]serotonin uptake). In addition, we determined the ability of RTI-4793-14 to block NMDA responses in cultured hippocampal neurons under voltage clamp. (+)-MK801 had high affinity for PCP site 1 (4.6 nM) and potently inhibited NMDA-induced responses, but was much less potent in the BAT-related measures (IC_{50}s > 10 μM). PCP had high affinity at PCP site 1 ($IC_{50} = 92$ nM) and PCP site 2 ($IC_{50} = 117$ nM), and was moderately potent in all BAT-related measures except [^{3}H]nisoxetine binding. Indatraline was potent in BAT-related measures (IC_{50}s, 2 to 5 nM), but weak in other measures (IC_{50}s > 1 μM). In contrast, RTI-4793-14 had high affinity for PCP site 2 (38 nM), low affinity for PCP site 1 (> 36 μM), moderate IC_{50}s for all BAT-related measures, and negligible activity at NMDA receptors. Viewed collectively, these data indicate that RTI-4793-14 binds with high affinity and selectivity to PCP site 2 and provide further support for an association between PCP site 2 and the BATs.

INTRODUCTION

[^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) and [^{3}H]MK-801 have been extensively used to label the phencyclidine binding site associated with NMDA receptors (PCP site 1). However, [^{3}H]TCP also binds to a

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site that is insensitive to MK-801 (PCP site 2) and [^{3}H]TCP in the presence of MK-801 can be used to label this site. Several lines of evidence suggest that this site may be associated with biogenic amine transporters (Rothman et al., 1989; Akunne et al., 1991; Akunne et al., 1992; Rothman et al., 1992). First, PCP, which inhibits the reuptake of dopamine (DA) and serotonin (5-HT) (Smith et al., 1977), has high affinity for site 2, whereas (+)-MK801, which is a very weak DA reuptake blocker (Snell et al., 1988), does not bind to this site. Second, as reported for the guinea pig (Rothman et al., 1989) and human brain (Akunne et al., 1991), high affinity serotonergic (fluoxetine) and dopaminergic (GBR12909 and BTCP) reuptake inhibitors bind selectively to site 2. Third, fluoxetine, GBR12909, and BTCP bind nearly irreversibly to PCP site 2 consistent with a high affinity interaction (Akunne et al., 1991). Fourth, MPTP-induced lesions of the dopaminergic innervation to the caudate nucleus decrease [^{3}H]TCP binding in this region (Akunne et al., 1992). The latter observation suggests a partial presynaptic localization for at least some of these sites.

Based upon the association of PCP site 2 with BATs, drugs with high affinity for PCP site 2 might be expected to inhibit the reuptake of biogenic amines. In the course of studying the interaction of a series of pyrroles with PCP binding sites, we observed that (2RS,3aSR,8bRS)-1,2,3,3a,4,8b-hexahydro-2-benzyl-1-methylindeno-[1,2-b]pyrrole resorcylate (RTI-4793-14) was a potent and selective ligand for PCP site 2 (Carroll et al., in press). The availability of this compound has enabled us to obtain further evidence supporting the concept that PCP site 2 is associated with BATs.

MATERIALS AND METHODS

Preparation of membranes

Since PCP site 1 and PCP site 2 are readily measured in guinea pig brain membranes (Rothman et al., 1989, 1992), the present study was conducted using this preparation. Large batches of frozen membranes were prepared with minor modifications of published procedures (Rothman et al., 1989). Frozen guinea pig brains with cerebellum (20–30) were thawed for 15 min and homogenized with a Polytron in ice-cold 5 mM Tris-HCl, pH 8.0 (10 ml/brain). The homogenate was centrifuged at 37,000 × g for 10 min, and the pellet was washed by resuspension in the same volume of buffer followed by recentrifugation. The pellets were resuspended in an equal volume of 5 mM Tris-HCl, pH 8.0, and the concentration was adjusted to 50 mM by the addition of 1 M Tris-HCl, pH 8.0. The homogenate was centrifuged for 10 min at 37,000 × g. The pellets were then washed three times by resuspension and centrifugation using 50 mM Tris HCl, pH 8.0. The final pellets were resuspended in the Tris-HCl buffer (0.5 ml/brain), pooled and 1 ml aliquots were distributed to microfuge tubes, which were stored at −80°C for assay. Membranes for the [^{3}H]nisoxetine assay were prepared as described (Rothman et al., 1993).

For [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT, WIN35,428) binding assays (see below), male Sprague-Dawley rats (200–300 g) (Charles River) were anesthetized with CO_2 gas and decapitated. Striata were dissected using glass manipulators, placed in small plastic containers, and then allowed to freeze by placing the container in dry ice. Striata collected in this way were stored at −80° C. On the day of the assay, each striatum was placed in ice-cold binding buffer (BB: 55.2 mM sodium phosphate buffer, pH 7.4, 5 ml per caudate) and homogenized while still frozen with a Polytron. The homogenate was centrifuged for 10 min at 30,000 × g, and the pellet was resuspended in an equal volume of BB. The homogenate was recentrifuged, and the pellet was resuspended in an equal volume of BB. An aliquot was saved for determination of protein (0.5 ml), and the remaining homogenate was brought up to a final volume of 50 ml/striatum using ice-cold BB. Typical final protein concentrations, determined with the method of Lowry et al., (1951), were 100 μg/ml. Initial experiments showed that at these protein concentrations, the specific binding was directly proportional to protein and that < 10% of the radioligand was bound (data not shown). Aliquots of membranes were then used in the radioligand binding assays (see below).

Binding assays

The [^{3}H]TCP binding assay for PCP site 2 was conducted with minor modifications of published protocols (Rothman et al., 1989). Briefly, 12 × 75 mm polystyrene test tubes were prefilled with 100 μl of [^{3}H]TCP (2 nM final concentration) in a protease inhibitor/antioxidant cocktail (PIC) containing 5,000 nM (+)-MK801, 100 μl of distilled H_2O or drug (in distilled H_2O), 50 μl of buffer (5 mM Tris-HCl, pH 8.0), and 750 μl of membrane (0.5–1.0 mg/ml protein, in 5 mM Tris-HCl, pH 8.0). The (+)-MK801 (500 nM final concentration) was included to block [^{3}H]TCP binding to PCP site 1. The protease inhibitor/anti-oxidant cocktail was composed of 25 μg/ml leupeptin, 25 μg/ml chymostatin,

Abbreviations

BTCP	benzo[b]thiophenylcyclohexylpiperidine
CFT WIN35,428	2β-carbomethoxy-3β-(4-fluorophenyl)tropane
GBR12909	1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenyl-propyl]piperazine
Indatraline (LU19-005)	(±)-trans-3-(3,4-dichlorophenyl)-N-methyl-1-indanamine
(+)-MK801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (+)-dizocilpine
RTI-4793-14	(2RS,3aSR,8aRS)-1,2,3,3a,8,8a-Hexahydro-2-benzyl-1-methylindeno-[1,2-b]pyrrole resorcylate
TCP	1-[1-(2-thienyl)cyclohexyl]piperidine

0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid (EGTA). The incubation time was for 18–24 hours at 4° C (steady state) in a final volume of 1 ml. Triplicate samples were filtered with an MR24 Brandel Cell Harvester and washed with two 5 ml aliquots of ice-cold buffer. Whatman GF/B filters were presoaked in buffer containing 2% polyethylenimine. The tritium retained on the filters was measured using a Taurus scintillation counter at 44% efficiency after an overnight extraction into ICN Cytoscint cocktail. Nonspecific binding was determined using 10 μM of TCP. Protein was determined using the method of Lowry et al. (1951). The [^{3}H](+)-MK801 assay (2 nM final concentration) proceeded as described above for [^{3}H]TCP except that (+)-MK801 was not included as a blocker, the incubation time was for 4–6 hours at 4° C, and nonspecific binding was determined with 1 μM TCP.

The [^{3}H]nisoxetine assay for the norepinephrine transporter (NE) was carried out as described (Tejani-Butt et al., 1990; Rothman et al., 1993). DA transporters were labeled with [^{3}H]CFT with minor modifications of published protocols (Madras et al., 1989). Briefly, 12 × 75 mm polystyrene test tubes were prefilled with 100 μl of drug, 100 μl of radioligand (1 nM final concentration), 50 μl of buffer. Drugs were made up in BB containing 1 mg/ml bovine serum albumin (BB/BSA). Radioligands were made up in a protease inhibitor cocktail containing 1 mg/ml BSA {BB containing chymostatin (25 μg/ml), leupeptin (25 μg/ml), EDTA (100 μM) and EGTA (100 μM)}. The assay was initiated by the addition of 750 μl of membranes, prepared as described above. Brandell cell harvesters were used to filter the samples over Whatman GF/B filters, which were presoaked in wash buffer (ice-cold 10 mM TRIS-HCl, pH 7.4 containing 150 mM NaCl) containing 2% polyethylenimine. In this procedure, samples were filtered and then washed with two 4 ml aliquots of ice-cold wash buffer. Nonspecific binding was defined using 10 μM (final concentration) GBR12909. All subsequent steps were identical to those described above.

Biogenic amine uptake assays

Synaptosomal uptake assays were carried out as described (Rothman et al., 1993). Briefly, synaptosomes were prepared by homogenization of rat caudate (for [^{3}H]DA reuptake) or whole rat brain minus cerebellum (for [^{3}H]5-HT reuptake) in ice-cold 10% sucrose, using a Potter-Elvehjem homogenizer. After a 1,000 × g centrifugation for 10 min at 4° C, the supernatants were retained on ice. The uptake assays were initiated by the addition of 100 μl of synaptosomes to 12 × 75 mm polystyrene test tubes, which were prefilled with 750 μl of [^{3}H]ligand (2 nM [^{3}H]5-HT or 5 nM [^{3}H]DA) in a Krebs-phosphate buffer (pH 7.4), which contained ascorbic acid (1 mg/ml) and pargyline (50 μM) (buffer), 100 μl of test drugs made up in buffer containing 1 mg/ml bovine serum albumin, and 50 μl of buffer. The [^{3}H]5-HT reuptake experiments were conducted in the presence of 100 nM nomifensine and 100 nM GBR12935 in order to block any possible reuptake into NE and DA nerve terminals. Although these agents were routinely included in the [^{3}H]5-HT reuptake assays, control studies showed that they had no discernible effect on [^{3}H]5-HT reuptake (data not shown). The nonspecific uptake of each [^{3}H]ligand was measured by incubations in the presence of 1 μM GBR12909 ([^{3}H]DA) and 10 μM fluoxetine ([^{3}H]5-HT). The incubations were terminated after a 15 min ([^{3}H]DA) or 30 min ([^{3}H]5-HT) incubation at 25° C by adding 4 ml of wash buffer (10 mM TRIS-HCl, pH 7.4 containing 0.9% NaCl at 25° C), followed by rapid filtration over Whatman GF/B filters and one additional wash cycle. Control studies indicated that specific uptake was (1) linear with time up to 30 min; and (2) was directly proportional to protein in the protein range used here. The Krebs-phosphate buffer contained 154.5 mM NaCl, 2.9 mM KCl, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, and 5 mM glucose. The tritium retained on the filters was counted, in a Taurus beta counter, after an overnight extraction into ICN Cytoscint cocktail.

Electrophysiological studies

Whole-cell recordings of NMDA-induced currents in cultured rat hippocampal neurons were carried out as described previously (Subramaniam et al., 1992). Hippocampal neurons grown in primary culture from 19-day-old Sprague-Dawley rat embryos were used 7–14 days after plating. The extracellular recording solution consisted of (in mM): 140 NaCl, 5 KCl, 0.1 $CaCl_2$, and 10 HEPES (osmolality, 315–325 mOsm; pH, 7.4). Tetrodotoxin (1 μM) and strychnine (1 μM) were added to block Na^+ channel currents and glycine-activated Cl^- currents, respectively. Patch pipettes (2-5 MΩ) were prepared from filament-containing thin wall glass capillary tubes and filled with an intracellular solution that consisted of (in mM): 145 CsCl, 2 $MgCl_2$, 5 HEPES, 0.1 $CaCl_2$, and 1 EGTA (osmolality 310 mOsm; pH, 7.4). Drugs were dissolved in recording solution and applied via a rapid gravity-fed perfusion system. Flow of the drug solutions was regulated by a microvalve operated by a programmable controller. Whole-cell recordings were performed with an Axopatch 1C patch-clamp amplifier (Axon Instruments, Burlingame, CA) and displayed on a high speed ink pen recorder (Gould Electronics, Cleveland, OH).

In vivo microdialysis experiments

Male Sprague-Dawley rats (350–400 g) were anesthetized with chloral hydrate (400 mg/kg; 100 mg/ml) and supplemented as required (90 mg/kg) to abolish the corneal reflex. Animals were then placed in a stereotaxic frame and dialysis probes (CMA 12, 2 mm) were

*TABLE I. Interaction of RTI-4793-14 and reference compounds with NMDA receptor- and biogenic amine transporter-related indices**

Drug	PCP site 2 IC_{50} (nM ± SD)	PCP site 1 IC_{50} (nM ± SD)	BAT related measures: [^{3}H]5-HT uptake IC_{50} (nM ± SD)	[^{3}H]DA uptake IC_{50} (nM ± SD)	[^{3}H]CFT binding IC_{50} (nM ± SD)	[^{3}H]Nisoxetine binding IC_{50} (nM ± SD)	NMDA-induced current IC_{50} (μM)
RTI-4793-14	37.9 ± 5.7	>36,000	1,024 ± 72	547 ± 20	850 ± 101	609 ± 48	768
PCP	92.4 ± 7.0	117 ± 3	1,424 ± 81	347 ± 40	1,546.74 ± 216.81	16,628 ± 98	2[1]
(+)-MK801	>10,000	4.58 ± 0.19	>4,700	>10,000	>15,000	6,576 ± 860	0.020[2]
Indatraline	2,529 ± 485	>20,000	4.49 ± .21	3.23 ± .45	4.32 ± .46	2.27 ± 0.28	95

*Synaptosomal uptake and ligand binding assays were conducted as described in Materials and Methods. Preliminary experiments were conducted to determine the dose-range required for 10–90% inhibition. Each [^{3}H]ligand was displaced by 10 concentrations of test drug. The data of two experiments were pooled and fit to the two parameter logistic equation for the best-fit estimates of the IC_{50} reported above; [1]tfrench-Mullen and Rogawski (1989); [2]Jones and Rogawski (1992). Typical total and nonspecific cpms obtained for each assay are as follows: [^{3}H](+)-MK801 for PCP site 1 (9774, 366), [^{3}H]TCP for PCP site 2 (3069, 580), [^{3}H]DA uptake (27121, 3486), [^{3}H]5-HT uptake (8694, 335), [^{3}H]CFT binding (760, 160), and [^{3}H]nosixetine (1026, 353).

implanted into the nucleus accumbens (AP:+2 mm, LAT:+1.5 mm, H:-7.5 mm relative to bregma) according to the atlas of Paxinos and Watson (Paxinos, 1982). The dialysis probes were perfused with a physiological medium containing 145 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, and 2 mM Na_2HPO_4, the final pH of the medium being pH 7.4. The probes were dialyzed at a flow rate of 2.34 ml/min and samples were collected every 15 mins.

The dialysis probes were allowed to equilibrate for 1–2 hr prior to collection of the baseline samples. Baseline samples were then collected until three consecutive samples did not differ significantly. RTI 4793-14 (1–100 μM) was then administered focally via the dialysis probe for a 15-min sampling period at the end of which normal dialysis medium was dialysed through the probe. Sampling continued for a further three samples before application of the subsequent concentration of RTI-4793-14. In a subsequent group of animals, RTI-4793-14 was administered systemically (5 mg/kg; 5 mg/ml) via a femoral vein catheter.

The samples were analyzed for DA using HPLC with electrochemical detection. The mobile phase [75 mM NaH_2PO_4, 1.5 mM sodium dodecyl sulphate, 20 mM EDTA, 15% acetonitrile, and 12% methanol, pH 5.6] was filtered and degassed before pumping at a rate of 0.75 ml/min through a HR-80 column (3 m BDS, 80 × 4.6 mm). Electrochemical detection was performed using a Coulochem II with Guard cell: +350mV, Det 1: −75mV and Det 2: +220mV.

Data analysis

Inhibition curves were fit to the two parameter logistic equation for the best-fit estimates of the IC50 using nonlinear least squares curve fitting methods as previously described (Jones and Rogawski, 1992; Rothman et al., 1993).

In vivo microdialysis data were analyzed by using a one-way ANOVA and posthoc Dunnetts t-test. In the focal RTI experiments in which three drug concentrations were administered the baseline value for dopamine was defined as the sample preceding the application of each drug concentration. Analysis of any drug effect was therefore made by comparing with the baseline value prior to the drug administration.

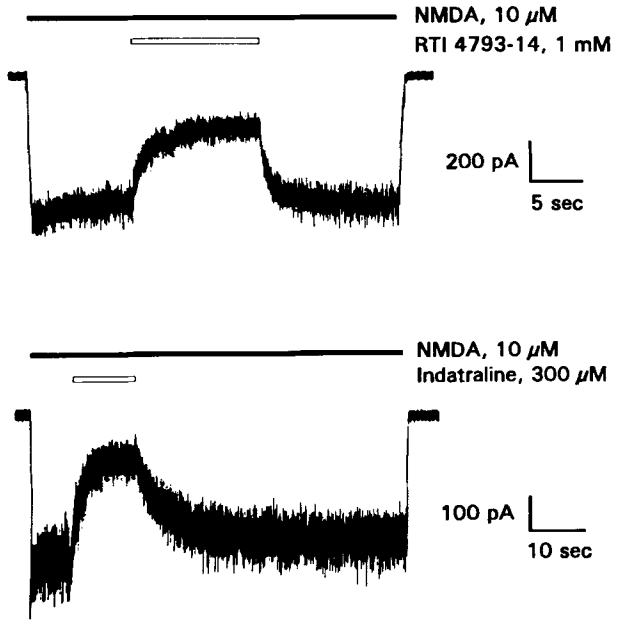
Chemicals

[^{3}H]TCP (40.8 Ci/mmol), [^{3}H]DA (47 Ci/mmol), [^{3}H]5-HT (28.2 Ci/mmol), [^{3}H](+)-MK801 (30 Ci/mmol), and [^{3}H]-CFT (80.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]Nisoxetine (SA = 82 Ci/mmol) was purchased from American Radiochemicals (St. Louis, MO). (+)-MK801 and indatraline were purchased from Research Biochemicals (Natick, MA). PCP was obtained from the NIDA Addiction Research Center pharmacy. RTI-4793-14 was synthesized as described (Blough et al., 1993; Carroll et al., in press). Frozen guinea pig brains were purchased from Pel-Freeze Laboratories (Rogers, AR). The sources of equipment and reagants required for the in vivo microdialysis studies are as published (Rothman et al., 1991). Rats were cared for according to NIH guidelines.

RESULTS

The IC_{50} values of RTI-4793-14, PCP, (+)-MK801, and indatraline in the binding assays are presented in Table I. As previously observed, PCP had moderate and about equal affinity for PCP site 1 and PCP site 2 (Rothman et al., 1989). Also as observed by others (Smith et al., 1977), PCP inhibited [^{3}H]DA (IC_{50}, 347 nM) and [^{3}H]5-HT (IC_{50}, 1424 nM) uptake with moderate potency. Consistent with its reported low affinity at the DA transporter labeled with [^{3}H]mazindol (Kuhar et al., 1990), PCP also had lower affinity at the [^{3}H]CFT binding site than would be predicted on the basis of its IC_{50} for inhibition of DA uptake. PCP also had very low affinity for the NE transporter as labeled by [^{3}H]nisoxetine. In contrast, (+)-MK801 had high affinity for PCP site 1 and negligible affinity for PCP site 2 and BAT-related measures. Consistent with its 25-fold greater potency at PCP site 1, (+)-MK801 has been shown to be much more potent than PCP in inhibiting NMDA-induced current responses (ffrench-Mullen and Rogawski, 1989; Jones and Rogawski, 1992).

As observed by others (Arnt et al., 1985; Hyttel and Larsen, 1985); indatraline potently inhibited [^{3}H]DA and [^{3}H]5-HT uptake, as well as [^{3}H]CFT binding to the DA transporter and [^{3}H]nisoxetine binding to the NE transporter. As is the case for other typical BAT ligands



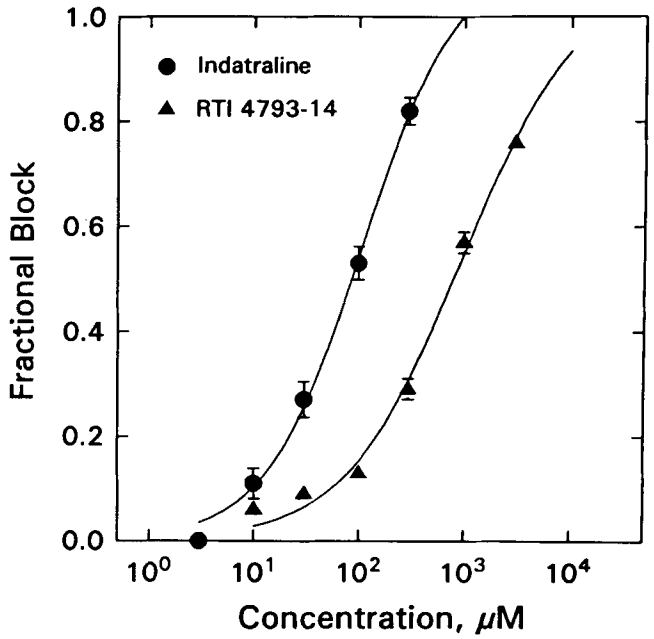


Fig. 1. Inhibition of NMDA-induced currents in voltage-clamped hippocampal neurons (holding potential, −60 mV) by RTI-4793-14 and indatraline. Traces on the **left** illustrate block of NMDA currents by 1 mM RTI-4793-14 (**top**) and 300 μM indatraline (**bottom**) (2 separate neurons). Concentration-response data from a series of similar experiments is shown to the **right**. Each point represents mean ± S.E.M. of steady-state fractional block values from 4–9 neurons. The data points were fit to a logistical function, $1/[1+(IC_{50}/[D])^n]$, where [D] is the concentration of drug and IC_{50} is the concentration of drug producing 50% block. The IC_{50} values are shown in Table I; the steepness parameter n was 0.83 and 0.96 for RTI-4793-14 and indatraline, respectively.

(Rothman et al., 1989; Akunne et al., 1991; Rothman et al., 1992), indatraline had low micromolar affinity for PCP site 2 (IC_{50}, 2.5 μM), and negligible affinity for PCP site 1 (IC_{50} > 100 μM). Consistent with its low affinity at PCP site 1, indatraline inhibited NMDA-induced current responses with low potency (Table I, Fig. 1).

RTI-4793-14 bound with high affinity to PCP site 2, and in addition, had moderate potency as an inhibitor of [^{3}H]DA uptake, [^{3}H]5-HT uptake, [^{3}H]CFT binding, and [^{3}H]nisoxetine binding. However, unlike PCP, RTI-4793-14 had low affinity for PCP site 1 (IC_{50} > 36 μM) and low potency as an inhibitor of NMDA-induced current responses (Table I, Fig. 1).

In vivo microdialysis experiments demonstrated that focal application of RTI-4793-14 via the microdialysis probe increased extracellular DA in a dose-dependent manner: 1 and 10 μM RTI-4793-14 increased extracellular DA by ~2- and 8-fold, respectively (Fig. 2). Additional experiments (Fig. 3) showed that intravenously administered RTI-4793-14 (5 mg/kg/i.v.) increased extracellular DA by ~2-fold.

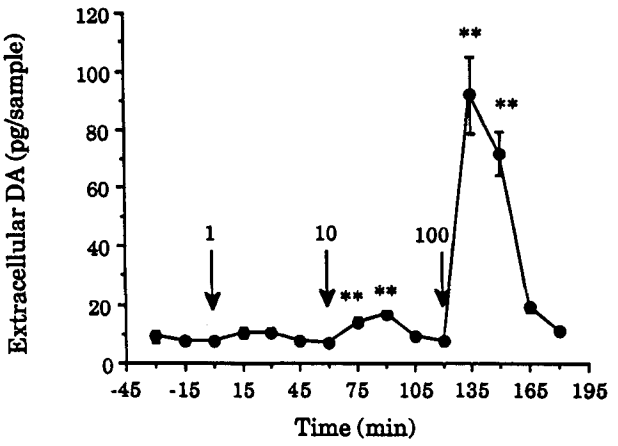


Fig. 2. Effects of focal application of RTI-4793-14 via the dialysis probe at concentrations of 1, 10, and 100 μM on extracellular dopamine in the nucleus accumbens. All values are mean ± SEM (n = 4), statistical analysis by one-way ANOVA with Dunnetts t-test. $^{**}P < 0.01$.

DISCUSSION

The results presented in this study demonstrate that RTI-4793-14 binds with high affinity and selectivity to the (+)-MK801-insensitive [^{3}H]TCP binding site (PCP site 2). Although most BAT ligands such as indatraline are selective for PCP site 2, they generally have low affinity for this site (K_i values > 1 μM). The only known exception is benztropine, which has a K_i value of 183 nM at PCP site 2 (Rothman et al., 1992). RTI-4793-14, which is at least 1,000-fold selective for PCP site 2 vs. PCP site 1, had the highest affinity for PCP site 2 of any of the drugs we tested and is the first ligand to be described that clearly distinguishes between the two PCP binding sites. Like PCP, which has moderate po-

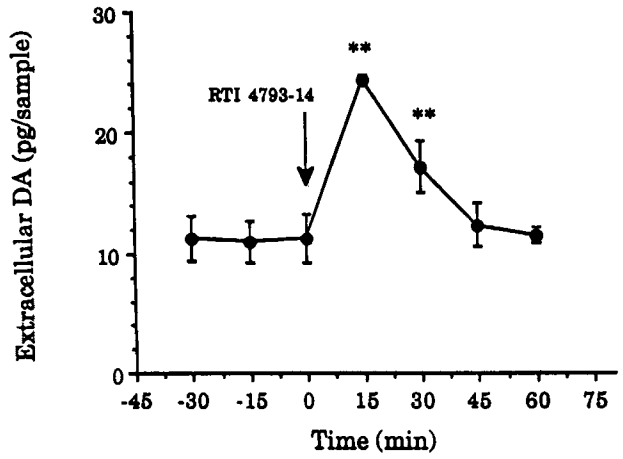


Fig. 3. Effects of systemic RTI-4793-14 (5 mg/kg, i.v.) on extracellular dopamine in the nucleus accumbens. All values are mean ± SEM (n = 3), statistical analysis by one-way ANOVA with Dunnetts posthoc t-test. $^{**}P < 0.01$.

tency in BAT-related measures, RTI-4793-14 inhibited [^{3}H]CFT binding and [^{3}H] 5-HT and [^{3}H]DA uptake with moderate potency, but unlike PCP was a very low potency inhibitor of NMDA-induced current responses. Thus, RTI-4793-14 has a pharmacological profile similar to that of PCP, except that it is largely devoid of PCP site 1 activity and has considerably higher affinity at the NE transporter.

In accord with previous reports (Arnt et al., 1985; Hyttel and Larsen, 1985); indatraline was a potent inhibitor of [^{3}H]CFT binding, [^{3}H]nisoxetine binding and [^{3}H]5-HT and [^{3}H]DA uptake (Rothman et al., 1992). However, as is typical of other previously investigated BAT ligands, the drug bound relatively weakly to PCP site 2. Although they both interact with biogenic amine transporters, RTI-4793-14 was distinguished from indatraline in having substantially higher binding affinity for PCP site 2.

DA uptake blockers, including PCP (Carboni et al., 1989) and indatraline (Hurd and Ungerstedt, 1989b), are known to increase extracellular DA levels. Consistent with its in vitro profile as a BAT inhibitor, RTI-4793-14 increased extracellular DA upon local or systemic administration in rats. The magnitude of the effect obtained with a 5 mg/kg IV dose was similar to that previously observed with a 1 mg/kg IV dose of cocaine, a potent DA uptake blocker (Hurd and Ungerstedt, 1989a). Thus, RTI-4793-14 acts in vivo as well as in vitro as a biogenic amine transport blocker.

The data presented here suggest that DA uptake inhibitors may fall into two classes: (1) classical BAT ligands, which exhibit high affinity Na^+-dependent binding, potent inhibition of [^{3}H]DA uptake, and weak but selective binding to PCP site 2, and (2) PCP site 2 ligands, which have Na^+-inhibited high affinity for PCP site 2, moderate potency for inhibiting [^{3}H]DA uptake, and moderate potency at the classical BAT binding site. It is of interest to note that the phencyclidine derivative BTCP, which potently inhibits DA uptake in vitro (Vignon et al., 1988) and in vivo (Maurice et al., 1992), falls into the category of classical BAT ligands. [^{3}H]BTCP exhibits high affinity Na^+-dependent binding to the DA transporter both in vitro (Vignon et al., 1988; Cerruti et al., 1991) and in vivo (Maurice et al., 1989). Like indatraline, it has low affinity and high selectivity for PCP site 2 (Rothman et al., 1989). Consistent with this neurochemical profile, BTCP has cocaine-like, not PCP-like, behavioral effects (Koek et al., 1989). Thus, although BTCP is chemically derived from PCP, it has the characteristics of a classical BAT ligand rather than a PCP site 2 ligand.

The results of the present study demonstrate that RTI-4793-14 is a potent and selective ligand for PCP site 2 and that it also has activity as a biogenic amine transport blocker. Unlike classical BAT ligands such as indatraline, but similar to PCP, RTI-4793-14 has only moderate activity as an uptake blocker. Nevertheless, RTI-4793-14 and PCP bind with high affinity to PCP site 2 and, as noted in the Introduction, there is substantial evidence linking this site with biogenic amine transporters. Thus, RTI-4793-14 may bind to a site on biogenic amine transporters that is distinct from the binding site for classical BAT ligands. If this is the case, RTI-4793-14 could represent the first of a novel class of ligands that bind with high affinity to biogenic amine transporters but are relatively less potent amine reuptake blockers than classical BAT ligands.

The high affinity and selectivity of RTI-4793-14 for PCP site 2 suggest that it or an analog may be useful in radioligand binding studies to label this site with greater precision than is currently possible. It should be possible to test the hypothesis that PCP site 2 is associated with biogenic amine transporters using the recently cloned DA and 5-HT transporters (Blakely et al., 1991; Kilty et al., 1991; Shimada et al., 1991). If the association is confirmed, RTI-4793-14 may represent a useful new tool for investigating biogenic amine transporters.

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REFERENCES

Akunne, H.C., Reid, A.A., Thurkauf, A., Jacobson, A.E., de Costa, B.R., Rice, K.C., Heyes, M.P., and Rothman, R.B. (1991) [^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine labels two high affinity binding sites in human cortex: Further evidence for phencyclidine binding sites associated with the biogenic amine reuptake complex. Synapse, 8:289–300.

Akunne, H.C., Johannessen, J.N., de Costa, B.R., Rice, K.C., and Rothman, R.B. (1992) MPTP lesions of the nigrostriatal dopaminergic projection decrease [^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine binding to PCP site 2: Further evidence that PCP site 2 is associated with the biogenic amine reuptake complex. Neurochem. Res., 17: 261–264.

Arnt, J., Christensen, A.V., and Hyttel, J. (1985) Pharmacology in vivo of the phenylindan derivative, LU 19-005, a new potent inhibitor of dopamine, noradrenaline and 5-hydroxytryptamine uptake in rat brain. Naunyn Schmiedeberg's Arch. Pharmacol., 329:101–107.

Blakely, R.D., Berson, H., Fremean, R.T. Jr., Caron, M.G., Peek, M.M., Prince, H.K., and Bradley, C.C. (1991) Cloning and expression of a functional serotonin transporter from rat brain. Nature, 354:66–70.

Blough, B.E., Mascarella, S.W., Rothman, R.B., and Carroll, F.I. (1993) Synthesis of 3-Halomethyl-2,3,4,5-tetrahydro-2,5-methano-1H-2-benzazepines and their rearrangement to 4-Halo-1,2,3,4,5,6-hexahydro-2,6-methano-2-benzocines. J. Chem. Soc. Chem. Comm., 758–760.

Carboni, E., Imperato, A., Perezzani, L., and Di Chiara, G. (1989) Amphetamine, cocaine, phencyclidine and nomifensine increase extracellular dopamine concentrations preferentially in the nucleus accumbens of freely moving rats. Neuroscience, 28:653–661.

Carroll, F.I., Blough, B., Mascarella, S.W., Xu, H., Goodman, C.G., and Rothman, R.B. (in press) Synthesis and ligand-binding at PCP site 1 and site 2 for hexahydro-2-substituted-1-methylindeno[1,2-b]pyrroles. Med. Chem. Res.

Cerruti, C., Kamenka, J.M., and Privat, A. (1991) Localization of dopamine uptake complex by [^{3}H]BTCP on rat brain sections and dopaminergic neurons in-vitro. In: Advances in the Biosciences, Vol. 82. Presynaptic Receptors and Neuronal Transporters. S.Z. Langer, A.M. Galzin, and J. Costentin, eds. Satellite Symposium to the Iuphar (International Union of Pharmacology) Congress, Rouen, 1991. pp. 165–166.

ffrench-Mullen, J.M., and Rogawski, M.A. (1989) Interaction of phencyclidine with voltage-dependent potassium channels in cultured rat hippocampal neurons: Comparison with block of the NMDA receptor-ionophore complex. J. Neurosci., 9:4051–4061.

Hurd, Y.L., and Ungerstedt, U. (1989a) Cocaine: an in vivo microdialysis evaluation of its acute action on dopamine transmission in rat striatum. Synapse, 3:48–54.

Hurd, Y.L., and Ungerstedt, U. (1989b) Ca^{2+} dependence of the amphetamine, nomifensine, and Lu 19-005 effect on in vivo dopamine transmission. Eur. J. Pharmacol., 166:261–269.

Hyttel, J., and Larsen, J.J. (1985) Neurochemical profile of LU 19-005: A potent inhibitor of uptake of dopamine, noradrenaline and serotonin. J. Neurochem., 44:1615–1620.

Jones, S.M., and Rogawski, M.A. (1992) The anticonvulsant (±)-5-aminocarbonyl-10,11-dihydro-5H-dibenz[a,d]cyclohepten-5,10-imine (ADCI) selectively blocks NMDA-activated current in cultured rat hippocampal neurons: kinetic analysis and comparison with dizocilpine. Mol. Neuropharmacol., 2:303–310.

Kitty, J.E., Lorang, D., and Amara, S.G. (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. Science, 254:578–579.

Koek, W., Colpaert, F.C., Woods, J.H., and Kamenka, J.M. (1989) The phencyclidine (PCP) analog N-[1-(2-benzo[b]thiophenyl)cyclohexyl]piperidine shares cocaine-like but not other characteristic behavioral effects with PCP, ketamine and MK-801. J. Pharmacol. Exp. Ther., 250:1019–1027.

Kuhar, M.J., Boja, J.W., and Cone, E.J. (1990) Phencyclidine binding to striatal cocaine receptors. Neuropharmacology, 29:295–297.

Lowry, O.H., Rosenbrough, N.H., Farr, A.L., and Randall, P.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265–275.

Madras, B.K., Spealman, R.D., Fahey, M.A., Neumeyer, J.L., Saha, J.K., and Milius, R.A. (1989) Cocaine receptors labeled by [^{3}H]2β-carbomethoxy-3-β-(4-fluorophenyl)tropane. Mol. Pharmacol., 36:518–524.

Maurice, T., Vignon, J., Kamenka, J.M., and Chicheportiche, R. (1989) In vivo labelling of the mouse dopamine uptake complex with the phencyclidine derivative [^{3}H]BTCP. Neurosci. Lett., 101:234–238.

Maurice, T., Roman, F.J., Pascaud, X., Kamenka, J.M., and Junien, J.L. (1992) Regional differences in the effect of N-{1-(2-benzo(b)thienyl)cyclohexyl}piperidine BTCP on extracellular dopamine levels an in-vivo microdialysis study. Neurosci. Lett., 138:63–66.

Paxinos, G. The Rat Brain in Stereotaxic Coordinates. New York: Academic Press, 1982.

Rothman, R.B., Reid, A.A., Monn, J.A., Jacobson, A.E., and Rice, K.C. (1989) The psychotomimetic drug phencyclidine labels two high affinity binding sites in guinea pig brain: Evidence for N-methyl-D-aspartate-coupled and dopamine reuptake carrier-associated phencyclidine binding sites. Mol. Pharmacol., 36:887–896.

Rothman, R.B., Mele, A., Reid, A.A., Akunne, H.C., Greig, N., Thurkauf, A., de Costa, B.R., Rice, K.C., and Pert, A. (1991) GBR12909 antagonizes the ability of cocaine to elevate extracellular levels of dopamine. Pharmacol. Biochem. Behav., 40:387–397.

Rothman, R.B., Reid, A.A., Silverthorn, M., de Costa, B.R., Monn, J.A., Thurkauf, A., Jacobson, A.E., Rice, K.C., Rogawski, M.A. Structure-activity studies on the interaction of biogenic amine reuptake inhibitors and potassium channel blockers with MK-801-sensitive (PCP site 1) and -insensitive (PCP site 2) [^{3}H]TCP binding sites in guinea pig brain. In: Multiple Sigma and PCP Receptor Ligands: Mechanisms for Neuromodulation and Protection. E.F. Domino and J.-M. Kamenka, eds. Ann Arbor, MI: NPP Books; 1992:137–146.

Rothman, R.B., Lewis, B., Dersch, C.M., Xu, H., Radesca, L., de Costa, B.R., Rice, K.C., Kilburn, R.B., Akunne, H.C., and Pert, A. (1993) Identification of a GBR12935 homolog, LR1111, which is over 4000-fold selective for the dopamine transporter, relative to serotonin and norepinephrine transporters. Synapse, 14:34–39.

Shimada, S., Kitayama, S., Lin, C.-L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M., and Uhl, G. (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science, 254:576–578.

Smith, R.C., Meltzer, H.Y., Arora, R.C., and Davis, J.M. (1977) Effects of phencyclidine on [^{3}H]catecholamine and [^{3}H]serotonin uptake in synaptosomal preparations from rat brain. Biochem. Pharmacol., 26:1435–1439.

Snell, L.D., Yi, S.J., and Johnson, K.M. (1988) Comparisons of the effects of MK801 and phencyclidine on catecholamine uptake and NMDA-induced norepinephrine release. Eur. J. Pharmacol., 145:223–226.

Subramaniam, S., Donevan, S.D., and Rogawski, M.A. (1992) 1,10-Diaminodecane and 1,12-diaminododecane block NMDA receptor currents by an open channel mechanism. Neurosci. Lett., 147:213–216.

Tejani-Butt, S.M., Brunswick, D.J., and Frazer, A. (1990) [^{3}H]Nisoxetine: A new radioligand for the norepinephrine uptake sites in brain. Eur. J. Pharmacol., 191:239–243.

Vignon, J., Pinet, V., Cerruti, C., Kamenka, J-M., and Chicheportiche, R. (1988) [^{3}H]-[1-(2-benzo[b]thiophenyl)cyclohexyl]piperidine ([^{3}H]BTCP): A new phencyclidine analog selective for the dopamine uptake complex. Eur. J. Pharmacol., 148:427–436.