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Solubilization and Characterization of σ-Receptors from Guinea Pig Brain Membranes

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Abstract: The σ-receptor, a distinct binding site in brain tissue that may mediate some of the psychotomimetic properties of benzomorphan opiates and phencyclidine, has been solubilized using the ionic detergent sodium cholate. Binding assays were performed with the solubilized receptor using vacuum filtration over polyethyleneimine-treated glass fiber filters. The pharmacological specificity of the solubilized binding site for σ-receptor ligands is nearly identical to the membrane-bound form of the receptor, with the order of potencies for displacement of the selective σ-ligand [^{3}H]di-*o*-tolylguanidine ([^{3}H]DTG) closely correlated. The stereoselectivity for (+)-benzomorphan opiate enantiomers was retained by the solubilized receptor. The soluble receptor retained high affinity for binding of [^{3}H]DTG (K_D = 28 ± 0.5 n*M*) and (+)-[^{3}H]3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine {(+)-[^{3}H]3-PPP} (K_D = 36 ± 2 n*M*). Photoaffinity labeling of the solubilized receptor by [^{3}H]*p*-azido-DTG, a σ-selective photoaffinity label, resulted in labeling of a 29-kilodalton polypeptide identical in size to that labeled in intact membranes. Estimation of the Stokes radius of the [^{3}H]DTG binding site was obtained by Sepharose CL-6B chromatography in the presence of 20 m*M* cholate and calculated to be 8.7 nm. This value was identical to the molecular size found for the binding sites of the σ-selective ligands (+)-[^{3}H]3-PPP and (+)-[^{3}H]SKF-10,047, supporting the hypothesis that all three ligands bind to the same macromolecular complex. **Key Words:** σ-Receptor—Solubilization—Benzomorphan opiates—Guinea pig brain. **Kavanaugh M. P. et al.** Solubilization and characterization of σ-receptors from guinea pig brain membranes. *J. Neurochem.* **53**, 1575–1580 (1989).

In order to account for the atypical psychotomimetic and autonomic effects observed following administration of benzomorphan opiates, particularly *N*-allylnormetazocine (SKF-10,047), Martin et al. (1976) proposed the existence of a distinct σ-"opiate" receptor that might mediate these effects. Radioligand binding studies in rodent brain membranes with the benzomorphan opiate (+)-[^{3}H]SKF-10,047 identified two related but distinct nonopiate binding sites, referred to as the phencyclidine (PCP) receptor and the σ-receptor (Vincent et al., 1979; Zukin and Zukin, 1979; Quirion et al. 1981; Su, 1982; Largent et al., 1984, 1986; Tam and Cook, 1984; Weber et al., 1986). Each of these binding sites is a candidate for mediating some of the behavioral effects of benzomorphans and PCP. The σ-receptor has a unique pharmacological profile, displaying moderately high affinity for PCP and high affinity for (+)-benzomorphan enantiomers. Two drugs that have been shown to display more selective binding to σ-receptors are (+)-[^{3}H]3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine {(+)-[^{3}H]3-PPP} (Largent et al., 1984) and [^{3}H]di-*o*-tolylguanidine ([^{3}H]DTG) (Weber et al., 1986). The σ-receptor also binds many neuroleptic drugs, such as haloperidol, with high affinity (Su, 1982; Tam and Cook, 1984). The σ-receptor displays a higher affinity for benzomorphans than for PCP, whereas the converse is true for the PCP receptor. An additional difference between the two sites is the very low binding affinity of the PCP receptor for neuroleptic drugs. Electrophysiological evidence suggests that the PCP receptor is related or identical to the voltage-sensitive ion channel associated with the *N*-methyl-D-asparate-type glutamate receptor, and that PCP and related drugs block currents associated with this receptor by binding in the ion channel (Anis et al., 1983; Honey et al., 1985). Drugs that exhibit specificity for the σ-receptor have been shown to enhance the twitch response of electrically stimulated vasa deferentia from

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Abbreviations used: [^{3}H]DTG, [^{3}H]di-*o*-tolylguanidine; kDa lodalton; PCP, phencyclidine; PEI, polyethyleneimine; PMSF nylmethylsulfonyl fluoride; (+)-[^{3}H]3-PPP, (+)-[^{3}H]3-(3-h phenyl)-*N*-(1-propyl)piperidine; SDS, sodium dodecyl sulf

guinea pig (Campbell et al., 1987) and mouse (Vaupel and Su, 1987), and to augment release of norepinephrine (Campbell et al., 1987). In addition, σ-ligands inhibit electrically stimulated contractions of the guinea pig ileum longitudinal muscle/myenteric plexus and noncompetitively inhibit serotonin-induced contractions in this preparation (Campbell et al., 1989). In order to gain insight into the detailed structure and function of the σ-receptor, it will be necessary to isolate and biochemically characterize the receptor protein. Here we describe a method for solubilizing σ-receptor from guinea pig brain membranes with retention of ligand binding characteristics of the native receptor.

EXPERIMENTAL PROCEDURES

Materials

Sodium cholate, phenylmethylsulfonyl fluoride (PMSF), polyethyleneimine (PEI), and molecular weight standards for gel filtration chromatography were obtained from Sigma (St. Louis, MO, U.S.A.). [^{3}H]DTG (custom synthesis, Amersham, Buckinghamshire, U.K.) had a specific activity of 52 Ci/mmol, (+)-[^{3}H]SKF-10,047 (New England Nuclear, Boston, MA, U.S.A.) had a specific activity of 25.6 Ci/mmol, and (+)-[^{3}H]3-PPP (New England Nuclear) had a specific activity of 110.1 Ci/mmol.

Brain membrane preparation and solubilization

Brain membranes were prepared as previously described (Weber et al., 1986) with some modifications. Frozen guinea pig brains (Biotrol, Indianapolis, IN, U.S.A.) were homogenized in 10 volumes (wt/vol) of ice-cold 0.32 *M* sucrose in the presence of 0.1 m*M* PMSF and 1 m*M* EDTA. The homogenate was spun at 1,000 *g* at 4°C for 10 min. The supernatant was then centrifuged at 20,000 *g* at 4°C for 40 min. The pellet was washed with 50 m*M* Tris (pH 7.4), 0.1 m*M* PMSF, and 1.0 m*M* EDTA, then centrifuged at 20,000 *g* and resuspended in Tris/PMSF/EDTA at a protein concentration of 10 mg/ml (Bio-Rad protein assay) and stored at −70°C. For solubilization experiments, frozen membranes were thawed and 1.0 *M* sodium cholate was added to give the appropriate final concentration. The suspension was stirred with a Teflon stir bar for 2 h at 4°C, followed by ultracentrifugation at 105,000 *g* for 2 h. The supernatant was then carefully removed and filtered through a 0.22 μm polysulfone filter.

Binding assays

Binding assays were performed at a final protein concentration of 600–700 μg/ml in 50 m*M* Tris Cl (pH 7.4) with 5 m*M* sodium cholate. To 12 × 75-mm polystyrene tubes was added 250 μl of solubilized extract, 550 μl of Tris, 100 μl of labeled drug in Tris, and 100 μl of Tris alone or with various concentrations of unlabeled competing drug. Incubations were performed for 2 h at 25°C. Using a Brandel 48-well cell harvester (Gaithersburg, MD, U.S.A.), the assay mixtures were diluted to 5 ml with Tris and rapidly filtered through Whatman GF/B filters that had been pretreated with 0.3% PEI (Bruns et al., 1983). The filters were then washed twice with 5 ml of Tris buffer. The filters were dissolved in Cytoscint (Westchem Products, San Diego, CA, U.S.A.), and radioactivity was measured with approximately 50% efficiency in a liquid scintillation counter. Scatchard analysis was performed using the EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) data analysis programs.

Sepharose CL-6B chromatography

Five milliliters of solubilized receptor preparation containing approximately 2.5 mg of protein/ml in 50 m*M* Tris with 20 m*M* cholate was incubated with 0.9 μCi/ml of either [^{3}H]DTG, (+)-[^{3}H]3-PPP, or (+)-[^{3}H]SKF-10,047 in the presence or absence of 10 μ*M* haloperidol for 2 h at 25°C. The sample was then applied to a 2 × 100-cm Sepharose CL-6B column equilibrated in 50 m*M* Tris with 20 m*M* cholate at 4°C. The column was eluted at a flow rate of 20 ml/h, and 2.5-ml fractions were collected. Radioactivity was determined by counting 1-ml aliquots dissolved in 12 ml of Ecolite scintillation cocktail (ICN Radiochemicals, Costa Mesa, CA, U.S.A.).

Photoaffinity labeling

Photoaffinity labeling of solubilized receptor was performed as described previously (Kavanaugh et al., 1988). Following incubation in the dark with 10 n*M* [^{3}H]*p*-azido-DTG, solubilized receptor was filtered and washed on 0.3% PEI-treated GF/B filters and then exposed to UV light. Protein on the filter was then solubilized by overnight incubation in 0.2% sodium dodecyl sulfate (SDS), lyophilized, and run on a 12% acrylamide gel under reducing conditions. Gel slices were incubated overnight in 10% Protosol (New England Nuclear) in Ecolite scintillation cocktail (ICN Radiochemicals) at 50°C, and radioactivity was counted.

RESULTS

Solubilization of [^{3}H]DTG binding sites from guinea pig brain membranes

In preliminary experiments, membrane suspensions at 10 mg/ml of protein were treated with various detergent concentrations followed by assay of yields of solubilized receptor and total protein. Increasing concentrations of sodium cholate gave increasing solubilization of protein and [^{3}H]DTG binding sites from guinea pig brain membranes (Fig. 1). For further experiments, 20 m*M* sodium cholate, which solubilized approximately 23% of the protein in the membrane suspension and 15% of the [^{3}H]DTG binding sites, was used. Binding of [^{3}H]DTG to the solubilized receptor was found to be linear with protein concentration up to 800 μg/ml (data not shown). In a typical experiment with 1.3 n*M* [^{3}H]DTG, total binding was approximately 3,000 cpm, whereas nonspecific binding, measured in the presence of 10 μ*M* haloperidol, was approximately 300 cpm. The kinetics of binding of [^{3}H]DTG to solubilized receptor are shown in Fig. 2. Linear transformation of the association and dissociation binding data gave values of $2.93 \times 10^5\ M^{-1}\ \mathrm{min}^{-1}$ and $7.39 \times 10^{-3}\ \mathrm{min}^{-1}$ for the association and dissociation rate constants, respectively. Figure 3 shows the results of Scatchard analysis of equilibrium binding to solubilized receptor. The Scatchard plot was linear and revealed a dissociation constant of 28 ± 0.5 n*M* and a B_{max} of 1.3 ± 0.2 pmol/mg of protein. This compared to a K_D of 25 n*M* calculated from the association and dissociation rate constants. In comparison, the mem-

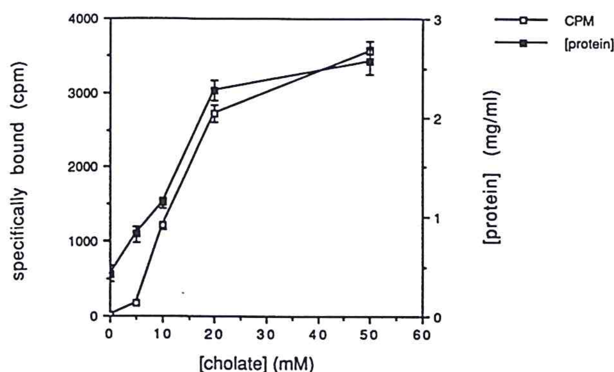

FIG. 1. Effect of cholate concentration on solubilization of protein (closed squares) and specifically bound [^{3}H]DTG (open squares). Guinea pig brain membranes were solubilized using various concentrations of cholate, and binding assays were performed as described in Experimental Procedures. Protein was determined with the Bio-Rad protein assay using bovine serum albumin as standard. Points represent means ± SE; n = 3.

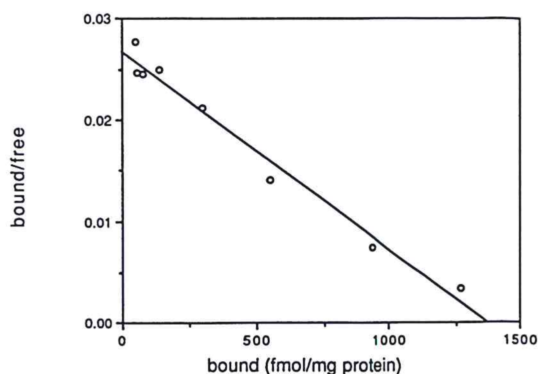

FIG. 3. Cold Scatchard analysis of [^{3}H]DTG binding to solubilized σ-receptor. Binding assays were performed as described in Experimental Procedures. Data analysis was performed using the EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) programs on an IBM AT PC.

brane-bound receptor exhibits a K_D of 28 ± 1 n*M* and a B_{max} of 2.0 ± 0.26 pmol/mg of protein.

Pharmacological characterization of solubilized σ-receptor

In order to evaluate the pharmacological specificity of the binding site labeled by [^{3}H]DTG in the solubilized extract, the potencies of a range of σ-receptor ligands were determined (Table 1). The solubilized σ-receptor displayed a specificity very similar to that of the membrane-bound receptor, with the order of potencies of the various drugs in displacing [^{3}H]DTG from membrane-bound versus soluble receptor exhibiting a correlation coefficient of 0.90 (Fig. 4). The stereoselectivity of the receptor for (+)-benzomorphan enantiomers was preserved (Table 1). Drug displacement curves for several representative σ-ligands are shown in Fig. 5. In addition to displacement studies with [^{3}H]DTG, binding experiments with the σ-selective ligand (+)-[^{3}H]3-PPP were performed (Table 1). The K_D for binding of (+)-[^{3}H]3-PPP to solubilized receptor was 36 ± 2 n*M* and the B_{max} was 1.6 ± 0.1 pmol/mg of protein. (+)-[^{3}H]3-PPP was also stereoselectively displaced from the solubilized receptor (IC_{50} for (+)-pentazocine = 15 ± 3 n*M*; (−)-pentazocine = 89 ± 10 n*M*).

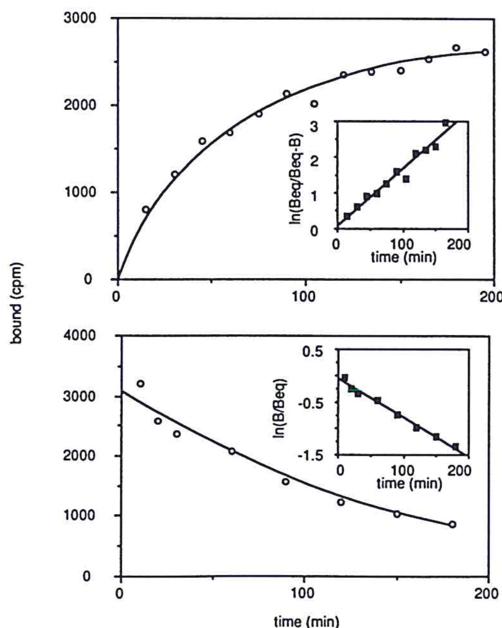

FIG. 2. Association and dissociation kinetics of [^{3}H]DTG with solubilized σ-receptor. Association kinetics (**top**). Binding to solubilized receptor was initiated by addition of 1.2 n*M* [^{3}H]DTG, and 1 ml-aliquots were removed at various times for filtration and washing on PEI-treated GF/B filters as described in Experimental Procedures. Nonspecific binding was determined by a parallel experiment in the presence of 10 μ*M* haloperidol. Dissociation kinetics (**bottom**). Haloperidol (10 μ*M*) was added 3 h after initiation of [^{3}H]DTG binding, and 1-ml aliquots were removed at various times for determination of specific binding. Points represent means of triplicate determinations.

Photoaffinity labeling of soluble σ-receptor

[^{3}H]*p*-Azido-DTG, a photoaffinity label that shows pharmacological selectivity for the σ-receptor (Kavanaugh et al., 1988), was used to label cholate-solubilized σ-receptor. Solubilization of derivatized receptor from GF/B filters with SDS followed by SDS-polyacrylamide gel electrophoresis revealed incorporation of radioactivity into a 29-kilodalton (kDa) polypeptide. Labeling of this polypeptide was completely blocked by 10 μ*M* haloperidol (Fig. 6).

Gel filtration chromatography of solubilized σ-receptor

Sepharose CL-6B chromatography was used to estimate the Stokes radius of the solubilized σ-receptor–cholate complex. Solubilized receptor was preincubated with [^{3}H]DTG, (+)-[^{3}H]3-PPP, or (+)-[^{3}H]SKF-10,047 in the absence or presence of 10 μ*M* haloperidol to determine nonspecific binding, and then chromato-

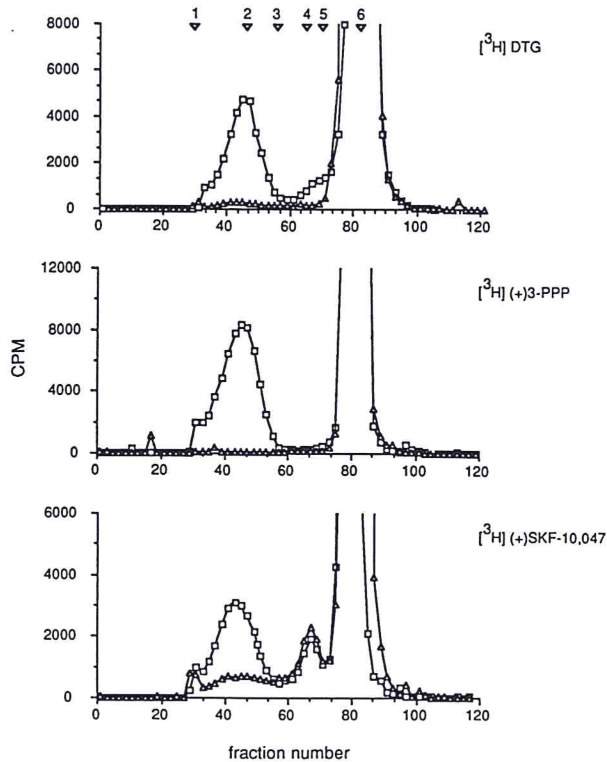

FIG. 7. Sepharose CL-6B chromatography of cholate-solubilized σ-receptor as described in Experimental Procedures. Before chromatography, solubilized receptor was preincubated with [^{3}H]DTG **(top)**, (+)-[^{3}H]3-PPP **(middle)**, or (+)-[^{3}H]SKF-10,047 **(bottom)** in the presence (triangles) or absence (squares) of 10 μM haloperidol. Standards (Sigma) were 1, blue dextran; 2, thyroglobulin (8.6 nm); 3, alcohol dehydrogenase (4.6 nm); 4, bovine serum albumin (3.6 nm); 5, carbonic anhydrase (2.0 nm); and 6, tryptophan.

and (+)-[^{3}H]3-PPP binding to a soluble receptor site could be measured using PEI-treated glass fiber filters. The soluble receptor retained the pharmacological specificity of the membrane-bound form. In addition, the binding affinities of a range of σ-receptor ligands were not significantly altered by solubilization. As with membrane-bound receptor (Kavanaugh et al., 1988), photoaffinity labeling of the solubilized receptor led to selective incorporation of radioactivity into a 29-kDa binding subunit (Fig. 6). These results suggest that the receptor has been solubilized in a form similar to that in membranes.

When [^{3}H]DTG was preincubated with solubilized receptor followed by chromatography on a Sepharose CL-6B column, a significant fraction of the radioactivity eluted in association with a peak whose Stokes radius was calculated to be 8.7 nm (Fig. 7). Binding to this peak was selective, as it was nearly completely blocked by the presence of 10 μM haloperidol in the preincubation volume. The σ-radioligands (+)-[^{3}H]3-PPP and (+)-[^{3}H]SKF-10,047 also labeled a peak of the same size. This result lends support to autoradiographic (Largent et al., 1986; McLean and Weber, 1988) and radioligand binding studies (Weber et al., 1986) suggesting that these drugs bind to the same site. The Stokes radius of the [^{3}H]DTG binding site determined here differs from that of the complex identified by the photoaffinity probe [^{3}H]*p*-azido-DTG, which labels a 29-kDa binding subunit noncovalently associated with a 4.6-nm complex (Kavanaugh et al., 1988). This may be due to dissociation of some components induced by the photolabeling process, or, alternatively, to aggregation of binding sites in the present protocol used for solubilization of the native complex.

In the case of (+)-[^{3}H]SKF-10,047, a smaller peak of radioligand binding apparently unrelated to the σ-receptor eluted with a Stokes radius of 0.5 nm. Binding to this component was not displaceable by haloperidol (Fig. 7). Because (+)-[^{3}H]SKF-10,047 also binds to the PCP receptor (Vincent et al., 1979), preincubation in the presence of the selective PCP receptor ligand MK-801 (Wong et al., 1986) was performed, but it, too, was ineffective at blocking binding of (+)-[^{3}H]SKF-10,047 to this smaller complex (data not shown). In addition, there was no detectable, specific [^{3}H]MK-801 binding to the cholate-solubilized material, indicating that solubilized PCP receptors were not present in an active form (data not shown).

In summary, we have demonstrated that the σ-receptor can be solubilized from guinea pig brain membranes using the ionic detergent sodium cholate in a form that retains the pharmacological characteristics of the membrane-bound form. This work should provide a basis for further studies in biochemical characterization and purification of the σ-receptor from brain membranes.

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TABLE 1. *Comparison of K_i values of various σ-receptor active drugs for native and cholate-solubilized receptor*

Drug	Solubilized	Nonsolubilized
	K_i, nM, against [^{3}H]DTG	
Haloperidol	9.8 ± 3	5 ± 0
DTG	28.1 ± 0.5	26 ± 2
Perphenazine	115 ± 6	41 ± 10
(+)-Pentazocine	7.4 ± 1.2	42 ± 2
(−)-Pentazocine	81 ± 2	131 ± 4
(+)-Butaclamol	764 ± 144	2087 ± 240
(−)-Butaclamol	175 ± 9	514 ± 89
(+)-3-PPP	25.2 ± 4.3	74 ± 4
(−)-3-PPP	220 ± 14	272 ± 21
(+)-Cyclazocine	67 ± 14	317 ± 42
(−)-Cyclazocine	1492 ± 313	2573 ± 240
(+)-SKF 10,047	77 ± 13	607 ± 86
(−)-SKF 10,047	3162 ± 346	3884 ± 549
1-[1-(2-Thienyl)cyclohexyl]-piperidine (TCP)	809 ± 104	1577 ± 395
Chlorpromazine	357 ± 45	1432 ± 257
Amitriptyline	230 ± 38	292 ± 8
Imipramine	290 ± 53	505 ± 14
Triflupromazine	1064 ± 306	1432 ± 257
	K_i, nM, against (+)-[^{3}H]3-PPP	
(+)-3-PPP	36 ± 2	32 ± 12
(−)-3-PPP	123 ± 21	229 ± 59
(+)-Pentazocine	15 ± 3	7.8 ± 2.9
(−)-Pentazocine	89 ± 10	79 ± 1

Values are means ± SEM (n ≥ 3).

graphed at 4°C. The elution profile for each σ-selective radioligand is shown in Fig. 7. In each case, there is a major peak of radioactivity eluting in the total volume of the column, which presumably represents free radioligand. Under these chromatographic conditions, the dissociation rate of radioligand is slow enough to allow detection of significant amounts of radioactivity associated with a peak eluting with a Stokes radius of 8.7 nm. When the solubilized receptor preparation was incubated in the presence of 10 μM haloperidol, radioligand binding to this complex was greatly inhibited, demonstrating that binding is specific and reversible. An additional peak of (+)-[^{3}H]SKF-10,047 binding eluted with a Stokes radius of 0.5 nm (Fig. 7). Binding associated with this peak was inhibited by neither 10 μM haloperidol nor 10 μM MK-801 (data not shown).

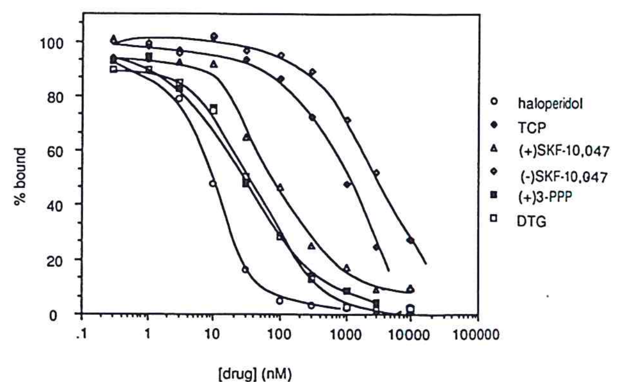

FIG. 5. Potencies of representative drugs in displacing [^{3}H]DTG from solubilized σ-receptors. Binding assays were performed as described in Experimental Procedures.

DISCUSSION

We have shown in the present study that the guinea pig brain membrane-bound σ-receptor can be solubilized using the ionic detergent sodium cholate. Solubilization of the receptor was demonstrated by lack of sedimentation at 100,000 g as well as by retention of the receptor on a Sepharose CL-6B column. [^{3}H]DTG

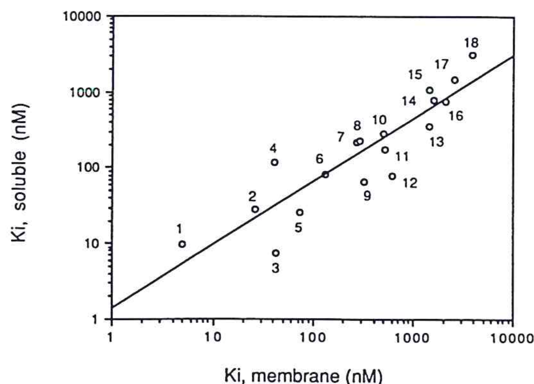

FIG. 4. Comparison of K_i values of various drugs for displacement of [^{3}H]DTG from membrane-bound (abscissa) and solubilized (ordinate) σ-receptor. The correlation coefficient is 0.90. 1, haloperidol; 2, DTG; 3, (+)-pentazocine; 4, perphenazine; 5, (+)-3-PPP; 6, (−)-pentazocine; 7, (−)-3-PPP; 8, amitriptyline; 9, (+)-cyclazocine; 10, imipramine; 11, (−)-butaclamol; 12, (+)-SKF-10,047; 13, chlorpromazine; 14, 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP); 15, triflupromazine; 16, (+)-butaclamol; 17, (−)-cyclazocine; 18, (−)-SKF-10,047.

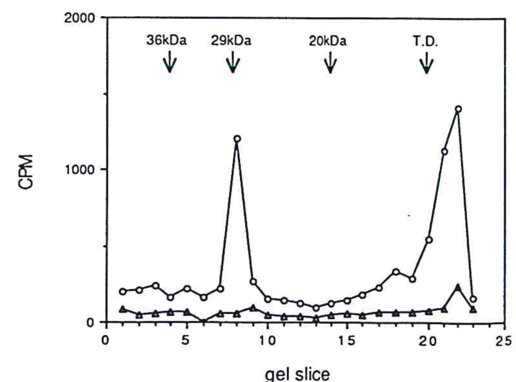

FIG. 6. SDS-polyacrylamide gel electrophoresis of photoaffinity-labeled cholate-solubilized σ-receptor. Soluble receptor was incubated in the presence (triangles) or absence (circles) of 10 μM haloperidol. Following incubation in the dark, solubilized receptor was filtered, washed, and exposed to UV light as described in Experimental Procedures. SDS-solubilized proteins from filters were electrophoresed on a 12% acrylamide gel under reducing conditions, followed by slicing and determination of radioactivity.

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