Induction of G Protein-Independent Agonist High-Affinity Binding Sites of D-1 Dopamine Receptors by β-Mercaptoethanol†

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Received February 28, 1994; Revised Manuscript Received July 15, 1994®

ABSTRACT: We have purified the D-1 dopamine receptor 8200-fold to 78% purity from rat striatal membranes. Critical to this purification was the *N*-ethylmaleimide (NEM)-mediated alkylation of all endogenous sulfhydryl groups, except those associated with the D-1 dopamine receptors, which were protected by the D-1 agonist SKF R-38393. Such NEM treatment of D-1 receptors abolished all agonist high-affinity binding sites of the receptors, but did not alter the antagonist binding properties. When NEM-treated D-1 receptors were affinity-purified by mercury–agarose columns, the pharmacological properties of these purified receptors were examined, after removal of β-mercaptoethanol (βME), which was used for elution of receptors from the affinity column. Purified D-1 receptors displayed typical dopaminergic antagonist binding values; however, agonists bound to the purified receptors with only high-affinity binding values, despite the prior absence of high-affinity sites in crude soluble extracts of NEM-treated receptors. The agonist high-affinity binding of purified D-1 receptors was insensitive to modulation by the GTP analog Gpp(NH)p and occurred in the absence of any G proteins. These Gpp(NH)p-insensitive high-affinity sites appeared to be induced by βME, since similar high-affinity binding was also restored by βME to crude soluble and membrane-bound receptors, which had been pretreated with NEM. The ability of D-1 dopamine receptors to bind with high-affinity to agonists in the absence of functionally active G proteins may be an intrinsic property of the reduced state of D-1 dopamine receptors.

Dysfunctional dopaminergic neurotransmissions have been implicated in several neuropathological conditions, including schizophrenia, Parkinson's and Huntington's diseases, drug (cocaine and amphetamine) addiction, hypertension, and hyperprolactemia. At the molecular level, the effects of dopamine are mediated through two receptor subtypes, D-1 and D-2, which are broadly classified according to different pharmacological and physiological properties (Kebabian & Calne, 1979). Recently, as many as five genetically distinct dopamine receptors have been identified through molecular cloning techniques (O'Dowd, 1993). D-1 dopamine receptors have been shown to stimulate adenylate cyclase (Kebabian & Calne, 1979), phospholipase C (Felder et al., 1989; Undie & Friedman, 1990), and protein kinase C (Bertorello & Aperia, 1989), to inhibit Na^+/K^+-ATPase stimulation (Bertorello et al., 1990), and to activate the arachidonic acid cascade system (Piomelli et al., 1991). The mechanism(s) by which D-1 dopamine receptors are coupled to these diverse signal transducing system remain(s) to be established. The elucidation of the mechanisms through which D-1 dopamine receptors are coupled to the diverse signalling pathways necessitates the cell-free reconstitution of the purified receptor with purified components of the signalling system. However, due to the extreme lability of the D-1 dopamine receptor after extraction from the membrane-bound state, very little progress has been made toward this end.

We have recently reported a novel purification procedure, which resulted in an 8200-fold purification of the D-1 dopamine receptor from rat striatal membranes to 78% purity, in a pharmacologically active state (Sidhu, 1990). The purification of the D-1 receptors was achieved by affinity chromatography on mercury–agarose columns, after inactivation of endogenous sulfhydryl (-SH)[1] groups, by treating membranes with the alkylating reagent *N*-ethylmaleimide, (NEM). The -SH groups associated with the D-1 dopamine receptors were specifically protected by occupation of agonist binding sites with the D-1-selective agonist SKF R-38393 prior to alkylation of membranes with NEM (Sidhu et al., 1986a, 1991). Both NEM-treated membranes and crude soluble extracts prepared from NEM-treated membranes failed to reveal the presence of any agonist high-affinity binding sites, and all these receptors bound agonists with only low-affinity values (Sidhu et al., 1986a; Sidhu, 1988). Crude soluble receptors, which were not pretreated with NEM, however, possessed high levels (40%) of high-affinity sites, which were coupled to G proteins (Sidhu, 1988). The absence of high-affinity binding sites in NEM-treated receptors was due to inactivation of G proteins by NEM, since the addition of exogenous sources of G proteins restored Gpp(NH)p-sensitive agonist high-affinity binding sites to the NEM-treated D-1 receptors (Sidhu et al., 1991).

To further characterize the molecular properties of the purified D-1 dopamine receptor, the current study was undertaken. Upon solubilization of NEM-treated membranes with sodium cholate (Sidhu, 1988), D-1 dopamine receptors present in the crude soluble extracts were adsorbed onto affinity columns, via interactions between the -SH groups of the

† This study was supported by a research grant from the National Institute of Neurological Disorders and Stroke, National Institutes of Health Grant NS-29685.

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® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: NEM, *N*-ethylmaleimide; βME, β-mercaptoethanol; -SH, sulfhydryl; G proteins, guanine nucleotide binding proteins; G_s, stimulatory G protein; G_i, inhibitory G protein; G_o, pertussis toxin-sensitive G protein; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; DTT, dithiothreitol.

0006-2960/94/0433-11246$04.50/0

Table 1: Specific Treatment and Preparation of Rat Striatal D-1 Dopamine Receptors Used in This Study[a]

source of receptor	specific treatment of D-1 receptors
control membrane-bound	crude membranes isolated as 18 000g pellet fraction
NEM-treated membrane-bound	crude membranes, treated with 5 mM NEM in presence of 10 μM SKF R-38393; membranes washed 3 times prior to use
crude soluble[b]	receptors solubilized and NEM-treated from membranes with 1% sodium cholate and 1.2 mg/mL phospholipids, isolated as 36 000g supernatant
purified[c]	crude soluble fraction purified by affinity chromatography on mercury–agarose columns

[a] The D-1 dopamine receptors used in this study were subject to different treatments, prior to incubation with 10 mM βME. D-1 receptors from both control membrane-bound and NEM-treated membrane-bound receptors were treated with 10 mM βME and then washed 3 times, prior to radioligand binding assays. [b] Crude soluble receptors were treated with 10 mM βME in the soluble state, and the reductant was removed by chromatography on Ultrogel AcA 202 columns. [c] Purified receptors were eluted from mercury–agarose columns with 10 mM βME and subject to chromatography on AcA 202 columns. Both crude soluble and purified D-1 receptors were reconstituted into phospholipid vesicles, prior to conducting any radioligand binding assays.

receptor and the methylmercury groups of the affinity column. The bound receptors were subsequently eluted with 10 mM βME, which was then removed by desalting the receptors on Ultrogel AcA 202 columns, prior to reconstitution of receptors into phospholipid vesicles (Sidhu, 1990). Competition studies indicated that the antagonist binding properties of the purified D-1 dopamine receptors were virtually identical to those of either membrane-bound (Sidhu et al., 1986b) or soluble D-1 dopamine receptors (Sidhu, 1988). By contrast, the purified receptor bound agonists with only high-affinity values, in a stereoselective manner, despite the absence of any such high-affinity binding in NEM-treated crude soluble receptors used as the starting source for these purifications. These high-affinity sites were not modulated by GTP analogs. Further, we show that the reducing agent βME induces the ability of the purified D-1 receptors to bind with high-affinity to agonists. The ability of βME to induce such Gpp(NH)p-insensitive high-affinity sites was also demonstrated in both membrane-bound and crude soluble extracts containing NEM-treated receptors. In the presence of functional G proteins, βME-induced high-affinity binding sites remained sensitive to modulation by Gpp(NH)p. These data suggest that whereas the Gpp(NH)p sensitivity of agonist high-affinity binding sites requires the presence of functional G proteins, the induction of these high-affinity sites by βME occurs independent of G proteins.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and reagents were from sources previously described (Sidhu, 1990); agonists and antagonists used in this study were purchased from Research Biochemicals Inc. (Natick, MA); [^{125}I]SCH 23982 (2200 Ci/mmol) was from Du Pont–New England Nuclear (Boston, MA). Antibodies 8729 and 9072 against G protein subtypes (G_i and G_o, respectively) were a kind gift of Dr. David Manning (University of Pennsylvania); the specificity of antibody 8729 was $G_i\alpha1 \sim G_i\alpha2 > G_i\alpha3$, and that of 9072 was $G_o\alpha1 = G_o\alpha2$. Anti-G_s antibodies, NEI-805, were purchased from Du Pont–New England Nuclear.

NEM Treatment of Membranes and Solubilization of D-1 Dopamine Receptors. Rat striatal membranes from male Sprague-Dawley rats were prepared by homogenizing striata in 100 volumes (w/v) of 50 mM Tris-HCl, pH 7.4, followed by centrifugation at 1000g for 5 min to pellet nuclei and cellular debris. The supernatant was recentrifuged at 18000g for 20 min, and the pellet was washed once in the Tris-HCl buffer and recentrifuged. The final pellet of crude plasma membranes was resuspended at a protein concentration of 0.8 mg/mL in buffer A (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$), prior to treatment with NEM (Sidhu, 1988). The membranes were pretreated with 30 μM SKF R-38393 for 30 min at 37 °C, and NEM (100 mM, w/v in H_2O) was added to a final concentration of 5 mM. After an additional 25 min of incubation, the membranes were washed 3 times with 50 mM Tris-HCl, pH 7.4 (Sidhu, 1988). The D-1 receptors in such preparations are referred to as NEM-treated membrane-bound receptors and are the starting source for crude soluble and purified receptors (see Table 1). In some experiments, membranes which were not treated with NEM were incubated with agonist for the same time period and processed as described above; the receptors in such preparations are referred to as control membrane-bound receptors.

D-1 dopamine receptors from both untreated (control) and NEM-treated striatal membranes were solubilized as described previously (Sidhu, 1990). Briefly, membranes were resuspended in buffer A containing 1 mM PMSF to a protein concentration of 1 mg/mL and treated with 10 μM SKF R-38393 for 20 min at 37 °C, to stabilize the receptor upon subsequent solubilization (Sidhu, 1988). The membranes were diluted with an equal volume of ice-cold 50 mM Tris-HCl, pH 7.4, centrifuged (18 000g, 20 min), and resuspended at a protein concentration of 2 mg/mL in buffer S (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, 1.5 mM PMSF, and 1 mM DTT). Sonicated phospholipids (type VII; Sigma, St. Louis, MO) were added to a final concentration of 1.2 mg/mL, followed by addition of sodium cholate (20% w/v stock in water) to a final concentration of 1%. After 15–20 min on ice, the mixture was centrifuged (36 000g, 45 min), and the clear supernatants containing the crude soluble receptors were stored frozen in aliquots at –80 °C.

Purification of D-1 Dopamine Receptors. D-1 dopamine receptors were purified using crude soluble extracts obtained from NEM-treated membranes (Table 1). The crude soluble extracts were applied to 5 mL volumes of mercury–agarose, as described before by us (Sidhu, 1990). Briefly, the soluble extracts were applied to activated mercury–agarose columns at room temperature, to ensure maximal binding of D-1 receptors to the affinity column. After extensive washing of the column at 4 °C, the bound receptors were eluted with buffer D (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, 0.5 mM PMSF, 5 μg/mL each of leupeptin and pepstatin, 1 mM EDTA, 0.4% sodium cholate, and 0.6 mg/mL sonicated phospholipids) containing 10 mM βME (Sidhu, 1990). The first 15 mL of eluate, which usually contained low [^{125}I]SCH 23982 binding activity, was discarded, and the next 10 mL of eluate was collected. The purified D-1 receptors were desalted on Ultrogel AcA 202 columns to remove βME.

Treatment of Membranes and Crude Soluble Extracts with β-Mercaptoethanol. Control or NEM-treated membranes in binding assay buffer A (see above) were preincubated with 10 mM (final concentration) βME at 37 °C. After 20 min,

2 volumes of ice-cold 50 mM Tris-HCl, pH 7.4, were added, and the membranes were centrifuged (18 000*g*, 20 min). The pellet was washed twice with the same buffer and resuspended in binding assay buffer A. βME treatment of crude soluble receptors was conducted by incubation of the soluble receptors with 10 mM βME for 30 min on ice. βME was then removed by chromatography on Ultrogel AcA 202, as described above for purified receptors (Sidhu, 1990). After chromatography, the crude soluble receptors were reconstituted into phospholipid vesicles prior to conducting radioligand binding assays.

Reconstitution of D-1 Dopamine Receptors into Phospholipid Vesicles. All D-1 dopamine receptors in the soluble state, both crude soluble and purified, were first reconstituted into phospholipid vesicles, prior to conducting any radioligand binding assays (Sidhu, 1988). The 1% sodium cholate, present in both the crude soluble and purified D-1 receptor fractions, was removed by adsorption of the detergent onto SM-2 BioBeads (Bio-Rad Laboratories, New York, NY), followed by simultaneous incorporation of the receptors into phospholipid vesicles; this was accomplished by readding type VII phospholipids (1.2 mg/mL) to the receptor extracts prior to addition of the SM-2 BioBeads (Sidhu, 1988). The mixture was shaken gently on ice for 1 h; the supernatant was removed by aspiration and used immediately in radioligand binding assays.

Radioligand Binding Assays. Binding assays with membrane-bound receptors were conducted as described before (Sidhu & Kebabian, 1985). Washed membranes were resuspended at 0.4 mg of protein/mL in binding assay buffer A, and the binding assays were conducted as described before using this buffer system for all drug and radioligand dilutions. Binding assays using crude soluble receptors, reconstituted into phospholipid vesicles, were performed as described elsewhere (Sidhu, 1988). Briefly, all receptor, drug, and radioligand dilutions were conducted in buffer B (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, and 1 mM EDTA); 20–50 μL aliquots of the reconstituted receptors were used in the binding assay. Binding assays with purified D-1 receptors, reconstituted into phospholipid vesicles, were conducted using buffer E (buffer B supplemented with 120 mM NaCl) (Sidhu, 1990). In binding assays with Gpp(NH)p, the nonhydrolyzable analog of GTP was added to a final concentration of 100 μM. In all binding assay experiments, [^{125}I]SCH 23982 (specific activity 2200 Ci/mmol) was dried under a stream of nitrogen and dissolved immediately in the appropriate binding assay buffer described above; the final concentration of the radioligand in all assays was 0.5 nM. Binding was conducted at 37 °C for 30 min with membranes, and at room temperature for 60 min for all other receptor preparations. The reactions were terminated by filtering the assay system onto glass fiber filters, pretreated with 200 μL of ice-cold 0.3% poly(ethylenimine solution). In all binding assays, specific binding was obtained by subtracting nonspecific binding (defined as binding obtained in the presence of 10 μM SCH 23390) from total binding (binding obtained in the presence of buffer alone). In a typical experiment, total binding obtained with membrane-bound receptors was 18 000–25 000 cpm, while nonspecific binding was 200–600 cpm; for reconstituted crude soluble receptors total binding was 12 000–14 000 cpm, while nonspecific binding was 1500–2000 cpm; for purified receptors, total binding was 10 000–15 000 cpm, and nonspecific binding was 1200–3000 cpm.

Other Procedures. SDS–PAGE (Laemmli, 1970) followed by silver staining (Morrisey, 1981) was performed as before (Sidhu, 1990). The presence of D-1 receptors in the 10 mL fraction was demonstrated by gel electrophoresis, by first lyophilizing the eluate followed by precipitation of the protein by the chloroform/methanol extraction procedure (Wessel & Flugge, 1984), as described previously by us (Sidhu, 1990). Western blot analyses were performed as described elsewhere (Ausubel et al., 1987). All competition curves were analyzed by the computer-fitted program LIGAND (Munson & Rodbard, 1980). In each case, a two-site model was considered to be a significantly better fit according to the F test at $p < 0.05$. All values are summarized as means ± SD from 3–5 separate experiments, from different receptor preparations.

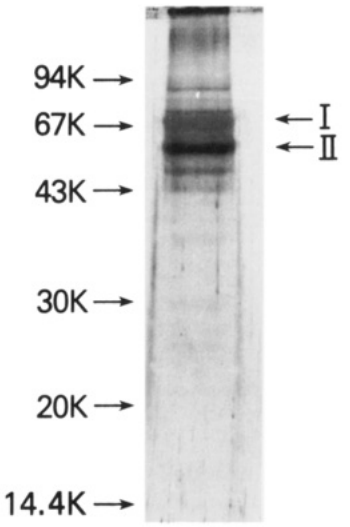

FIGURE 1: SDS–PAGE analysis of affinity-purified D-1 dopamine receptors. Crude soluble extracts, prepared from NEM-treated membranes, were applied to a 5 mL mercury–agarose affinity column, and the column was washed extensively as described under Experimental Procedures. The bound receptors were eluted with 10 mM βME; the first 15 mL of eluate was discarded, and the next 10 mL of eluate was collected and analyzed by SDS–PAGE, followed by silver staining (Sidhu, 1990). Bands I and II indicate the major polypeptides of interest.

RESULTS

Purification of D-1 Dopamine Receptors. In the present study, we purified D-1 dopamine receptors from NEM-treated rat striatal membranes, using a batch procedure for receptor purification. Briefly, crude soluble extracts prepared from NEM-treated membranes solubilized with 1% sodium cholate were applied onto mercury columns, and after the columns were washed, the bound receptors were eluted with 10 mM βME, as described before (Sidhu, 1990). However, instead of collecting small fractions of the βME-eluted material, the receptors were collected in a single batch. The first 15 mL of βME-eluted fraction was discarded, since this fraction did not usually display any appreciable [^{125}I]SCH 23982 binding activity. The next 10 mL of βME-eluted fraction was collected in a single batch and was desalted on a single AcA 202 column, to remove βME, as described before (Sidhu, 1990). After reconstitution of the desalted, purified receptors into phospholipid vesicles, radioligand binding studies revealed that approximately 60–75% of the applied binding activity was routinely recovered in this fraction.

To verify the purity of the D-1 dopamine receptors in the 10 mL fraction, this fraction was analyzed by SDS–PAGE, followed by silver staining of the gels (Sidhu, 1990). As seen in Figure 1, the 10 mL eluted fraction appeared to consist of two major polypeptides with masses of 70 and 52 kDa for bands I and II, respectively, well within the reported range of molecular masses of 67–74 kDa for polypeptide I and 50–54 kDa for polypeptide II (Sidhu, 1990). In addition, the 70

Table 2: Antagonist Dissociation Constants for Membrane-Bound and Affinity-Purified D-1 Dopamine Receptors[a]

antagonist	K_d (nM) of binding	
	membrane-bound	purified
SCH 23390	0.36 ± 0.12	1.1 ± 0.4
SCH 23388	18.6 ± 0.11	51.3 ± 1.8
(+)-butaclamol	19.0 ± 3.23	11.7 ± 2
SKF 103108-A	2.68 ± 1.04	1.69 ± 1.0
YM 09151	2360 ± 540	1820 ± 611

[a] Competition curves for all fractions were performed using 0.5 nM [^{125}I]SCH 23982, under the standard binding assay conditions described under Experimental Procedures. Membrane-bound receptors are control receptors, which were not treated with NEM. Purified receptors were reconstituted into phospholipid vesicles prior to conducting the radioligand binding assays. The competition curves were analyzed using the curve-fitting program LIGAND. Values are the means ± SD from 3–5 independent experiments.

kDa polypeptide stained with a characteristic translucent yellow color, representing the glycosylated form of the receptor, while the dark-staining 52 kDa protein was consistent with the deglycosylated form of the receptor (Sidhu, 1990). From densitometric scannings with a CS-9000 dual-wavelength scanner (Schimadzu, Columbia, MD) of the gels, an average purity of 70–80% of the receptor protein was estimated in this fraction.

Antagonist Binding Properties of Purified D-1 Dopamine Receptors. The purified D-1 dopamine receptors bound dopaminergic antagonists with appropriate stereoselectivity and affinity values (Table 2) typical of D-1 dopamine receptors in crude soluble receptors prepared from either control or NEM-treated membranes (Sidhu & Kebabain, 1985; Sidhu et al., 1986a). The iodo isomer of SCH 23390, SKF 103108-A, a potent D-1-selective antagonist, also bound to the purified D-1 receptors with affinity values typical of the membrane-bound receptors (Figure 2A). The binding of (+)-butaclamol to the purified receptor was similar to that of the membrane-bound receptor, while YM 09151, a D-2-selective antagonist, bound to the purified receptors only at high concentrations. The typical rank order of potency for the binding of antagonists to purified D-1 receptors was SCH 23390 = SKF 103108-A > (+)-butaclamol > SCH 23388 >> YM 09151. These data suggest that the D-1 dopamine receptor was isolated in a pharmacologically active state and that the antagonist binding properties of the purified receptors were unaltered as a consequence of NEM treatment of membranes or solubilization processes, and the different experimental manipulations employed during the course of purification.

Agonist Binding Properties of Purified D-1 Dopamine Receptors. When the agonist binding properties of the purified D-1 dopamine receptors were analyzed, a surprisingly different result was obtained. Competition curves with agonists were uniphasic, suggesting a single affinity state of the receptor (Figure 2A); Hill's plot of the binding data using purified D-1 receptors and SKF R-38393 gave a value of 0.97, confirming that all the binding sites of the purified receptor were composed of a single receptor population. The affinity binding values obtained with the purified receptor preparations, however, corresponded only to the agonist *high*-affinity state of the D-1 dopamine receptor. Thus, when using SKF R-38393, 100% of the purified D-1 receptors were in the high-affinity state and displayed an affinity value of binding of 13.7 ± 5.4 nM (n = 4), which is similar to the high-affinity binding value (K_h) obtained for the receptor in the control crude soluble state, prepared from membranes not treated with NEM (10.5 ± 4.4 nM, n = 9) (Table 3). The presence of high-affinity binding sites in the purified D-1 receptors was unexpected,

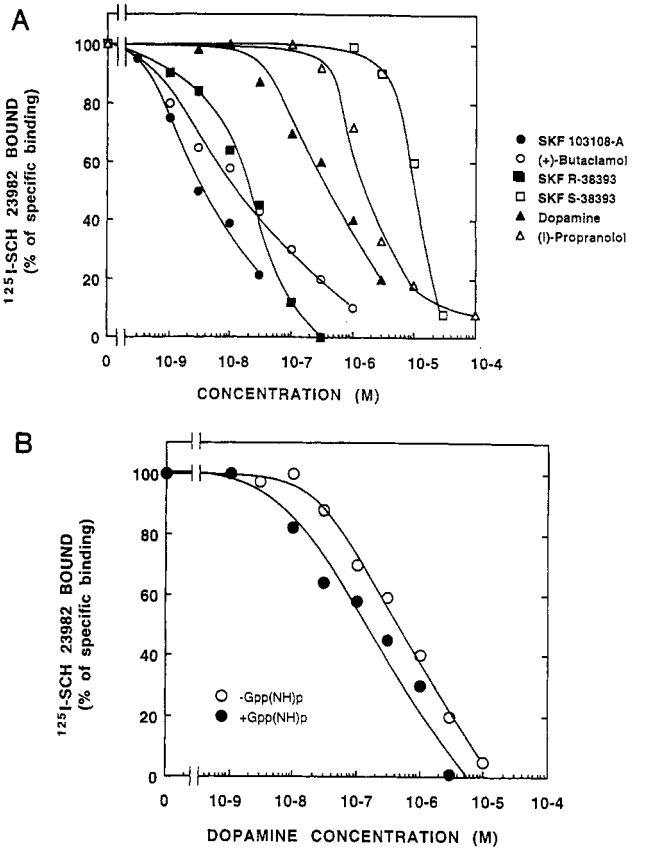

FIGURE 2: Pharmacological properties of ligand binding to purified D-1 dopamine receptors. The affinity-purified D-1 dopamine receptors contained in the 10 mL βME-eluted fraction of Figure 1 were desalted on Ultrogel AcA 202 columns, reconstituted into phospholipid vesicles, and tested for the ability of various drugs to compete for [^{125}I]SCH 23982 binding. Competition studies were conducted using 0.5 nM [^{125}I]SCH 23982 and increasing concentrations of different drugs (A), as described under Experimental Procedures. (B) Dopamine competition curves were obtained using increasing concentrations of dopamine in the presence or absence of a 100 μM sample of the nonhydrolyzable analog of GTP, Gpp(NH)p. The curves are from a representative experiment performed in triplicate; each competition curve was repeated at least 3 times, using different purified receptor fractions.

Table 3: Agonist Dissociation Constants of Control and NEM-Treated Crude Soluble, and Purified D-1 Dopamine Receptors[a]

agonist	K_d (nM) of binding		
	control crude soluble[b]	NEM-treated crude soluble	purified
SKF R-38393	10.5 ± 4.4 (K_h) (40)		13.7 ± 5.4 (100)
	286 ± 131 (K_l)	630 ± 150	
SKF S-38393	25000 ± 15500	11800 ± 1860	10300 ± 762
dopamine	173.4 ± 65.9		172.3 ± 96.7
	(K_h) (48.5)		(100)
	48000 ± 25000 (K_l)	11900 ± 2800	

[a] Competition curves were performed and analyzed as described in the legend to Table 2 and under Experimental Procedures. Control crude soluble receptors were not treated with any NEM; NEM-treated crude soluble receptors represent receptors solubilized from NEM-treated membranes. All crude soluble and purified fractions were reconstituted into phospholipid vesicles prior to radioligand binding assays. The value in parentheses represents the percent of receptor population which exhibits high-affinity binding for agonists. Values represent the means ± SD from 3–5 independent experiments. [b] Data presented with control crude soluble receptors are from Sidhu (1988).

since we had earlier demonstrated that NEM treatment of membranes resulted in abolition of high-affinity binding sites (Sidhu et al., 1986a, 1991). Thus, in crude soluble extracts prepared from NEM-treated membranes, SKF R-38393

bound to a single site on the receptor, with a binding value of 630 ± 150 nM (n = 5), corresponding to the *low*-affinity state of the receptor (Table 3). Since these crude soluble extracts from NEM-treated membranes were used as the source for receptor purification, the subsequent reemergence of high-affinity binding sites in the purified receptor was puzzling.

In order to determine if these results were due to anomalous binding between the purified D-1 receptors and the partial agonist SKF R-38393, the above studies were repeated using the full agonist dopamine (Figure 2B). To our surprise, similar results were also obtained with dopamine, whereby all the purified receptors were in a single affinity state, which exhibited a binding value of 172.3 ± 96.7 nM (n = 5) for dopamine. This corresponds to the high-affinity binding values of the D-1 receptor for dopamine in the control crude soluble state, where the K_h of binding was 173.4 ± 65.9 nM (n = 5) (Table 3). In crude soluble extracts prepared from NEM-treated membranes, however, dopamine bound to a single site with affinity values (K_d = 11 900 ± 2800 nM, n = 3) corresponding to the low-affinity state of the receptor (Table 3).

Interestingly, the ability of the purified receptors to bind with high affinity to agonists was seen only with pharmacologically active agonists, since the inactive isomer of SKF R-38393, SKF S-38393, bound to purified receptors with a K_d value of 10 300 ± 762 nM (n = 3), which is similar to the low-affinity values seen in crude soluble receptors isolated from either control or NEM-treated membranes (Table 3). The data with SKF S-38393 also indicate that the purified D-1 receptor was pharmacologically active in retaining the ability to discriminate between active and inactive isomers of the agonist.

Purified Receptor and G Protein Interactions. Since agonist high-affinity binding sites of receptors represent coupling between receptors activated in the presence of agonist and guanine nucleotide binding proteins (G proteins), we decided to investigate the possibility that G proteins may have been copurified with the D-1 dopamine receptors. Although this hypothesis seemed unlikely, since NEM causes an irreversible covalent alkylation of -SH groups associated with these G proteins (Sidhu et al., 1986a, 1991), disrupting the ability of these proteins to bind to mercury–agarose columns, we decided to, nonetheless, test the validity of this hypothesis. When competition curves (Figure 2B) with the purified receptor were performed using dopamine in the presence of 100 μM Gpp(NH)p, there were no rightward shifts of the competition curve, indicating that the high-affinity sites of the purified receptor were insensitive to modulation by the GTP analog. The affinity value for the competition curves obtained in the presence of Gpp(NH)p was 142 ± 16 nM (n = 4) and is within the standard error of mean of the value obtained for purified receptors binding to dopamine in the absence of Gpp(NH)p (172.3 ± 96.7 nM). The inability of Gpp(NH)p to convert the high-affinity binding of the purified receptor to dopamine to low affinity suggests that these high-affinity binding sites were not due to coupling between the receptor and G proteins.

To confirm this, we tested for the presence of G proteins in our purified receptor preparations using highly specific antibodies (Law et al., 1991), by Western blot techniques (Ausubel et al., 1987). Since D-1 receptors are able to couple to both G_s and G_i (Sidhu et al., 1991), we also used antibodies against both these G proteins, as well as to G_o. As seen in Figure 3A, anti-G_s antibodies, NEI-805, failed to bind to any proteins present in the βME-eluted fractions containing the D-1 dopamine receptor (lanes 3–6). To verify the ability of NEI-805 to bind to G_s, Western blots were conducted using

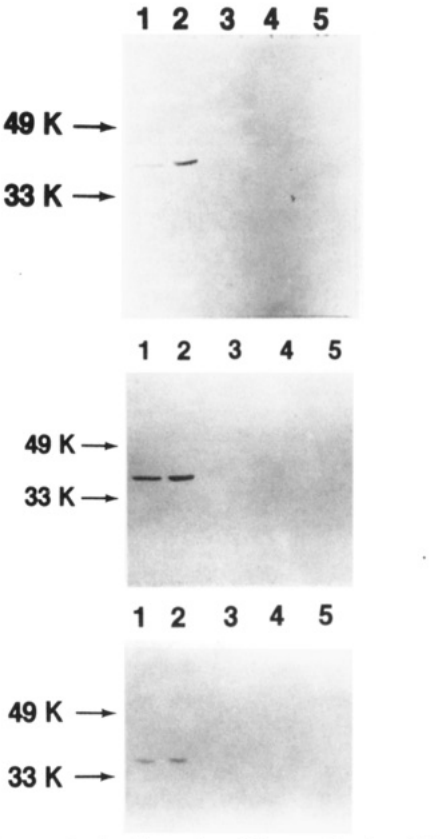

FIGURE 3: Immunological detection of the α-subunits of G proteins. The presence of G proteins in various receptor preparations was analyzed using the following specific antibodies against the α-subunits of the G proteins: (top) NEI-805; (middle) 8729; (bottom) 9072. Crude soluble extracts (5 μg of protein) were loaded in lanes 1; flow-through fractions (5 μg of protein) representing proteins which were not adsorbed by mercury–agarose columns were analyzed in lanes 2. Lanes 3–5 show 1 mL fractions specifically eluted by βME (Sidhu, 1990). Each fraction was lyophilized and precipitated by the chloroform/methanol extraction procedure (Wessel & Flugge, 1984). After SDS–PAGE on 10% polyacrylamide gels, Western blot analysis was conducted as described under Experimental Procedures. The positions of prestained molecular weight markers are indicated.

crude soluble extracts from NEM-treated membranes (lane 1) and to the flow-through fraction containing proteins which were unadsorbed by the mercury–agarose column (lane 2). In both instances, NEI-805 recognized a 45 kDa protein, of molecular mass similar to the α-subunit of G_s reported by others (Freissmuth et al., 1989). Similarly, antibody 8729 failed to detect the presence of any G_i subunit in the purified fractions (Figure 3B, lanes 3–6), although it recognized G_i in both the crude soluble (lane 1) and the flow-through fraction (lane 2). Similar negative results were also seen with antibody 9072, suggesting an absence of $G_o\alpha$ in the purified fraction (Figure 3C). These results indicate that G proteins were not copurified with the D-1 receptor and that the high-affinity binding values occurred independent of the presence of G proteins.

Effect of β-Mercaptoethanol on Agonist Binding Properties of Crude Soluble D-1 Dopamine Receptors. Since purified D-1 dopamine receptors were eluted from the mercury–agarose columns by the use of 10 mM β-mercaptoethanol (βME), we decided to investigate the possibility that the agonist high-affinity binding sites may have been induced by βME, by modulation of essential -SH groups localized at the agonist binding site of the receptor (Sidhu et al., 1986a). We tested for this by incubating crude soluble extracts prepared from NEM-treated membranes in the absence and presence of 10 mM βME for 30 min on ice. The soluble receptors were then

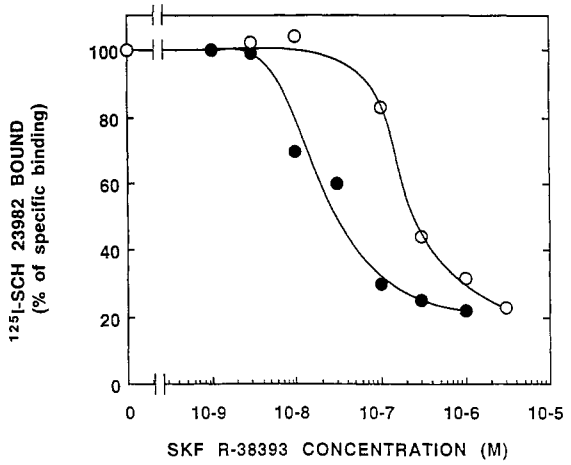

FIGURE 4: Effect of β-mercaptoethanol in inducing high-affinity agonist binding sites in D-1 receptors of crude soluble extracts. Crude soluble extracts, prepared from NEM-treated membranes, were incubated in the absence (○) and presence (●) of 10 mM βME for 30 min at 4 °C. Following incubation, the receptors of both preparations were subject to chromatography on Ultrogel AcA 202 columns and reconstituted into phospholipid vesicles. Binding of [^{125}I]-SCH 23982 to the reconstituted preparations was conducted in the presence of increasing concentrations of SKF R-38393, as described under Experimental Procedures; the curves are from a representative experiment performed in triplicate. Each experiment and competition curve were repeated 3 times.

Table 4: Effect of β-Mercaptoethanol in Inducing High-Affinity Agonist Binding Sites in NEM-Treated Crude Soluble D-1 Dopamine Receptors[a]

treatment of D-1 dopamine receptors	K_d of binding (nM)	
	-Gpp(NH)p	+Gpp(NH)p
-βME	566.5 ± 48.5	515.5 ± 29.5
+βME	21.0 ± 10.7	50.0 ● 11

[a] Crude soluble receptors were isolated from membranes which were treated with 5 mM NEM in the presence of 10 μM SKF R-38393. Soluble receptors were incubated in the absence and presence of 10 mM βME at 4 °C for 30 min, followed by chromatography on AcA 202 columns. The receptors were then reconstituted into phospholipid vesicles, and binding studies were performed. Values represent the means ± SD from 3 independent experiments.

desalted on AcA 202 columns and reconstituted into phospholipid vesicles, and the binding of SKF R-38393 to these receptors was analyzed. As expected, in the absence of any βME treatment, SKF R-38393 bound to a single low-affinity site on the D-1 receptors, with a K_d of binding of 566.5 ± 48.5 nM (Figure 4), which was not modulated by 100 μM Gpp(NH)p (K_d = 515.5 ± 29.5 nM, Table 4). When the crude soluble receptors were treated with 10 mM βME, the competition curves were shifted leftward, and SKF R-38393 bound with much higher affinity values (K_d = 21.0 ± 10.7 nM) to a single site on the receptors (Figure 4). These induced high-affinity sites were also not due to coupling to G proteins, since competition curves performed in the presence of Gpp(NH)p failed to significantly ($p > 0.05$) alter the K_d of binding of SKF R-38393 to the βME-treated, reconstituted receptors (Figure 4 and Table 4).

Effect of β-Mercaptoethanol on Agonist Binding Properties of Membrane-Bound D-1 Dopamine Receptors. Since βME induced Gpp(NH)p high-affinity binding sites in crude soluble receptors, we felt it worthwhile to determine whether βME could also induce the formation of similar binding sites in membranes. In addition, by using such a system, we would eliminate any artifacts which may have been inadvertently introduced during NEM treatment or during solubilization/reconstitution. We had previously shown that in the native

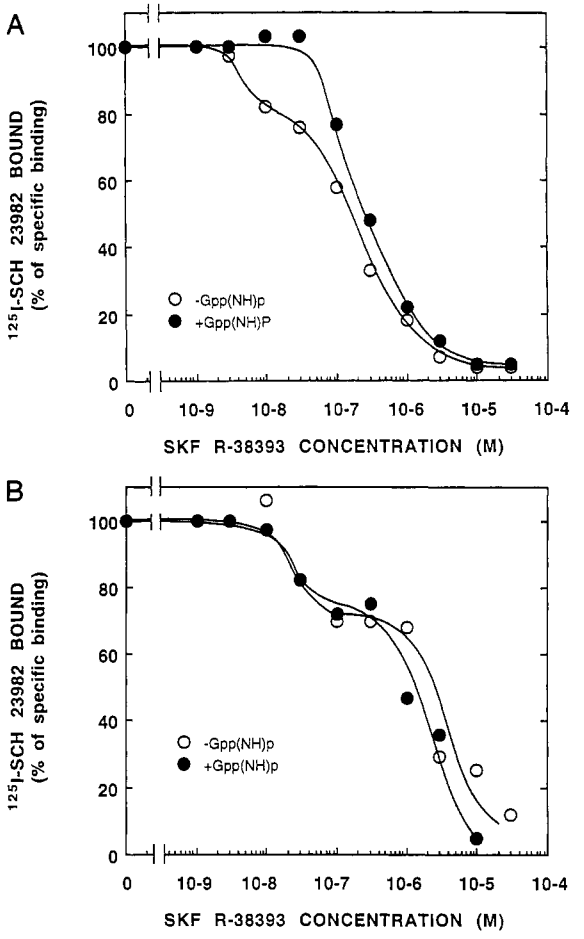

FIGURE 5: Induction of high-affinity binding sites of membrane-bound D-1 dopamine receptors by β-mercaptoethanol. (A) Control (not NEM-treated) membranes and (B) NEM-treated membranes were incubated with 10 mM βME for 20 min at 37 °C. The membranes were washed 3 times with 50 mM Tris-HCl, pH 7.4, prior to conducting radioligand binding assays with 0.5 nM [^{125}I]SCH 23982, in the presence of increasing concentrations of SKF R-38393. When present, Gpp(NH)p was added to a final concentration of 100 μM. The curves are from a representative experiment; all experiments were repeated 2–3 times, using different membrane preparations. The competition curves were analyzed by the curve-fitting program LIGAND, and in each case, a two-site model was considered to be a significantly better fit according to the F test at $p < 0.05$.

membrane-bound state, only 6% of the total D-1 receptor population was in the high-affinity state (Sidhu, 1988). These control (not treated with NEM) membranes were incubated with 10 mM βME for 20 min at 37 °C and washed to remove the reducing agent. Competition curves with βME-treated membranes using SKF R-38393 revealed the presence of both high- and low-affinity binding sites, with affinity values of 5.95 ± 0.35 and 291.0 ± 9.0 nM (n = 3), respectively (Figure 5A and Table 5). Compared to membranes not treated with βME, there was a 4-fold induction of D-1 receptors in the high-affinity state, and approximately 25% (n = 3) of the receptors were in the high-affinity state. In the presence of 100 μM Gpp(NH)p, these high-affinity sites were abolished, and all the binding sites displayed low-affinity binding values (K_d = 348.5 ± 97.5 nM, n = 4).

The above studies were repeated using membranes which were pretreated with 5 mM NEM to inactivate endogenous G proteins (Sidhu et al., 1986a), followed by treatment in the absence or presence of 10 mM βME. Treatment of membranes with 5 mM NEM abolished all agonist high-affinity binding sites, and 100% of the D-1 receptors were in a low-affinity binding state (Sidhu et al., 1986a). Competition studies of the NEM- and βME-treated membranes with increasing

Table 5: Effect of β-Mercaptoethanol and *N*-Ethylmaleimide on High-Affinity Agonist Binding Sites of Membrane-Bound D-1 Dopamine Receptors[a]

treatment of membranes	affinity (nM)			
	–Gpp(NH)p		+Gpp(NH)p	
	K_h	K_l	K_h	K_l
+βME	5.95 ± 0.35 (25 ± 0)	291.0 ± 9.0 (75 ± 0)	0	348.5 ± 97.5 (100)
+βME, +NEM	31.8 ± 1.8 (27.3 ± 3.8)	5100 ± 2840 (72.7 ± 3.8)	43.6 ± 3.8 (27.5 ± 2.5)	3180 ± 180 (72.5 ± 2.5)

[a] Rat striatal membranes, pretreated with 5 mM NEM and protected with SKF R-38393, were treated with 10 mM βME at 37 °C. After 30 min, the membranes were washed 3 times with 50 mM Tris-HCl, pH 7.4, and competition studies were conducted using the agonist SKF R-38393, as described under Experimental Procedures. The results represent the mean ± SD from 3–4 independent experiments, using different tissue preparations. Values in parentheses denote the percent of receptors in the high- or low-affinity state.

concentrations of SKF R-38393 showed the presence of both high- and low-affinity (K_h = 31.8 ± 1.8 and K_l = 5100 ± 2840 nM, n = 4) binding sites (Figure 5B and Table 5), with 27.3 ± 3.8% (n = 4) of the receptors in the high-affinity state. However, unlike the results obtained above for βME-treated membranes, the addition of 100 μM Gpp(NH)p failed to convert the high-affinity sites to low affinity, and SKF R-38393 continued to bind to the receptors with both high- and low-affinity values (K_h = 43.6 ± 3.7 and K_l = 3180 ± 180 nM, n = 3); 27.5 ± 2.5% (n = 3) of the receptors were in the high-affinity state.

Interestingly, in the studies with membranes, 72–75% of the total receptor binding sites persisted in the low-affinity state after βME treatment in the absence of Gpp(NH)p (Figure 5), whereas in soluble receptor preparations (both crude soluble and purified) low-affinity sites were completely absent after βME treatment of receptors. The lack of complete conversion of low-affinity sites to high-affinity in membranes may be due to inaccessibility of -SH groups to the reducing agent, perhaps due to constraints imposed by the membrane structure. In soluble systems where such constraints are absent, all available -SH may be equally accessible to the reducing agent. Taken together, the data suggests that βME can cause an induction of high-affinity binding sites in D-1 dopamine receptors, independent of functional G proteins. Gpp(NH)p sensitivity of these βME-induced high-affinity sites, however, is dependent on the presence of functional G proteins.

DISCUSSION

In the present study, we have shown that D-1 dopamine receptors can be rapidly purified, by a modified "batch" method, to a high state of purity and that the purified receptors retain antagonist binding properties which are virtually identical to the receptors in either the control membrane-bound or the crude soluble states. We have also shown that agonists bind to the purified receptors with high-affinity values. Since NEM treatment of membranes inactivates G proteins, resulting in a loss of Gpp(NH)p-sensitive agonist high-affinity binding sites (Sidhu et al., 1986a, 1991), the presence of high-affinity sites in the purified receptors was unexpected and puzzling. The inability of GTP analogs to modulate these high-affinity sites suggests that the purified D-1 receptors were not coupled to G proteins. Furthermore, there were no G proteins copurified with the D-1 receptors, as confirmed by Western blots using specific antisera against the various α-subunits of the G proteins.

In order to determine the molecular basis for the induction of high-affinity binding sites in the purified D-1 receptor preparation, we examined the possibility that these sites may have resulted as a consequence of the purification procedures. It was unlikely that the solubilization/reconstitution procedure itself resulted in the formation of such sites, since crude soluble receptors prepared from NEM-treated membranes exhibited only low affinity for agonists (Table 3). We focused our efforts on the reducing agent, βME, used for elution of purified receptors from the affinity column, and speculated that βME may have caused changes in the -SH groups located at or near the agonist binding region of the receptor, resulting in the formation of such sites. Incubation of NEM-treated receptors in the crude soluble state with 10 mM βME also caused a conversion of the entire receptor population from a low-affinity to a high-affinity, Gpp(NH)p-insensitive state. The prior lack of high-affinity sites in crude soluble receptors, which were prepared from NEM-treated membranes, but not incubated with βME (Table 3), strongly supports our conclusion that βME itself is the causative agent responsible for the induction of such sites in the purified receptors.

When these studies were repeated in membranes, βME treatment of membranes resulted in only a partial conversion of receptors to the high-affinity state, raising the possibility that constraints imposed by the membrane structure may cause only a certain population of the receptors to be accessible to βME. Interestingly, Gpp(NH)p sensitivity was only seen in those membranes which were not pretreated with NEM. Since NEM treatment of membranes cause alkylation of G protein-associated -SH groups resulting in inactive G proteins (Sidhu et al., 1986a), the presence of functional G proteins is a prerequisite for conferring Gpp(NH)p-sensitivity to receptor high-affinity binding sites. However, the formation and existence of the D-1 receptor in the high-affinity state appear to be solely dependent on the molecular interactions between the receptor and βME, and appear to occur independent of the presence or absence of active or inactive G proteins. Thus, the ability of D-1 dopamine receptors to exist in a high-affinity state, independent of G proteins, may be an intrinsic function of the receptor.

The mechanism by which βME induces these high-affinity sites in the D-1 receptors remains to be established, but this effect may be directly related to the reducing properties of βME. We had earlier shown that D-1 dopamine receptors possess essential -SH groups which are located at or near the agonist binding site (Sidhu et al., 1986a). Modification of these -SH groups by either NEM or 5,5′-dithiobis(2-nitrobenzoic acid) resulted in the loss of ligand binding activity, suggesting that these -SH groups are essential for receptor function. Subsequent to our findings, others have confirmed the involvement of these essential -SH groups in the functional properties of D-1 dopamine receptors (Dewar & Reader, 1989). Although NEM-treated receptors lack high-affinity Gpp(NH)p-sensitive sites, treatment of these receptors with βME may directly affect these -SH groups, resulting in some conformational change in the receptor protein, such that it can now bind to agonists with high-affinity, but in a manner which is insensitive to Gpp(NH)p. Such a change may result in a reversion of the conformation of the purified receptor to

that of the active state of the receptor, since the affinity *value* of binding of purified receptors to agonists remained the same as that seen for receptors in either the membrane-bound (Sidhu et al., 1986b) or the control crude soluble state (Sidhu, 1988). The observed lack of differences for antagonist binding values between purified and membrane-bound or crude soluble receptors also supports the hypothesis that βME treatment did not prevent the purified receptor from existing in a pharmacologically active state.

It is unlikely that βME acts on the phospholipids themselves, since solubilization and reconstitution of control (not treated with NEM) receptors, in the presence of 1 mM DTT, show substantial enhancement of Gpp(NH)p-sensitive, agonist high-affinity binding (Sidhu, 1988), irrespective of whether βME is present. It is equally unlikely that βME acts on the G proteins themselves, causing a reversal of alkylated -SH groups in NEM-treated G protein preparations, since βME failed to restore Gpp(NH)p-sensitivity to the high-affinity binding sites of crude soluble extracts (Table 3). As mentioned above, in our earlier studies in which D-1 dopamine receptors were solubilized from membranes not treated with NEM, in the presence of 1 mM DTT, we also demonstrated the induction of high-affinity binding sites (Sidhu, 1988). The solubilization procedure resulted in 40% of the receptor population to exist in the high-affinity state, which maintained complete sensitivity to Gpp(NH)p, due to the presence of active G proteins. It is likely that at a lower concentration, reducing agents are only able to partially induce the formation of these high-affinity binding sites. Alternately, the presence of functional G proteins may cause changes in the receptor structure, rendering some of the -SH sites of the receptor to be inaccessible to reducing agents. In this regard, it should be noted that DTT was added to membranes prior to solubilization (Sidhu, 1988), and the full effect of DTT on soluble receptors remains to be studied.

Interestingly, the existence of agonist high-affinity sites in dopamine receptors which are not coupled to G proteins is not without precedence. Digitonin-solubilized D-1 dopamine receptors exhibited high-affinity agonist binding values, which were higher than those found for membrane-bound receptors (Gingrich et al., 1988). This is in contrast to our own studies, whereby the crude soluble or purified D-1 receptors retained the identical high-affinity binding values seen for membrane-bound receptors. In other studies, approximately half the agonist high-affinity sites were found to be uncoupled from G proteins after solubilization and were not modulated by guanine nucleotides (Niznik et al., 1986). In both these studies, however, the antagonist affinity values were significantly lower (5–33-fold) than those seen in the membrane-bound state, raising the possibility that these receptors may not be completely pharmacologically active.

The high-affinity binding of receptors has been widely believed to represent the active state of the receptor, and it is well accepted that these high-affinity sites are due to formation of the heterotrimeric complex of agonist, receptor, and G proteins. However, the existence of high-affinity receptor sites in the presence of inactive G proteins, or in the complete absence of G proteins, has not been shown to the best of our knowledge. These studies with D-1 receptors raise the possibility that certain receptors are intrinsically able to exist in a high-affinity state, independent of G proteins. The induction and abolition of such high-affinity sites have an important and significant regulatory role in the functional activity of these receptors, especially in the etiology of pathological states. In Parkinson's disease, enhanced oxidative effects, manifest by reduction in glutathione peroxidase activity and reduction in glutathione levels (an endogenous reducing agent), have been suggested to have an important role in the clinical manifestation of the disease (Youdim et al., 1993). In other studies, decreases in levels of the antioxidant ascorbic acid have been shown to increase production of hydrogen peroxide (Spina & Cohen, 1989). As a corollary to our findings that βME induces high-affinity binding, increases in the oxidative state of neural cells in Parkinson's disease may lead to decreases in the high-affinity binding of the receptor, resulting in a receptor which is less active. In a recent study, we have shown that under oxidizing conditions, 50% of receptor binding activity was lost, but this change was accompanied by a compensatory increase in the high-affinity binding value of the remaining receptors (Kimura & Sidhu, 1994). Thus, the -SH groups which are located at the ligand binding site of D-1 receptors, and which demonstrate exquisite sensitivity to both oxidizing and reducing agents, may provide the key for understanding the mechanism of D-1 dopamine receptor function in neuronal systems.

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