Specific Phospholipid Requirements for the Solubilization and Reconstitution of D-1 Dopamine Receptors from Striatal Membranes†

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ABSTRACT: We have reported the solubilization and reconstitution of functional D-1 dopamine receptors from rat striatal tissue, using sodium cholate as detergent [Sidhu, A. (1988) *Biochemistry 27,* 8768–8776]. Critical to our method of extraction was the absolute requirement for the persistent presence of a crude extract of phospholipids (PLs) from bovine brain, during both solubilization of membranes and reconstitution of the soluble extract into PL vesicles. In the absence of PLs, fewer than 10% of the receptors were recovered, while in the presence of PLs, 40% of the receptors were reconstituted into vesicles. To probe the composition of PLs required by D-1 dopamine receptors during these extraction procedures, specific PLs of a defined composition were used during either solubilization or reconstitution alone or during both solubilization and reconstitution. Phosphatidylcholine (PC), when used during the solublization procedure alone or during both solubilization and reconstitution, resulted in recovery of 41–48% of the D-1 dopamine receptors but was 3.7-fold less effective when present during reconstitution alone (11%). Phosphatidylethanolamine (PE), when used during reconstitution alone, resulted in recovery of nearly 25% of the D-1 dopamine receptors. When PE was present during either solubilization or both solubilization and reconstitution, 6–11% of the receptors were recovered. If PE was used with PC in ratios of 1:1 or 2:1, respectively, 28–38% of the receptors were recovered. When PL vesicles of PE:PC were present in ratios of 1:2 during both solubilization and reconstitution, the maximum theoretical (74–87%) recovery of total receptor binding sites was achieved. These reconstituted receptors were pharmacologically active, with 53.5% of the receptor population present in the high-affinity state.

Neuronal dopaminergic transmissions have been implicated in several neuropathological diseases including Parkinsonism, depression, drug addiction, schizophrenia, hyperprolactemia, hypertension, and possibly Huntington's chorea. Dopamine exerts its effects via two receptor subtypes, D-1 and D-2, which have been classified according to their ability to either stimulate or inhibit adenylate cyclase activation, respectively (Kebabian & Calne, 1979). D-1 dopamine receptors have also been linked to diverse signaling pathways other than the adenylate cyclase system. Thus, D-1 receptors are reported to mediate the stimulation of phospholipase C (Felder et al., 1989; Undie & Friedman, 1990) and protein kinase C (Bertorello & Aperia, 1989), to inhibit Na^+/K^+-ATPase stimulation (Bertorello et al., 1990), and to activate the arachidonic acid cascade system (Piomelli et al., 1991). However, the precise mechanisms by which D-1 receptors are coupled to multiple signaling systems remains to be deduced.

In order to define the mechanisms, effectors, and regulatory factors through which D-1 dopamine receptors are coupled to these diverse signaling pathways, it is essential to purify these receptors and reconstitute functional activity with purified effectors of these pathways in cell-free systems. To this end, we have developed protocols for the solubilization and reconstitution into phospholipid (PL)[1] vesicles of pharmacologically active D-1 dopamine receptors (Sidhu, 1988; Sidhu et al., 1991). We have also described an 8200-fold purification of D-1 dopamine receptors from rat striata to 78% purity in a pharmacologically active state (Sidhu, 1990). These procedures were performed using sodium cholate to extract proteins from membranes and required the persistent presence of exogenously added crude mixtures of PLs. The reconstitution of the soluble receptors into PL vesicles was achieved by readdition of these crude PLs and the simultaneous removal of detergent with SM-2 Bio-Beads (Sidhu, 1988). Our extraction method resulted in a 40% recovery of D-1 receptors, with complete retention of agonist and antagonist binding properties typical of the membrane-bound receptors.

Our method remains the only method for the solubilization, reconstitution, and purification of pharmacologically active D-1 dopamine receptors. A different method described for the solubilization of 10–24% of D-1 receptors, using digitonin as detergent, has not been used for reconstitution of these receptors into PL vesicles (Niznik et al., 1986). These digitonin-solubilized receptors displayed altered pharmacological properties resulting in affinity values for antagonists which were 33-fold lower than those of membrane-bound receptors. Moreover, the agonist high-affinity sites may have been partially inactivated by this procedure, since approximately half these sites were uncoupled from G proteins and were not modulated by guanine nucleotides.

Since the requirement for PLs in the cholate method of extraction during the extraction procedures appeared to be

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[1] Abbreviations: PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PMSF, phenylmethanesulfonyl fluoride; BHT, butylated hydroxytoluene; G proteins, guanine nucleotide binding proteins; NEM, *N*-ethylmaleimide; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

critical for optimal recovery of D-1 dopamine binding sites, we decided to further investigate the effect of specific PLs when present in a defined composition during the solubilization and/or reconstitution process. In this study, we report that the PL requirements of the D-1 dopamine receptor during solubilization and reconstitution procedures are quite specific. Some PLs appear to be more effective in stabilizing the ligand binding properties of the D-1 receptors during solubilization, while others are more effective during the reconstitution procedure. Certain PLs in a defined molar ratio, when present during both solubilization and reconstitution, stabilize the D-1 receptor to levels which result in maximum theoretical receptor yields. Additionally, these PLs may be better able to preserve the functional state of the reconstituted D-1 dopamine receptors, as evidenced by enhanced potentiation of the receptor population existing in the agonist high-affinity state.

EXPERIMENTAL PROCEDURES

Materials. SKF R- and S-38393, SCH 23390, and SCH 23388 were from Research Biochemicals Inc. (Natick, MA), while guanyl-5′-yl imidodiphosphate [Gpp(NH)p] was from Boehringer Mannheim (Indianapolis, IN). Sodium cholate and all PLs used in this study were from Sigma Chemical Co. (St. Louis, MO); Type I PL consists primarily of PI (10–20%) and PS (50–60%), while Type VII PL consists of the major PLs and glycolipids of the brain. [^{125}I]SCH 23982 (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA), while SM-2 Bio-Beads were from Bio-Rad Laboratories (New York). All other chemicals and reagents were obtained as described elsewhere (Sidhu, 1988, 1990).

PLs (PE, PC, PI, PS, Type I, and Type VII PL brain extract) were sonicated in 10 mM Tris-HCl, pH 7.4, as a concentration of 20 mg/mL, containing 10 μg of BHT/mg of PL and then solubilized with 1% sodium cholate after sonication. PLs which were stored in chloroform were first evaporated to dryness under a stream of nitrogen prior to sonication. PL vesicles in defined ratios were obtained by mixing the PLs to a final concentration of 20 mg/mL either prior to or after sonication.

Membrane Preparation. Freshly excised bovine brains from the slaughterhouse were kept on ice and the striata were dissected within 2 h. Excess fat was trimmed from the striatal lobes and the tissue was homogenized in 50 mM Tris-HCl, pH 7.4, and 1 mM PMSF. Membranes were isolated by differential centrifugation as described before for rat striata (Sidhu et al., 1986a,b). The membranes were washed twice in the same buffer and were either used immediately after resuspension in buffer A (see below) or were stored frozen in aliquots at −80 °C after resuspension in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 5 mM $MgCl_2$.

Solubilization Procedures. The washed membranes were resuspended at a protein concentration of 4 mg/mL and solubilized, after incubation with the agonist SKF R-38393, as described before (Sidhu, 1988). Briefly, membranes were resuspended in buffer A (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$) containing 1 mM PMSF. SKF R-38393 was added to 10 μM, and after an incubation period of 20 min at 37 °C, the membranes were diluted with an equal volume of ice-cold 50 mM Tris-HCl, pH 7.4, and centrifuged (18000g for 20 min) at 4 °C. The membranes were then resuspended at a protein concentration of 4 mg/mL in buffer S (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 250 mM sucrose, 1.5 mM PMSF, 1 mM DTT, and 1 M NaCl). Various sonicated PLs were added to a final concentration of 1.2 mg/mL; sodium cholate [20% (w/v) in water] was added to a final concentration of 1%. After a 15–20-min incubation on ice, the mixture was centrifuged at 31300g for 45 min. The clear supernatant was removed and stored frozen at −80 °C for up to 1 year without appreciable loss in ligand binding activity.

Reconstitution Procedures. Soluble D-1 receptors were reconstituted into PL vesicles, as described previously (Sidhu, 1988, 1990). Briefly, the soluble protein samples were first diluted 1:1 with buffer B (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, and the following protease inhibitors: 0.5 mM PMSF and 5 μg/mL each leupeptin and pepstatin). The appropriate sonicated PLs were added to a final concentration of 1.2 mg/mL and the detergent was removed as described before, by adsorption onto SM-2 Bio-Beads (Sidhu, 1988).

Radioligand Binding Assays. The ability of D-1 dopaminergic drugs to compete for binding sites on the reconstituted receptors with the D-1 selective antagonist [^{125}I]SCH 23982 was assayed as described by this laboratory (Sidhu & Kebabian, 1985; Sidhu et al., 1986a,b), by filtration onto glass fiber filters. The binding assay was performed using 50-μL aliquots of receptor preparations, varying concentrations of the competing drug, and a final concentration of 0.5 nM [^{125}I]-SCH 23982. For saturation binding studies, [^{125}I]SCH 23982 was used to a final concentration of 0.1–2.5 nM. The radioligand was dried under a stream of nitrogen gas prior to usage; all ligand and drug dilutions were performed using buffer B. When present, Gpp(NH)p was added to the binding assays to a final concentration of 100 μM. After the samples were incubated at room temperature for 60–90 min, the reaction was terminated by filtering the assay system onto glass fiber filters pretreated with 200 μL of ice-cold 0.3% poly(ethylenimine) solution. In all binding assays, nonspecific binding was determined in the presence of 1 μM SCH 23390 and represented 10–30% of total binding.

Other Procedures. Protein was determined by the method of Lowry, using bovine serum albumin as standard (Lowry et al., 1951). The computer fitting program LIGAND was used to analyze the binding data (Munson & Rodbard, 1980). All values are summarized as means ± SEM from 3–6 separate experiments.

RESULTS

We have previously demonstrated that D-1 dopamine receptors extracted and purified from rat striatal membranes with sodium cholate required the persistent presence of exogenously added PLs (Type VII) during all aspects of the experimental manipulations, including the affinity chromatography and desalting procedures (Sidhu, 1988, 1990). The presence of PLs was also necessary during the solubilization and reconstitution of peripheral DA-1 dopamine receptors extracted from proximal tubules of rat kidney (Sidhu et al., 1992). Given the absolute requirement for PLs during the extraction of D-1 dopamine receptors into cell-free systems, we decided to assess the effect of using specific PLs of a defined composition during each step of the extraction procedure (solubilization and reconstitution) on the ligand binding properties of the recovered D-1 dopamine receptors. The PLs selected for this study are the major PLs normally found in brain. All studies were performed in conjunction with, and relative to, Type VII PLs, since this is the prototype of PLs previously used in our studies. Type VII PLs is a chloroform–methanol (1:2) extract of whole bovine brain and contains the major PLs and glycolipids normally found in brain tissue.

Table 1: Effect of Different PLs on the Recovery of [^{125}I]SCH 23982 Binding Activity Obtained after Solubilization and Reconstitution of D-1 Dopamine Receptors[a]

	[^{125}I]SCH 23982 binding (% of membrane-bound receptors)		
phospholipids	expt A[b]	expt B[c]	expt C[d]
Type VII	40 ± 2	40 ± 2	40 ± 2
Type I	9 ± 1	13.6 ± 2	14 ± 2
PE	11 ± 7	24.9 ± 2.8	6 ± 1.2
PC	41.2 ± 1.8	11.3 ± 5.9	48 ± 5.5
PE:PC	38.3 ± 4.8	28 ± 5.1	34.2 ± 0.4
2 PE:1 PC	30.6 ± 0.2	38 ± 6.4	33.6 ± 5.2
1 PE:2 PC	33.2 ± 0.4	43.8 ± 1.8	74.4 ± 3.3

[a] Bovine striatal membranes were solubilized in the presence of 1.2 mg/mL PLs and reconstituted with 1.2 mg/mL PLs. Binding activity of [^{125}I]SCH 23982 to the reconstituted D-1 sites was determined as described in Experimental Procedures and was computed relative to binding activity obtained with membrane-bound receptors. In the absence of added PLs, 12.2% ± 4% of the total receptor binding activity was recovered. [b] D-1 dopamine receptors were solubilized with different PLs and reconstituted with Type VII PLs. [c] D-1 dopamine receptors were solubilized with Type VII PLs and reconstituted with different PLs.[d] D-1 dopamine receptors were solubilized and reconstituted with the indicated PLs.

The effect of different PLs on D-1 receptors during *solubilization* was analyzed by solubilizing bovine striatal membranes with sodium cholate in the presence of defined PLs, added to a final concentration of 1.2 mg/mL, as described under Experimental Procedures. After high-speed centrifugation to remove insoluble material, Type VII PLs were added to the extracts (1.2 mg/mL) and the receptors were reconstituted into PL vesicles. The specific binding of [^{125}I]-SCH 23982 to these proteoliposomes was determined and the data were computed relative to binding activity of the starting preparation of membrane-bound D-1 dopamine receptors. In the absence of any added PLs during solubilization and reconstitution, only 12.2% ± 4% of total receptor binding activity was detected after reconstitution of the solubilized extracts into Type VII PL vesicles (Table 1). If both solubilization and reconstitution were performed in the presence of type VII PLs, 40% ± 2% of membrane-bound D-1 dopamine receptor binding activity was recovered (Table 1), which is similar to the recovery seen for D-1 receptors from rat striatal membranes using these Type VII PLs (Sidhu, 1988, 1990).

When another undefined mixture of PLs, Type I, which primarily contains PI and PS, was used during solubilization or reconstitution, or during both solubilization and reconstitution, 9–14% of the total binding activity was recovered in the vesicles (Table 1). Other crude mixtures of PLs or individual PLs (PI and PS) tested did not give significantly higher receptor yields than those obtained in the absence of added PLs (not shown).

Since type VII PLs are primarily composed of PE and PC, we decided to specifically investigate the effect of these PLs when used individually during the *solubilization* procedure. The addition of PE to the solubilizing mixture resulted in only 11% ± 7% recovery of D-1 receptor binding activity. By contrast, the addition of PC during solubilization resulted in receptor yields (41.2% ± 1.8%) equal to those seen with Type VII (Table 1), suggesting that PC may be an important component in the stabilization of the D-1 receptor during solubilization. When defined ratios of PE and PC (1:1, 2:1, or 1:2) were used during the solubilization procedure, 30–38% of the D-1 receptor binding activity was recovered.

In order to test the PL requirements of soluble D-1 dopamine receptors during the *reconstitution* procedure, striatal membranes were first solubilized in the presence of Type VII PLs and then reconstituted into vesicles upon the addition of different PLs. The use of PE during reconstitution resulted in 2.3-fold higher receptor yields (24.9% ± 2.8%) over the yield obtained when PE was used during solubilization alone. Surprisingly, the addition of PC during reconstitution yielded a 3.7-fold *lower* recovery of D-1 dopamine receptor (11.3% ± 5.9%) than the yield obtained when PC was used during solubilization. Although Type VII PLs were present during solubilization, and presumably conferred some receptor stability, the subsequent addition of PC during reconstitution appeared to result in an overall inhibitory effect on recovery of receptor activity. Taken together, these data with PE and PC indicate that PE may be better able to promote receptor recovery during reconstitution, while PC may be better able to promote receptor recovery during solubilization. When different ratios of PE and PC were used during reconstitution, between 28 and 43% of total receptor binding activity was recovered in the PL vesicles (Table 1).

Table 2: B_{max} and K_d Values for [^{125}I]SCH 23982 Binding to Membrane-Bound or Solubilized and Reconstituted D-1 Dopamine Receptors in Either Type VII or 1 PE:2 PC PLs[a]

source of receptors	B_{max} (fmol/mg of protein)	K_d (nM)	% R_h
membrane-bound	307 ± 77.3	1.6 ± 0.5	
Type VII-extracted	144 ± 14	3.05 ± 0.35	40.7 ± 0.9
1 PE:2 PC-extracted	268.1 ± 22.5	3.02 ± 0.5	53.5 ± 1.5

[a] The extraction of D-1 dopamine receptors was performed using the indicated PL persistently present during both solubilization and reconstitution into PL vesicles. Binding to membrane-bound and reconstituted receptors was assayed with [^{125}I]SCH 23982 (0.1–2.5 nM) essentially as described under Experimental Procedures. B_{max} and K_d values were obtained from Scatchard analyses of the saturation binding curves. The percent receptors in the high-affinity state (R_h) were obtained from competition curves with SKF R-38393 (see Figure 1B).

Since the data seemed to suggest that some PLs were better able to stabilize receptor binding activity during either solubilization of reconstituion, we decided to test the effects of these PLs when consistently present during *both solubilization and reconstitution.* The persistent presence of PE was unable to stabilize D-1 receptor binding activity during the extraction and reconstitution procedures, despite its effectiveness when used during reconstitution alone (Table 1). PC, when present during both solubilization and reconstitution, gave a receptor yield of 48% ± 5.5%, which was similar to the yield obtained when this PL was used during solubilization alone (41.2% ± 1.8%). To our surprise, the highest receptor yields were obtained with vesicles of PE:PC in a ratio of 1:2, and 74.4% ± 3.3% of the membrane-bound ligand binding activity of the D-1 dopamine receptor was recovered. This value was nearly 2-fold higher than that obtained with Type VII PLs or when other PLs were used during either solubilization or reconstitution alone. The requirement for PE and PC in a ratio of 1:2, respectively, appeared to be quite specific, since varying this ratio to 1:1 or 2:1 resulted in substantially lower receptor yields (Table 1).

Since the exceptionally high binding obtained with 1 PE:2 PC was unexpected, we decided to quantitate the receptor yield from saturation binding studies. The binding of [^{125}I]-SCH 23982 to D-1 receptors extracted with 1 PE:2 PC was specific and saturable, with a maximum binding capacity (B_{max}) of 268.1 ± 22.5 fmol/mg of protein and an affinity constant (K_d) value of 3.02 ± 0.5 nM (Table 2). This compared favorably with K_d values of 1.6 ± 0.52 nM obtained for the membrane-bound D-1 receptor. Since the D-1 receptor density

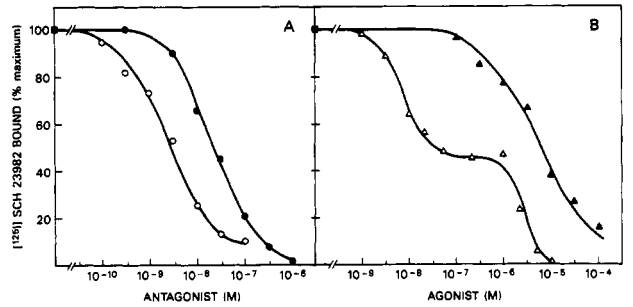

FIGURE 1: Ability of D-1 selective compounds to compete for [^{125}I]-SCH 23982 binding to reconstituted D-1 receptors isolated with 1 PE:2 PC. Under the standard assay binding conditions, membranes that were both solubilized and reconstituted with 1 PE:2 PC were incubated with increasing concentrations of various drugs: (A) SCH 23390 (○) and SCH 23388 (●); (B) SKF R-38393 (△) and SKF S-38393 (▲), as described under Expermiental Procedures. After subtraction for nonspecific binding, the specific counts bound at each drug concentrations were expressed as percent total of specific binding.

Table 3: Agonist and Antagonist Dissociation Constants for Membrane-Bound and Reconstituted D-1 Dopamine Receptors Extracted with Type VII or 1 PE:2 PC PLs[a]

	K_d (nM)		
compound	membrane-bound	Type VII	1 PE:2 PC
SCH 23390	3.5 ± 1.6	4.0 ± 2.3	4.19 ± 1.1
SCH 23388	58.6 ± 4.2	233 ± 118	32.5 ± 12.4
SKF R-38393	607 ± 10.2	2.5 ± 1.6 (K_h) 1700 ± 1000 (K_l)	9.4 ± 3.1 (K_h) 4100 ± 1600 (K_l)
SKF R-38393 + Gpp(NH)p	nd	550 ± 122.6	1020 ± 877.8
SKF S-38393	20700 ± 360	9570 ± 1630	8420 ± 480

[a] Membrane-bound and reconstituted D-1 receptors solubilized and reconstituted in either Type VII or 1 PE:2 PC PLs were incubated with 0.5 nM [^{125}I]SCH 23982 and increasing concentrations of the indicated compounds. Gpp(NH)p (100 μM) was added in the presence of SKF R-38393. Values are the means ± SD from 3–5 separate experiments; nd = not done.

in bovine striatal membranes is 307 ± 77.3 fmol/mg of protein, 87% of the total binding sites were apparently recovered after extraction with 1 PE:2 PC. When Type VII PLs were used, 46.9% of the membrane-bound sites were recovered (Table 2), confirming that the efficacy of receptor recovery is nearly 2-fold higher with 1 PE:2 PC than with Type VII PLs.

In order to eliminate the possibility that these high receptor recoveries were due to increased extraction of receptors other than D-1 dopamine receptors, the pharmacological properties of the extracted receptors were examined from competition binding studies. Since [^{125}I]SCH 23982 has been shown to cross-react with 5-HT_2 and 5-HT_{1c} serotonin binding sites (Hoyer & Karpf, 1988), it was important at the outset to demonstrate that these receptors were not serotonergic. Displacement curves with the extracted receptors were performed using serotonergic drugs (serotonin, mianserin, and ketanserin), and in all instances, K_d values >1000 nM were obtained (not shown), suggesting that these sites were not serotonergic.

From competition studies with SKF R-38393 (see Figure 1), the percent receptors in the high-affinity state was calculated (Table 2). Using these values as a function of the calculated B_{max} values of Table 2, the total number of receptors present in the high-affinity state was estimated. Thus, when D-1 receptors were solubilized and reconstituted with Type VII PLs, approximately 58.6 fmol of receptors/mg of protein was in the high-affinity state, which represented 40.7% of the total reconstituted receptor population. When receptors were solubilized and reconstituted with 1 PE:2 PC, 53.5% ± 1.5% of the receptor population was present in the high-affinity state and the total number of high-affinity receptor sites was increased 2.5-fold to approximately 143.4 fmol/mg of protein. An increase of only 1.5-fold of the low-affinity sites of the receptor was seen when 1 PE:2 PC was used (125 fmol/mg of protein), as compared to receptors isolated exclusively with Type VII PLs (86 fmol/mg of protein). Thus, the substantial increase in the high-affinity sites of receptors reconstituted with 1 PE:2 PC suggests that these PLs may be better able to preserve these high-affinity sites.

The D-1 receptors solubilized and reconstituted in the presence of 1 PE:2 PC displayed pharmacological properties typical of membrane-bound D-1 dopamine receptors and those receptors which were isolated in the presence of Type VII PLs. Thus, displacement curves of 1 PE:2 PC-isolated receptors with D-1 selective antagonist SCH 23390 were uniphasic (Figure 1A) with K_d values of 4.19 ± 1.1 nM, which compares favorably with K_d of binding for membrane-bound (3.5 ± 1.6 nM) and Type VII-isolated (K_d = 4.0 ± 2.3 nM) D-1 receptors (Table 3). With the D-1 selective agonist SKF R-38393, displacement curves for membrane-bound D-1 receptors were uniphasic with a K_d value of 607 ± 10.2 nM (Table 3). Upon solubilization and reconstitution of these receptors in the presence of Type VII PLs, the competition curves were biphasic, suggestive of high- and low-affinity binding states of the receptor, with K_h and K_l values of 2.5 ± 1.6 and 1700 ± 1000 nM, respectively (Table 3). When D-1 receptors were isolated in the persistent presence of 1 PE:2 PC, the K_h and K_l values obtained were 9.4 ± 3.1 and 4100 ± 1600 nM, respectively (Figure 1B and Table 3). The reconstituted receptors extracted in either Type VII of 1 PE:2 PC were also able to discriminate between the active and inactive isomers of dopaminergic compounds; the inactive isomers of both SCH 23390 (SCH 23388) and SKF R-38393 (SKF S-38393) were much less potent than the active from of these drugs for both the membrane-bound and reconstituted receptor (Figure 1 and Table 3).

The potentiation of high-affinity binding sites seen above has been reported for rat striatal D-1 receptors when using Type VII PLs (Sidhu, 1988) and is due to enhanced coupling between the D-1 receptors and G proteins in the cell-free state (Sidhu et al., 1991, 1992). In order to determine if the high-affinity binding sites of receptors isolated with 1 PE:2 PC are coupled to G proteins and thus sensitive to modulation by guanine nucleotides, agonist competition curves were performed with the nonhydrolyzable guanine nucleotide analog Gpp(NH)p. Thus, in the presence of 100 μM Gpp(NH)p, the high-affinity binding sites were abolished and the resulting competition curves were uniphasic, with a single affinity site (K_d = 1020 ± 877.8 nM), corresponding to the low-affinity state of the receptor (Table 3). This suggests that these high-affinity sites of 1 PE:2 PC-isolated D-1 receptors were also due to increased coupling of the reconstituted receptors to G proteins. The agonist high-affinity sites of Type VII isolated receptors were similarly sensitive to modulation by Gpp(NH)p (Table 3).

DISCUSSION

As stated in the Introduction, the ability of D-1 dopamine receptors to couple to diverse signaling pathways is not clearly understood. Whether these different signaling responses are due to multiple D-1 receptors or to receptors capable of coupling to multiple effectors, or both (i.e., multiple receptors

coupling to multiple effectors), is unclear. We recently demonstrated that, in reconstituted states, striatal D-1 receptors were able to couple to multiple effectors of the adenylate cyclase system (Sidhu et al., 1991). Thus, the D-1 dopamine receptors were able to couple to both the stimulatory guanine nucleotide-binding protein (G_s) and the inhibitory guanine nucleotide-binding protein (G_i). Moreover, the ability of D-1 receptors to couple to G_i in the simultaneous presence of G_s may be of interest in view of the multiple signal transducing ability of these receptors.

As part of our ongoing studies to investigate the coupling mechanisms between D-1 receptors and different effectors in cell-free states, we have been investigating different aspects of the solubilization and reconstitution conditions of D-1 receptors, in order to precisely define the experimental conditions for the isolation of these receptors from the membrane-bound state. Our primary interest was to obtain high yields of functional receptors, which can be used in future studies for assaying physiological properties of the D-1 dopamine receptor. In this paper, we have examined the effect of different PLs on D-1 dopamine receptors during solubilization and reconstitution. Based on our findings, it appears that PLs may play an essential role in stabilizing and protecting the D-1 dopamine receptor from inactivation during these experimental procedures. Further, certain PLs appear to be more effective in preserving the ligand binding activity of the receptor during either reconstitution or solubilization. PC resulted in a recovery of 41.2% ± 1.8% of the receptor binding sites when present during the solubilization procedure but was 3.7-fold less effective when used during reconstitution. If PC was used during both solubilization and reconstitution, the receptor yield was increased to 48% ± 5.5% of total. The combined data suggest that PC appears to be essential for stabilization of receptors during the initial solubilization and that, once stabilized, they remain stable during the reconstitution procedure. The decrease in receptor yields observed when PC was used during the reconstitution may suggest a somewhat inhibitory effect of this PL during reconstitution. Whether this inhibition occurs directly at the receptor level or is due to improper configuration of the vesicles remains to be established. Interestingly, this inhibitory effect appears to be abolished in the presence of added PE, since PL vesicles of PE and PC (in ratios of 1:1 or 2:1, respectively) yield increased and equivalent amounts of receptors regardless whether these PLs were used during solubilization or reconstitution or during both of these procedures (Table 1).

Although PE and PC in ratios of 1:2 resulted in respectable receptors yields (33.2–43.8%) when used in various stages of solubilization and reconstitution, the best results were obtained when these PLs were consistently present during both solubilization and reconstitution. Thus, under these optimal conditions 74.4% ± 3.3% of the total binding activity of the D-1 receptors were recovered; from Scatchard plots of saturation binding studies, 87% of total binding activity was obtained. Since up to 90% of striatal membrane proteins can be extracted with 1% sodium cholate (Sidhu & Fishman, 1986), this recovery of D-1 dopamine receptor binding activity obtained with 1 PE:2 PC may be close to the theoretical maximum obtainable under these experimental conditions. This suggests that PE and PC in a ratios of 1:2, rather than Type VII PLs, may be better suited for extraction of D-1 receptors from striatal tissues. However, the opposite may be true for D-1 dopamine receptors located in peripheral tissues. Thus, in other studies using renal proximal tubule membranes, Type VII PLs resulted in receptor yields that were close to the theoretical maximum after solubilization and reconstitution (Sidhu et al., 1992).

From the pharmacological studies, the D-1 dopaminergic properties of the receptors isolated in either Type VII or 1 PE:2 PC PLs appear to have been preserved under the experimental conditions. Further, competition curves with the D-1 selective agonist SKF R-38393 revealed the presence of both high- and low-affinity receptor binding sites. Due to low levels (4–6%) of agonist high-affinity sites in the membrane-bound state of striata (Sidhu & Kebabain, 1985), we have not been able to detect agonist high-affinity sites in bovine striatal membranes. The 2.5-fold increase in the high-affinity binding sites seen when D-1 dopamine receptors were isolated in the presence of 1 PE:2 PC, relative to Type VII PLs, suggests that 1 PE:2 PC may be better able to preserve these sites during the isolation procedures. Since agonist high-affinity sites represent the functional state of the receptor, the use of 1 PE:2 PC during the isolation of D-1 receptors may result in receptors which are more functionally active than those receptors which are isolated in the presence of an unknown composition of PLs, such as Type VII. Further, the modulation of these high-affinity sites by guanine nucleotide analogs is consistent with our earlier observations (Sidhu, 1988), indicating enhanced coupling between the receptors and G proteins in the cell-free state (Sidhu et al., 1991).

The primary role of PLs during the extraction procedures may be in the stabilization of hydrophobic stretches of the receptor protein. The PLs also appear to play an essential role during reconstitution itself and may be essential for inserting the receptors in a proper configuration within the lipid vesicle, similar to its membrane-bound state. The results demonstrated in this paper may be useful in those studies which reconstitute the functional coupling of these receptors with second messenger systems in cell-free states. In such studies, the isolation of functionally active receptors is especially important. Due to the enhanced preservation of high-affinity sites of receptors isolated in the presence of 1 PE:2 PC, these PLs may be the PLs of choice for isolating functional receptors. Further, by using PLs of a known and defined composition, sources of artifacts which could otherwise arise when using crude mixtures of PLs can be eliminated. Finally, the high yields of D-1 receptors obtained using 1 PE:2 PC will be useful for maximal extraction of D-1 receptors from those tissues which contain low amounts of these receptors, as well as from cultured cell lines expressing the cloned D-1 dopamine receptor gene.

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