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CORRESPONDENCY BETWEEN DIFFERENT AFFINITY STATES AND TARGET SIZE OF THE BOVINE STRIATAL D2 DOPAMINE RECEPTOR

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SUMMARY : Target size analysis of the D_2 dopamine receptor in the bovine striatum revealed the presence of two populations of this receptor, in terms of apparent molecular size. The size of large target was approximately 150 x 10^4 daltons, while that of small target was 11 x 10^4 daltons. The antagonist [^{3}H]spiperone labeled both large and small sized D2 receptors, while agonist [^{3}H]n-propylapomorphine (NPA) labeled only the former. In addition, the apparent molecular size of a functional unit for the GTP effect was calculated to be 150 x 10^4 daltons, such appearing to be identical to that of large target sized D_2 dopamine receptors. Therefore, the large sized D_2 receptor, probably an oligomeric complex consisting of D_2 receptor recognition protein and guanine nucleotide regulatory protein, has a high affinity for both agonist and antagonist, while the small sized receptor, probably a monomeric or dimeric receptor recognition protein, has a high affinity for only the antagonist.

Existence of two different affinity states of the same D_2 dopamine receptor populations has been proposed using labeled antagonist and agonist (1-3). The agonist high-affinity form of the receptor, which represents about half of the total receptor, is reportedly modulated by guanine nucleotides (1-3). Recently, we found that islet-activating protein (IAP), a B. pertussis toxin, induced a selective decrease in the affinity of D_2 dopamine receptor for agonist and a loss of GTP effect associated with ADP-ribosylation of the 40,000 dalton protein in the bovine striatal membrane (4). These observations suggest a bidirectional coupling of inhibitory guanine nucleotide regulatory protein (Ni) to the receptor and to adenylate cyclase. However, molecular mechanisms of these phenomena have not been elucidated. We have now used target size analysis to evaluate the functional molecular sizes of these D_2 receptor related membrane macromolecules.

MATERIALS AND METHODS

Materials : [^{3}H]spiperone (31.7 Ci/mmol) and [^{3}H]n-propylapomorphine (NPA, 58.8 Ci/mmol) were purchased from New England Nuclear. GTP, dopamine hydro-

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chloride and apomorphine hydrochloride were purchased from Sigma Chemical Co. Sulpiride (Fujisawa, Japan), (-)- and (+)-butaclamol (Ayerst, Canada) and ketanserin (Kyowa Hakko, Japan) were gifts from the respective companies.

Membrane preparation and radiation inactivation : Crude synaptic membrane preparations from bovine striatum were prepared as described previously (4, 5). The membrane preparations (2-5 mg protein) in 1 ml of 50 mM Tris-HCl buffer, pH 7.4, were lyophilized in ampoules and sealed in vacuo. Samples were irradiated with gamma ray from the source of ^{60}Co (34,400 Ci) at a dose of approximately 5.0 Mrad/hr at 0-10°C. Irradiated samples were rehydrated and assayed for D_2 receptor binding and acetylcholinesterase. Size of the target was calculated from the following formula, as described by Schlegel et al. (6) : molecular size (daltons) = $6.4 \times 10^{11}/D_{37}$, where D_{37} is the radiation dose (rad) giving a residual binding activity of 37 %.

D_2 receptor binding assay : Assay of [^{3}H]NPA binding was carried out by incubating the membrane preparations (0.5-0.8 mg protein) with 1 nM [^{3}H]NPA in 0.6 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 2.5 mM $MgCl_2$, 0.1 % ascorbic acid and indicated concentrations of drugs. Tubes were incubated for 60 min at 25°C and the reaction terminated by vacuum filtration of the membrane through Whatman GF/B filters followed by three washings with 5 ml of ice cold Tris buffer. Assay of [^{3}H]spiperone binding was carried out by incubating the membrane preparations (0.3-0.6 mg protein) with 1 nM [^{3}H]-spiperone in 0.6 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl and indicated concentrations of drugs. Tubes were incubated for 30 min at 25°C and the incubation was terminated as described for [^{3}H]NPA binding. Radioactivity in the filters was counted in a toluene-base scintillator by LS-7000 Beckman scintillation spectrometer at 48 % efficiency. Stereospecific binding was defined as the difference in the binding obtained with incubation in the presence of 1 μM (-)- and (+)-butaclamol. D_2 specific binding was defined as that which occurred in the presence of 0.1 μM ketanserin (to occlude S2 serotonergic sites) but which was displaced by 1 μM (+)-butaclamol for [^{3}H]-spiperone binding, or 0.05 μM spiperone for [^{3}H]NPA binding. The difference between the [^{3}H]spiperone binding, with and without 100 μM GTP at 10 μM dopamine, was taken as the value for the GTP effect.

Acetylcholinesterase assay : Acetylcholinesterase activity was determined by a decrease in the amount of acetylcholine which had been added to the enzymic reaction mixture as substrate. The enzymic reaction was performed by addition of 0.1 ml of the rehyrated lyophilized membrane preparations into a test tube containing 1 ml of acetylcholine-buffer-salt mixture reagent. This reagent was composed of 8 volumes of 1/15 M phosphate buffer and 1 volume of each 0.5 M acetylcholine and salt mixture containing 4.2 g $MgCl_2$ and 0.2 g of KCl per 100 ml. After incubation sample for 1 hr at 37°C, the content of acetylcholine remaining in the samples was determined colorimetrically by the method of Hestrin (7).

RESULTS AND DISCUSSION

We first carried out a binding study using [^{3}H]NPA and [^{3}H]spiperone with crude synaptic membrane preparations from the bovine striatum and determined the effect of lyophilization on these bindings. The inclusion of 0.1 μM ketanserin in all incubation tubes and the definition of nonspecific bindings by 1 μM (+)-butaclamol for [^{3}H]spiperone binding and by 0.05 μM spiperone for [^{3}H]NPA binding made feasible labeling of only the D_2 receptor, as validated by drug displacement experiments (data not shown). Scatchard plots of D_2 receptor spec-

ific binding of these ligands to crude synaptic membrane preparations were linear and the calculated receptor density (B_{max}) and dissociation constant (K_D) values were 81.2 fmol/mg protein and 0.31 nM, respectively, for [^{3}H]NPA binding and 200 fmol/mg protein and 0.32 nM, respectively, for [^{3}H]spiperone binding. These K_D values are all but identical to those given in previous reports (1-3). The B_{max} value ratio of ^{3}H-labeled agonist to ^{3}H-antagonist (81.2 : 200) was also similar to those in previous reports (1-3), thus indicating the presence of interconvertible high- and low-affinity states of the D_2 receptor. When the striatal membrane preparations were lyophilized before binding assays, no loss of binding of either ligand occurred and the same K_D and B_{max} values were obtained (data not shown). Next, we performed radiation inactivation of an enzyme of known molecular weight, acetylcholinesterase, in the same membrane preparations to verify the appropriate condition of the radiation inactivation. Acetylcholinesterase activity decreased linearly on a semilogarithmic scale with an increase in the dose of irradiation (Fig. 1), and the molecular size was calculated to be 85,000 daltons (Table 1). This value is all but identical to that determined by gel filtration (mol. wt. 80,000) of acetylcholinesterase

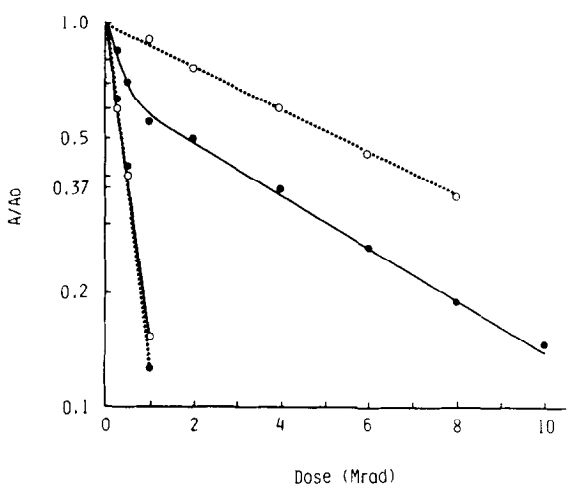

Figure 1. Representative radiation inactivation curves for [^{3}H]NPA binding, [^{3}H]spiperone binding, GTP effect and acetylcholinesterase. Samples were irradiated with gamma ray from the source of ^{60}Co, and a assay of [^{3}H]NPA binding, [^{3}H]spiperone binding, GTP effect and acetylcholinesterase were performed as described in the text. 0.1 μM ketanserin was included in all incubation tubes for [^{3}H]NPA binding and [^{3}H]spiperone binding to occlude S_2 serotonergic sites. The logarithm of percent of surviving activity was plotted against radiation dose. (●·····●) [^{3}H]NPA binding; (●——●) [^{3}H]spiperone binding; (○——○) GTP effect; (○·····○) acetylcholinesterase.

Table 1. Average target sizes for D_2 dopamine receptor in bovine striatal membranes

Target	Size (daltons x 10^4)	
[^{3}H]NPA binding	150	(120-170)
GTP effect	150	(135-160)
[^{3}H]spiperone binding		
small	11	(8.5-13)
large	150	(128-160)
Acetylcholinesterase	8.5	(7.6-9.4)

The data are derived from a series of experiments such as those described in Fig. 1. Size of the target was calculated as described in the text. Each value represents the mean of three independent experiments each of which was done in triplicate. Values in parentheses are range of sizes.

in calf brain (8). The specific [^{3}H]NPA binding decayed linearly with the dose of radiation (Fig. 1). The calculated target size of the [^{3}H]NPA binding sites averaged 150 x 10^4 daltons (Table 1). On the other hand, as shown in Fig. 1, the radiation inactivation curve for specific [^{3}H]spiperone binding was non-linear. For analysis of this nonlinear curve we assumed that there were two target sizes and the inactivation curve was resolved into two simple exponential functions from which both molecular sizes were calculated. The contribution of the smaller target to the total binding activity at zero dose was approximately 60 %. The ratio of the contribution of large to small target (40 : 60) was similar to the ratio of agonist high-affinity to low-affinity D_2 receptor densities, as described above (81.2 : 118.8). As shown in Table 1, the calculated size of the large target was 150 x 10^4 daltons whereas that of the small target was 11 x 10^4 daltons. These results suggest that there are two distinct sub-population of D_2 receptors labeled by [^{3}H]spiperone and that the larger sized sub-population corresponds to the receptors labeled by [^{3}H]NPA. Whether or not the large sized sub-population of the D_2 receptor is identical to the agonist high-affinity state, that is, [^{3}H]NPA binding sites and to the [^{3}H]spiperone binding sites in the guanine nucleotide sensitive state had to be determined. Therefore, we carried out drug displacement experiment of [^{3}H]-spiperone binding in the presence or the absence of 100 μM GTP, using the lyophilized striatal membranes irradiated with 3 Mrad, a minimal dose inducing a selective destruction of the large sized sub-population of D_2 receptor. As

Table 2. Effect of GTP on the displacement of [^{3}H]spiperone binding to irradiated and nonirradiated bovine striatal membranes by dopamine agonists and antagonists

Drugs	IC50 (μM)			
	Control	Irradiation	GTP	Irradiation + GTP
Dopamine	32 ± 4	110 ± 10*	120 ± 10*	120 ± 10*
Apomorphine	1.9 ± 0.2	4.0 ± 0.4*	4.4 ± 0.3*	4.0 ± 0.5*
Sulpiride	9.1 ± 1.0	8.2 ± 1.0	8.0 ± 0.6	8.6 ± 0.9
(+)-butaclamol	0.012 ± 0.002	0.012 ± 0.001	0.014 ± 0.002	0.011 ± 0.002

Lyophilized bovine striatal membranes were irradiated with 3 Mrad by gamma ray as described in the text. Displacement of 1 nM [^{3}H]spiperone binding to irradiated and nonirradiated membranes was performed in the presence or absence of 100 μM GTP, as described in the text. Membrane preparations were lyophilized before irradiation and/or binding experiment. Inhibition was measured at five different concentrations and expressed as the IC50 value (the drug concentrations which inhibited stereospecific [^{3}H]spiperone binding 50 %). Stereospecific [^{3}H]spiperone binding was defined as described in the text. Each value represents the mean ± S.E.M. of three independent experiments. * Significantly different from the control value ($P < 0.05$).

shown in Table 2, IC_{50} values of agonists, dopamine and apomorphine for [^{3}H]-spiperone binding with the irradiated membranes were significantly higher than that with control membranes, while IC_{50} values of antagonists, sulpiride and (+)-butaclamol were not significantly changed by irradiation. These results clearly indicate that the small sized sub-population of the D_2 receptor has a low affinity for agonists, and are consistent with our data that the agonist [^{3}H]NPA labeled only the large sized sub-population of the D_2 receptor. Table 2 also shows that the small sized sub-population of the D_2 receptor is insensitive for GTP, thereby indicating guanine nucleotide sensitive [^{3}H]spiperone binding sites have a larger target size. The target size of the functional unit for GTP effect on [^{3}H]spiperone binding was then determined, based on the difference between [^{3}H]spiperone binding with and without 100 μM GTP at 10 μM dopamine. A representative decay curve for the GTP effect, as shown in Fig. 1, was linear with the dose and the target size was calculated to be 150 x 10^4 daltons. This is practically identical to the target size of the [^{3}H]NPA binding and of the large sized sub-population of [^{3}H]spiperone binding sites. These results support the data obtained in our previous study indicating that

the zwitter-ionic detergent-solubilized D_2 receptor is sensitive to guanine nucleotides and has a extremely larger Stokes radius compared with that of digitonin-solubilized D_2 receptor which is insensitive to guanine nucleotides (5). Therefore, this large size (150 x 10^4 daltons) appears to represent the minimal assembly of units required for a high affinity and for the GTP sensitive state of the D_2 receptor. Rodbell proposed that neurotransmitter and hormone receptors regulated by guanine nucleotides form oligomeric complexes with guanine nucleotide regulatory protein in the cell membrane, as determined in a target size analysis of the receptor-adenylate cyclase system (6,9). According to this hypothesis and our previous result (4), the agonist high-affinity state of the D_2 receptor has a large target size and may be an oligomeric complex consisting of the D_2 receptor recognition protein and Ni, and that the agonist low-affinity state of the D_2 receptor has a small target size which may be the monomeric or dimeric receptor protein which binds with antagonsit only. Most recently, Lilly et al. (10) reported data on radiation inactivation of the canine and human striatal D_2 dopamine receptor, using high-energy electrons. The molecular size was calculated to be 123,000 daltons, such being consistent with that of small sized D_2 receptor in our study.

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