MOLECULAR PHARMACOLOGY, 27:193–199

A Photoaffinity Ligand for Dopamine D_2 Receptors: Azidoclebopride

H. B. NIZNIK, J. H. GUAN, J. L. NEUMEYER, AND P. SEEMAN

Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada M5S 1A8 (H.B.N., P.S.), Section of Medicinal Chemistry, College of Pharmacy and Allied Health Professions, Northeastern University, Boston, Massachusetts 12115 (J.L.N.), and Research Biochemicals Inc., Wayland, Massachusetts 01778 (J.H.G.)

Received August 2, 1984; Accepted November 28, 1984

SUMMARY

In order to label D_2 dopamine receptors selectively and covalently by means of a photosensitive compound, azidoclebopride was synthesized directly from clebopride. The dissociation constant (K_D) of clebopride for the D_2 dopamine receptor (canine brain striatum) was 1.5 nM, while that for azidoclebopride was 21 nM. The affinities of both clebopride and azidoclebopride were markedly reduced in the absence of sodium chloride. In the presence of ultraviolet light, azidoclebopride inactivated D_2 dopamine receptors irreversibly, as indicated by the inability of the receptors to bind [^{3}H]spiperone. Maximal photoinactivation of about 60% of the D_2 dopamine receptors occurred at 1 μM azidoclebopride; 30% of the receptors were inactivated at 80 nM azidoclebopride (pseudo-IC_{50}). Dopamine agonists selectively protected the D_2 receptors from being inactivated by azidoclebopride, the order of potency being (−)-*N*-*n*-propylnorapomorphine > apomorphine > (±)-6,7-dihydroxy-2-aminotetralin > (+)-*N*-*n*-propylnorapomorphine > dopamine > noradrenaline > serotonin. Similarly, dopaminergic antagonists prevented the photoinactivation of D_2 receptors by azidoclebopride with the following order of potency: spiperone > (+)-butaclamol > haloperidol > clebopride > (−)-sulpiride > (−)-butaclamol. The degree of D_2 dopamine receptor photoinduced inactivation by azidoclebopride was not significantly affected by scavengers such as *p*-aminobenzoic acid and dithiothreitol. Furthermore, irradiation of striatal membranes with a concentration of azidoclebopride sufficient to inactivate dopamine D_2 receptors by 60% did not significantly reduce dopamine D_1, serotonin (S_2), benzodiazepine, α_1- or β-noradrenergic receptors. This study describes the use of a novel and selective photoaffinity ligand for brain dopamine D_2 receptors. The molecule, in radiolabeled form, may aid in the molecular characterization of these receptors.

INTRODUCTION

Two distinct types of receptors for dopamine, termed D_1 and D_2 receptors, exist in brain striatum (1–4). The D_1 receptors stimulate adenylate cyclase and they are virtually insensitive to substituted benzamide neuroleptics (e.g., sulpiride). D_2 dopamine receptors, however, inhibit adenylate cyclase activity (5, 6) and have a picomolar or nanomolar affinity for all neuroleptics including the benzamides. The D_2 dopamine/neuroleptic receptor has many functional correlates with a number of dopaminergic behaviors, including rotation, locomotion, emesis, and stereotypy (1).

While considerable progress has been made in the molecular characterization of β-adrenoceptors (7), attempts to isolate dopamine D_2 receptors (8) have been hampered by the lack of specific and suitable ligands. Irreversible ligands, such as *N*-chloroethylapomorphine (9–13), phenoxybenzamine (14, 15), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (16), and flupenthixyl chloride (17, 18), have been too low in affinity and/or selectivity for the receptor to be of value in dopamine D_2 receptor isolation and purification. Similarly, attempts to purify dopamine D_2 receptors selectively by affinity chromatography (19), preparative isoelectric focusing (20), and immunoaffinity chromatography (21) have resulted in only low enrichment of receptors.

The development of photoaffinity probes as covalent labels for both the α- and β-adrenergic receptors (7, 22, 23) has served to elucidate the molecular structure of these receptors. Although suitable photoaffinity probes for brain dopamine receptors have not previously been developed, attempts have been made to photolabel dopamine receptors with existing ligands, such as dopamine and chlorpromazine (24–26). There is no convincing evidence, however, that these photolabeling interactions

This work was supported by the Ontario Mental Health Foundation, the Medical Research Council of Canada, Research Biochemicals Incorporated, and by National Institutes of Health Research Grant NS 15439 (J. L. N.) at Northeastern University. H. B. N. is a recipient of a Research Studentship from the Ontario Mental Health Foundation.

0026-895X/85/020193-07$02.00/0

correspond specifically with either dopamine D_1 or D_2 receptors (27, 28).

In order to obtain a selective photoaffinity probe for brain dopamine D_2 receptors, we prepared a photolabile derivative (azidoclebopride) of the substituted benzamide clebopride. We describe here the ability of azidoclebopride to photolabel selectively brain dopamine D_2 receptors. A preliminary report of this work has appeared elsewhere (29).

MATERIALS AND METHODS

Drugs. [^{3}H]Spiperone (21–23 Ci/mmol), [^{3}H]flupenthixol (10.8 Ci/mmol), [^{3}H]ketanserin (64.6 Ci/mmol), [^{3}H]prazosin (28 Ci/mmol), [^{3}H]rauwolscine (84.4 Ci/mmol), [^{3}H]dihydroalprenolol (105 Ci/mmol), and [^{3}H]flunitrazepam (84 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

Drugs were generously donated by the following pharmaceutical firms: Ayerst Research Laboratories (Montreal, Quebec), (+)-butaclamol; CIBA-Geigy Corporation, (Dorval, Quebec), phentolamine hydrochloride; Hoffmann-LaRoche (Vaudreuil, Quebec), clonazepam; Imperial Chemical Industry, (U. K.), propranolol; Janssen Pharmaceutica (Beerse, Belgium), spiperone, haloperidol; Merck Frosst Laboratories (Dorval, Quebec), (−)-apomorphine; Schering Corporation (Bloomfield, NJ), SCH-23390; E. R. Squibb and Sons Incorporated (Princeton, NJ), cinanserin; Ravizza (Milan, Italy), (*S*)-sulpiride.

We thank Dr. R. Spickett and Labortorios Almirall, Barcelona, for their generous donation of clebopride (*N*-(*N*-benzyl-4-piperidinyl)-4-amino-5-chloro-2-methoxybenzamide).

(±)-6,7-ADTN,[1] (−)-butaclamol, and (−)- and (+)-NPA were purchased from Research Biochemicals (Wayland, MA). Dopamine hydrochloride, (−)-noradrenaline hydrochloride, serotonin hydrochloride, *p*-aminobenzoic acid, and dithiothreitol were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals and reagents were purchased from either Sigma Chemical Co. or Fisher Scientific Company (Pittsburgh, PA).

Preparation of N-(1′-benzyl-4′-piperidyl)-2-methoxy-4-azido-5-chlorobenzamide (azidoclebopride). Clebopride (190 mg/0.51 mmol) was dissolved in 1.2 ml of concentrated hydrochloric acid and 2 ml of water, cooled to 0–5°, sodium nitrate (365 mg) in 1.3 ml of water was added, and the reaction mixture was stirred for 0.5 hr at 0–5°. Sodium azide (325 mg) in 1.3 ml of water was then added. The solid that precipitated out of solution was recrystallized from 0.8 ml of absolute ethanol to give 80 mg (42%) of azidoclebopride (m.p. 200–201° dec.) (mass spectra, *m/z* 399; analysis calculated for $C_{20}H_{22}ClN_5O_2$: C 53.46; H 5.79; N 15.59; found: C 53.73; H 5.42; N 15.79). The structure of clebopride and azidoclebopride are illustrated in Fig. 1.

Membrane preparation. Striata were dissected from frozen canine brains (Pel-Freeze Biologicals, Rogers, AR) and homogenized (Brinkmann Polytron; PT-10, setting 7) for 20 sec in 20 ml of 50 mM Tris-HCl buffer containing: 1 mM EDTA-acid, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $mgCl_2 \cdot 6H_2O$ and 120 mM NaCl (when present), pH 7.7 at 22°.

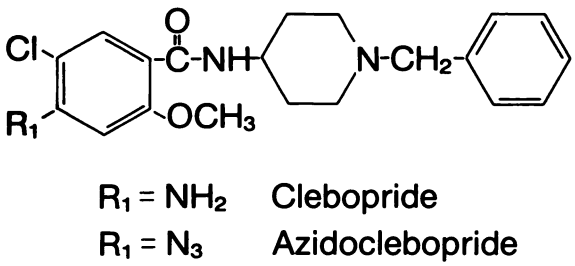

FIG. 1. *Structure of clebopride and azidoclebopride*

[1] The abbreviations used are: ADTN: (±)-2-amino-6,7-dihydroxy-1,2,3,4,-tetrahydronaphthalene; NPA: *N*-*n*-propylnorapomorphine; *hv*, light.

Homogenates were centrifuged (0-4°) for 30 min at 49,000 × *g*; the resulting pellets were resuspended in 20 ml of buffer and recentrifuged. The final pellets were resuspended in buffer to yield a tissue concentration (original wet weight) of 4.5 mg/ml (or 15 mg/ml for irradiation experiments), homogenized for an additional 10 sec, and preincubated for 10 min at 37°.

[^{3}H]Spiperone-binding assay. For competition experiments, assays were initiated by the addition of 0.5 ml of cooled membrane suspension (2.25 mg of tissue) to a mixture of 0.25 ml of 50 mM Tris-HCl buffer, as above, with or without 120 mM NaCl (pH 7.4 at 22°), 0.25 ml of [^{3}H] spiperone (80–100 pM, final concentration) and 0.5 ml of buffer or competing drug. Mixtures were incubated in triplicate for 120 min (22°) under subdued light. Incubations were terminated by rapid filtration under vacuum (450–550 mm Hg) through a Titertek cell harvester (Skatron; Sterling, VA) using glass fiber filter mats (Skatron). The filters were rinsed for 15 sec (approximately 10 ml) with 50 mM Tris-HCl buffer, pH 7.4 (at 22°), placed in plastic miniscintillation vials, equilibrated with 4 ml of Scint-A (Packard, Chicago, IL) scintillation fluid for at least 12 hr by shaking, and monitored for tritium in a Packard 460 C scintillation spectrometer with 43% efficiency.

For saturation experiments, aliquots of tissue homogenates (0.5 ml) were incubated in triplicate with 15 concentrations of [^{3}H]spiperone (10–2000 pM) for 45 min at 37° in a total volume of 1.5 ml of the Tris buffer described above, at pH 7.4. Incubations were terminated by rapid filtration, as previously described. For all experiments, the specific binding of [^{3}H]spiperone was defined as that binding which was inhibited by 1 μM (+)-butaclamol.

Data from both competition and saturation experiments were analyzed by the nonlinear least squares curve-fitting computer program LIGAND (written by Munson and Rodbard of National Institutes of Health) as previously described (30).

Other ^{3}H-ligand-binding assays. [^{3}H]Ketanserin (1 nM), [^{3}H]prazosin (0.9 nM), [^{3}H]rauwolscine (2.75 nM), and [^{3}H]dihydroalprenolol (5.0 nM, final concentration) binding to striatal serotonergic (S_2), α_1-, α_2-, and β-adrenergic receptors, respectively, were assayed in quadruplicate (30-min incubation at 37°) under conditions identical to those described for [^{3}H]spiperone.

[^{3}H]Flunitrazepam (1 nM, final concentration) binding to striatal benzodiazepine receptors was measured in quadruplicate in Tris buffer (as above) in the presence of 100 μM γ-aminobutyric acid. Assay mixtures were incubated for 90 min at 0–4°.

[^{3}H]Flupenthixol (0.7 nM, final concentration) binding was measured in quadruplicate (30 min at 37°) in the presence of 30 nM spiperone to preclude this D_1 ^{3}H-ligand from labeling D_2 receptors.

Nonspecific binding for [^{3}H]ketanserin, [^{3}H]prazosin and [^{3}H]rauwolscine, [^{3}H]dihydroalprenolol, [^{3}H]flunitrazepam, and [^{3}H]flupenthixol were defined by 1 μM (final concentration) cinanserin, phentolamine, (−)-propranolol, clonazepam, and (+)-butaclamol, respectively.

Photolysis. Aliquots of cooled tissue suspension were incubated in the dark with azidoclebopride (1 μM, final concentration, unless stated otherwise) for 60–90 min at 22° in the absence or presence of protecting drugs. For photolysis, a 15-ml suspension was placed in an uncovered plastic Petri dish (100 × 13 mm, Fisher) 11 cm away from a light source (85-W Hg lamp: Gates, Thomas Co., Philadelphia) and irradiated for 30 sec without stirring (fluid depth, 3–4 mm). In preliminary experiments, photolysis was also obtained with a hand-held Mineralight UVS-11 (254 nm). Aliquots (10 ml) of the irradiated tissue were placed in aluminum foil-covered centrifuge tubes and centrifuged for 15 min at 49,000 × *g* (0–4°). Pellets were resuspended in 15–20 ml of Tris buffer (as above) containing 0.5% bovine serum albumin, incubated for 10 min at 37°, and recentrifuged. This washing and incubation procedure was repeated one additional time followed by resuspension and centrifugation in buffer containing 0.25% bovine serum albumin. The albumin was removed from the pellets by resuspension and recentrifugation in 20 ml of fresh buffer. Finally, the resulting pellets were resuspended in buffer to yield a tissue concentration (original wet weight) of 4.5 mg/ml and assayed for D_2 receptors with [^{3}H]spiperone (1 nM, final

concentration, or 0.01–2.0 nM for Scatchard analysis) as described above.

RESULTS

Competition of [^{3}H]spiperone binding by azidoclebopride. In competition binding experiments with [^{3}H]spiperone (80–100 pM), both clebopride and azidoclebopride were found to bind (in the presence of 120 mM sodium chloride) with high affinity to D_2 receptors, the dissociation constants (K_D) being 1.5 and 21 nM, respectively (Fig. 2). As with all the substituted benzamides, the affinity of these drugs was markedly dependent on the presence of sodium ions. Thus, as shown in Fig. 2, the K_D of both clebopride and azidoclebopride was increased by a factor of 25 in the absence of physiological concentrations of sodium chloride (to 48 and 476 nM, respectively).

Photoinduced inactivation of [^{3}H]spiperone binding by azidoclebopride. Concentration effect curves for the photoinactivation of striatal dopamine D_2 receptors by azidoclebopride are in Fig. 3. Irradiation of brain membranes for 30 sec in the presence of varying concentrations of azidoclebopride, followed by extensive tissue washing, reduced the specific binding of [^{3}H]spiperone (1 nM) in a concentration-dependent fashion.

Preliminary characterization of the continuous UV absorption spectra of azidoclebopride (in Tris-ion buffer) revealed that the characteristic shoulder observed at 225–230 nm was no longer present or was greatly diminished after irradiation for 30 sec, indicating that the azido group was inactivated at this time period (data not shown).

The duration of *hv* exposure was chosen to maximize the photolysis-induced inactivation of dopamine D_2 receptors by azidoclebopride without loss of D_2 receptor activity due to *hv* exposure alone (data not shown).

In the presence of 120 mM NaCl, photoinduced inactivation of D_2 receptors by azidoclebopride proceeded with a pseudo-IC_{50} of 80 nM and corresponded with the IC_{50} value obtained in reversible competition experiments (see Fig. 2). In the absence of sodium ions, the ability of azidoclebopride to photoinactivate D_2 receptor activity was markedly reduced to a pseudo-IC_{50} value of 4000 nM (Fig. 3). This value was in close agreement with the IC_{50} value obtained in competition experiments with [^{3}H]spiperone in the absence of NaCl (see Fig. 2).

Maximal photoinactivation was attained with 1 μM azidoclebopride and typically represented a loss of 60% of the total number of sites labeled by [^{3}H]spiperone. Reducing the tissue concentration to 7 mg/ml buffer (from 13 mg/ml) during irradiation did not significantly improve the efficiency of photoinactivation (data not shown).

Maximal photoinactivation of striatal dopamine D_2 receptors with azidoclebopride (1 μM) reduced the B_{max} for [^{3}H]spiperone binding by 55–60% without significantly affecting the K_D of [^{3}H]spiperone for the receptor (Table 1). Preincubation of brain membranes with azidoclebopride without irradiation did not result in any loss of [^{3}H]spiperone-binding activity. In addition, as shown in Table 1, membranes which were incubated with both azidoclebopride and clebopride and then subjected to photolysis showed no loss of [^{3}H]spiperone binding. Other control preparations were carried out and are described in the figure legends.

Dopamingeric protection of [^{3}H]spiperone binding from photoinactivation by azidoclebopride. The ability of various dopamine agonists and antagonists to protect against *maximal* photoinactivation by azidoclebopride is illustrated in Fig. 4. Incubation of brain membranes with varying concentrations of dopamine agonists and antagonists clearly protected the specific photoinactivation of D_2 receptors by azidoclebopride. Protection was both stereoselective and concentration-dependent. Agonist protection displayed a dopamine D_2 receptor profile with the following rank order of potency: (−)-NPA > (−)apomorphine > (±)-ADTN > (+)-NPA > dopamine > noradrenaline > serotonin.

Similarly, dopaminergic antagonists prevented the photoinactivation of dopamine D_2 receptors with the following order of potency: spiperone > (+)-butaclamol > haloperidol > clebopride > (−)-sulpiride > (+)-butaclamol. The dopamine D_1 selective receptor antagonist SCH-23390 was virtually without protective effects. The receptor protection afforded by a variety of dopaminergic agonists and antgonists clearly suggests that the photoinactivation by azidoclebopride occurred via a receptor-specific mechanism.

Specificity of photoinactivation by azidoclebopride. The selectivity of azidoclebopride for dopamine D_2 receptors following irradiation was investigated. As shown in Fig. 5, preincubation of brain membranes with a concentration of azidoclebopride (1 μM) sufficient to photoinactivate 60% of the total number of [^{3}H]spiperone-binding sites did not significantly reduce striatal dopamine D_1, serotonin S_2, benzodiazepine, or α_1- or β-adrenergic receptors under the assay conditions used in the present experiments. A consistent reduction, however, in striatal [^{3}H]rauwolscine binding to α_2-adrenoceptors was observed (control: 6.15 fmol/mg of tissue; photolysed: 4.8 fmol/mg of tissue; $n = 5$, $p > 0.05$ as determined by Student's two-tailed t test for independent samples).

Mechanism of photoaffinity labeling of dopamine D_2 receptor by azidoclebopride. The ability of azidoclebopride to photoinactivate D_2 receptors was largely dependent on the integrity of the azido moiety and the generation of short-lived reactive intermediates within the vicinity of the receptor during photolysis. As shown in Fig. 6, preillumination of a 15 μM solution of azidoclebopride in Tris-ion buffer for various time periods (in the absence of receptors) severely limited the subsequent photoinactivation of D_2 receptors, suggesting that aryl nitrene intermediates generated during photolysis were scavenged by the surrounding solvent.

The mechanism of photoaffinity labeling by azidoclebopride was also investigated in the presence of the scavengers *p*-aminobenzoic acid and dithiothreitol. Aliquots of striatal membranes were exposed to either azidoclebopride (1 μM), *p*-aminobenzoic acid (1 mM), or dithiothreitol (1 mM) for 90 min (at 22°), photolysed, and extensively washed as described. Subsequent [^{3}H]spiperone binding revealed that *p*-aminobenzoic acid or dithiothreitol alone did not inactivate D_2 receptors (Ta-

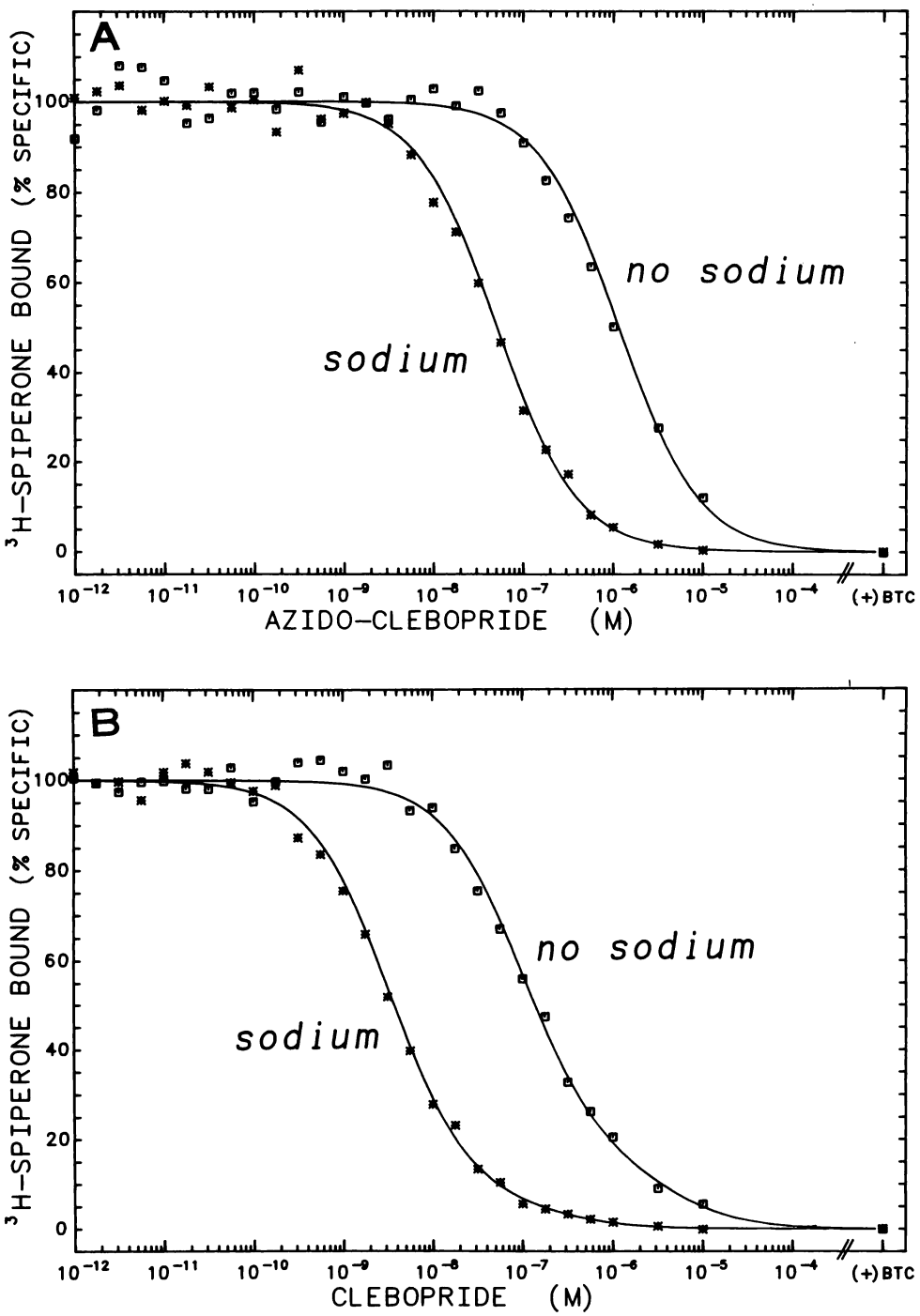

FIG. 2. *Competition of [^{3}H]spiperone binding to striatal dopamine D_2 receptors by azidoclebopride and clebopride*

Canine striatal membranes were incubated under reduced light, with varying concentrations of competing ligand and 80–100 pM [^{3}H]spiperone for 120 min at 22° in the presence or absence of 120 mM NaCl. The K_D values for [^{3}H]spiperone (80 pM) were determined in a separate series of experiments by Scatchard analysis. Data were analyzed by the nonlinear least squares curve-fitting program LIGAND. Each experimental point is the mean of triplicate determinations with standard errors of less than 4% and is representative of two such independent experiments.

ble 2). More significantly, however, both scavengers did not prevent the photoinactivation of dopamine D_2 receptors by azidoclebopride.

DISCUSSION

These experiments suggest that azidoclebopride binds specifically and with high affinity to striatal dopamine D_2 receptors, and that upon photoinactivation covalent interactions with the receptor are formed.

First, in reversible competition experiments, azidoclebopride inhibited the binding of [^{3}H]spiperone to dopamine D_2 receptors with a K_i value of 21 and 476 nM in the presence or absence of sodium chloride, respectively. The affinity of azidoclebopride for dopamine D_2 receptors was approximately 10-fold lower than the parent com-

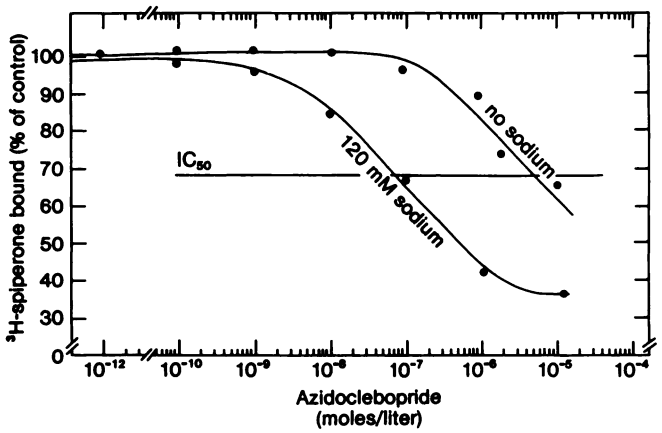

FIG. 3. *Concentration effect curves for the photoinactivation of striatal dopamine D_2 receptors by azidoclebopride*

Aliquots of tissue suspension (15 ml) were incubated under reduced light with increasing concentrations of azidoclebopride (10^{-12}–10^{-5} M) for 90 min at 22° in the presence or absence of 120 mM sodium chloride. The mixtures were then placed in uncovered plastic Petri dishes, photolysed for 30 sec, and extensively washed to remove noncovalently bound ligand as described in Materials and Methods. D_2 receptor activity remaining after photolysis was measured using [^{3}H]spiperone (1 nM, final concentration) as described in the text. Control tissues were irradiated (in the presence or absence of 1×10^{-5} M clebopride), washed, and assayed for [^{3}H]spiperone as described above. Each point represents the mean of quadruplicate determinations with standard error of less than 5%.

TABLE 1

Irreversible blockade of dopamine D_2 receptors by azidoclebopride

Striatal membranes were incubated for 90 min at 22°, in the absence or presence of acidoclebopride and protecting drug as indicated, photolysed for 30 sec, and extensively washed to remove both noncovalently bound azidoclebopride and protecting agent. Aliquots of washed brain membranes were incubated with 15 concentrations of [^{3}H]spiperone (10–2000 pM) for 45 min at 37° and assayed for accessible dopamine D_2 receptor sites. B_{max} (pmol/g original wet weight) and K_D (pM) values (±SE) were estimated by the nonlinear least squares computer program LIGAND.

Treatment/addition	Dopamine D_2 receptor concentration	Receptor affinity K_D
	pmol/g tissue	*pM*
1. Nonirradiated	18 ± 0.72	98 ± 14
2. Irradiated	17 ± 1.0	100 ± 13
3. Irradiated in presence of azidoclebopride (1 μM)	7 ± 0.40	110 ± 19
4. Irradiated in presence of azidoclebopride (1 μM) and clebopride (2 μM)	17 ± 0.86	99 ± 13
5. Nonirradiated in presence of azidoclebopride (1 μM) and clebopride (2 μM)	19 ± 1.0	113 ± 11

pound and probably reflects the decreased lipophilic nature of the molecule. Furthermore, azidoclebopride behaved as a classical substituted benzamide in that sodium chloride was essential for high affinity binding to D_2 dopamine receptors.

Second, evidence for the covalent interaction of azidoclebopride following photolysis was shown by the fact that [^{3}H]spiperone-binding activity was not restored to control values even after extensive membrane washing.

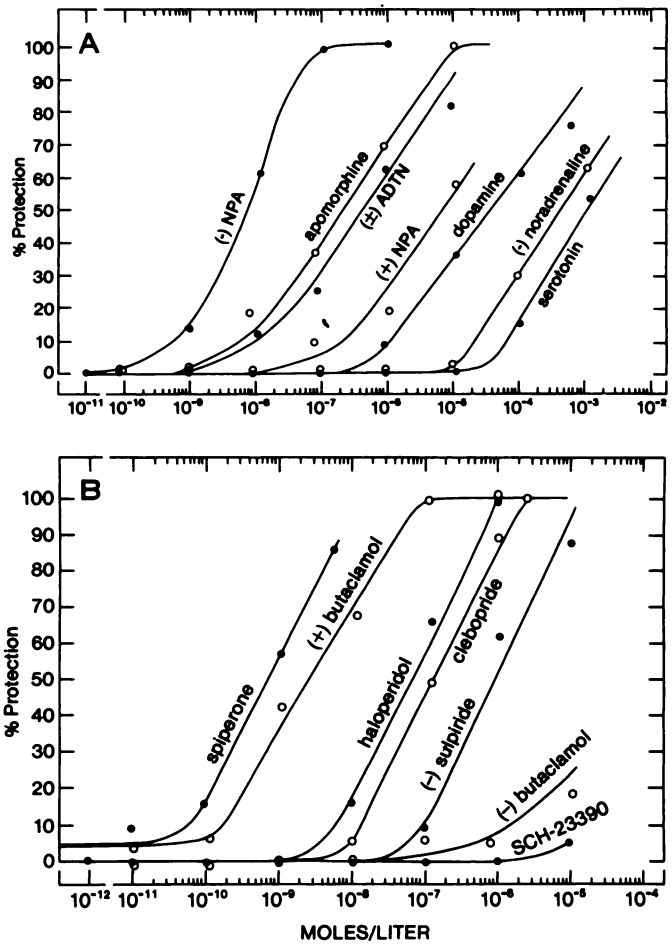

FIG. 4. *Protection of [^{3}H]spiperone-binding sites against photoinactivation by azidoclebopride*

Canine striatal membranes were co-incubated with azidoclebopride (1 μM, final concentration) and various concentrations of protecting agent for 60–90 min at 22°, photolysed for 30 sec, and extensively washed to remove noncovalently bound azidoclebopride and the protecting drug. D_2 receptors remaining after photolysis were measured using [^{3}H]spiperone (1 nM, final concentration) as described in the text. Each experimental point represents the mean of one to three independent determinations each in quadruplicate with standard error of less than 8%. Protection of [^{3}H]spiperone binding = $(B_{prot} - B_{photo})/(B_{cont} - B_{photo}) \times 100\%$, where B_{photo} is the specific binding of [^{3}H]spiperone after photolysis in the presence of azidoclebopride alone, B_{prot} is the binding after irradiation with azidoclebopride and protecting drug, and B_{cont} is that after photolysis in the presence of the highest concentration of protecting drug used without azidoclebopride. Inclusion of azidoclebopride and protecting drug but without photolysis, or irradiating membranes with 1 μM clebopride and protecting drug, followed by extensive membrane washing yielded specific binding values similar to B_{cont}.

Similarly, dopamine receptor inactivation was still evident in fractions of photolysed and extensively washed membranes which were subsequently solubilized in 3-[3-cholamidopropyldimethylammonio]-1-propanesulfonate (10 mM) detergent and assayed for [^{3}H]spiperone binding activity by Sephadex G-50 chromatography.[2]

Third, the degree of dopamine D_2 receptor blockade by azidoclebopride was both concentration- and photodependent.

[2] H. B. Niznik and P. Seeman, unpublished observations.

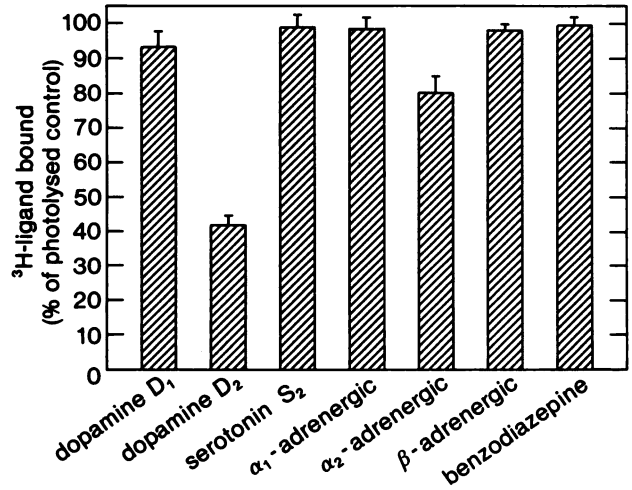

FIG. 5. *Selectivity of azidoclebopride for dopamine receptor photoinactivation*

Canine striatal membranes were incubated (90 min at 22°) with azidoclebopride (1 μM), photolysed for 30 sec, and extensively washed as described under Materials and Methods. Control membranes were treated in an identical manner but without azidoclebopride. Dopamine D_1, D_2, serotonergic (S_2), benzodiazepine, α_1-, α_2-, and β-adrenoceptors were subsequently assayed with an appropriate concentration of ^{3}H-ligand sufficient to occupy at least 50% of the total number of binding sites as described in the text. Results are the mean of three to five independent experiments each determined in quadruplicate. Only [^{3}H] rauwolscine binding to striatal α_2 receptors was found to be affected (approximately 20% reduction) by azidoclebopride.

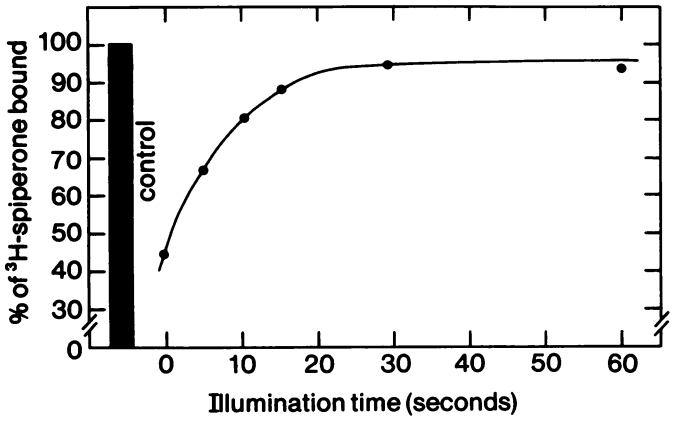

FIG. 6. *Preillumination of azidoclebopride: effect on subsequent dopamine D_2 receptor photoinactivation*

Aliquots of a 15 μM solution of azidoclebopride were irradiated with a 85-W Hg lamp for various time periods. Aliquots (1 ml) of the irradiated solution were then added to striatal tissue suspensions and incubated in the dark for 90 min (22°), photolysed for an additional 30 sec, and extensively washed as described in Materials and Methods. Control membranes were photolysed in the absence of azidoclebopride. Aliquots of washed brain membranes were collected and assayed for D_2 receptor activity as described in the text ([^{3}H]spiperone, 1 nM, final concentration). Each experimental point is the means of triplicate determinations with standard error of less than 5%. The experiment was repeated with similar results.

Fourth, the photoinactivation of dopamine receptors by azidoclebopride could be protected by dopaminergic agonists and antagonists with the appropriate pharmacological profile. Although most protection curves (Fig. 4) were right-shifted, the calculated K_i values for antagonist protection correlated with K_i values obtained from antagonist competition experiments with [^{3}H]YM-09151-2 (Fig. 7) in canine striatal membranes.[3]

TABLE 2

Effect of p-aminobenzoic acid and dithiothreitol on dopamine D_2 receptor photoinactivation by azidoclebopride

Aliquots of striatal membrane suspensions were exposed to azidoclebopride (Az-Clebo) and/or *p*-aminobenzoic acid (*p*-ABA) or dithiothreitol (DTT) for 60 min at 22°, irradiated for 30 sec, and extensively washed as described. Dopamine D_2 receptor activity was measured with [^{3}H]spiperone (1 nM, final concentration). Results are the means (±SE) of two independent experiments, each determined in quadruplicate.

hv	Treatment/additions			Specific bound	
	Az-Clebo (1 μM)	*p*-ABA (1 mM)	DTT (1 mM)		
				cpm filter	*% control*
+	−	−	−	667 ± 10	100
+	+	−	−	321 ± 15	48[a]
−	−	+	−	658 ± 13	99
+	−	+	−	651 ± 30	98
−	+	+	−	620 ± 23	93
+	+	+	−	421 ± 17	63[a]
−	−	−	+	647 ± 22	97
+	−	−	+	674 ± 11	101
−	+	−	+	663 ± 21	99
+	+	−	+	306 ± 14	46[a]

[a] $p < 0.01$

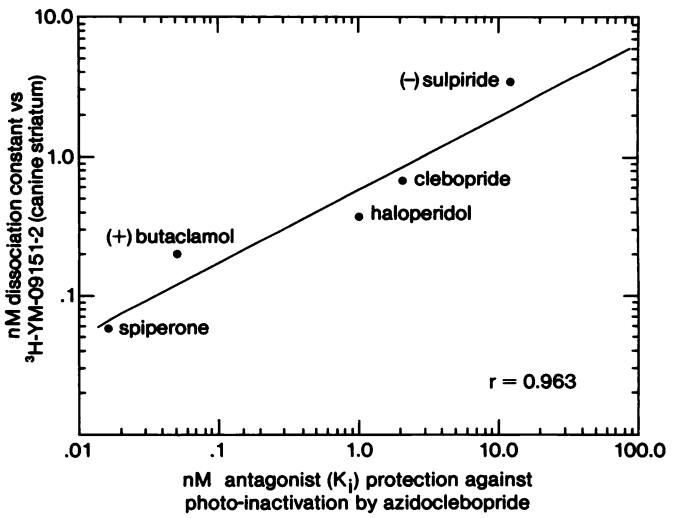

FIG. 7. *Correlation between K_i values for antagonist protection of [^{3}H]spiperone-binding activity during photolysis and the K_D values of these ligands for [^{3}H]YM-09151-2 binding to dopamine D_2 receptors in canine striatal membranes*

Approximate K_i values for dopamine antagonist protection curves were derived from the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [AZ\text{-}CLEBO]/K_D$ of AZ-CLEBO), where IC_{50} is the concentration of dopaminergic antagonist which inhibited dopamine D_2 receptor photoinactivation by azidoclebopride by 50%, [AZ-CLEBO] is the concentration of azidoclebopride (1 μM) used during photolysis, and K_D [AZ-CLEBO] is the dissociation constant of azidoclebopride for striatal dopamine D_2 receptors (21 nM) as determined by competition experiments with [^{3}H] spiperone.

[3] H. B. Niznik, D. Grigoriadis, I. Pri-Bar, O. Buchman, and P. Seeman, submitted for publication.

Finally, azidoclebopride appears to be a selective photoaffinity ligand for striatal dopamine D_2 receptors, since the specific binding of ^{3}H-ligands to dopamine D_1, serotonin S_2, benzodiazepine, and α_1- and β-adrenoceptors was not significantly reduced following photolysis with 1 μM azidoclebopride. Some reduction (approximately 20%) in α_2-adrenoceptors was noted and suggests that azidoclebopride (1 μM) may have some affinity for these sites.

Although azidoclebopride (1 μM), in reversible competition experiments, completely reduced [^{3}H]spiperone-binding activity, complete photoinactivation of dopamine D_2 receptors by this same concentration of azidoclebopride could not be achieved. Similar findings have been reported for other photoaffinity ligands (e.g., Ref. 22) and may be in part due to the: (i) biological screening effect of membranes in the photolysed solution and/or (ii) photoinactivation of azidoclebopride in solvent buffer rather than at specific receptor sites (see Fig. 6), thereby effectively reducing the concentration of reactive photoligand at the receptor.

The mechanism of photoaffinity labeling by azidoclebopride was studied in the presence of the scavengers *p*-aminobenzoic acid and dithiothreitol. It has been suggested that aryl nitrene intermediates formed during photolysis may be generated at the occupied receptor site (true photoaffinity labeling; see Ref. 31) or may be free in solution and simply diffuse to both specific and nonspecific binding sites (pseudo-photoaffinity labeling). Since pseudo-photoaffinity labeling is merely another form of affinity labeling, it is useful to distinguish between these two mechanisms by photolysing membranes in the presence of high concentrations of scavengers. These provide an alternative site for covalent attachment of reactive intermediates that are generated free in solution (low affinity binding site) but not those generated within the vicinity of the receptor itself (high affinity binding site). Inclusion of *p*-aminobenzoic acid or dithiothreitol did not reduce the ability of azidoclebopride to inactivate dopamine D_2 receptors during photolysis and suggests that dopamine receptors are photolabeled by a true photoaffinity mechanism.

In summary, the present results demonstrate that azidoclebopride is a specific photoaffinity label for dopamine D_2 receptors. The incorporation of ^{125}I into azidoclebopride[4] may yield a photoactive ligand of sufficiently high specific activity and affinity to be of use in the isolation of the D_2 dopamine receptor. This work is currently in progress.

[4] J. L. Neumeyer, J. H. Guan, H. B. Niznik, A. Dumbrille-Ross, P. Seeman, S. Padmanabhan, and D. Elmaleh, submitted to *J. Med. Chem.*

REFERENCES

1. Seeman, P. Brain dopamine receptors. *Pharmacol. Rev.* **32**:229–313 (1980).
2. Seeman, P. Nomenclature of central and peripheral dopaminergic sites and receptors. *Biochem. Pharmacol.* **31**:2563–2568 (1982).
3. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).
4. Creese, I. M., W. Hamblin, S. E. Leff, and D. R. Sibley. CNS dopamine receptors, in *Biochemical Studies of CNS Receptors, Handbook of Psychopharmacology*, (L. L Iversen, S. D. Iversen, and S. H. Snyder, eds.), Vol. 17. Plenum Press, New York, 81–138 (1983).
5. Stoof, J. C., and J. W. Kebabian. Opposing roles for D_1 and D_2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. *Nature (Lond.)* **294**:366–368 (1981).
6. Onali, P., M. C. Olianas, and G. L. Gessa. Selective blockade of dopamine D_1 receptors by SCH-23390 discloses striatal dopamine D_2 receptors mediating the inhibition of adenylate cyclase in rats. *Eur. J. Pharmacol.* **99**:127–128 (1984).
7. Stiles, G. L., M. G. Caron, and R. J. Lefkowitz. β-Adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiol. Rev.* **64**:661–743 (1984).
8. Lilly, L., C. M. Fraser, C. Y. Jung, P. Seeman, and J. C. Venter. Molecular size of the canine and human brain D_2 dopamine receptors as determined by radiation inactivation. *Mol. Pharmacol.* **24**:10–14 (1983).
9. Neumeyer, J. L., S. J. Law, R. J. Baldessarini, and N. S. Kula. (−)*N*-(2-chloroethyl)-10,11 dihydroxynoraporphine (chloroethylnorapomorphine) a novel irreversible dopamine receptor antagonist. *J. Med. Chem.* **23**:595–599 (1980).
10. Costall, B., D. H. Fortune, S. J. Law, R. J. Naylor, J. L. Neumeyer, and V. Nohria. (−)*N*-Chloroethylnorapomorphine inhibits striatal dopamine function via irreversible receptor binding. *Nature (Lond.)* **285**:571–573 (1980).
11. Guan, J. H., J. J. Neumeyer, C. N. Filer, D. G. Ahern, L. Lilly, M. Watanabe, D. Grigoriadis, and P. Seeman. Aporphines. 58. *N*-(2-chloroethyl)[8,9-^{3}H] norapomorphine, an irreversible ligand for dopamine receptors: synthesis and application. *J. Med. Chem.* **27**:806–810 (1984).
12. Baldessarini, R. J., and N. S. Kula. Irreversible binding of ^{3}H-(−)*N*-chloroethylnorapomorphine to mammalian brain tissue. *Life Sci.* **33**:769–778 (1983).
13. Lilly, L., and P. Seeman. D_2 dopamine receptor purification using [^{3}H]-NCA: identification of a 40,000 dalton binding protein. *Soc. Neurosci. Abstr.* **9**:30 (1983).
14. Hamblin, M. W., and I. Creese. Phenoxybenzamine treatment differentiates dopaminergic ^{3}H-ligand binding sites in bovine caudate membranes. *Mol. Pharmacol.* **21**:44–51 (1982).
15. Lilly, L., J. Magnan, A. Davis, and P. Seeman. Irreversible ^{3}H-ligands for dopamine receptor purification. *Soc. Neurosci. Abstr.* **8**:719 (1982).
16. Hamblin, M. W., and I. Creese. Behavioral and radioligand binding evidence for irreversible dopamine receptor blockade by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. *Life Sci.* **32**:2247–2255 (1983).
17. Schuster, D. I., W. L. Holden, A. P. S. Narula, and R. B. Murphy. Irreversible blockade of striatal dopamine receptors *in vivo* by a derivative of α-flupenthixol. *Eur. J. Pharmacol.* **77**:313–316 (1982).
18. Cross, A. J., J. L. Waddington, and S. T. Ross. Irreversible interaction of β-haloalkylamine derivatives with dopamine D_1 and D_2 receptors. *Life Sci.* **32**:2733–2740 (1983).
19. Ramwani, J., and R. K. Mishra. Purification of dopamine (D_2) receptor by affinity chromatography. *Fed. Proc.* **41**:1325 (1982).
20. Lilly, L., A. Davis, B. Madras, C. M. Fraser, J. C. Venter, and P. Seeman. Dopamine D_2 receptors: partial purification. *Soc. Neurosci. Abstr.* **7**:10 (1981).
21. Davis, A., C. M. Fraser, L. Lilly, B. K. Madras, J. C. Venter, and P. Seeman. Monoclonal antibodies to partially purified D_2 dopamine receptors. *Soc. Neurosci. Abstr.* **7**:10 (1981).
22. Hess, H. J., R. M. Graham, and C. J. Homcy. Photoaffinity label for the α_1-adrenergic receptor: synthesis and effect on membrane and affinity purified receptors. *Proc. Natl. Acad. Sci. U. S. A.* **80**:2102–2106 (1983).
23. Leeb-Lundberg, L. M. F., K. E. G. Dickinson, S. L. Heald, J. E. S. Wikberg, P. O. Hagen, J. F. Debernardis, M. Winn, D. L. Arendsen, R. J. Lefkowitz, and M. G. Caron. Photoaffinity labeling of mammalian α_1-adrenergic receptors: identification of the ligand binding subunit with a high affinity radioiodinated probe. *J. Biol. Chem.* **259**:2579–2587 (1984).
24. Nishikori, K., O. Noshiro, K. Sano, and H. Maeno. Characterization, solubilization, and separation of two distinct dopamine receptors in canine caudate nucleus. *J. Biol. Chem.* **255**:10909–10915 (1980).
25. Thermos, K., R. B. Murphy, and D. I. Schuster. Photoincorporation of [^{3}H]-chloropromazine into solubilized bovine striatal preparation. *Biochem. Biophys. Res. Commun.* **106**:1469–1477 (1982).
26. Hsu, L. L., and K. Moroi. A photoaffinity probe for the brain dopamine receptor: synthesis and characterization. *Soc. Neurosci. Abstr.* **9**:1115 (1983).
27. Davis, B., L. Abood, and A. M. Tometsko. Utilization of [^{3}H]-dopamine as a photoaffinity label of brain synaptosomes. *Life Sci.* **26**:85–88 (1980).
28. Testylier, G., D. Daveloose, F. Leterrier, O. Buchmann, and M. Shimoni. Photochemical binding of phenothiazines on biological membrane proteins. *Photochem. Photobiol.* **39**:273–276 (1984).
29. Niznik, H. B., J. H. Guan, J. L. Neumeyer, and P. Seeman. A dopamine D_2 receptor photolabel: azidoclebopride. *Eur. J. Pharmacol.* **104**:389–390 (1984).
30. Grigoriadis, D., and P. Seeman. Complete conversion of brain D_2 dopamine receptors from the high to the low affinity state for dopamine agonists, using sodium ions and guanine nucleotides. *J. Neurochem.*, in press (1985).
31. Chowdhry, V., and F. H. Westheimer. Photoaffinity labeling of biological systems. *Annu. Rev. Biochem.* **48**:293–325 (1979).

Send reprint requests to: H. B. Niznik, Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada, M5S 1A8.