Sulfhydryl Group(s) in the Ligand Binding Site of the D-1 Dopamine Receptor: Specific Protection by Agonist and Antagonist

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ABSTRACT: An iodinated compound, [^{125}I]-8-iodo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepin-7-ol, has been recently reported [Sidhu, A., & Kebabian, J. W. (1985) *Eur. J. Pharmacol. 113*, 437–440] to be a specific ligand for the D-1 dopamine receptor. Due to its high affinity and specific activity, this ligand was chosen for the biochemical characterization of the D-1 receptor. Alkylation of particulate fractions of rat caudate nucleus by *N*-ethylmaleimide (NEM) caused an inactivation of the D-1 receptor, as measured by diminished binding of the radioligand to the receptor. The inactivation of the receptor sites by NEM was rapid and irreversible, resulting in a 70% net loss of binding sites. On the basis of Scatchard analysis of binding to NEM-treated tissue, the loss in binding sites was due to a net decrease in the receptor number with a 2-fold decrease in the affinity of the receptor for the radioligand. Receptor occupancy by either a D-1 specific agonist or antagonist protected the ligand binding sites from NEM-mediated inactivation. NEM treatment of the receptor in the absence or presence of protective compound abolished the agonist high-affinity state of the receptor as well as membrane adenylate cyclase activity. The above-treated striatal membranes were fused with HeLa membranes and assayed for dopamine-stimulated adenylate cyclase activity. When the sources of D-1 receptors were from agonist-protected membranes, the receptors retained their ability to functionally couple to the HeLa adenylate cyclase. In contrast, when unprotected and antagonist-protected membranes were used as the donors, the response to dopamine after fusion was very low. These results suggest that the D-1 dopamine receptor contains NEM-sensitive sulfhydryl group(s) either at or near the vicinity of the ligand binding sites, which are critical for both receptor binding and function.

The dopamine receptor has been classified into two distinct categories of receptors, D-1 and D-2, on the basis of differential pharmacological and physiological functions (Kebabian & Calne, 1979). The D-1 dopamine receptor stimulates adenylate cyclase activity and increases parathyroid hormone release from parathyroid tissue (Brown et al., 1977), while the D-2 dopamine receptor causes inhibition of adenylate cyclase and prolactin release from anterior pituitary tissue (Ben-Jonathan et al., 1977). Although a great deal of information on the D-2 receptor has been obtained through the use of selective agonists and antagonists, comparable progress with the D-1 receptor has been severely hampered, in part, by the lack of availability of similarly selective compounds.

The best available ligands used to study the D-1 receptor had been the thioxanthene neuroleptics, [^{3}H]-*cis*-(*Z*)-flupentixol (Setler et al., 1978) and [^{3}H]-*cis*-(*Z*)-piflutixol (Seeman, 1980), which show similar affinities for the D-1 and D-2 sites. The recent identification of SCH 23390, (*R*)-(+)-8-chloro-7-hydroxy-2,3,4,5-tetrahydro-3-methyl-1-phenyl-1*H*-3-benzazepine, as a potent and selective antagonist of the D-1 dopamine receptor (Hyttel, 1983; Iorio et al., 1983) provided the first generation of D-1 specific antagonists. With the availability of the ligand in its tritiated form (Billard et al., 1984), the pharmacology (Billard et al., 1984) and regional distribution (Dawson et al., 1985) of the D-1 site in the brain have been studied.

In order to develop a ligand of higher specific activity than tritiated compounds, we recently reported the synthesis of [^{125}I]SCH (*RS*)-23982,[1] the 8-iodo analogue of SCH 23390 (Sidhu & Kebabian, 1985). Due to the higher specific activity of the iodo ligand (2200 Ci/mmol) over [^{3}H]SCH 23390, we decided to use the [^{125}I]SCH (*RS*)-23982 as a probe to investigate the biochemical and molecular functions of the D-1 receptor in rat striatum.

The alkylating agent NEM[2] has been used extensively to probe the potential role of sulfhydryl groups in the maintenance of the structure and function of β-adrenergic receptors (Stadel & Lefkowitz, 1979; Vauquelin et al., 1980; Korner et al., 1982; Heidenreich et al., 1982). Although NEM treatment had no effect on antagonist binding, it abolished high-affinity agonist binding to the β-adrenergic receptors, presumably by preventing the formation of the ternary complex between agonist, receptor, and G protein (Stadel & Lefkowitz, 1979). When membranes were exposed to NEM in the presence of agonist, approximately half of the antagonist binding sites were lost (Vauquelin et al., 1980). Under these conditions, NEM appears to stabilize the agonist–receptor–G protein complex

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[1] In this paper, reference is made to several forms of the substance 8-iodo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepin-7-ol: [^{125}I]SCH (*RS*)-23982 refers to the racemate of this substance; [^{125}I]SCH 23982 refers to the 5*R* enantiomer of this substance; SK & F 103108A refers to the nonradioactive, racemic form of this substance.

[2] Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); G protein, guanyl nucleotide binding protein; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; GTP, guanosine 5′-triphosphate.

(Heidenreich et al., 1982) and to actually "lock" the agonist on the receptor (Korner et al., 1982). In this regard, NEM appears to have no effect on β-adrenergic receptor function (Korner et al., 1982) or structure (Moxham & Malbon, 1985). In the present study we demonstrate that, in contrast to the β-adrenergic receptor, the D-1 dopamine receptor has sulfhydryl group(s) associated with it that are critical for both ligand binding and function.

Experimental Procedures

Materials. Drugs were obtained from the following sources: all SK & F compounds, Smith Kline and French Laboratories (Philadelphia, PA); SCH 23390 and SCH (*S*)-23390, Schering-Plough (Bloomfield, NJ). Poly(L-lysine), NEM, and DTNB were purchased from Sigma Chemical Co. (St. Louis, MO). [^{125}I]SCH (*RS*)-23982 (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA) by the custom iodination of SK & F (*RS*)-83692, the racemic dehalogenated benzazepine. [^{125}I]SCH 23982, the pharmacologically active *R* isomer of the iodo ligand, was a gift from Russell Garlick of New England Nuclear. All other reagents were of the highest purity commercially available.

Tissue Preparation. A particulate preparation of fresh caudate nucleus of male Sprague-Dawley rats was prepared by homogenizing the tissue in 100 volumes (w/v) of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 18000*g* for 10 min, and the pellet was washed once in the Tris-HCl buffer and centrifuged again. The final pellet was resuspended in 100 volumes (w/v) of binding assay buffer, containing 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. The homogenate was either used immediately or stored at −70 °C for up to 60 days without appreciable loss in ligand binding activity.

Treatment with Sulfhydryl Reagents. Tissue homogenate preparations (200 μL) were placed in 1.5-mL Eppendorf tubes in the presence of the indicated concentrations of NEM or DTNB. After incubation at 37 °C for 20 min, the samples were diluted with 1 mL of Tris-HCl, pH 7.4, and centrifuged in a Beckman microfuge at 15000*g* for 10 min. The pellets were washed 3 times and suspended in either the binding assay buffer or the adenylate cyclase assay buffer. To demonstrate the ability of the presence of either agonist or antagonist to protect against the effect of NEM, the tissue homogenates were preincubated with the appropriate drugs for 15–30 min at 37 °C prior to the addition of NEM.

Radioligand Binding. Washed tissue homogenates were resuspended in 200 volumes (w/v) of binding assay buffer (see above), and the binding assay was performed in quadruplicate essentially as described previously (Sidhu & Kebabian, 1985). Briefly, 50 μL of the tissue homogenate, containing 250 μg of tissue, was incubated with 0.5 nM of the radioligand in the presence or absence of agonists or antagonists, in a total volume of 150 μL in a 96-well microtiter plate (Flow Labs, McLean, VA). For saturation binding, the concentration of the (*R*)-iodo ligand varied from 0.01 to 2.5 nM. The radioligand and drugs were diluted in the binding assay buffer. After incubation for 30–60 min at 37 °C, the assay mixtures were transferred with a 12-channel Titertek pipet (Flow Labs) onto no. 30 glass-fiber filters contained in a 96-well microfiltration manifold (Schleicher and Schuell, Keene, NH). The bound ligand was separated from the free ligand by washing each filter with 1.5 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.4. The filters were counted in a Micromedic γ counter (75% efficiency). For the saturation binding curves, nonspecific binding was defined as binding in the presence of 10 μM SK & F (*RS*)-103108A; for all other experiments, nonspecific binding was determined in the presence of 10 μM SCH 23390. Prewetting the filters with 0.2% solution of poly(L-lysine) reduced the amount of ligand bound nonspecifically to the filters. Under these assay conditions, nonspecific binding represented less than 5% of the total radioactivity. In a typical experiment using 0.5 nM of the (*RS*)-iodo ligand, the total bound radioactivity was approximately 7000 cpm, while the nonspecific counts were less than 200 cpm.

Adenylate Cyclase Assays. Adenylate cyclase activity was assayed as previously described (Côte et al., 1980) in the presence of 1.5 mM ATP and 6 mM $MgSO_4$. Forskolin and SK & F (*R*)-38393 were present at a concentration of 10 μM, while 100 μM of dopamine was used. The amount of protein present in the assays was determined by the method of Lowry et al. (1951).

Membrane Fusion. Striatal membranes were fused with HeLa membranes by means of established procedures (Kassis et al., 1984; Kassis & Fishman, 1984). Briefly, NEM-treated striatal membranes (1.2–1.35 mg of protein) were mixed with membranes (1 mg of protein) from butyrate-treated HeLa cells, pelleted by centrifugation, and fused with poly(ethylene glycol). The fused membranes were then washed and assayed for adenylate cyclase activity with [^{32}P]ATP as substrate (Kassis et al., 1984).

Data Analysis. Analysis of binding saturation and competition curves was accomplished by using the computer fitted program LIGAND (Munson & Rodbard, 1980). This modeling technique provides values for the affinity constants of the competing ligand for the receptor sites and the proportion of the sites present when a two-state fit is appropriate. A two-state fit is considered appropriate only when the fit is significantly improved ($p < 0.05$) over that for a one-state fit. All values are summarized as mean ± SE from three to six separate experiments.

Results

Inactivation of D-1 Dopamine Receptors by NEM. Pretreatment of rat striatal particulate fractions with the sulfhydryl alkylating reagent NEM caused an apparent inactivation of the D-1 dopamine receptor, resulting in a decrease in the binding of [^{125}I]SCH (*RS*)-23982 (Figure 1). The loss of ligand binding sites by NEM was dependent on the concentration of the sulfhydryl reagent, with an IC_{50} for receptor inactivation of $(1.4 \pm 0.47) \times 10^{-4}$ M. Although NEM reacts specifically and irreversibly with free sulfhydryl residues of proteins, it seemed important to establish at the outset of this study that the apparent inactivation of the D-1 receptor is indeed due to an attack on sulfhydryl groups. For that purpose, the effect of another sulfhydryl reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), (DTNB) on the ligand binding site of the receptor was tested. As seen in Figure 1, DTNB also caused a reduction in the binding sites of the D-1 receptor, and the reagent appeared to be almost as potent as NEM, with an IC_{50} of $(4.5 \pm 0.55) \times 10^{-4}$ M. At the highest concentration of the sulfhydryl reagents tested (3 mM), approximately 70% of the receptor binding sites disappeared; in other experiments the apparent loss of binding sites varied from 65% to 75%. Inactivation of the binding sites was irreversible, and binding activity of NEM-treated striatal tissue homogenates was not restored by up to 10 mM DTT (data not shown).

The attack by NEM on sulfhydryl groups was rapid (Figure 2); the $t_{1/2}$ of receptor inactivation with 0.1 and 3 mM NEM was 60 and 75 s, respectively, and maximum receptor inactivation was achieved in 20 min. Prolonging the incubation time to 40 min did not result in further increase in the loss of binding sites (data not shown).

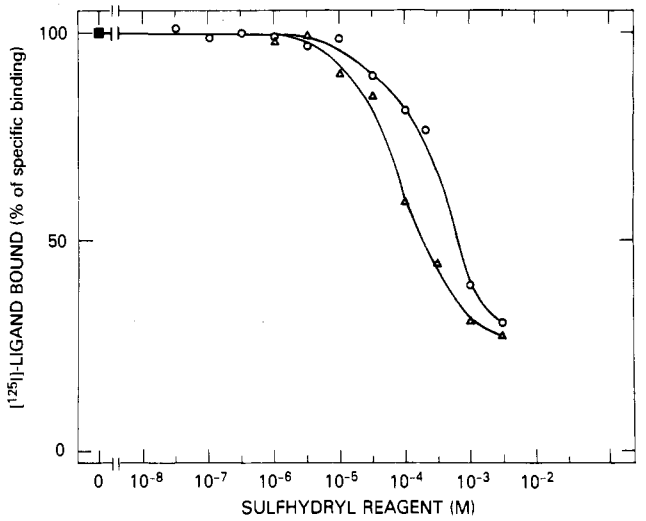

FIGURE 1: Inactivation of [^{125}I]SCH (*RS*)-23982 binding sites by sulfhydryl reagents. Particulate fractions from rat striatal tissue, suspended in 100 volumes (w/v) of binding assay buffer was incubated with increasing concentrations of NEM (△) or DTNB (○). After 20 min at 37 °C, the lysates were washed 3 times and the final pellet was resuspended in 200 volumes (w/v) of binding assay buffer. The binding of [^{125}I]SCH (*RS*)-23692 (0.5 nM) was performed for 30 min as described under Experimental Procedures. The data are representative of at least three separate experiments and are expressed as percentage of control binding. Control binding (■) refers to binding of the radioligand to fractions pretreated with buffer alone, after subtracting for nonspecific binding (determined in the presence of 10 μM SCH 23390).

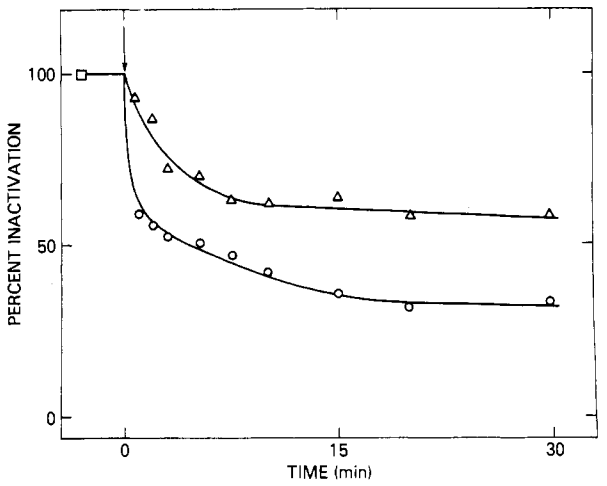

FIGURE 2: Time course of inactivation of [^{125}I]SCH (*RS*)-23982 binding sites by NEM. Particulate fractions were treated with 0.1 mM (△) or 3 mM (○) NEM. After incubation for the indicated time at 37 °C, the assay was terminated by the addition of 1 mL of stopping buffer (50 mM Tris-HCl, pH 7.4, 10 mM DTT) and centrifuged. The lysates were washed 3 times with 50 mM Tris-HCl, pH 7.4, in the absence of DTT, and radioligand binding was assayed as described in the legend to Figure 1. Control binding (□) refers to specific counts bound in the absence of NEM.

In order to characterize the binding parameters of the receptor after alkylation by NEM, analysis of saturation binding curves were performed. Initially, such studies were conducted with the (*RS*)-iodo ligand (data not shown). However, since the racemic radioligand mixture sometimes bound to the receptor in a biphasic manner (Sidhu & Kebabian, 1985), we felt that a more accurate estimation of the binding parameters would be obtained with the pharmacologically active *R* form of the ligand. With the recent availability of [^{125}I]SCH 23982, the *R* enantiomer of the antagonist, the saturation binding curves were performed with the latter ligand, and the data obtained are presented in Figure 3. [^{125}I]SCH 23982 bound

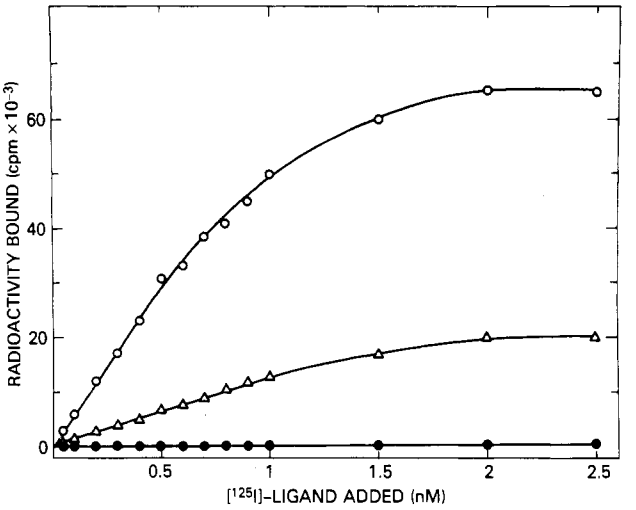

FIGURE 3: [^{125}I]SCH binding to control and NEM-treated striatal tissue. Particulate fractions were incubated in the presence (△) and absence (○) of 3 mM NEM for 20 min at 37 °C and washed 3 times as described under Experimental Procedures. The washed particulate fractions were resuspended in 200 volumes (w/v) of binding assay buffer and incubated with increasing concentrations of [^{125}I]SCH 23982 (0.01–2.5 nM) for 60 min at 37 °C. Nonspecific binding was determined in the presence of 10 μM SK & F (*RS*)-103108A. Since the nonspecific binding was almost identical in both control and NEM-treated tissue, the counts were averaged and are shown as a single line (●).

Table I: Dissociation Constants of Control and NEM-Treated Striatal Particulate Fractions[a]

compound	dissociation constants (nM)	
	control tissue	NEM-treated tissue
SK & F (*R*)-38393	19.4 ± 8.2, 640 ± 240	269 ± 40.4
SK & F (*S*)-38393	13700 ± 2200	11900 ± 1800
SCH 23390	0.36 ± 0.12	5.8 ± 1.0
SCH (*S*)-23390	18.6 ± 0.11	363 ± 79.9

[a] After treatment with 3 mM NEM, washed particulate tissue was incubated with 0.5 nM [^{125}I]SCH (*RS*)-23982 and increasing concentrations of agonists or antagonists. The dissociation constants (K_d) were obtained by computer analysis of the competition curves. The K_d of the untreated control fractions, except for the value obtained for SCH (*S*)-23390, were from Table 1 of Sidhu and Kebabian (1985). When two affinity sites were detected, the K_d for the high (K_h) and low (K_l) affinity states were calculated.

to a single site on the D-1 receptor in both control and NEM-treated tissue homogenates. After treatment with 3 mM NEM, the number of receptors decreased 70% from B_{max} = 1.35 ± 0.05 pmol/mg of protein in control tissue to B_{max} = 0.442 ± 0.11 pmol/mg of protein in NEM-treated tissue. Alkylation of the D-1 receptor also resulted in a 2-fold decrease in affinity of the ligand for the receptor, from K_d = 0.7 ± 0.05 nM (in control tissue) to K_d = 1.51 ± 0.14 nM (in NEM-treated tissue).

Pharmacology of NEM-Resistant Ligand Binding Sites. Since NEM did not totally abolish receptor binding activity, it seemed worthwhile to investigate the pharmacological profile of the surviving binding sites in order to determine the impact of alkylation on these sites. Radioligand binding assays were performed in the presence of D-1 specific agonist and antagonist, by using washed striatal preparations pretreated with 3 mM NEM. From computer analysis of these displacement curves, the K_ds of the tested drugs were calculated and are presented in Table I. Although the NEM-resistant sites retained the ability to distinguish between the selective stereoisomers of the agonst and antagonist, the affinity of these drugs for the D-1 receptor was altered. Thus, NEM-treatment

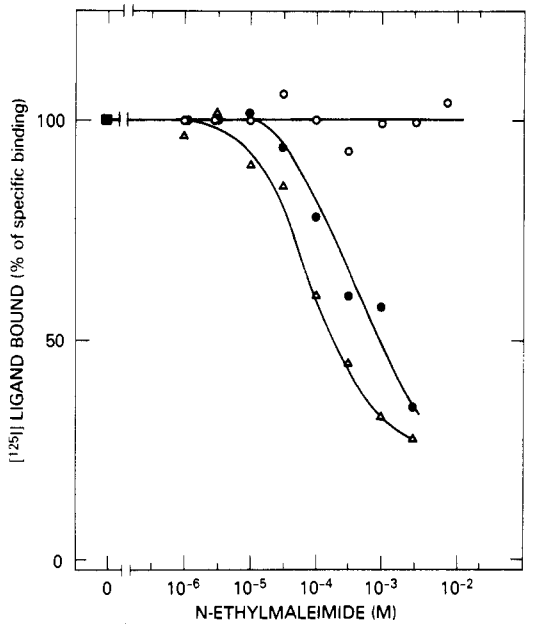

FIGURE 4: Selective protection of D-1 dopamine binding site by agonist from inactivation by NEM. Washed striatal particulate fractions were preincubated at 37 °C for 15 min in buffer alone (△) or in the presence of 1×10^{-5} M SK & F (*R*)-38393 (○) or SK & F (*S*)-38393 (●). Increasing amounts of NEM were then added, and the samples were incubated for an additional 20 min. The samples were washed 3 times, and the radioligand binding assay was performed as described under Experimental Procedures. Control binding (■) was determined in the absence of added NEM.

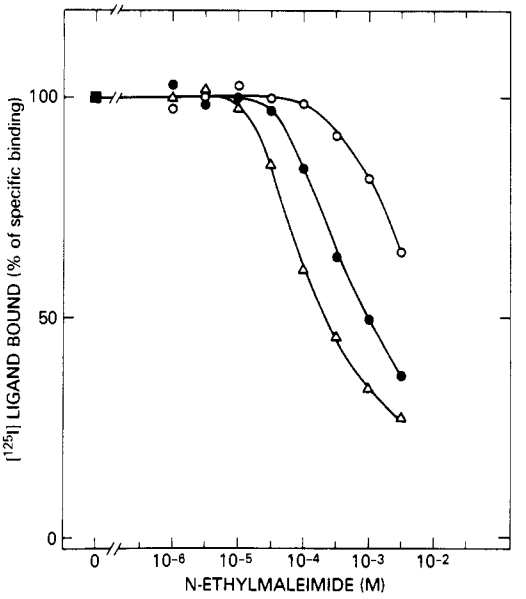

FIGURE 5: Selective protection of D-1 dopamine binding site by antagonist from inactivation by NEM. Washed striatal particulate fractions were preincubated for 15 min at 37 °C in the absence (△) and presence of 1×10^{-7} M SCH 23390 (○) or SCH (*S*)-23390 (●). Increasing amounts of NEM were then added, and the samples were processed as described in the legend to Figure 4. Control binding (■) was determined in the absence of added NEM.

of the D-1 receptor resulted in a 16-fold decrease in the potency of SCH 23390, the selective D-1 antagonist, while the potency of the pharmacologically inactive enantiomer SCH (*S*)-23390 was decreased 20-fold. It is unclear why NEM treatment of the striatal tissue affected the K_d of SCH 23390 binding to a greater extent than the K_d of the iodo analogue of the antagonist. Such differential effects may be reflective of the molecular properties of the substituted halogens on the antagonist molecule itself.

Previously, SK & F (*R*)-38393, 7,8-dihydroxy-2,3,4,5-tetrahydro-1-phenyl-1*H*-3-benzazepine, the most selective D-1 agonist currently available, has been shown to bind to two sites on the D-1 receptor with differing affinity (Sidhu & Kebabian, 1985). NEM treatment of the D-1 receptor, however, appeared to selectively abolish the high-affinity state of the receptor, and only a single category of binding sites was detected with $K_d = 269 \pm 40.4$ nM. The affinity of the inactive enantiomer of the agonist, SK & F (*S*)-38393, was not significantly affected by NEM treatment.

Effect of Receptor Occupancy by Agonist or Antagonist on NEM-Mediated Inactivation of Binding Sites. In order to study the effect of receptor occupancy by agonist on NEM-mediated alkylation of the D-1 receptor, striatal homogenates were incubated with 10 μM of either SK & F (*R*)-38393 or SK & F (*S*)-38393 prior to the addition of an increasing amount of NEM. After an additional incubation of 20 min, the tissue was washed extensively to remove bound agonist and NEM, and the residual binding sites were assayed as described under Experimental Procedures. As seen in Figure 4, preincubating the receptors with SK & F (*R*)-38393 resulted in complete protection of the sulfhydryl groups from alkylation by NEM. In four such experiments, 101 ± 2.65% of the receptor binding sites were protected by the agonist at the highest concentration of NEM tested (3 mM). By contrast, the inactive *S* isomer of the agonist failed to provide protection of the binding sites, and only 29 ± 6.51% ($n = 4$) of the sites were resistant to 3 mM NEM, a value almost identical with that obtained by the alkylating reagent alone in the absence of any drug. In order to determine if the protection of binding sites by SK & F (*R*)-38393 was agonist-specific, the protective effect, if any, of receptor occupancy by antagonist was also tested by using the stereoisomers of SCH 23390. Initially, when samples were preincubated with 1 μM SCH 23390, it was very difficult to wash the bound drug from the receptor, making the interpretation of the resultant data difficult. It was necessary to use concentrations of SCH 23390 <1 μM in the reaction mixtures, followed by extensive washings of the tissue. As seen in Figure 5, the D-1 binding sites were only partially protected by SCH 23390; at 3 mM NEM, the maximum recovery of binding sites in the presence of the antagonist was 66.67 ± 6.39% ($n = 3$). As in the case with the agonist, the partial protection of the sulfhydryls was dependent on the stereoselectivity of the antagonist; SCH (*S*)-23390 was much less effective, and only 37.67 ± 1.33% of the binding sites were resistant to NEM.

In order to determine if the agonist/antagonist-dependent protection of the sulfhydryl groups was due to receptor occupancy by these compounds, protection was correlated with the affinity of the receptor for the drug. To this end, the striatal homogenate was preincubated with increasing concentrations of either agonist or antagonist prior to the addition of 3 mM NEM. As seen in Figure 6A, increasing the concentration of the agonist in the reaction mixture led to a concomitant increase in the protective effect of the agonist on the binding site. The partial protection by SCH 23390 of the D-1 site also corresponded favorably to the affinity of the antagonist for the receptor (Figure 6B).

Pharmacology of the D-1 Receptor Binding Sites Protected by Agonist and Antagonist. In order to examine if the receptor binding sites protected from NEM inactivation by agonist or antagonist had undergone any modifications, the pharmacology of the protected sites was studied. Table II shows that irrespective of the protective reagent used, the binding sites retained the ability to distinguish between the stereoisomers of

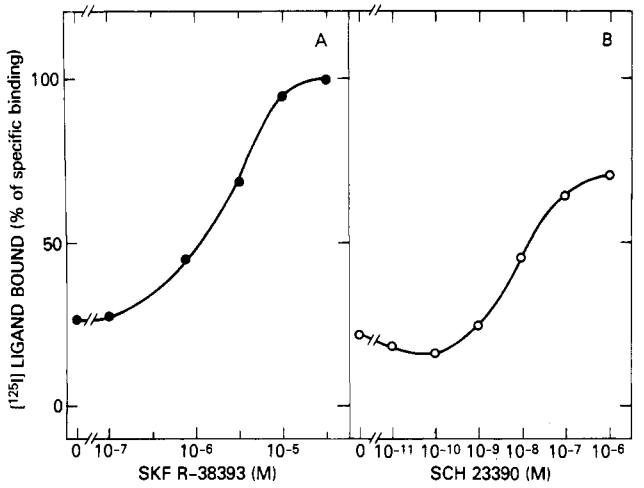

FIGURE 6: Protection of ligand binding sites as a function of receptor occupancy. Striatal particulate fractions were preincubated for 30 min with increasing concentrations of SK & F (*R*)-38393 (A) or SCH 23390 (B). NEM (3 mM) was added and the fractions were processed and assayed for receptor binding sites, as described in the legend to Figure 4.

Table II: Effect of Agonist or Antagonist Protection on Binding Properties of the NEM-Treated D-1 Receptor[a]

compound	K_d (nM) of binding in tissue protected by SK & F (*R*)-38393	SCH 23390
SK & F (*R*)-38393	188 ± 48.9	192 ± 34.5
SK & F (*S*)-38393	15690 ± 1570	28300 ± 4245
SCH 23390	0.37 ± 0.12	0.93 ± 0.20
SCH (*S*)-23390	41.6 ± 3.3	63.9 ± 9.5

[a] Striatal homogenates were preincubated for 15 min at 37 °C in the presence of either 1×10^{-5} M SK & F (*R*)-38393 or 1×10^{-7} M SCH 23390, prior to the addition of 3 mM NEM. After NEM treatment, displacement curves were obtained and the K_d values were calculated by computer analysis of the resultant data.

SK & F 38393 and SCH 23390. Furthermore, computer analysis of agonist displacement curves suggested that the high-affinity state of the receptor was lost regardless if the agonist or antagonist was used as the protective reagent. Additionally, the affinity of the remaining single site of the receptor for SK & F (*R*)-38393 when protected by either agonist or antagonist (K_d = 188 ± 48.9 nM and K_d = 192 ± 34.5 nM, respectively) corresponded to the low-affinity state of the unprotected receptor (K_d = 269 ± 40.4 nM, Table I).

Displacement curves by SCH 23390 of agonist-protected sites yielded a K_d of 0.37 ± 0.12 nM, a value almost identical with the K_d obtained from untreated tissue (K_d = 0.36 ± 0.12 nM). This suggests that protection of the binding sites by agonist preserved the binding site such that the binding of the antagonist to the receptor was unaffected. On the other hand, when the binding sites were protected by the antagonist, the affinity of SCH 23390 was reduced 3-fold (K_d = 0.93 ± 0.2 nM). This suggests that partial alkylation of the receptor sites in the presence of the antagonist leads to an accompanying reduction in the affinity of the antagonist for the receptor. The affinity of the inactive isomers of the agonist and antagonist tested was slightly reduced in membranes treated with NEM in the presence of the protective compounds.

Adenylate Cyclase Activity of NEM-Treated D-1 Receptors. As seen in Table III, after NEM treatment of striatal tissue, D-1 mediated adenylate cyclase activity was greatly diminished. Since the G protein and the catalytic component of adenylate cyclase have been reported to contain essential NEM-sensitive sulfhydryl groups (Ross & Gilman, 1979; Stadel & Lefkowitz, 1979), this loss of activity was not surprising. Indeed, after NEM treatment of striatal tissue, forskolin-mediated adenylate cyclase activity was also lost.

Table III: Adenylate Cyclase Activity of NEM-Treated Tissue[a]

compound	adenylate cyclase activity [pmol of cAMP (10 min)$^{-1}$ (mg of protein)$^{-1}$] −NEM	+NEM
SK & F (*R*)-38393	346.7 ± 21.9	113.3 ± 34.8
dopamine	467.7 ± 32.1	80.2 ± 28.3
forskolin	4500 ± 364	108.1 ± 4.9

[a] Striatal particulate fractions were incubated in the absence or presence of 3 mM NEM, and acenylate cyclase activity was determined on washed fractions. The results are expressed as the net amount of cAMP produced, after subtracting the basal value from the stimulated value for each data point. The basal values for untreated and NEM-treated tissue were 283 ± 39 and 33 ± 14 pmol of cAMP (10 min)$^{-1}$ (mg of protein)$^{-1}$, respectively.

Table IV: Transfer of D-1 Receptors to an Active Adenylate Cyclase by Membrane Fusion[a]

source of D-1 receptors	adenylate cyclase activity[b] [pmol (10 min)$^{-1}$ (mg of acceptor protein)$^{-1}$] basal	dopamine	isoproterenol	NaF	Δ-dopamine
NEM	279 ± 8	327 ± 14	758 ± 43	1550 ± 49	48 ± 15
NEM + antagonist	244 ± 4	306 ± 7	701 ± 25	1649 ± 13	62 ± 14
NEM + agonist	274 ± 6	403 ± 27	750 ± 23	1535 ± 78	129 ± 27

[a] Striatal membranes were treated with NEM in the presence or absence of agonist or antagonist as described in Table II. The membranes were fused with HeLa membranes and assayed for adenylate cyclase activity as described under Experimental Procedures. Values represent the mean ± SD of triplicate determinations. Similar results were obtained in a separate experiment. Although the basal activities were somewhat higher, the net response to dopamine (Δ-dopamine) was 30 (no ligand), 32 (antagonist), and 109 (agonist) pmol (10 min)$^{-1}$ (mg of acceptor protein)$^{-1}$, respectively. [b] Activity was determined with the following effectors: basal, 50 μM GTP and 1 μM (−)-alprenolol; dopamine, 100 μM plus GTP and alprenolol; isoproterenol, 10 μM plus GTP; NaF, 10 mM.

Protection of the binding site by either agonist or antagonist prior to receptor inactivation by NEM had no effect in protecting adenylate cyclase activity (data not shown). Hence, in order to establish the physiological role of the sulfhydryls associated with the D-1 receptor, we coupled the receptor with a functionally active adenylate cyclase, using the membrane fusion technique originally developed by Schramm (1979). We prepared striatal membranes treated with NEM in the absence and presence of agonist or antagonist, fused them with membranes from HeLa cells and assayed the fusates for dopamine-stimulated adenylate cyclase activity. In our initial experiments, we observed that the HeLa adenylate cyclase responded to dopamine. This response, however, was totally blocked by (−)-alprenolol, a potent and specific β-adrenergic antagonist, and is consistent with dopamine being a weak, partial agonist for the β-adrenergic receptor. We therefore assayed dopamine-stimulated activity in the presence of alprenolol. As shown in Table IV, when NEM-treated membranes were used as the donor, the response of adenylate cyclase in the fused membranes to dopamine was very poor. Antagonist-protected membranes were only slightly more effective than unprotected membranes. In contrast, when the donor membranes were protected with agonist, dopamine-stimulated activity was substantial and similar to that obtained in the original membranes (see Table III). The response of the acceptor HeLa adenylate cyclase in the fused membranes

to isoproterenol or NaF was the same irrespective of the source of the donor membranes. Thus, dopamine-stimulated activity in the fused membranes appears to reflect the functional state of the D-1 receptor.

Discussion

The results presented here show that the treatment of rat striatal membranes with the sulfhydryl alkylating reagent, NEM, caused an inhibition in the binding of the antagonist ligand, [^{125}I]SCH 23982, to the D-1 dopamine receptor. NEM treatment resulted in an apparent decrease in the B_{max} and K_d of the radioligand for the receptor, suggesting that an essential sulfhydryl group(s) may be located in the proximity of or at the active binding site of the D-1 dopamine receptor.

The protection of the sulfhydryls from NEM by the agonist suggests that receptor occupancy by the agonist somehow shielded these groups from alkylation. Furthermore, since the protective effect of the agonist showed a clear correlation with its concentration, receptor occupancy is clearly required. It appears unlikely that the protection of the sulfhydryls is due to inactivation of the NEM by the agonist itself, since the stereoisomer of the agonist, SK & F (*R*)-38393, failed to provide significant protection of the sulfhydryls. Rather, it appears that since the protection by agonist corresponds to the affinity of the molecule at the D-1 site, the agonist must prevent the action of NEM by shielding these groups. The protective effect of the agonist could be either due to an agonist-dependent conformational change of the receptor protein or due to a better fit of the *R* isomer at the binding site such that the sulfhydryls are no longer exposed to attack by NEM.

The antagonist, SCH 23390, was less effective, protecting only 50% of the total pool of NEM-sensitive sites while providing greater protection than its *S* isomer. Again, there was a clear correlation between the degree of protection and the affinity of the antagonist towards the binding site. It is unclear why the antagonist did not protect the sulfhydryls from NEM alkylation as efficiently as the agonist. The partial protection provided by the antagonist is probably reflective of the differential mechanism of interaction between the binding site and the agonist or antagonist. It is tempting to speculate that the agonist and antagonist shield two different subpopulations of the sulfhydryls; the antagonist protecting 50% and the agonist protecting 100% of the NEM-sensitive sites.

The recent investigations of hormone receptor systems linked to adenylate cyclase produced strong experimental evidence for the concept that the primary action of the hormone receptor consists of an interaction with G protein, the guanyl nucleotide binding protein. In binding studies, in the absence of guanyl nucleotides, the receptor demonstrates a high affinity for the hormone and low affinity for guanyl nucleotides (Maguire et al., 1976; Stadel et al., 1980; De Lean et al., 1980). It has been suggested that the high-affinity state is an expression of the formation of an activated hormone–receptor–G protein ternary complex and that a low-affinity state represents dissociation of the ternary complex by GTP. NEM has been reported to mimic the effect of nucleotides on β-adrenergic receptors by preventing the formation of the agonist high-affinity receptor complex (Stadel & Lefkowitz, 1979). With the localization of sulfhydryls on the G protein, it has been suggested that agonist binding to the receptor induces a conformational change in the G protein, exposing an essential sulfhydryl (Korner et al., 1982). In the present study, the selective abolition of the high-affinity state of the D-1 receptor seen in (a) agonist-protected, (b) antagonist-protected, (c) unprotected receptor suggests that NEM also prevented the formation or caused a destabilization of the high-affinity form of the receptor, perhaps by alkylation of a sulfhydryl located on G. Furthermore, it is unlikely that the sulfhydryls on the D-1 receptor-linked G protein are masked from NEM action, since when protection was performed by either agonist or antagonist, the high-affinity site of the receptor was, nonetheless, lost. Finally, in contrast to Korner et al. (1982), who observed that NEM treatment "locked" the agonist in the β-adrenergic receptor, there was no evidence of irreversible or persistent binding of the agonist to the D-1 receptor in the presence of NEM.

The role of these sulfhydryl groups located on the D-1 receptor has yet to be established. Since the adenylate cyclase system has been known to contain NEM-sensitive sulfhydryls, it was not surprising that NEM treatment of the D-1 receptor resulted in a loss of dopamine- and forskolin-stimulated adenylate cyclase activity. Protection of the sulfhydryls by either agonist or antagonist had no effect in preventing the inactivation of cyclase by NEM. In order to establish a functional role for the sulfhydryls associated with the receptor, we transferred the alkylated D-1 receptors to an active adenylate cyclase system by membrane fusion (Schramm, 1979; Kassis et al., 1984; Kassis & Fishman, 1984). When unprotected, NEM-treated membranes were used as the source of the D-1 receptors, only a marginal dopamine-stimulated activity was recovered in the fused membranes.[3] Antagonist-protected membranes were only slightly better as a source of D-1 receptors. In contrast, the D-1 receptors in agonist-protected membranes appeared to remain completely functional as the response to dopamine in the fused membranes was similar to that observed in the striatal membranes. The difference between protection by agonist and antagonist is quite striking, as the antagonist protects most of the [^{125}I]SCH 23982 binding sites. From these results, it is clear that NEM alkylates sulfhydryl group(s) associated with the D-1 receptor that are critical both for ligand binding and for receptor function. The observation that the agonist protected both of these properties whereas the antagonist only partially protected the former is consistent with the possibility that more than one sulfhydryl group is being alkylated by NEM. Thus, the sensitivity of the D-1 receptor to alkylation by NEM is very different from that observed with the β-adrenergic receptor. After NEM treatment, the latter receptor retains its ability to bind antagonists and to functionally couple to adenylate cyclase (Korner et al., 1982).

The functional importance of these sulfhydryls may lie in the delineation of a common pathway in the molecular mechanism of receptor function and, in particular, adenylate cyclase stimulation. The D-1 receptor linked sulfhydryls could also be potentially useful in the synthesis of mono- or bifunctional D-1 receptor specific cross-linking reagents and in the affinity chromatography of the solubilized D-1 receptor.

Registry No. Adenylate cyclase, 9012-42-4; dopamine, 51-61-6.

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[3] Olasmaa and Terenius (1985) recently reported that dopamine-stimulated adenylate cyclase was obtained after fusion of NEM-treated rat striatal membranes with Friend erythroleukemia cells. They did not measure ligand binding or determine whether there was inactivation or modification of the D-1 receptors by NEM. Furthermore, NEM treatment was done at 4 °C in contrast to the 37 °C used in our studies.

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Fraction of Cholesterol Undergoing Spontaneous Exchange between Small Unilamellar Phosphatidylcholine Vesicles†

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ABSTRACT: The kinetics of the spontaneous exchange of [^{3}H]cholesterol between small unilamellar vesicles of phosphatidylcholine has been reexamined. Although first-order exchange kinetics were observed (k = 0.0117 min^{-1}), in good agreement with previous studies, about 20% of the total cholesterol was found to be nonexchangeable in the 8-h time frame of the experiments. The size of this nonexchangeable pool was found to depend on the type of phospholipid and the temperature. It seems probable that the two pools of cholesterol defined in these experiments reflect the complex phase structure of the cholesterol–phosphatidylcholine vesicles.

The spontaneous exchange of cholesterol between plasma and red blood cells was first reported by Hagerman and Gould (1951). Since then, exchange has been demonstrated to occur between a variety of biological membranes, lipoproteins, and liposomes (Bruckdorfer & Graham, 1976; Bell, 1978). The mechanism of cholesterol exchange has been studied in biological membranes (Phillips et al., 1981; Clejan & Bittman, 1984; Davis et al., 1984) as well as in model membranes, such as unilamellar vesicles (Backer & Dawidowicz, 1979; McLean & Phillips, 1981). It has been shown in these systems that the transfer of cholesterol proceeds by diffusion of cholesterol through the aqueous phase and follows single-exponential kinetics.

Although several investigators have found only a single pool of cholesterol (Backer & Dawidowicz, 1979; McLean & Phillips, 1981; Bloj & Zilversmit, 1977), others have observed a significant nonexchangeable portion, leading to the conclusion that membrane cholesterol may exist in two or more kinetically distinguishable pools (Bell & Schwartz, 1971; d'Hollander & Chevallier, 1972; Poznansky & Lange, 1978). Furthermore, a number of studies in a variety of systems have attempted to correlate this nonexchangeable pool with cholesterol on the inner vesicle surface by assuming a very slow transbilayer movement of cholesterol (Smith & Green, 1974; Poznansky & Lange, 1976; Lenard & Rothman, 1976).

In the investigation reported in this paper, we reexamine the magnitude of the fraction of exchangeable cholesterol in small vesicles formed from mixtures of cholesterol and several

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