Solubilization and Reconstitution of the D-1 Dopamine Receptor: Potentiation of the Agonist High-Affinity State of the Receptor

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ABSTRACT: The D-1 dopamine receptor was extracted from rat striatal membranes with sodium cholate and NaCl in the presence of a specific agonist and phospholipids. The soluble receptor then was reconstituted into phospholipid vesicles by further addition of phospholipids prior to detergent removal. Of the total membrane receptors, up to 48% were extracted and 36% were reconstituted into phospholipid vesicles. Yields were greatly reduced if the agonist was omitted or replaced with an antagonist. The solubilized and reconstituted D-1 receptors retained the pharmacological properties of the membrane-bound receptors, including the ability to discriminate between active and inactive enantiomers of specific agonists and antagonists. In this regard, the affinity of the reconstituted receptors for the D-1 specific antagonist ^{125}I SCH 23982 was similar to that of the membrane-bound receptors with a K_d of 1.5 nM. Both the soluble and reconstituted forms of the D-1 receptor exhibited two affinity states for the D-1 specific agonist SK&F *R*-38393. In contrast to the low proportion of the receptors that had a high affinity for the agonists in striatal membranes (<6%), there was a dramatic increase following solubilization (22%) and reconstitution (40%). Similar results were obtained by using dopamine; the proportion of high-affinity sites increased from 4% (membrane-bound) to 48% (reconstituted) of the total receptor population. These high-affinity sites were coupled to G proteins, as guanyl nucleotides completely abolished them. Addition of guanyl nucleotides prior to solubilization or to reconstitution, however, had no effect on the subsequent yield of the reconstituted receptors. Similarly, pretreatment of membranes with *N*-ethylmaleimide, which abolished the high affinity for the agonist, did not reduce the recovery of the reconstituted receptors. Although high recoveries of soluble and reconstituted receptors required the initial formation of a complex with the agonist, the coupling to G proteins was not necessary for their extraction and reconstitution into phospholipid vesicles. Once the D-1 receptors and G proteins were solubilized and reconstituted into phospholipid vesicles, however, they were readily able to interact to form the high-affinity state.

On the basis of differential pharmacological and physiological functions, the neural dopamine receptors have been classified into two different categories of receptors, D-1 and D-2 (Kebabian & Calne, 1979). The D-1 dopamine receptor stimulates adenylate cyclase, while the D-2 dopamine receptor inhibits adenylate cyclase and prolactin release from anterior pituitary tissue (Brown et al., 1977). Dopaminergic neurotransmissions in the central nervous system have been intensively studied and linked to several neurological disorders including Parkinsonism, schizophrenia, hyperprolactemia, and hypertension. The D-2 receptor has been extensively characterized at the molecular level and purified to homogeneity (Gorissen & Laduron, 1979; Hall et al., 1983; Kilpatrick & Caron, 1983; Lew & Goldstein, 1984; Ramwani & Mishra, 1986). Although D-1 receptors have also been the subject of intensive investigation, very little is known about the molecular or physiological properties of this receptor. Recent evidence suggests that D-1 stimulation elicits behavioral effects (Arnt, 1985) and may have a modulatory influence on the activity of D-2-mediated events (Herrera-Marschitz & Ungerstadt, 1984; Meuer et al., 1985). Since D-1 receptors have a lower affinity for dopamine than D-2 receptors and a poor ablity to stimulate adenylate cyclase, the sequence of events triggering the modulatory action of D-1 remains to be established. The recent identification of SCH 23390 as a potent and selective antagonist of the D-1 receptor (Hyttel, 1983; Billard et al., 1984) and the subsequent synthesis of the 8-iodo analogue of SCH 23390, ^{125}I SCH 23982 (Sidhu & Kebabian, 1985; Sidhu et al., 1986a), provided the first generation of D-1-specific radioligands. We have used ^{125}I SCH 23982 to investigate the pharmacology, regional distribution, and biochemical functions of the D-1 receptor in rat brain (Sidhu & Kebabian, 1985; Sidhu et al., 1986a,b; Dawson et al., 1986).

Critical to the understanding of receptor function and activation is the solubilization, purification, and subsequent reconstitution of reconstituent components. Recently, this laboratory (Sidhu & Fishman, 1986) and Seeman's group (Niznik et al., 1986) independently reported the first successful solubilization of the D-1 receptor using cholate and digitonin, respectively. The yield of digitonin-solubilized receptors was low, and the receptors appeared to be altered, since 50% of the high-affinity sites were unable to undergo modulation by guanine nucleotides (Niznik et al., 1986). The cholate-solubilized receptors, on the other hand, retained full pharmacological function, including the ability to be completely modulated by guanine nucleotides (see Results). In this paper we present a detailed account of the molecular properties of the detergent-extracted receptor and report the first successful reconstitution of solubilized D-1 receptors. We demonstrate that under the right experimental conditions the receptor is fairly stable and able to withstand several biochemical manipulations. We also show that the process of solubilization and reconstitution results in an unusual potentiation of the agonist high-affinity sites of the receptor, which remain completely sensitive to modulation by guanine nucleotides. The

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possible implication of the potentiated high-affinity sites as a mechanism for in vivo receptor activation is discussed.

The high yield and stability of cholate-soluble receptors coupled with a simple one-step method for both detergent removal and reconstitution into phospholipid vesicles should prove to be extremely useful in future research aimed at elucidating D-1 function. Furthermore, the potentiated high-affinity sites should provide valuable information on the processes controlling receptor activation and coupling to G proteins.

Experimental Procedures

Materials. SK&F *R*- and *S*-38393 were from Smith, Kline and French Laboratories (Philadelphia, PA); SCH *R*- and *S*-23390 were from Schering-Plough (Bloomfield, NJ); *cis*(*Z*)- and *trans*(*E*)-flupenthixol were from Lundbeck (Copenhagen, Denmark). ^{125}I SCH 23982 (2200 Ci/mmol) was obtained from New England Nuclear. Poly(L-lysine), dopamine, sodium cholate, and crude phospholipids (bovine brain extract, type VII) were purchased from Sigma Chemical Co. (St. Louis, MO), while Gpp(NH)p[1] was from Boehringer Mannheim (Indianapolis, IN); SM-2 Bio-Beads were from Bio-Rad Laboratories (New York, NY). All other reagents were of the highest purity commercially available.

Membrane Preparation. Striatal membranes from male Sprague-Dawley rats were prepared as described previously (Sidhu et al., 1986b) and resuspended at a protein concentration of 1–3 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 $MgCl_2$) and 1 mM PMSF. For agonist pretreatment, membranes were routinely incubated with 10 μM SK&F *R*-38393 or with the appropriate concentrations of the indicated agonists. For antagonist pretreatment, the membranes were incubated with varying concentrations or with 1 nM of ^{125}I SCH 23982. After a 20-min incubation with the ligands at 37 °C, the membranes were diluted with 2–4 volumes of buffer A and isolated as described before (Sidhu et al., 1986b).

For NEM pretreatment, striatal membranes (0.8 mg of protein/mL) in buffer A were incubated with 10 μM SK&F *R*-38393 for 15 min at 37 °C. NEM (5 mM) was then added to the membranes, and after an additional incubation of 15 min at 37 °C, the membranes were diluted with 2–4 volumes of 50 mM Tris-HCl, pH 7.4. The membranes were isolated upon centrifugation at 18000*g* for 15 min and washed three times with 50 mM Tris-HCl, pH 7.4. The washed membranes were then resuspended at 2 mg/mL in buffer A and processed as described above.

Solubilization and Reconstitution Procedures. The ligand-occupied membranes isolated above were routinely resuspended at a protein concentration of 2 mg/mL (except where noted in text) in buffer S (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 250 mM sucrose, 1.5 mM PMSF, 1 mM DTT, and 1 M NaCl). Crude phospholipids, sonicated at 20 mg/mL in 10 mM Tris-HCl, pH 7.4, containing 10 μg of BHT/mg of phospholipid and solubilized with 1% sodium cholate, were added to a final concentration of 1.2 mg/mL. After a 10-min incubation on ice, sodium cholate was added to a final concentration of 1%. The suspension was maintained on ice for an additional 10–15 min and centrifuged at 31300*g* for 40 min. The clear, yellowish supernatant containing the soluble membrane proteins was either used immediately or stored frozen at −70 °C for up to 3 months without appreciable loss in ligand binding activity. This centrifugation procedure was routinely used since prolonged centrifugation (150000*g* for 45 min) did not sediment additional receptors. Cholate solubilization of striatal membranes extracted 75–80% of total membrane proteins, and approximately 50% of the ^{125}I SCH 23982 binding activity was detected in the supernatant after removal of cholate.

In order to reconstitute the cholate-solubilized D-1 receptors into proteoliposomes, sonicated phospholipids, 0.6–1.2 mg of phospholipids/mL of soluble protein, were readded to the soluble extract. Cholate was removed by the addition of moist SM-2 Bio-Beads (1.2 g/mL of extract), and the mixture was shaken gently for 1 h at 4 °C. After the beads were allowed to settle, the supernatant was removed and used directly in binding studies.

Radioligand Binding Assays. ^{125}I SCH 23982 binding to membrane-bound and reconstituted receptors was assayed by filtration onto glass fiber filters. The binding assay was performed by using 20 μL of protein extract, 0.5 nM ^{125}I SCH 23982, and unlabeled agonists, antagonists, or effectors in a final volume of 150 μL. The radioligand and drugs were diluted in buffer B (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 250 mM sucrose); the final concentration of NaCl in the assay was 133 mM. After the samples were incubated at room temperature for 90 min (or the indicated time), binding was determined by filtering the assay system on glass fiber filters (Sidhu & Kebabian, 1985).

Binding of ^{125}I SCH 23982 to solubilized receptors was performed by using the PEG precipitation/filtration procedure (Sidhu & Fishman, 1986). In all binding assays, nonspecific binding was determined in the presence of 10 μM SCH 23390 and represented 10–30% of total binding. In a typical experiment using 0.5 nM radioligand and 25 μg of proteoliposomes, total and nonspecific binding to reconstituted D-1 receptors was 12000 and 2000 cpm, respectively.

Gel Filtration. Samples (750 μL) of reconstituted or soluble receptors were applied to a 0.9 × 34 cm column of Ultrogel AcA 34 (LKB, Uppsala, Sweden) and eluted with buffer A containing 250 mM sucrose in the absence of presence of 0.2% cholate. The elution was performed at a flow rate of 11 mL/h at 4 °C. Column fractions (1.0 mL) were assayed for radioligand binding activity either immediately (reconstituted) or after detergent removal/reconstitution with SM-2 Bio-Beads (soluble).

Other Procedures. Protein was determined by the method of Lowry et al. (1951), and when cholate was present in the samples, a correction was made for the presence of the detergent. ^{125}I radioactivity was assayed in a Beckman γ counter (counting efficiency of 80%). The computer-fitted program LIGAND (Munson & Rodbard, 1980) was used to analyze the binding data. In each case a two-site model was considered to be a significantly better fit according to the *F* test at $p < 0.05$. With increased data points for some of the binding curves, the level of significance for a two-site model was increased to $p < 0.01$. All values are summarized as means ± SEM from separate experiments where *n* equals the number of experiments.

Results

Reconstitution of Solubilized D-1 Dopamine Receptors into Phospholipid Vesicles. We have previously demonstrated that cholate extraction of rat striatal membranes, in the presence of agonist and phospholipids, specifically solubilized 48% of

[1] Abbreviations: Gpp(NH)p, guanyl-5′-yl imidodiphosphate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; G proteins, guanyl nucleotide binding proteins; PEG, poly(ethylene glycol); GTP, guanosine 5′-triphosphate; BHT, butylated hydroxytoluene; K_d, dissociation constant; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate.

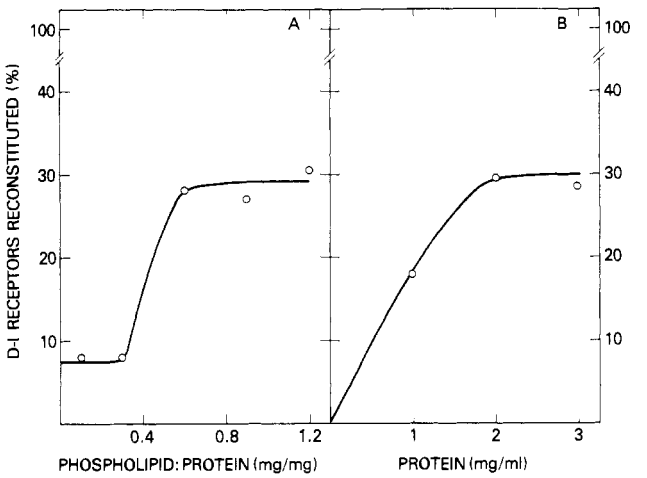

FIGURE 1: Effect of phospholipid:protein ratios and membrane protein concentration on reconstitution of D-1 receptors. Agonist-pretreated membranes were resuspended at either 2 mg/mL (A) or 1–3 mg/mL (B) in buffer S, and the proteins the proteins were extracted as described under Experimental Procedures. After centrifugation, the soluble extract was mixed with either varying amounts of sonicated phospholipids (A) or with 0.6 mg of phospholipid/mg of soluble protein (B). Sodium cholate was removed with SM-2 Bio-Beads, and binding of ^{125}I SCH 23982 was assayed by trapping the reconstituted proteoliposomes onto glass fiber filters. Specific binding to proteoliposomes is expressed as percent of total specific binding to the original membrane-bound receptors.

D-1 dopamine binding sites (Sidhu & Fishman, 1986). After removal of cholate by SM-2 beads, 62% of these solubilized binding sites appeared to be associated with lipid vesicles and were sedimented at 100000*g*. Trapping of these solubilized, lipid-associated receptors onto glass fiber filters resulted in recovery of only 10% of total binding sites, suggesting an inefficient reconstitution of the receptors into large-sized phospholipid vesicles. These cholate-free receptors, referred to as the solubilized D-1 receptors in this paper, were maximally detected only by the PEG precipitation/filtration technique. In order to improve the efficiency of proteoliposome formation, it was necessary to add back sonicated crude phospholipids to the soluble cholate extract. The subsequent removal of detergent by SM-2 beads appeared to permit the formation of proteoliposomes that were large enough to be trapped onto glass fiber filters (Figure 1). Reconstitution of the ^{125}I SCH 23982 binding sites was dependent on the phospholipid:protein ratio, and optimal recovery was obtained at 0.6 mg of phospholipids/mg of soluble protein (Figure 1A). Although there was some variation within different experiments, at this ratio approximately 36 ± 4.1% (n = 8) of the original membrane-bound D-1 receptors could be trapped onto filter papers. Attempts to substitute other phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine) in place of the crude phospholipid mixture were met with limited success, and the yield of proteoliposomes was poor (<10%) and variable (data not shown).

The success of the reconstitution procedure was also dependent on the membrane protein concentration prior to solubilization, and the highest yields of reconstituted proteoliposomes were obtained when initial protein concentrations exceeded 2 mg/mL (Figure 1B). Since proteoliposome formation was dependent on the adsorption of cholate on SM-2 beads, it also became necessary to reduce the concentration of cholate in the extraction mixture from 1.4% (Sidhu & Fishman, 1986) to 1%. The lower detergent concentration had no effect on the amount of protein extracted from membranes (data not shown).

Table I: Effect of Receptor Occupancy by D-1 Dopaminergic Antagonists on the Yield of Reconstituted D-1 Dopamine Receptors[a]

	recovery of binding sites concentration added (M)			
antagonist	10^{-9}	10^{-8}	10^{-7}	10^{-6}
SCH 23390	20.7 ± 3.2	9.6 ± 3.9	11.2 ± 4	2.5 ± 2
SCH *S*-23390	8.8 ± 5.5	4.9 ± 2.9	6.7 ± 6.4	4.0 ± 1.2
cis(*Z*)-flupenthixol	ND[b]	5.9 ± 4.1	4.5 ± 2.5	4.8 ± 0.7
trans(*E*)-flupenthixol	ND[b]	7.3 ± 5.8	7.0 ± 3.1	11.6 ± 3.8

[a] Rat striatal membranes (2 mg/mL) were incubated with the indicated concentrations of D-1 antagonists for 20 min at 37 °C. The reactions were teminated upon the addition of 2 vol of buffer A and antagonist-bound membranes were recovered by centrifugation. D-1 receptors were subsequently solubilized, reconstituted, and assayed for ^{125}I SCH 23982 binding activity. Nonspecific binding was determined in the presence of 10 μM SCH 23390. Results are expressed as a percent of the total specific binding obtained in untreated membrane-bound D-1 receptors. [b] ND, not determined.

Receptor occupancy by D-1 specific compounds prior to solubilization was essential in obtaining a high yield of receptor recovery; in the absence of any added ligand, only 10% of the binding sites were recovered in proteoliposomes. If, however, striatal membranes were preincubated with 10 μM D-1 specific agonist SK&F *R*-38393 prior to solubilization, 37 ± 5.5% of the total receptors were incorporated into phospholipid vesicles (data not shown). Solubilization in the presence of the inactive *S*-isomer of the agonist gave a low (12%) and variable yield of receptor. Dopamine (5 mM) also resulted in a favorable yield (29 ± 1.5%, n = 2) of the reconstituted receptor binding activity. Solubilization of antagonist-occupied receptors, however, resulted in low recovery of the binding sites (Table I). At concentrations of SCH 23390 >10^{-8} M, the yield of reconstituted binding sites was low and not significantly higher than that obtained in the presence of the inactive *S*-isomer. At 10^{-9} M SCH 23390, the yield of the reconstituted receptor was 2-fold higher than the *S*-isomer but was lower than the yield obtained with agonists. The use of another dopaminergic antagonist, *cis*(*Z*)-flupenthixol, as well as its inactive *trans*-*E*-isomer, resulted in similar poor yields of reconstituted receptor. The low yield of reconstituted receptor is not explained by less efficient solubilization, since the balance of the binding activity was not found in the cholate-insoluble pellet (data not shown).

To test the possibility that low receptor yields were a result of "trapping" of antagonist by the soluble receptor, leading to interference in the binding assay, D-1 receptors were labeled with 10^{-9} M ^{125}I SCH 23982 prior to solubilization. At this concentration, the K_d of the ligand, 50% of the membrane-bound receptors should be occupied by antagonist. However, the radioactivity bound to either soluble or reconstituted receptors represented less than 10% of the radioligand. Furthermore, the radioligand was bound reversibly and could be easily displaced by 10 μM of the unlabeled drug. Therefore, the antagonist does not appear to be trapped by the receptors. Since in the presence of 10^{-9} M unlabeled SCH 23390 (representing 50% receptor occupancy), the yield of receptors was nearly 21% (Table I), the effective yield of receptor would be over 40% had all the membrane receptors been occupied by antagonist. The data therefore suggest that at this concentration of the antagonist an antagonist-stabilized receptor may be isolated with yields similar to those of agonist-stabilized receptors. At higher antagonist concentrations limitations on receptor yields may be due to the high affinity of the antagonist and the inability of the experimental procedure to effectively remove it.

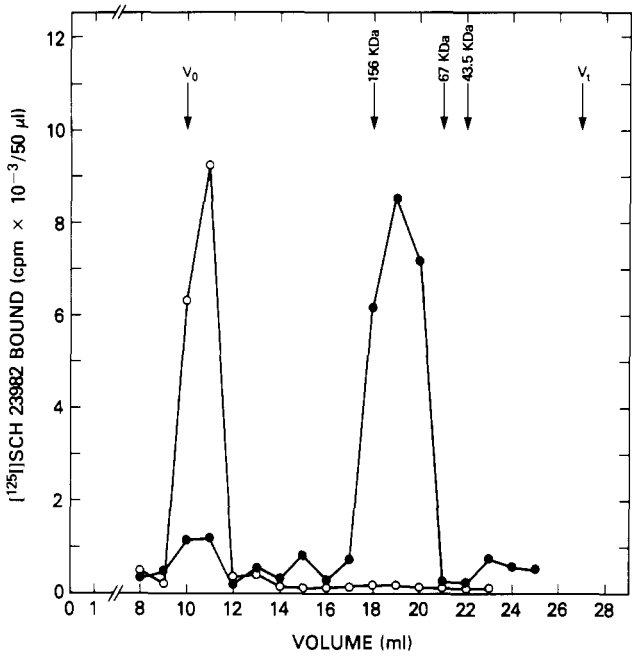

FIGURE 2: Ultrogel AcA 34 chromatography of soluble and reconstituted D-1 receptors. Aliquots (750 μL) of soluble (●) or reconstituted (○) preparations were chromatographed on an Ultrogel AcA 34 column as described under Experimental Procedures. Fractions of 1 mL were collected and either reconstituted (soluble preparations) or used immediately (reconstituted preparations) in radioligand binding assays. The results shown are representative of five different experiments. The molecular weight standards used for column calibration are blue dextran (V_0), γ-globulin (156 000), bovine serum albumin (67 000), ovalbumin (43 500), and galactose (V_t).

Molecular and Thermal Properties of Soluble and Reconstituted D-1 Dopamine Receptors. Soluble receptor preparations were chromatographed on an Ultrogel AcA 34 column in the presence to 0.2% cholate, and the elution profile of a typical run is presented in Figure 2. The ^{125}I SCH 23982 binding activity was eluted as a major peak with an apparent molecular weight of approximately 130 000. In some experiments, a small peak of binding activity was also present in the void volume (data not shown). A brief centrifugation of the thawed soluble receptors prior to chromatography and/or increasing the concentration of cholate to 0.5% during chromatography substantially reduced the binding activity of the peak at the void volume, suggesting aggregation of receptors either during thawing or during chromatography.

If the receptors were first reconstituted into phospholipid vesicles and then chromatographed on the column in the absence of cholate, the ^{125}I SCH 23982 binding activity was abolished in the 130 000-dalton range. Instead, all binding activity was present in a single peak eluting just after the void volume, suggesting the incorporation of the D-1 receptors into high molecular weight proteoliposomes.

The thermal stability of soluble and reconstituted receptors was tested by incubating the two receptor preparations for 30 min at 22 °C. At this temperature, the soluble receptors were thermally unstable, losing 83% of binding activity within 30 min. The reconstituted receptors, however, retained full binding capacity. Both the soluble and reconstituted receptors remained fairly stable at 0–4 °C (>16 h) (data not shown).

^{125}I SCH 23982 Binding to Solubilized and Reconstituted Receptor Preparations. ^{125}I SCH 23982 bound rapidly and reversibly to the reconstituted D-1 dopamine receptors. Association experiments revealed that ^{125}I SCH 23982 binding reached equilibrium within 90 min at room temperature (Figure 3A). The mean apparent association rate constant

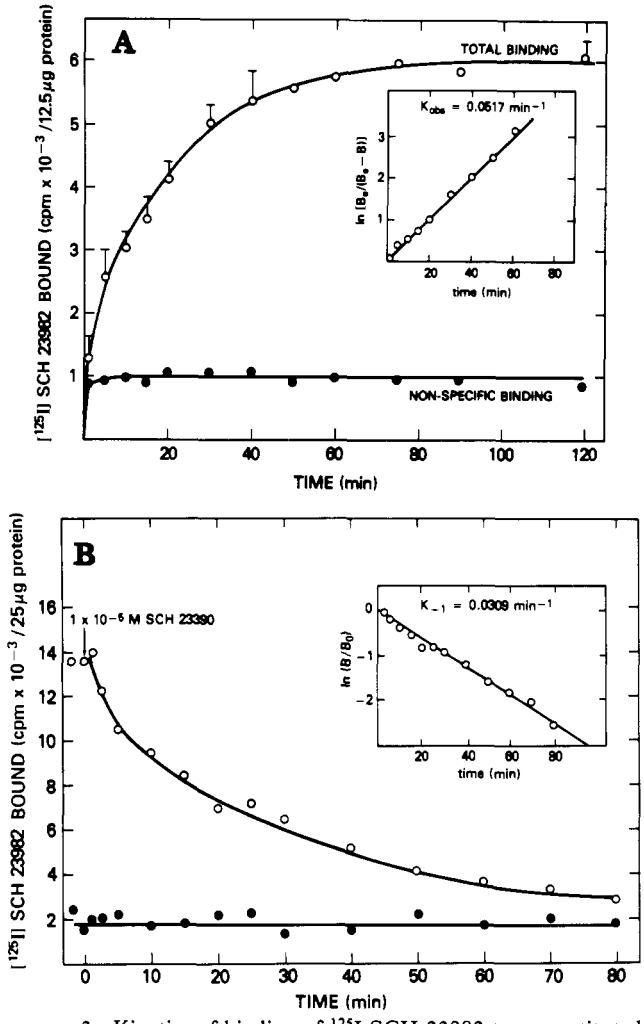

FIGURE 3: Kinetics of binding of ^{125}I SCH 23982 to reconstituted D-1 dopamine receptors. (A) Association experiments were performed by incubating the reconstituted proteoliposomes (12.5 μg of protein/assay) with 0.5 nM ^{125}I SCH 23982 for the indicated periods of time, in the absence (○) and presence (●) of 10 μM SCH 23390. The mean association rate constant for ^{125}I SCH 23982, K_{obs} was determined by assuming pseudo-first-order kinetics, by plotting in $B_e/(B_e - B)$ versus time, where B_e is the specific amount bound at equilibrium and B is the specific amount bound at any given time (see inset). From the slope of this line (K_{ob}) and the dissociation constant (K_{-1}), the association constant (K_{+1}) was calculated from the equation $K_{ob} - K_{-1} = K_{+1}L_t$, where L_t is the concentration of radioligand. (B) To determine the dissociation rate, proteoliposomes were prepared as described above and incubated with 0.5 nM [^{125}I SCH 23982 in the absence (○) and presence (●) of 10 μM SCH 23390 for 90 min at room temperature to achieve binding equilibrium. Specifically bound ^{125}I SCH 23982 was then measured at increasing times following addition of unlabeled SCH 23390 (final concentration 10 μM). The dissociation rate constant (K_{-1}) was calculated by the relationship $\ln (B/B_0) = -K_{-1}t$ (see inset), where B_0 is the amount bound at time zero, B is the amount bound at various times, t, following the addition of unlabeled SCH 23390.

(K_{ob}) was calculated to be 0.066 ± 0.02 min^{-1} (n = 2). The dissociation rate constant (K_{-1}) of ^{125}I SCH 23982 binding to the reconstituted receptor (Figure 3B) was estimated to be 0.041 ± 0.01 min^{-1} (n = 2), while the association constant (K_{+1}) was calculated to be 0.05 nM^{-1} min^{-1}. The K_d value obtained from these experiments (K_{-1}/K_{+1}) was 0.82 nM and correlated favorably with the K_d values obtained from Scatchard analyses of the saturation binding data of D-1 receptors in the reconstituted state (Figure 4).

The binding of ^{125}I SCH 23982 to reconstituted receptors was specific and saturable (Figure 4). Specific binding represented the bulk of total binding, and even at high radioligand

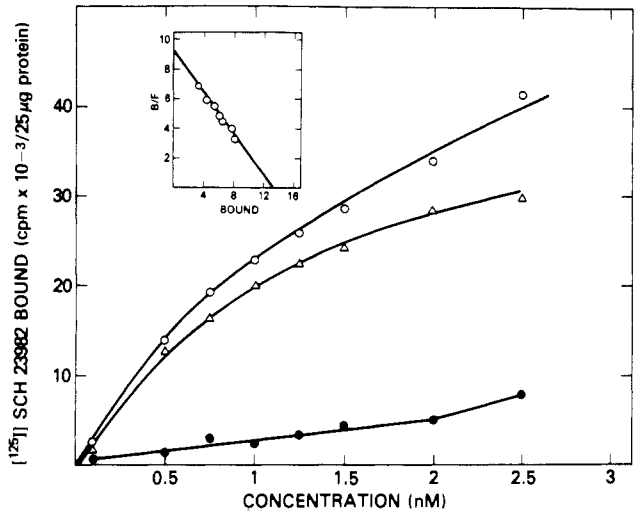

FIGURE 4: Concentration dependence of ^{125}I SCH 23982 binding to reconstituted D-1 dopamine receptors. Proteoliposomes containing the reconstituted D-1 dopamine receptors (25 μg of protein/assay) were incubated in triplicate with increasing concentrations of ^{125}I SCH 23982 for 120 min at room temperature in the absence (○) and presence (●) of 10 μM SCH 23390. Specific binding (△) was obtained by subtracting nonspecific binding from total binding, and the data were analyzed by a Scatchard plot. The amount of bound and free ligand was calculated in fentomoles and nanomolar, respectively.

Table II: B_{max} and K_d Values for ^{125}I SCH 23982 Binding to Membrane-Bound, Solubilized, and Reconstituted D-1 Receptors[a]

source of D-1 dopamine receptors	B_{max} (pmol/mg of protein)	K_d (nM)
membranes	1.35 ± 0.05	0.7 ± 0.05
solubilized	0.83 ± 0.17	1.56 ± 0.6
reconstituted	0.58 ± 0.06	1.46 ± 0.03

[a] D-1 dopamine receptors from rat striatal membranes or solubilized preparations were assayed for ^{125}I SCH 23982 (0.1–2.5 nM) binding in the presence or absence of 10 μM SCH 23390. Binding data of reconstituted receptors were from Figure 4. Values represent the mean ± SEM of two to three independent experiments.

concentrations, nonspecific binding was between 15 and 20% of total binding. Scatchard analysis (Figure 4, inset) of the saturation data revealed that the ligand bound to a single site with an apparent K_d of 1.46 ± 0.03 nM (n = 2) and a B_{max} of 0.58 ± 0.06 pmol/mg of protein (n = 2). The 2-fold shift in the affinity of both solubilized and reconstituted receptors over that of the membrane-bound receptor is probably reflective of the specific environmental requirements that have been disrupted upon solubilization (Table II).

Specificity of ^{125}I SCH 23982 Binding to Solubilized and Reconstituted D-1 Receptor. We demonstrated earlier that the cholate-solubilized receptors retained the pharmacological properties of membrane-bound D-1 receptors, including the ability to discriminate between the active and inactive isomers of drugs specific for the D-1 dopamine receptor (Sidhu & Fishman, 1986). The D-1 receptors reconstituted into phospholipid vesicles also displayed stereoselectivity typical of the membrane-bound D-1 receptors (Figure 5). Thus, the *S*-enantiomers of both D-1 specific agonist and antagonist were 200–250-fold less potent than the corresponding *R*-forms of these drugs. The K_d values of these drugs were calculated from displacement curves of both solubilized and reconstituted receptors and compared with the values obtained for the native, membrane-bound receptors (Table II). In all three forms of the receptor, the displacement curves of the antagonist SCH 23390 were uniphasic with K_d values ranging from 1.2 to 2.9 nM. The displacement curves of the agonist SK&F *R*-38393

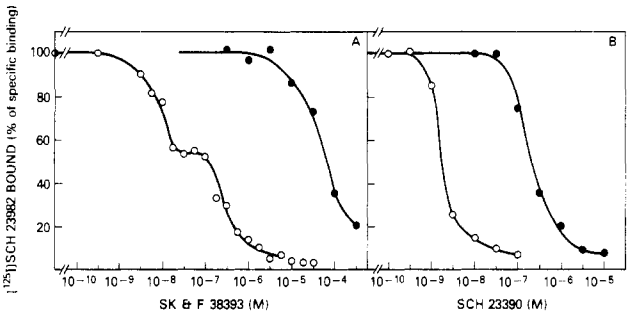

FIGURE 5: Ability of D-1 specific enantiomers of agonist (A) and antagonist (B) to compete for ^{125}I SCH 23982 binding to reconstituted D-1 dopamine receptors. Under the standard assay conditions, the reconstituted D-1 receptors were incubated with increasing concentrations of the pharmacologically active *R*-enantiomers (open symbols) and the pharmacologically inactive *S*-enantiomres (filled symbols). Total binding was determined in the absence of any drug, while nonspecific binding was determined in the presence of 10 μM SCH 23390. After subtraction for nonspecific binding, the specific counts bound at each drug concentration were expressed as percent of total specific binding.

Table III: Agonist and Antagonist Dissociation Constants for Membrane-Bound, Solubilized, and Reconstituted D-1 Dopamine Receptors[a]

compound	membrane-bound	solubilized	reconstituted
	K_d (nM) of D-1 Dopamine Receptors		
expt A			
SK&F *R*-38393 (K_h)	19.4 ± 8.2	11.3 ± 6.6	10.5 ± 4.4
SK&F *R*-38393 (K_l)	640 ± 240	307 ± 198	286 ± 131
SK&F *S*-38393	13 700 ± 2200	10 800 ± 5000	25 000 ± 15 000
SCH 23390	1.2 ± 0.12	2.1 ± 0.9	2.9 ± 1.6
SCH *S*-23390	18.6 ± 0.11	49.2 ± 26.5	214 ± 101
	Relative Proportions (%)		
expt B			
R_h	6.0 ± 1.3	22.5 ± 2.9	40.0 ± 8.2
R_l	94.0 ± 1.3	77.5 ± 2.9	60.0 ± 8.2

[a] For experiment A, membrane-bound, solubilized, and reconstituted D-1 receptors were incubated with 0.5 nM ^{125}I SCH 23982 and increasing concentrations of the indicated compounds, as described in the legend to Figure 4. For experiment B, the relative proportions of the high- (K_h) and low- (K_l) affinity states of the receptor were calculated by using SK&F *R*-38393 as the displacing reagent. The values are expressed as percent of the D-1 receptor population within each receptor preparation.

were also similar in all three receptor forms; the curves were shallow and best fit to a two-site model (see Figure 5), with a high-affinity site (K_h) between 10.5 and 19.4 nM and a low-affinity site (K_l) between 286 and 640 nM. These data suggest that the solubilization and subsequent incorporation of the D-1 receptor into proteoliposomes did not result in modification of the pharmacological properties of the receptor.

The most dramatic change upon solubilization and reconstitution of the receptors appeared to occur in the relative proportions of the agonist high- (R_h) and low- (R_l) affinity sites of the receptor (Table III). In native membrane-bound receptors, only 6% of the total receptor population was found to exist in the high-affinity state (Sidhu & Kebabian, 1985). Upon solubilization of the membranes, the relative proportion of the receptors in the high-affinity state increased dramatically to 22.5 ± 2.9% (n = 4). Since the agonist high-affinity state of a receptor is reflective of the coupling between the receptor and G proteins, it would appear that the process of solubilization and removal of detergent promotes the interaction

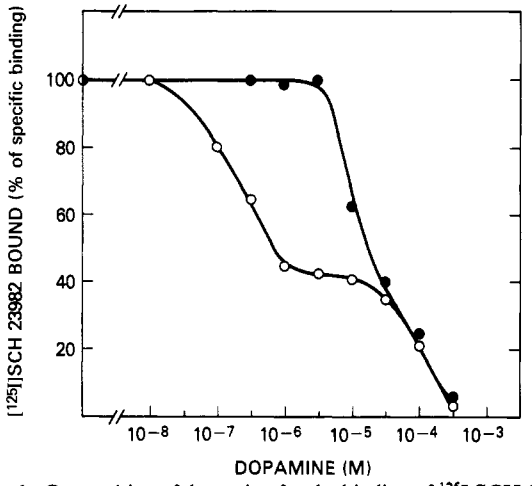

FIGURE 6: Competition of dopamine for the binding of ^{125}I SCH 23982 in reconstituted D-1 receptors. Competition curves were obtained by incubating the reconstituted D-1 receptors with increasing concentrations of dopamine in the absence (○) or presence (●) of 100 μM Gpp(NH)p. The radioligand binding assay was performed as described under Experimental Procedures.

between D-1 receptor and G proteins. When the solubilized D-1 receptors were reconstituted into phospholipid vesicles, the relative proportion of the receptors in the high-affinity state was further potentiated 2-fold to 40 ± 8.2% (n = 4). The formation of proteoliposomes, in creating a membranous environment, probably further induced and stabilized the coupling between the D-1 receptor and G proteins.

Regulation of the High-Affinity State of the D-1 Receptor. In order to investigate the high-affinity sites of the reconstituted receptors, displacement studies were performed with dopamine, the native neurotransmitter for this receptor. The results obtained with the full agonist (dopamine) were very similar to those seen for the partial agonist (SK&F *R*-38393). The competition curves were shallow and fit best to a two-site model with a K_h of 173.4 ± 65.9 nM (n = 5) and a K_l of 48 000 ± 25 000 nM (n = 5) (Figure 6). These values compare favorably to the value obtained for the membrane-bound receptors where the affinity constants were 635 ± 75 and 34 500 ± 5500 nM for the high- and low-affinity sites, respectively (Sidhu & Kebabian, 1985). Again, the most dramatic change was the increase in the relative proportions of agonist high-affinity sites. We had earlier reported that only 4% of the membrane-bound D-1 receptors existed in a high-affinity state (Sidhu & Kebabian, 1985). Upon solubilization and reconstitution, the receptors in the high-affinity state were increased to 48.5 ± 10% (n = 5), and only 51.5 ± 10% (n = 5) remained in the low-affinity state.

In order to verify that the potentiated high-affinity state of the reconstituted D-1 receptor is due to coupling between the receptor and G proteins, it was essential to demonstrate the ability of the high-affinity state to undergo modulation by GTP or its analogues. Thus, agonist displacement curves (Figure 6) were obtained in the absence or presence of Gpp(NH)p, the nonhydrolyzable analogue of GTP. The dopamine high-affinity state of the reconstituted receptor was tightly coupled to G proteins, and in the presence of Gpp(NH)p the high-affinity site was completely abolished and a single low site with a K_d of 30 000 ± 11 000 nM (n = 4) was seen. Studies using SK&F *R*-38393 as agonist also yielded similar results. As seen in Figure 7, the high-affinity states of both the solubilized (A) and reconstituted (B) D-1 receptors were modulated by the GTP analogue; 100 μM Gpp(NH)p completely abolished the high-affinity states, converting these ligand binding sites to

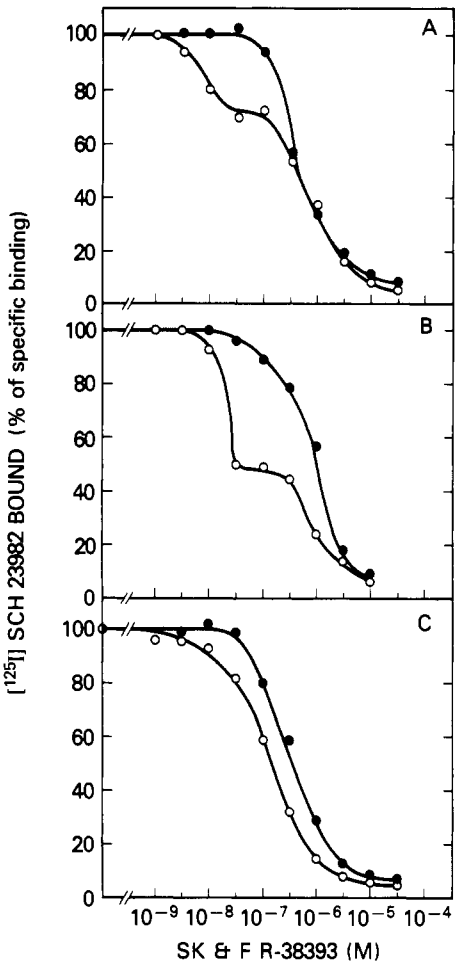

FIGURE 7: Effect of Gpp(NH)p on agonist competition for ^{125}I SCH 23982 binding to solubilized (A), reconstituted (B), and membrane-bound (C) D-1 dopamine receptors. D-1 receptors were mixed with 0.5 nM radioligand and increasing concentrations of the SK&F *R*-38393 in the absence (open symbols) or presence (filled symbols) of 100 μM Gpp(NH)p as described under Experimental Procedures. After 90 min at room temperature, the counts bound to membranes and proteoliposomes were assayed by filtration onto glass fiber filters, while binding to solubilized fractions was assayed by the PEG precipitation/filtration method. The results shown are representative of the three to five independent experiments.

a single low-affinity site with a K_d of 250 ± 80 nM (n = 2) (solubilized) and 1200 ± 300 nM (n = 2) (reconstituted).

Recent studies with β-adrenergic receptors appear to suggest that low temperatures (4 or 25 °C) potentiate the formation of the high-affinity state of the receptor (Schwartz et al., 1986). Since our earlier agonist displacement curves of membrane-bound D-1 receptors were performed at 37 °C (Sidhu & Kebabian, 1985) instead of 22 °C (room temperature) used in the current studies, it is possible that the potentiation of the high-affinity sites of the solubilized and reconstituted receptors may have been a consequence of the lower temperatures. In order to investigate this, agonist displacement curves of native membrane-bound receptors in the absence or presence of Gpp(NH)p were generated at room temperature (Figure 7C). Lowering the temperature of the binding assay, however, did not enhance the percent population of receptors in the high-affinity state, thus eliminating the possibility of a temperature-dependent promotion of coupling between the membrane-bound D-1 receptor and G proteins.

Effect of G Proteins on the Stability and Yield of the D-1 Dopamine Receptor. Since agonist occupancy of the D-1 receptor is absolutely essential for recovering high yields of the solubilized and reconstituted D-1 receptor, it is reasonable

Table IV: Effect of Guanyl Nucleotides and *N*-Ethylmaleimide on the Yield of Reconstituted D-1 Receptors[a]

membrane pretreatment	% yield of reconstituted D-1 dopamine receptors
expt A	
control	45.5 ± 5
+Gpp(NH)p (before solubilization)	47.1 ± 3.5
+Gpp(NH)p (before reconstitution)	45.3 ± 8.1
expt B	
control	37.3 ± 4.1
+NEM	36.5 ± 3.2

[a] For experiment A, membranes were incubated with 10 μM SK&F 38393 either in the absence (control) or in the presence of 100 μM of Gpp(NH)p for 20 min at 37 °C. The membranes were subsequently processed and solubilized as described under Experimental Procedures. Prior to reconstitution, a portion of the solubilized control fractions (not treated with Gpp(NH)p) were mixed with 100 μM Gpp(NH)p before the addition of phospholipids. All three fractions were constituted into proteoliposomes and assayed for ^{125}I SCH 23982 binding activity. For experiment B, membranes that were pretreated with 10 μM SK&F *R*-38393 (37 °C, 15 min) were incubated in the absence (control) or presence of 5 mM NEM. After 15 min at 37 °C, the membranes were washed four times, incubated with 10 μM SK&F *R*-38393 (37 °C, 20 min), and the D-1 receptors were solubilized and reconstituted as described under Experimental Procedures. Results are expressed as percent specific binding of ^{125}I SCH 23982 to total specific binding in membranes.

to assume that perhaps the agonist induces a conformational change in the receptor protein, leading to a tight coupling between the receptor and G proteins, which is preserved during solubilization. Hence, it is possible that G proteins might be required for conferring additional stability to the receptor protein during solubilization and that the resulting species is a ternary complex consisting of hormone–receptor–G proteins. Several approaches were thus designed to investigate the role of G proteins in the solubilization and reconstitution processes. The first approach was to perform the solubilization of the receptors under conditions that would promote the destabilization of any ternary complex formed before or during solubilization. Since guanyl nucleotide binding to G proteins results in the uncoupling of receptors from G proteins, D-1 receptors were solubilized prior to reconstitution into phospholipid vesicles. As seen in Table IV, addition of Gpp(NH)p membranes prior to solubilization did not effect the yield of reconstituted receptors. Therefore, it is unlikely that the extraction of receptors from membranes is dependent on the formation of an agonist–receptor–G proteins ternary complex within membranes prior to solubilization. When Gpp(NH)p was added to the soluble receptors just prior to reconstitution, the yield of reconstituted receptors was the same as the control levels. From these results, it also appears unlikely any ternary complex formed after solubilization is essential for the stability and assembly of receptor into phospholipid. Instead, the data provide strong support for the concept that solubilization and reconstitution of the D-1 receptor and G proteins proceed independently of one another. However, once assembled into proteoliposomes, these proteins are able to couple tightly and specifically with each other.

To further pursue the involvement of G proteins in the recovery of the binding sites after solubilization, striatal membranes, in the presence of SK&F *R*-38393, were treated with 5 mM NEM prior to solubilization. We had earlier shown that under these conditions the D-1 binding sites are protected by agonist, while G proteins are inactivated by the alkylating reagent, resulting in an uncoupling of the G proteins from the receptor (Sidhu et al., 1986b). Table IV shows that inactivation of G proteins, by NEM, prior to solubilization had

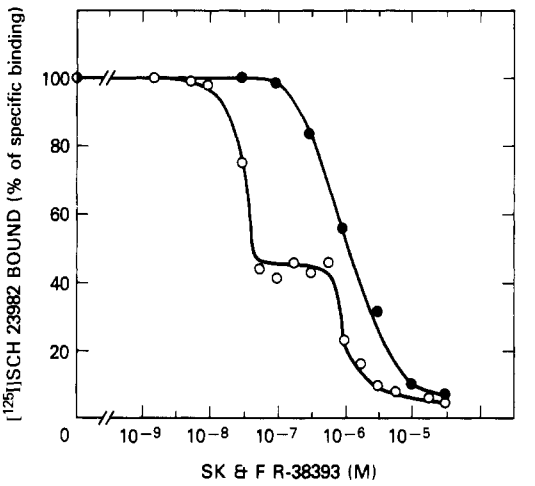

FIGURE 8: Effect of NEM pretreatment on the potentiated agonist high-affinity state of the reconstituted D-1 receptor. Striatal membranes, pretreated with 10 μM SK&F *R*-38393, were incubated in the absence (○) or presence (●) of 5 mM NEM. The D-1 receptors were processed and reconstituted and assayed for agonist competition as described in the legend to Table III.

no significant effect on the yield of reconstituted receptors and 36.5 ± 3.2% (n = 2) of the sites were recovered. In order to examine the effect of NEM inactivation of G proteins on the two affinity states of the reconstituted receptors, agonist displacement curves of NEM-treated membranes from Table IV were studied. In these experiments, the high and low dissociation constants of the untreated control reconstituted receptors were 15.3 ± 3.8 and 278.4 ± 138 nM (n = 2), respectively (Figure 8). Upon NEM inactivation of G proteins, the potentiated high-affinity sites of the reconstituted dopamine receptors were completely abolished, and the reconstituted receptor population was composed entirely of the agonist low-affinity state, with a K_d of 630.1 ± 150 nM (n = 2). Thus, the presence of the agonist high-affinity site does not appear to be essential for the stabilization and subsequent recovery of the reconstituted receptor.

Discussion

In this paper we report that D-1 dopamine receptors from rat striatum can be solubilized in an active form by cholate and reconstituted into phospholipid vesicles upon removal of the detergent. The D-1 receptors, in both the solubilized and reconstituted forms, exhibit several critical features: (a) the receptors retain the pharmacological properties typical of the membrane-bound D-1 receptor, including the ability to distinguish between the stereoisomers of both agonists and antagonists; (b) the receptors display kinetic binding parameters similar to the membrane-bound species; (c) the receptors retain the agonist high-affinity form of the membrane-bound receptors; (d) the agonist high-affinity sites maintain full capacity to undergo modulation by guanyl nucleotides. These observations suggest that the isolated receptor species are identical with the membrane-bound receptors and that the experimental conditions did not grossly alter the properties of the receptors.

During our initial attempts to solubilize the D-1 receptors from rat striatum, several detergents (Trition X-100, Lubrol-PX, CHAPS, deoxycholate, and digitonin) were used with limited success. The best results were obtained with sodium cholate as detergent. The ability of cholate to extract and reconstitute functional D-1 receptors was dependent on several key factors: phospholipids, NaCl, and agonist occupancy of the binding site. The addition of exogenous phospholipids to

solubilizing systems has been reported for the nicotinic acetylcholine receptor, where it was suggested that the phospholipids, by interacting with the hydrophobic regions of the receptor, helped stabilize it (Epstein & Racker, 1978; Anholt et al., 1981). The inclusion of increasing concentrations of NaCl, a chaotropic reagent, in the solubilizing mixture resulted in a corresponding increase in the amount of membrane protein solubilized by cholate, and maximal extraction occurred at NaCl concentrations >0.7 M (data not shown). Thus, NaCl permitted the usage of lower concentrations of the detergent. The precise role of the agonist, SK&F *R*-38393, in the solubilization of the D-1 receptor is unclear. The primary action of the agonist may be to promote a conformational change in the receptor, resulting in greater stability of the receptor in the detergent. An alternative, and more likely, explanation may be that occupancy of the binding sites by agonist protects these sites from denaturation upon solubilization by cholate. The data obtained from SCH 23390 also provide evidence for this concept.

The most unique feature of this system is the potentiation of the high-affinity site of the D-1 receptor upon solubilization and reconstitution. The potentiated sites do not have an increased affinity for the agonists, and the K_h of the three forms of the receptor remain virtually identical (Table III). Since the high-affinity site of the D-1 receptor is indicative of coupling between the receptor and G proteins, the potentiation of the sites is suggestive of enhanced coupling between these two proteins. Furthermore, the ability of these potentiated high-affinity sites to be completely modulated by Gpp(NH)p suggests that these sites are tightly and specifically coupled to G proteins.

Recently, the D-1 dopamine receptors, from bovine and canine caudate nuclei, have been solubilized by using digitonin as the detergent (Niznik et al., 1986). These digitonin-solubilized receptors retained the agonist high-affinity sites but without any potentiation of these sites. Additionally, these sites were not completely sensitive to guanyl nucleotides, and only 50% of the high-affinity sites were modulated by Gpp(NH)p. Several other receptor systems, linked to either the stimulation (β-adrenergic) or inhibition (α2-adrenergic, D-2 dopaminergic, muscarinic cholinergic) of adenylate cyclase, have been solubilized with either the loss or retention of the agonist high-affinity binding site (Gorissen & Laduron, 1979; Leff & Creese, 1982; Smith & Limbird, 1981; Limbird & Lefkowitz, 1978; Florio & Sternweis, 1981). To date, however, only the muscarinic cholinergic receptors, solubilized from bovine brain by deoxycholate, have displayed an increase in both the relative proportion and the K_h of the agonist high-affinity site (Florio & Sternweis, 1985). The potentiated high-affinity sites of these muscarinic receptors retained full sensitivity to modulation by guanyl nucleotides.

The mechanism of potentiation of the high-affinity sites of either muscarinic or D-1 dopaminergic receptors is not clear. Under our experimental conditions, approximately 4–6% of the total receptor population in membranes exist in the high-affinity state (Sidhu & Kebabian, 1985). If the mechanism of potentiation is due to the selective enrichment of existing high-affinity sites during solubilization/reconstitution, then the percent of high-affinity sites in the reconstituted receptor would not be expected to exceed 10–15% of the total receptor population (assuming an optimal receptor recovery of 40%). However, the actual levels of the high-affinity sites of the reconstituted receptors are severalfold higher than the predicted levels. Hence, it appears unlikely that enrichment of the high-affinity sites is the mechanism of potentiation. Since potentiation of the high-affinity sites of membrane-bound D-1 receptors has not been observed, potentiation does not appear to occur as a prerequisite to solubilization. Rather, all evidence suggests that potentiation of the sites occurs only after the receptors have been extracted from membranes.

The precise mechanism of coupling between the solubilized or reconstituted receptor and G proteins is not clear. Striatal tissues consist of a heterogeneous population of cells. The distribution of cell types may be such that in membranes the D-1 receptor has access to only a few molecules of G proteins. The disruption of membrane structures upon solubilization coupled with experimental conditions favoring the isolation of functionally active G proteins may result in a greater accessibility of the D-1 receptors to a functionally active pool of G proteins. In this context, it is interesting to note that some of the features of the solubilization protocol, cholate as detergent and inclusion of high concentrations of NaCl, are routinely used in the purification of active forms of G proteins (Hanski et al., 1981; Sternweis & Robishaw, 1984). While it is clear that 22.5–40% of the receptor population in the solubilized and reconstituted states, respectively, are coupled to G proteins, it is not clear why the remaining receptor population continues to exist in the uncoupled low-affinity state. It is not known whether the low-affinity sites are due to defective receptors that no longer interact with G proteins but still bind dopaminergic ligands or due to experimental constraints that do not allow all of the receptors to exist in a high-affinity state. The existence of two independent populations of D-1 receptors or trace quantities of endogenous guanyl nucleotides also cannot be ruled out.

Apart from physical constraints on coupling between receptor and G proteins, an alternative explanation for the potentiation could be that certain component(s) exist in membranes as part of a regulatory mechanism that promotes that inefficient coupling between the D-1 receptor and G proteins. The solubilization of the membranes may result in loss or inactivation of this factor(s), resulting in enhanced coupling between the receptor and G protein.

An intracellular separation of receptors and G proteins remains a possibility with potentially interesting implications for regulation. It should be noted that D-1 receptors from striatal tissue stimulate adenylate cyclase rather poorly, and under the most optimal conditions only a doubling of the response of the cyclase is obtained (Clement-Cormier et al., 1975). It is unclear whether the poor stimulation of adenylate cyclase is due to an intracellular separation of D-1 receptors and G proteins, as a possible means of receptor regulation. Alternatively, the stimulation of adenylate cyclase in striatal tissue may not be the primary physiological function of the D-1 receptor.

The reconstituted D-1 receptors in this paper should provide a useful model for studying the interaction and control mechanisms that regulate agonist activation of receptor proteins and the subsequent physiological manifestation of this interaction, namely, the stimulation of adenylate cyclase. Also, the higher yields of solubilized D-1 receptors obtained with cholate and the ease with which the detergent can be subsequently removed should be of great value in studies aimed at the ultimate purification and elucidation of the role of these receptors in regulating brain functions.

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Registry No. SCH 23390, 87134-87-0; SCH *S*-23390, 116669-85-3; ^{125}I SCH 23982, 116780-39-3; SK&F *R*-38393, 62751-59-1; SK&F

S-38393, 81702-43-4; Gpp(NH)p, 34273-04-6; NaCl, 7647-14-5; dopamine, 51-61-6; *cis*(*Z*)-flupenthixol, 53772-82-0; *trans*(*E*)-flupenthixol, 53772-85-3; sodium cholate, 361-09-1.

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Modulation of the Bilayer to Hexagonal Phase Transition and Solvation of Phosphatidylethanolamines in Aqueous Salt Solutions†

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ABSTRACT: Several salts affect the temperature of the bilayer to hexagonal phase transition of phosphatidylethanolamines. Their effects are dependent on the anion as well as the cation of the salt. Salt effects on this transition can be explained by preferential hydration and ion binding. Those salts which are excluded from the solvation sphere of the membrane promote hexagonal phase formation. For example, Na_2SO_4 promotes preferential hydration and is a hexagonal phase promoter while NaSCN does not do this and is a bilayer stabilizer. Unlike amphiphiles and hydrocarbons, salts can shift the bilayer to hexagonal phase transition temperature without altering the cooperativity of the transition. The effect of these salts on the gel to liquid-crystal transition is opposite to their effect on the bilayer to hexagonal phase transition. We also find that $MnCl_2$ markedly raises the gel to liquid-crystal transition temperature. This effect is due to binding of the cation to the membrane surface. The effect is reduced with $MnSO_4$ because of preferential hydration. Our results demonstrate that the nature of the anion as well as the cation can alter the effect of salts on lipid phase transition properties. The observed effects can be explained as resulting from preferential hydration and ion binding.

Bilayers composed of phosphatidylethanolamines are particularly prone to rearrange into the hexagonal phase (Cullis & de Kruijff, 1979). The temperature at which the bilayer phase is destabilized is very sensitive to the presence of small amounts of certain hydrophobic or amphiphilic compounds (Epand, 1985a). In addition to modulation of this phase transition by substances which partition primarily into the

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