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Molecular Size of the Canine and Human Brain D_2 Dopamine Receptor as Determined by Radiation Inactivation

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SUMMARY

Target-size analysis (radiation inactivation) has been utilized for determination of the molecular size of the striatal D_2 dopamine receptor of both canine and human membranes. The dog and human receptors were found to have a molecular size of 123,000 daltons. The identity of molecular size values is consistent with available pharmacological and biochemical evidence supporting D_2 dopamine receptor identity in canine and human tissues. These data suggest that the canine receptor may be a valid model for molecular and structural investigation of the human D_2 dopamine receptor.

INTRODUCTION

The D_2 dopamine receptor is the dopamine-sensitive site in the brain and pituitary to which neuroleptics bind with high (nanomolar) affinities. The D_2 receptor is thought to be involved in a variety of dopaminergic behaviors, such as rotation and stereotypy in rats and emesis in dogs and humans. It has also been linked to diseases in humans (1). While D_2 receptors from a variety of mammalian sources have been extensively investigated pharmacologically with radioligand binding studies (see ref. 1 for review), few data on the molecular properties of D_2 receptors have been forthcoming. This report provides the first estimate of the molecular size of this receptor.

Canine brains were obtained from Pel-Freeze Biologicals (Rogers, Ark.) and were shipped frozen on dry ice. Human brains were obtained at autopsy from local hospitals from donors diagnosed as neurologically normal, and were frozen 12–24 hr after death. [^{3}H]Spiperone (26.7 Ci/mmole) and Econofluor were from New England Nuclear Corporation (Boston, Mass.). Enzymes and associated reagents were obtained from Sigma Chemical Company (St. Louis, Mo.), and of the highest purity available; all other reagents were from Sigma Chemical Company or Fisher Scientific Company (Pittsburgh, Pa.).

Membranes were prepared, as has previously been described (2), by homogenization of striata dissected from previously frozen canine or human brains in 0.25 M sucrose at 4° in a 10-fold (weight to volume) ratio. This mixture was centrifuged at 1,100 × *g* for 10 min, and the supernatant fluid was collected. The pellet was again homogenized in an additional 10 volumes of 0.25 M sucrose and again centrifuged. The second supernatant was combined with the first, and the mixture was centrifuged at 100,000 × *g* for 60 min. The resulting pellet was suspended in TEAN buffer (50 mM Tris/5 mM EDTA/0.01% ascorbate/100 mM NaCl/10 μM nialamide, pH 7.4 at 4°) at a protein concentration of 4 mg/ml (3) and stored at −80° until use.

The procedure for molecular weight calculation is an adaption of the method described by Lo *et al.* (4), except that frozen rather than lyophilized samples were used (5). Dopamine receptor binding has been shown to be the same in control frozen samples as in control nonfrozen samples (2). For molecular size calibration of radiation inactivation, a number of enzymes with known molecular weights were used as internal standards. Enzyme molecular weight and subunit composition were analysed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis as described (4, 6), with results essentially identical to those reported (4). When a mixture of these standard enzymes was irradiated with 1.5 MeV electrons, each enzyme activity declined as a single exponential of the radiation dose (Figs. 1 and 2). The inactivation ratio, defined as the ratio of the slope of a semilogarithmic

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inactivation plot of an enzyme (S_X) to the slope for a reference standard enzyme β-galactosidase (S_G), was calculated for each enzyme. The inactivation ratio for each standard enzyme X was found to be equal to the ratio of its molecular weight (M_X) to the molecular weight (M_G) of the reference standard enzyme (Fig. 2). This finding conforms to the simple prediction of the classical radiation target theory, which asserts that radiation sensitivity (the slope of the semilogarithmic plot, or S_X) is proportional to the molecular size. It follows that M_X can be calculated from S_X/S_G given that M_G is known.

Radiation inactivation was performed essentially as described by Venter (5) for the muscarinic cholinergic receptor. One milliliter of the sample, consisting of enzymes alone or striatal membranes mixed with enzymes, was layered 0.5 mm deep into open aluminum trays, which were then frozen by immersion in liquid nitrogen. Samples were housed in a closed aluminum chamber (target chamber) and irradiated at doses ranging from 0 to 10 Mrad; during irradiation, the chamber was cooled by a stream of liquid nitrogen flowing under the sample, and chamber temperature was monitored as described (7). The sample was maintained between −45° and −52° during irradiation, and was subsequently stored at −80°

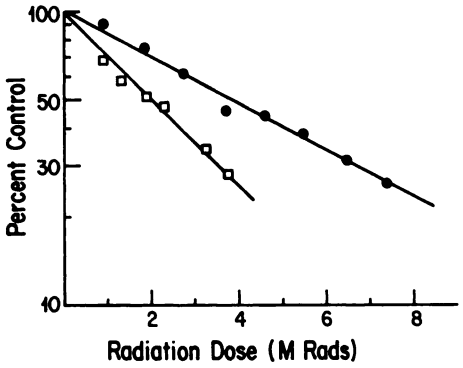

FIG. 1. *Radiation inactivation: target size analysis of canine striatal D_2 dopamine receptors and internal enzyme standard*

D_2 dopamine receptor-containing canine striatal membranes were frozen in thin layers in aluminum trays with enzyme standards and subjected to high-energy electron bombardment as described in the text. Radiation-induced loss of the D_2 dopamine receptor was assessed by measuring [^{3}H]spiperone binding at four concentrations of ligand ranging from 0.2 nM to 1.7 nM. The survival of specific [^{3}H]spiperone binding is represented by ●; each point represents the mean of triplicate determinations for four ligand concentrations. The degree of inactivation measured is independent of ligand concentration used (see text).

The radiation inactivation of the D_2 dopamine receptor is compared on the same plot with the inactivation of yeast ADH (□) from the same radiation experiment. Lines were drawn by least-squares linear regression. The molecular size of the D_2 dopamine receptor was calculated as described in the text by determining the inactivation ratio $S_{\text{dopmaine receptor}}/S_{\text{yeast ADH}}$, which permits a direct comparison to enzyme standards on a linear plot (Fig. 2) of the relationship between the inactivation ratio S_X/S_G and molecular weight ratio M_X/M_G determined in a series of radiation inactivation experiments. The inactivation ratio of the D_2 dopamine receptor was related to the standard plot by

$$\frac{S_{\text{dopamine receptor}}}{S_G} = \frac{S_{\text{dopamine receptor}}}{S_{\text{ADH}}} \cdot \frac{S_{\text{ADH}}}{S_G}$$

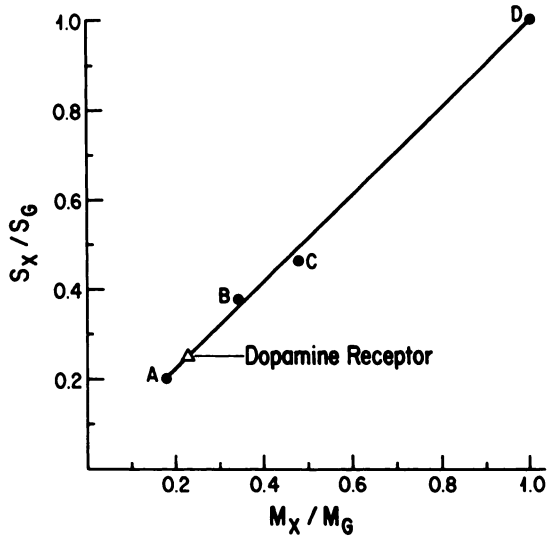

FIG. 2. *Radiation inactivation molecular weight calibration curve*

Standard enzymes [horse liver ADH (*A*), 84,000 daltons; yeast ADH (*B*), 160,000 daltons: pyruvate kinase, (*C*), 224,000 daltons; and *Escherichia coli* β-galactosidase (*D*), 464,000 daltons (4)] were inactivated individually, in groups, and with membrane samples with identical results, and their inactivation ratios were determined as described in the text. The least-squares slope of the line is 0.97. The D_2 dopamine receptor (△) was related to the standard plot as described in Fig. 1, and these data indicate a molecular size of 123,000 daltons for the canine D_2 dopamine receptor.

until assayed. Dosimetry was performed at the sample irradiation temperature with the bleaching of Blue Cellophane (Dupont MSC-300) calibrated against Fricke's dosimetry (8).

Yeast ADH[4] was dissolved in the striatal membrane suspension at a concentration of 3 mg of ADH per milliliter of suspension for internal standardization. Horse liver ADH, yeast ADH, pyruvate kinase, and β-galactosidase over a range of 0.4 mg/ml to 4.0 mg/ml were irradiated as above, either alone or mixed.

D_2 dopamine receptors were quantitated using [^{3}H] spiperone (see ref. 1). For determination of [^{3}H]spiperone binding, the trays were first warmed to room temperature, and the samples were recovered using TEAN buffer and then diluted to a protein concentration of 0.7 mg/ml. Four concentrations of [^{3}H]spiperone were used for each sample, and specific binding was defined as that remaining in the presence of 10 μM (−)-sulpiride (9). Culture tubes in triplicate received, in order, 0.2 ml of [^{3}H]spiperone (one of four concentrations), 0.2 ml of buffer or (−)-sulpiride, and 0.2 ml of membrane suspension. Following an incubation of 4 hr at 4° (required to approach equilibrium) (2), the mixture was vacuum-filtered over Whatman GF/B filters, which were then washed with 14 ml of ice-cold TEAN buffer. Scintillation cocktail (9 ml) was added, and the vials were monitored for tritium in a scintillation counter at an efficiency of 40%. On average, specific [^{3}H]spiperone binding represented 85% of the total binding. Scatchard analysis of

[4] The abbreviation used is: ADH, alcohol dehydrogenase.

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saturation binding was performed in control membranes and at all radiation doses used.

Enzyme activity was measured on a recording Gilford spectrophotometer. β-Galactosidase was measured by the method of Craven *et al.* (10), ADH as described by Vallee and Hoch (11), and pyruvate kinase as described by Bueher and Pfleiderer (12). To prevent pyruvate kinase aggregation upon freezing and thawing, bovine serum albumin (10 mg/ml) was included with the enzyme (6).

When canine or human striatal membranes were frozen and subjected to high-energy irradiation, there was an exponential loss of [^{3}H]spiperone binding as a function of the radiation dose (Fig. 1). The linearity of the semilogarithmic plot of receptor survival over the range of radiation doses tested indicated that, based on size, a single population of sites was affected. However, due in part to the limited signal when greater than 75% of receptors are inactivated, an accurate determination of the existence of a minor receptor fraction with a molecular size less than 123,000 was not feasible. The decrease in the amount of specific [^{3}H]spiperone binding was assessed at four ligand concentrations which bracketed the K_d value for [^{3}H]spiperone binding. The percentage reduction in [^{3}H]spiperone binding caused by a given radiation dose was independent of ligand concentration used to assess that change, and the means of these changes were used in Fig. 1. Scatchard analysis of these data is shown in Fig. 3. K_d values of 0.74 nM and 0.76 nM were obtained for control and irradiated membranes, respectively. Receptor number decreased by 35% at the dose of radiation shown. This illustrated that the irradiation produces a change in receptor number without affecting receptor affinity.

The functional molecular size of the D_2 dopamine receptor was determined from the slope ratios as described above by the inclusion of an enzyme standard

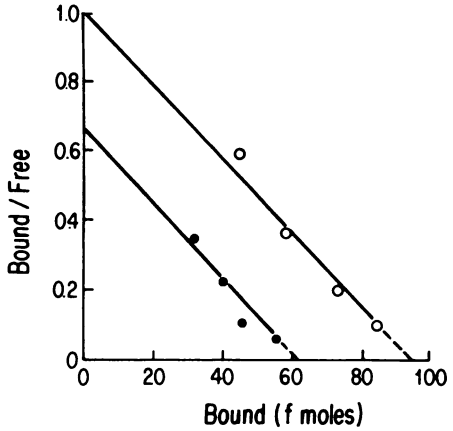

FIG. 3. *Scatchard analysis of [^{3}H]spiperone binding in nonirradiated and irradiated canine striatal membranes*

Illustrated are representative Scatchard analyses of specific [^{3}H] spiperone binding to canine D_2 dopamine receptors. Nonirradiated samples (○) had a K_d value of 0.74 nM; samples that received a radiation dose which reduced the mean amount of specific binding to 69% of control values (Fig. 1) had a K_d value of 0.76 nM (●). Determinations were made at identical protein concentrations. The parallel shift in the curves reflects a reduction in the bound value to about 65% of control.

TABLE 1

Molecular properties of D_2 dopamine receptors

Parameter[a]	Dog	Human
Receptor size (radiation inactivation) (daltons)	123,000	123,000
pI (isoelectric point) (13, 29)	5.06	5.0
K_d[b] (nM), this study (2, 14)	0.74	—
	0.40	0.37
B_{max}[b] (fmoles/mg of protein) (2, 14)	410	220

[a] Numbers in parentheses are references.

[b] [^{3}H]Spiperone binding.

(yeast ADH) with the membrane preparation (Fig. 1). The calculated molecular weight calibration curve (Fig. 2) indicates a molecular size of 123,000 daltons for the canine striatal D_2 dopamine receptor (Table 1). Experiments performed using human brain striatum (Fig. 4) also showed a single exponential receptor loss over the range of doses used. The identical inactivation ratio was obtained for both the canine and human striatal D_2 dopamine receptors, indicating that the human receptor also has a functional molecular size of 123,000 daltons (Table 1).

The present results indicate that the D_2 dopamine receptor from both canine and human striatal membranes has a functional molecular size of 123,000 daltons. This identity is consistent with a number of other biochemical parameters that have been evaluated (Table 1) and supports the use of the canine receptor in the study of human brain dopamine receptors.

Target size analysis is the only method known for the estimation of the functional molecular size of a protein in intact membranes. It does not require purified material, and, under appropriate conditions, the molecular weight calculated from target size analysis is the oligomeric size of a multisubunit structure. This method has been used to determine the molecular size for several membrane-bound proteins, including the (Na^+,K^+)-ATPase and mitochondrial Mg^{2+}-ATPase (15), (H^+,K^+-ATPase (7), glucose carrier protein (16), the insulin receptor (17), benzodiazepine receptor (18, 19), nicotinic (4) and muscarinic (5) cholinergic receptors, and *alpha-* (20) and *beta*-adrenergic (21, 22) receptors, in examining the hormone-activated adenylate cyclase (23) in addition to studying many other enzymes (see ref. 24 for review).

Lo *et al.* (4), in studies on the nicotinic acetylcholine receptor, reported a novel method for the determination of protein molecular weight from radiation inactivation. This procedure, which is based upon the irradiation of enzymes of known molecular weights and subunit composition, overcomes the use of temperature correction factors that have been, in part, necessitated by decreased radiation inactivation at reduced temperatures. The inclusion of enzyme standards within the receptor-containing membranes being irradiated also corrects for any radiation quenching attributable to the sample itself. This laboratory has recently extended the approach of Lo *et al.* to evaluation of the muscarinic cholinergic receptor (5) and the slow, inward calcium channel (6).

It has been reported that [^{3}H]spiperone labels serotonin (S_2) as well as dopamine sites in the striatum (9).

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The use in the present investigation of (−)-sulpiride to delineate specific [^{3}H]spiperone binding allows us to examine the D_2 dopamine receptor population alone. Preliminary experiments using (+)-butaclamol, which has been shown to affect D_2 and S_2 sites (25, 26), to define specific binding also yielded simple exponential decay curves for radiation inactivation (results not shown). This suggests that the contribution of S_2 sites to the total [^{3}H]spiperone binding is minimal. Comparison of the potencies of (−)-sulpiride and (+)-butaclamol of S_2 sites to the total signal in canine striatum is 4.6 ± 0.8% (data not shown), and it has recently been shown that the quantity of S_2 sites in human striatum is even lower (27).

Scatchard analysis of [^{3}H]spiperone binding in nonirradiated and irradiated membranes shows a parallel shift in the curves, with no change in affinity. These data indicate that the radiation damage is not due to secondary ionization events which might produce only minor modification of receptor function; rather, the high-energy electrons are destroying the receptors, as predicted by radiation target theory (28). This parallel shift also supports the use of the means of decay determinations at a number of ligand concentrations to obtain the semilogarithmic plot from which the inactivation ratio is determined. The K_d values reported here (0.74 nM for control membranes) are slightly higher than those reported by others for [^{3}H]spiperone binding in a variety of mammalian tissues (1); however, the use of high tissue concentrations in the receptor binding assays causes an artificial increase in the apparent K_d value (14) and does not affect the validity of the comparisons made.

There are now a number of neurotransmitters whose molecular size has been determined using radiation inactivation as well as by standard biochemical means (Table 2). The results indicate that target-size analysis is a useful tool in the estimation of receptor functional molecular size. In the case of the *beta*$_2$-adrenergic receptor, for example, this technique has provided supporting evidence for the existence of a functional dimer (21). The size of the D_2 dopamine receptor determined in this study suggests a similarity to other neurotransmitter receptors (Table 2), and, as the task of purifying the brain D_2 dopamine receptors proceeds, the present data should serve as a base for subsequent comparison and receptor structure determinations.

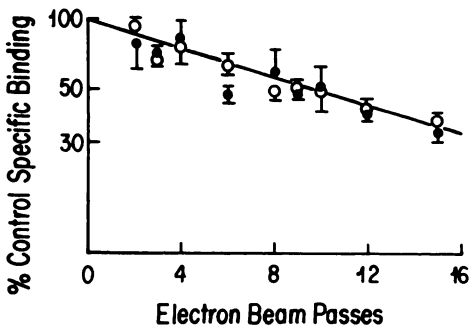

FIG. 4. *Comparison of D_2 dopamine receptor radiation inactivation profiles from canine and human brain striata*

Human straital membranes were prepared using the procedure outlined for canine brain (see text) and irradiated along with the canine striatal membranes. The semilogarithmic plots of the decrease in specific [^{3}H]spiperone binding with increasing radiation dose have identical slopes for canine and human striatal membranes. This illustrates that the D_2 dopamine receptors from the two species have identical functional sizes. Samples were irradiated with a 0.5-mamp beam of 1.5 MeV electrons produced with a Van de Graaf generator. The cumulative dose was a linear function of the number of passes of the beam over the sample.

TABLE 2

Target size analysis of neurotransmitter receptors

Receptor[a]	Molecular size	
	Radiation inactivation	SDS/PAGE[b]
	daltons	
Rat liver *alpha*$_1$-adrenergic (20, 30)	160,000[c]	80,000
Turkey red blood cell *beta*$_1$ adrenergic (31, 32)	90,000	65–70,000
Dog heart *beta*$_1$-adrenergic[d]	130,000[c]	64,000
Dog lung *beta*$_2$-adrenergic (21)	109,000 ± 5,300[c]	59,000
Nicotinic acetylcholine receptor (5 subunits) (4)	300,000 ± 5,900	270,000
Rat brain muscarinic acetylcholine receptor (5)	80,000	80,000
Human muscarinic acetylcholine receptor (5)	80,000	80,000
Benzodiazepine (18)	90–100,000	100,000
Canine brain D_2 dopamine	123,000	
Human brain D_2 dopamine	123,000	

[a] Numbers in parentheses are references.
[b] Sodium dodecyl sulfate/polyacrylamide electrophoresis.
[c] Suggests the presence of a dimer.
[d] Fraser and Venter, unpublished observation.

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