ELSEVIER

European Journal of Pharmacology 304 (1996) 201–210

ejp

Differential solubilization of rat liver σ_1 and σ_2 receptors: retention of σ_2 sites in particulate fractions

Cheryl Torrence-Campbell, Wayne D. Bowen *

Unit on Receptor Biochemistry and Pharmacology, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892, USA

Received 30 January 1996; accepted 6 February 1996

Abstract

Rat liver membranes (crude P_2 membranes) were solubilized in 10 mM Tris-HCl, pH 7.4 containing 7 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The soluble fraction was designated the Extract 1. The 105 000 × *g* pellet was washed once, and then extracted a second time (Extract 2). The various resulting fractions were assayed for sigma (σ) binding characteristics, using [^{3}H](+)-pentazocine to label σ_1 sites and [^{3}H]1,3-di-*o*-tolylguanidine (DTG) in the presence of 1 μM dextrallorphan to label σ_2 sites. Both of the extracts and resultant pellets (Pellet 1 and Pellet 2) contained σ_1 and σ_2 receptors, as indicated by the pharmacological profiles upon competition studies. The K_d and B_{max} values for σ_1 activity in the original P_2 membranes were 8.3 ± 0.73 nM and 5333 ± 572 fmol/mg protein; K_d and B_{max} for σ_2 activity was 19 ± 0.17 nM and 9190 ± 800 fmol/mg protein. There were no changes in the radioligand K_d values of the two sites in the subsequent soluble and particulate fractions. However, while the σ_1 and σ_2 B_{max} values in extracts and pellets were generally on the same order as those of P_2 membranes, the actual σ_2 to σ_1 B_{max} ratio varied markedly across the fractions. The ratio of σ_2/σ_1 binding in Extract 1 and Extract 2 was 0.86 and 0.68, respectively, compared to a ratio of 1.7 in the original P_2. However, the ratio in Pellet 2 was 3.8, twice that of the original P_2 membranes. Furthermore, the B_{max} value for σ_1 sites in Pellet 2 did not change, whereas the σ_2 B_{max} increased 1.8 fold relative to the original P_2 membranes. The changes in σ_2/σ_1 binding ratio in extracts were observed using two different assay methods for soluble receptors (retention on polyethyleneimine-coated filters and polyethylene glycol precipitation) and is therefore not an artifact of assay procedure. These data suggest that, relative to σ_1 receptors, σ_2 receptors are more resistant to solubilization and become somewhat enriched in the particulate fractions. This supports the notion that σ_1 and σ_2 receptors are distinct macromolecules and may indicate different modes of association with the cell membrane.

Keywords: σ Receptor; Liver, rat; Solubilization; CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); (+)-Pentazocine; DTG (1,3-di-*o*-tolylguanidine)

1. Introduction

Sigma (σ) receptors have attracted much interest due to their high affinity for a number of psychotropic drug classes, particularly neuroleptics, and their potential involvement in the therapeutic action or side effects of these compounds (for reviews see Musacchio et al., 1989; Walker et al., 1990; Itzhak and Stein, 1990; Su, 1991, 1993; Ferris et al., 1991; Bowen, 1993, 1994; Debonnel, 1993; Walker et al., 1993). Though first postulated by Martin et al. (1976) as an opiate receptor type, the definition and nomenclature has undergone extensive revision since that time, beginning with the discovery of the non-opioid nature of these binding sites (Su, 1982; Tam, 1983). These sites are characterized by their high affinity for the prototypic σ ligands haloperidol, 1,3-di-*o*-tolylguanidine (DTG), and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)3-PPP) (Largent et al., 1986; Tam and Cook, 1984; Weber et al., 1986).

It has been demonstrated that σ receptors exist in multiple forms, presently termed sigma-1 (σ_1) and sigma-2 (σ_2) (Hellewell and Bowen, 1990), each of which has a pharmacological profile distinct from any other known receptor (reviews: Walker et al., 1990; Quirion et al., 1992; Bowen, 1993; Itzhak, 1994). Though they both bind haloperidol and DTG with high affinity, the two sites can be readily differentiated from each other by their affinity

* Corresponding author. Unit on Receptor Biochemistry and Pharmacology, Laboratory of Medicinal Chemistry, Bldg. 8, Rm. B1-23, 8 Center Dr., MSC 0815, Bethesda, MD 20892-0815, USA. Tel.: (301) 402-3375; fax: (301) 402-0589.

Elsevier Science B.V.
PII S0014-2999(96)00109-4

and stereoselectivity for opiate benzomorphans and morphinans (Hellewell and Bowen, 1990; Di Paolo et al., 1991; Bowen et al., 1993). Also, σ_1 and σ_2 binding sites reside on distinct polypeptides. For example, σ_1 sites are enriched in guinea pig brain, exhibit a high affinity for (+)-benzomorphans, and have been demonstrated to have an apparent molecular weight of about 25 kDa by [^{3}H]azido-DTG photolabeling (Kavanaugh et al., 1988; Hellewell and Bowen, 1990). σ_2 sites were first characterized in pheochromocytoma (PC12) cells, display a markedly lowered affinity for the (+)-benzomorphans as compared to σ_1 sites, and have an apparent molecular weight of 18–21 kDa (Bowen and Hellewell, 1988; Hellewell and Bowen, 1988; 1990). Since (−)-benzomorphans do not discriminate the two subtypes, they can also be distinguished by enantioselectivity for enantiomeric pairs of benzomorphans, with (+)-benzomorphans > (−)-benzomophans at σ_1 sites and (−)-benzomorphans > (+)-benzomorphans at σ_2 sites (Hellewell and Bowen, 1990; Di Paolo et al., 1991). Recently, 'σ_3' and 'σ_4' subtypes have been proposed, with properties distinct from σ_1 and σ_2 sites (Booth et al., 1993; Bowen et al., 1995).

A majority of the studies on σ receptors have utilized the brain of either guinea pig, rat, or mouse. However, σ sites are also distributed outside the central nervous system in various cell lines (Vilner and Bowen, 1992; Vilner et al., 1995) and in various peripheral tissues. σ receptors have been localized in the rat pituitary gland, adrenal gland, testis, ovary (Wolfe et al., 1989); in tissues of the immune system (Wolfe et al., 1988); kidney (Musacchio et al., 1988); and in the liver (Musacchio et al., 1988; Samovilova et al., 1985, 1988; Ross, 1991). However, little is known about the σ receptor subtypes in these peripheral tissues.

Using a combination of radioligand binding and photoaffinity labeling, we have carried out a detailed analysis of σ receptor subtypes in rat liver and kidney, and demonstrated that these tissues contain high densities of both σ_1 and σ_2 receptors (Bruce et al., 1990; Hellewell et al., 1990, 1994). Three highly selective σ probes were used to label σ sites in rat liver. [^{3}H](+)-Pentazocine, a selective σ_1 probe (Bowen et al., 1993), bound to rat liver membranes with a $K_d = 7.5$ nM and $B_{max} = 2929$ fmol/mg protein. [^{3}H]DTG and [^{3}H](+)3-PPP bound to rat liver membranes with K_d values of 17.9 nM and 51.9 nM and B_{max} values of 11 895 fmol/mg protein and 11 070 fmol/mg protein, respectively. [^{3}H]DTG and [^{3}H](+)3-PPP would label the total population of σ sites ($\sigma_1 + \sigma_2$), whereas [^{3}H](+)-pentazocine labels only the σ_1 population. Thus, based on B_{max} values the rat liver appears to contain 75% σ_2 sites and 25% σ_1, with σ_2 sites occurring in particularly high density compared to other tissues such as brain. The presence of both subtypes was supported by the pharmacological profiles obtained upon competition of various σ ligands vs. [^{3}H](+)-pentazocine and vs. [^{3}H]DTG and [^{3}H](+)3-PPP under conditions where 1 μM dextrallorphan was used to mask labeling of σ_1 sites by the latter two radioligands. Finally, photoaffinity labeling using [^{3}H]azido-DTG resulted in labeling of polypeptides of 25 kDa and 21.5 kDa, similar to those photolabeled in the guinea pig brain and PC12 cells (Hellewell and Bowen, 1990).

In order to help elucidate function of σ sites, several laboratories have solubilized σ receptor activity in a first attempt to purify the receptors (Kavanaugh et al., 1989; McCann and Su, 1991). Kavanaugh et al. (1989) solubilized σ-like binding activity from guinea pig brain membranes with sodium cholate and labeled a 29 kDa protein with [^{3}H]azido-DTG. Similarly, McCann and Su (1991) solubilized an estimated 450 kDa [^{3}H]*N*-allylnormetazocine ([^{3}H](+)-SKF 10047) binding site complex from rat liver membranes using 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and molecular sizing chromatography. In both of these studies, the soluble binding sites retained the pharmacological properties of σ sites in the intact membranes. However, no attempt was made to determine the identity and amount of specific σ subtypes in the extracts. Schuster and coworkers have reported CHAPS solubilization and purification from rat liver of 28, 40, and 65 kDa polypeptides which bind [^{3}H]haloperidol with the pharmacological properties of σ_1 receptors (Schuster et al., 1994). Partial sequencing of the 28 kDa polypeptide revealed similarity to cyclophilins. These workers have also reported solubilization and purification of σ_1-like receptors from rat and bovine cerebellum (Schuster et al., 1995).

The present study investigates solubilization of σ binding activity from rat liver membranes using CHAPS, with emphasis on selective assay conditions for detecting and characterizing σ_1 and σ_2 receptors. We report solubilization of both subtypes in active form, but with preferential extraction of σ_1 sites over σ_2.

2. Materials and methods

2.1. Membrane preparation

Male Sprague-Dawley rats (150–200 g, Charles River, MA) were killed by decapitation. Livers were removed and immediately frozen in powdered dry ice for storage at −80°C until use. Frozen livers were weighed and thawed slowly in a small amount of ice cold 10 mM Tris-HCl/0.32 M sucrose, pH 7.4 (Tris-sucrose buffer) at 4°C and subsequently homogenized in Tris-sucrose buffer (10 ml/g tissue wet weight) with a Potter-Elvehjem homogenizer by 10 strokes with a Teflon pestle. The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was collected and centrifuged again at 31 000 × *g* for 15 min at 4°C. The resulting pellet was resuspended in ice-cold 10 mM Tris, pH 7.4 at a concentration of 3 ml/g original

tissue wet weight. The suspension was allowed to incubate at 25°C for 15 min, followed by centrifugation at 31 000 × *g* for 15 min. The pellet (P_2) was again resuspended in ice-cold 10 mM Tris, pH 7.4 by Potter-Elvehjem homogenization to a final volume of 0.75 ml/g. The volume of the P_2 membrane fraction was measured and a portion was stored at −80°C until use. Protein concentration was determined by the bicinchoninic acid method of Smith et al. (1985), using bovine serum albumin as standard.

2.2. Solubilization and fractionation

For solubilization, fresh (unfrozen) P_2 preparation was diluted to an average protein concentration of 24 mg/ml in 10 mM Tris-HCl, pH 7.4 containing 7 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The suspension was incubated on ice for 20–30 min with stirring. Soluble proteins were separated from particulate matter by centrifugation at 105 000 × *g* for 70 min at 4°C. The supernatant (Extract 1) was collected and saved. The pellet (Pellet 1) was resuspended in Tris-HCl buffer (pH 7.4) equal to the volume of original P_2, and a fraction of it saved for binding assays. The remaining pellet was washed by dilution with 10 mM Tris-HCl, pH 7.4 to a volume four times the original P_2, and centrifugation at 105 000 × *g* for 70 min as above. The supernatant was saved and labeled as 'wash'. The resultant pellet was suspended to two-thirds the original P_2 volume in 10 mM Tris-HCl, pH 7.4 and CHAPS was added to a final concentration of 7 mM for a second detergent extraction. This suspension was incubated for 20–30 min at 4°C, and centrifuged as described for the first CHAPS solubilization step, resulting in Extract 2 and Pellet 2. All fractions were stored at −80°C in 0.5 ml aliquots until use for binding assays. The wash fraction was concentrated to approximately 10% of the original volume with Centriprep-10 concentrators (Amicon, Danvers, MA). Protein concentration in each fraction was determined by the bicinchoninic acid method, using bovine serum albumin as standard.

2.3. Binding assays

Each fraction was diluted in 50 mM Tris-HCl (pH 8.0) to a final protein concentration of 100–180 μg per 50 μl. 50 μl of membrane suspensions, extracts, or extracted pellets were incubated in 50 mM Tris-HCl, pH 8.0 for 120 min at 25°C, with either [^{3}H](+)-pentazocine to label σ_1 binding sites (Bowen et al., 1993) or [^{3}H]DTG in the presence of 1 μM dextrallorphan to label σ_2 sites (Hellewell et al., 1994). Radioligand concentrations were as specified in table legends. Nonspecific binding was defined as that measured in the presence of 10 μM haloperidol. Binding assays and competition experiments were conducted in a final volume of 0.5 ml. With CHAPS extracts, the final detergent concentration was less than 0.35 mM. The incubations were terminated by dilution with 5 ml of ice-cold 10 mM Tris-HCl buffer (pH 7.4), unless otherwise stated. The samples were then vacuum filtered through glass fiber filters, which had been presoaked in 0.5% polyethyleneimine for a minimum of 30 min at 25°C. Filters were then washed twice with 5 ml ice-cold Tris buffer. Radioactivity retained on the filters was measured by liquid scintillation spectrometry. This method was suitable for both particulate fractions and soluble extracts, the latter being due to the adsorption of soluble proteins onto the polyethyleneimine coated filter (Bruns et al., 1983). Filtration was carried out using a Brandel cell harvester (Gaithersburg, MD).

2.4. Polyethylene glycol precipitation

Where specified, binding activity in soluble extracts was determined by the precipitation method described by Cuatrecasas (1972). Incubations were terminated by adding 0.5 ml of ice-cold 0.1% bovine γ-globulin in 50 mM Tris pH 7.7, and vortexing quickly. This was immediately followed by addition of 0.5 ml of ice-cold 24% polyethylene glycol (PEG, MW 8000) in 50 mM Tris-HCl, pH 7.7 and vortexing for at least 15 s to precipitate protein. The protein was applied to glass fiber filters that were pre-wetted with 8% polyethylene glycol in 50 mM Tris pH 7.0, and vacuum filtered. The tubes were then rinsed three times with 5 ml of ice-cold 8% polyethylene glycol in Tris-HCl, pH 7.0 and vacuum filtered after each rinse. Radioactivity retained on the filters was measured by liquid scintillation spectrometry.

2.5. Chemicals

[^{3}H](+)-Pentazocine (51.7 Ci/mmol) was synthesized as described previously (De Costa et al., 1989; Bowen et al., 1993). [^{3}H]DTG (39.1 Ci/mmol) was purchased from DuPont/New England Nuclear (Boston, MA). Haloperidol, Tris-HCl, polyethyleneimine, polyethylene glycol (MW 8000), Triton X-100, sodium cholate, dimethyl maleic anhydride, ethylene diamine tetraacetic acid (EDTA) and phenyl methane sulfonyl fluoride were purchased from Sigma Chemicals (St. Louis, MO). CHAPS and bacitracin were purchased from Calbiochem (LaJolla, CA). Leupeptin was purchased from Boehringer Mannheim (Indianapolis, IN). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). DTG was purchased from Aldrich Chemicals (Milwaukee, WI). Dextrallorphan and enantiomers of pentazocine and *N*-allylnormetazocine (SKF 10047) were kindly provided by Kenner C. Rice (NIDDK, Bethesda, MD)

3. Results

Incubation in 0.2% (3.3 mM) CHAPS for 2 h has been shown to be effective in solubilizing [^{3}H](+)-SKF-10047

Table 1
Pharmacological profile of σ_1 sites in rat liver P_2 and fractions resulting from solubilization

Ligand	K_i (nM) vs. [^{3}H](+)-pentazocine				
	P_2	Extract 1	Pellet 1	Extract 2	Pellet 2
Haloperidol	2.44 ± 1.13	3.4 ± 1.5	3.4 ± 1.7	9.4 ± 5.5	2.1 ± 0.3
DTG	65 ± 34	62 ± 25	31 ± 7.9	99 ± 58	66 ± 22
(+)-Pentazocine	4.8 ± 1	25 ± 10	47 ± 28	7.5 ± 2.9	11 ± 5.9
(−)-Pentazocine	23 ± 11.7	27 ± 16	11 ± 3	19 ± 8.4	33 ± 16
(+)-SKF 10,047	264 ± 113	250 ± 102	403 ± 248	116 ± 68	335 ± 155
(−)-SKF 10,047	4 124 ± 1 815	4 143 ± 1 343	5 103 ± 1 599	1 324 ± 309	3 648 ± 1 158

Solubilization of σ_1 sites was confirmed by determining pharmacological profile for competition against the σ_1 selective probe, [^{3}H](+)-pentazocine. Rat liver P_2 membranes, soluble CHAPS extracts, and the resulting pellets were incubated with nine concentrations of unlabeled ligand ranging from 0.01 nM to 100 000 nM and 3 nM [^{3}H](+)-pentazocine as described in Materials and methods. IC_{50} values were determined using the curve fitting program GraphPAD InPlot (San Diego, CA). K_i values were then calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and K_d values as shown in Table 3. Data are from three different preparations, with two experiments carried out in each preparation (values are average of six curves, ±S.E.M.). Each experiment was carried out in duplicate.

binding activity from rat liver membranes as demonstrated by McCann and Su (1991). To further optimize solubilization conditions, we assessed protein solubilization at several CHAPS concentrations and incubation times (data not shown). CHAPS concentrations ranging from 2 mM to 13 mM and incubation times ranging from 15 min to 2 h all resulted in concentration- and time-dependent solubilization of σ binding activity. However, after 20 min there was no significant increase in σ_1 or σ_2 binding activity of protein solubilized in 7 mM CHAPS or 13 mM CHAPS. Furthermore, we found that final concentrations of CHAPS above 0.7 mM in the binding assay significantly inhibited σ_1 and σ_2 binding. Therefore, we used 7 mM CHAPS to minimize subsequent detergent inhibition of binding and a shorter incubation time (20 min) than previously reported by others (McCann and Su, 1991).

Solubilization of σ_1 binding sites was confirmed by determining pharmacological profiles for competition against the σ_1-selective probe, [^{3}H](+)-pentazocine in CHAPS extracts as shown in Table 1. [^{3}H](+)-Pentazocine labeled sites with a high affinity for DTG, haloperidol, and (+)-pentazocine, and moderate affinity for (+)-SKF 10047. The (+)-enantiomers of pentazocine and SKF 10047 generally had higher affinities than the corresponding (−)-enantiomers. The profile in the CHAPS extract (Extract 1) was very similar to the profile in the original P_2 membranes, showing that solubilization did not significantly alter the binding sites. This profile is similar to that described by McCann and Su (1991) for soluble rat liver σ sites labeled by [^{3}H](+)-SKF 10047, another σ_1 radioligand.

Solubilization of σ_2 sites has not yet been described. It was therefore of interest to determine the properties of soluble σ_2 sites. Table 2 shows that σ_2 sites, labeled using [^{3}H]DTG in the presence of dextrallorphan, were also present in the CHAPS extracts (Extract 1). These sites displayed a high affinity for DTG and haloperidol. The affinities of (−)-pentazocine and (−)-SKF 10047 were substantially greater than those of the corresponding (+)-enantiomers. Furthermore, the affinities of (+)-pentazocine and (+)-SKF 10047 were greatly reduced when compared to their affinities at σ_1 sites (Table 1), whereas the affinities of (−)-pentazocine and (−)-SKF 10047 are virtually the same. These findings are all consistent with

Table 2
Pharmacological profile of σ_2 sites in rat liver P_2 and fractions resulting from solubilization

Ligand	K_i (nM) vs. [^{3}H]DTG and 1 μM dextrallorphan				
	P_2	Extract 1	Pellet 1	Extract 2	Pellet 2
Haloperidol	38 ± 11	120 ± 59	142 ± 71	21 ± 4.8	75 ± 39
DTG	29 ± 14	28 ± 6.3	19.7 ± 2.7	25 ± 7.6	49 ± 11
(+)-Pentazocine	1 936 ± 125	2 797 ± 503	1 009 ± 364	3 938 ± 926	2 251 ± 776
(−)-Pentazocine	13 ± 7	48 ± 19	72 ± 41	35 ± 4.7	30 ± 5.7
(+)-SKF 10047	> 15 000	10 545 ± 384	> 27 000	> 9 000	> 20 000
(−)-SKF 10047	4 236 ± 1 916	2 311 ± 405	3 703 ± 2 000	919 ± 241	805 ± 541

Solubilization of σ_2 sites was confirmed by determining pharmacological profile for competition against [^{3}H]DTG in the presence of 1 μM dextrallorphan. Rat liver P_2 membranes, soluble CHAPS extracts, and the resulting pellets were incubated with 5 nM [^{3}H]DTG in the presence of 1 μM dextrallorphan and nine concentrations of unlabeled test ligand ranging from 0.01 nM to 100 000 nM as described in Materials and methods. IC_{50} values were determined using the curve-fitting program GraphPAD InPlot (San Diego, CA). K_i values were then calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and K_d values as shown in Table 3. Data are from at least three different preparations, with at least one experiment carried out in each preparation (values are average of 4–6 curves, ±S.E.M.).Each experiment was carried out in duplicate.

σ_2 sites. This profile in the extract was also similar to that of the original P_2 membranes, again suggesting that detergent solubilization did not alter the σ_2 binding site characteristics.

However, it was observed that the ratio of 3 nM [^{3}H](+)-pentazocine binding to 5 nM [^{3}H]DTG binding was markedly different in the CHAPS extract compared to the native P_2 membranes. The extracts appeared to contain a reduced [^{3}H]DTG:[^{3}H](+)-pentazocine ratio relative to P_2 membranes, which suggested differential extraction of the binding sites. This was investigated in more detail by carrying out Scatchard analysis of the binding of [^{3}H](+)-pentazocine and [^{3}H]DTG (in the presence of 1 μM dextrallorphan) to determine the relative densities of σ_1 and σ_2 sites, respectively, in the soluble and particulate fractions. The effect of multiple detergent extractions was investigated by subjecting CHAPS-extracted membranes to washing and then a second extraction with 7 mM CHAPS (Extract 2 and Pellet 2). These results are shown in Table 3. Ligand selectivity profiles were also determined in the various fractions to monitor any changes in σ_1 or σ_2 profiles. These data are shown in Tables 1 and 2. Tables 1 and 2 show that the pharmacological profiles of Extract 2 and Pellet 2 remained similar to the profiles of the original P_2, indicating there were no significant changes in σ_1 and σ_2 binding site chacteristics upon repeated treatment with CHAPS.

Table 3 shows that Scatchard plots of equilibrium binding of [^{3}H](+)-pentazocine and [^{3}H]DTG + 1 μM dextrallorphan ([^{3}H]DTG/dextrallorphan) to the rat liver P_2 membranes were both linear, with K_d values of 8.3 ± 0.73 nM and 19 ± 0.17 nM, respectively, and B_{max} values of 5333 ± 572 fmol/mg protein and 9190 ± 800 fmol/mg protein, respectively. It should be noted that whereas the B_{max} value for σ_2 sites reported here in the original P_2 fraction is the same as the calculated σ_2 B_{max} reported previously (Hellewell et al., 1994), the σ_1 B_{max} obtained here is nearly twice that previously reported. The reason for this is not clear, but may possibly be related to the use of frozen rat livers in the current study vs. freshly dissected rat livers in the previous study, which was the only major difference in the procedures.

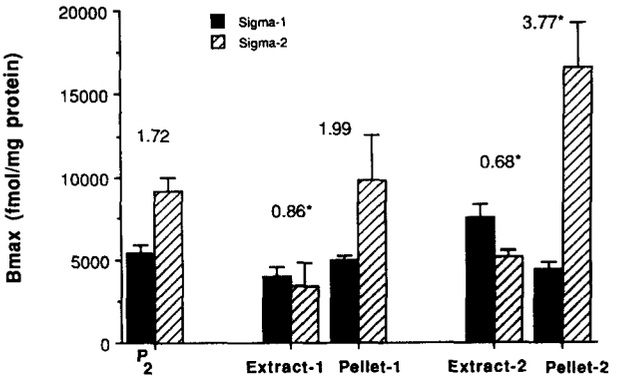

Fig. 1. Comparison of σ_1 and σ_2 concentrations in P_2 membranes and CHAPS-treated fractions. The B_{max} values for σ_1 and σ_2 receptors are taken from Table 3. The values above each column represent the σ_2 to σ_1 ratio determined by dividing the B_{max} for σ_2 receptors by the B_{max} for σ_1 receptors in the indicated fraction. All ratios, with the exception of that exhibited in Pellet 1, were significantly different from the ratio found in the original P_2 membranes, as indicated by asterisk (Extract 1: $P = 0.0001$; Extract 2: $P = 0.01$; Pellet 2: $P = 0.03$). Significance was determined at $P = 0.05$ using Student's t test.

Scatchard analysis data of the solubilized receptors and resulting particulate fractions are also shown in Table 3. Treatment of membranes with CHAPS did not significantly alter the K_d values of radioligands for σ_1 or σ_2 sites. The K_d values of soluble receptors (CHAPS extracts) and CHAPS-treated membranes (resultant CHAPS pellets) were similar when compared to native P_2 membranes. However, there were marked changes in the B_{max} values across the various fractions, which resulted in dramatic changes in the ratio of σ_1 to σ_2 sites. These B_{max} values are compared in Fig. 1. As shown previously (Hellewell et al., 1994), the original P_2 membranes contain more σ_2 receptors than σ_1. The σ_2/σ_1 B_{max} ratio obtained here for the P2 membrane fraction was 1.72. However, after solubilization, the σ_2/σ_1 B_{max} ratios in Extract

Table 3
Saturation analysis of σ_1 and σ_2 binding in fractions resulting from solubilization

	P_2	Extract 1	Pellet 1	Extract 2	Pellet 2
σ_1					
K_d (nM)	8.3 ± 0.73	7.3 ± 1.44	8.2 ± 1.5	8.7 ± 0.76	7.7 ± 1.7
B_{max} (fmol/mg prot.)	5333 ± 572	3939 ± 644	4939 ± 229	7560 ± 820	4381 ± 421
σ_2					
K_d (nM)	19 ± 0.17	34 ± 6.3	91 ± 55	27 ± 6.2	27 ± 3.7
B_{max} (fmol/mg prot.)	9190 ± 800	3368 ± 1407	9804 ± 2760	5144 ± 378	16527 ± 2641

Saturation analysis of the binding of [^{3}H]DTG and [^{3}H](+)-pentazocine to the various fractions resulting from CHAPS treatment. Scatchard analysis was performed on rat liver P_2 membranes, soluble CHAPS extracts, and the resulting pellets. Binding parameters for σ_1 receptors were determined by incubating the fractions in 0.1–100 nM [^{3}H](+)-pentazocine under the conditions described in Materials and methods. Binding parameters for σ_2 receptors were determined by using 3 nM [^{3}H]DTG in the presence of 1 μM dextrallorphan and various concentrations of unlabeled DTG ranging from 5 to 400 nM. Data were analyzed using the iterative curve fitting program BDATA (Baltimore, MD), and were best fit to a one site model. Data are from three different preparations, with at least one experiment carried out in each preparation (values are average of five plots, ± S.E.M.). Each experiment was carried out in duplicate.

1 and Extract 2 decreased significantly to 0.86 and 0.68, respectively. These changes in ratio were due largely to a decrease in the relative amount of σ_2 sites present. Conversely, the σ_2/σ_1 ratio in the particulate fractions appeared to increase relative to the original P_2 fraction. The increase did not reach significance in Pellet 1 but was marked in Pellet 2, increasing from 1.72 to 3.77. This increase was due to an increase in the relative proportion of σ_2 sites in the fraction. Observation of the actual B_{max} values across fractions relative to the original P_2 membranes (Table 3) shows that the density of σ_1 sites is reasonably comparable across the various fractions. However, the B_{max} value for σ_2 sites appears to consistently decrease in soluble fractions and increase in particulate fractions (especially Pellet 2) relative to the original P_2 membranes. Taken together, these data suggest that σ_1 sites are more readily solubilized than σ_2 sites, with σ_2 sites becoming somewhat enriched in the particulate fractions relative to other proteins.

The σ_2 and σ_1 B_{max} values in Table 3 show a relative decrease in σ_2 sites in the soluble extracts relative to the particulate fractions, indicating an apparent resistance to extraction. However, another possibility to consider is that the σ_2 receptors were solubilized by CHAPS, but were not detected in the binding assay. For example, the soluble receptor assay using polyethyleneimine-coated filters takes advantage of the acidic nature of most membrane proteins by retention of soluble proteins on the filter due to electrostatic attraction for the positively charged polyethyleneimine (Bruns et al., 1983). Thus, if σ_2 receptors are neutral or basic proteins they may not be retained and detected, despite efficient extraction. This was addressed by assaying receptor binding using a method involving precipitation of soluble proteins with polyethylene glycol and filtration trapping of precipitated proteins (Cuatrecasas, 1972). Table 4 compares the σ_1 and σ_2 binding in polyethylene glycol precipitated fractions with σ_1 and σ_2 binding of soluble preparations using the 0.5% polyethyleneimine method of Bruns et al. (1983). We first determined whether polyethylene glycol treatment alone would have a general effect on binding of 3 nM [^{3}H](+)-pentazocine (σ_1) and 5 nM [^{3}H]DTG + 1 μM dextrallorphan (σ_2). This was done by subjecting original P_2 membranes to the polyethylene glycol procedure and comparing the results to membranes assayed in the normal manner. Since the P_2 membranes are already particulate, any effect of polyethylene glycol would be on receptor activity and not related to ability to precipitate and trap the proteins. Polyethylene glycol itself was found to have a significant inhibitory effect on σ_2 binding (31% inhibition) but only a small effect on σ_1 activity (6.4% inhibition). This amount of inhibition of σ_1 or σ_2 binding by polyethylene glycol in the P_2 membranes was used as a correction factor to calculate the specific binding of 3 nM [^{3}H](+)-pentazocine and 5 nM [^{3}H]DTG/dextrallorphan in Extract 1 and Extract 2 when the polyethylene glycol method was used. These figures were then used to calculate the σ_2 to σ_1 binding ratios. After adjusting for inhibition, the σ_2 to σ_1 ratios in P_2 membranes were 1.2 in control (polyethyleneimine filter method) and 1.1 in the polyethylene glycol-precipitated fractions. The ratios in Extract 1 were 0.29 and 0.22, while the Extract 2 ratios were 0.98 and 0.93 for polyethyleneimine and polyethylene glycol methods respectively. Thus, there was no difference in any of the ratios of the polyethylene glycol-precipitated fraction after adjustment as compared to fractions assayed by the

Table 4
Polyethylene glycol precipitation of P_2 membranes and CHAPS extracts

Fraction/ subtype	0.5% PEI Method		PEG Method			
	fmol/mg protein	σ_2:σ_1 ratio	fmol/mg protein	% inhibition by PEG	Adjusted fmol/mg	σ_2 : σ_1 ratio
P_2						
σ_1	1 603 ± 195	1.2	1 500 ± 161	(−6.4%)	1 596	1.1
σ_2	1 896 ± 517		1 300 ± 166	(−31%)	1 703	
Extract 1						
σ_1	1 646 ± 217	0.29	1 412 ± 16	–	1 503	0.22
σ_2	480 ± 144		255 ± 89	–	334	
Extract 2						
σ_1	1 661 ± 285	0.98	721 ± 50	–	767	0.93
σ_2	1 629 ± 444		544 ± 139	–	713	

σ_1 and σ_2 binding in solubilized fractions and P_2 membranes were assayed using a single concentration of radioligand, 3 nM [^{3}H](+)-pentazocine for σ_1 and 5 nM [^{3}H]DTG/1 μM dextrallorphan for σ_2. Following incubation, binding activity (fmol bound/mg protein) was determined using either the polyethyleneimine (PEI)-coated filter method or the polyethylene glycol (PEG) precipitation method as described in Materials and methods. In order to assess any general effect of polyethylene glycol treatment on σ binding activity, intact P_2 membranes were subjected to the polyethylene glycol procedure and compared to P_2 membranes assayed by the routine method of trapping on polyethyleneimine-coated filters. Polyethylene glycol itself was found to have a significant inhibitory effect on σ_2 binding in P_2 membranes (31% inhibition) and only a small effect on σ_1 activity. Therefore, σ_1 and σ_2 binding values in polyethylene glycol-precipitated CHAPS extracts were adjusted to correct for this amount of inhibition. The values for the polyethyleneimine method are the mean ± S.E.M. of five experiments from three different preparations. Each experiment was carried out in duplicate. The unadjusted values for the polyethylene glycol method are the average ± S.E.M. of two experiments, each carried out in duplicate. Note that σ_2/σ_1 ratios are for binding of a single concentration of radioligand and not B_{max} values as in Fig. 1.

polyethyleneimine method. This shows that the decrease in σ_2 binding activity in soluble fractions cannot be due to an artifact of loss of binding activity through the filters, since the same results are obtained when receptors are assayed as trapped precipitates. The data thus point to more retention of σ_2 activity in the particulate fractions upon solubilization.

The total yield of receptors (pmol of receptor) in each fraction was calculated, based on starting with 806 mg of P_2 membrane protein. This data is shown in Table 5. The preferential extraction of σ_1 sites over σ_2 receptors is also born out here, and can be seen at each extraction of a particulate fraction. Extraction of the original P_2 membranes (Extract 1) gave a 24% yield of σ_1 receptors, but only a 12% yield of σ_2 sites. Subsequent extraction of Pellet 1 (Extract 2) gave a 15% yield of the σ_1 sites remaining in Pellet 1, whereas a 5% yield of σ_2 receptors was obtained at this step. Totalling activity in the soluble fractions (Extract 1 + Extract 2), it is found overall that 29% of the original σ_1 activity present in P_2 membranes was extracted whereas only 14% of the original σ_2 activity was extracted. Furthermore, assessment of the amount of original P_2 membrane activity remaining particulate after two CHAPS extractions (Pellet 2) reveals 32% of σ_2 receptors and only 15% of σ_1 activity. Interestingly, an assessment of recovery (relative to original P_2 membranes) of total activity across all fractions reveals that 82% of the original σ_2 activity is accounted for, whereas 74% of σ_1 activity is recovered across all fractions. This suggests that during the manipulations, σ_1 activity may be slightly more labile than σ_2. Thus, lability of σ_2 activity cannot account for the differential extraction. Furthermore, if it is assumed that loss of receptor activity occurs evenly across fractions, one can correct the σ_1 and σ_2 values in each fraction to account for this loss. When this is done, it is found that the differential extraction of the σ subtypes could actually be even more pronounced, with an estimated 37% extraction for σ_1 activity and only 16% extraction for σ_2 activity.

Other attempts were made to effect more efficient solubilization of σ_2 sites (data not shown). σ_2 receptor activity was not altered in the presence of a protease inhibitor cocktail (1 mM EDTA, 2.5 μg/ml bacitracin, 5 μg/ml leupeptin, and 0.1 mM phenyl methane sulfonyl fluoride in 10 mM Tris, pH 7.4), thus arguing against loss of solubilized receptors due to proteolysis. Extraction with 1% Triton X-100 or 0.65% (15 mM) cholate gave similar results as with CHAPS, showing that the phenomenon is not specific to CHAPS. Extraction of P_2 membranes with 0.5 M NaCl yielded little or no soluble σ activity. The combination of 0.5 M NaCl and 7 mM CHAPS yielded soluble σ_1 and σ_2 activity, with an increase in the σ_2/σ_1 ratio. However, the increase in σ_2/σ_1 ratio was partly due to a decrease in σ_1 activity, suggesting that in the presence of 0.5 M NaCl, σ_1 activity is less soluble in CHAPS. We have also attempted to solubilize σ binding activity using dimethyl maleic anhydride to extract peripheral membrane proteins (Shanahan and Czech, 1977) and chloroform:methanol to extract proteolipids (Hampson and Poduslo, 1986). There was little or no soluble σ_1 or σ_2 activity resulting from these procedures.

4. Discussion

Samovilova et al. (1985, 1988) demonstrated a high concentration of σ sites in rat hepatic membrane fractions. Previously, Bowen and colleagues (Bruce et al., 1990; Hellewell et al., 1990, 1994) demonstrated that rat liver possesses both σ_1 and σ_2 receptors, with a higher density of σ_2 sites present. These sites also displayed biochemical and pharmacological characteristics very similar to the σ_1 sites in guinea pig brain and the σ_2 receptor sites characterized in PC12 cells, C6 glioma cells, and several other cell lines (Hellewell and Bowen, 1990; Vilner and Bowen, 1992; Bowen et al., 1993; Vilner et al., 1995).

McCann and Su (1991) have previously demonstrated solubilization of σ receptors from rat liver using CHAPS. These investigators used [^{3}H](+)-SKF 10047 as the radiolabeled probe to characterize the receptors. Since this is a (+)-benzomorphan with very low affinity for σ_2 sites (Hellewell and Bowen, 1990) this radioligand would label σ_1 receptors and not σ_2 sites. The pharmacological pro-

Table 5
Total yield of binding activity in P_2 and CHAPS extracts

Fraction	mg of protein ± S.E.M.	σ_1		σ_2	
		Total activity (pmol)	Extraction yield (%)	Total activity (pmol)	Extraction yield (%)
P_2	806 ± 38	4297 ± 203	-	7405 ± 349	—
Extract 1	250 ± 57	984 ± 224	24 ± 4	841 ± 191	12 ± 2
Pellet 1	275 ± 107	1356 ± 528	36 ± 0.7	2692 ± 1048	41 ± 1
Extract 2	27 ± 9	206 ± 68	5 ± 0.1 {15%}	140 ± 46	2 ± 0.1 {5%}
Pellet 2	147 ± 30	642 ± 133	15 ± 2	2423 ± 503	32 ± 5

The total yield of σ_1 and σ_2 activity was calculated for the original P_2 membranes, soluble extracts, and their respective pellets. The total activity in pmol of receptor was calculated by multiplying the total protein yield for the given fraction by the σ_1 or σ_2 B_{max} for that fraction, as given in Table 3. The extraction yield is the percent of total binding activity in the various fractions relative to the original P_2 membranes (806 mg protein). The figures in brackets {}, represent the percent of binding activity in Extract 2 relative to Pellet 1. All averaged values ±S.E.M. are the combined data from at least three separate preparations.

file of solubilized sites obtained by these investigators is consistent with σ_1 receptors, and the binding characteristics were not significantly different from those of the intact rat liver membranes (McCann and Su, 1991). Also, Schuster and co-workers have recently reported solubilization from rat liver and purification to homogeneity of [^{3}H]haloperidol binding activity with the properties of σ_1 receptors (Schuster et al., 1994).

As a prelude to attempts to isolate the individual σ receptor subtypes, here we have investigated the effect of detergent solubilization on these subtypes. We found the optimal CHAPS concentration to be 7 mM, which yielded a large number of active σ receptors. The binding properties of σ_1 receptors labeled by [^{3}H](+)-pentazocine were retained in the solubilized preparation. Furthermore, here demonstrated for the first time, σ_2 receptors labeled by [^{3}H]DTG in the presence of dextrallorphan were solubilized with retention of the pharmacological profile of the original P_2 membranes. CHAPS treatment produced no significant changes in the affinity of σ_1 or σ_2 receptors for their respective radioligands. However, after solubilization, Extract 1 had a completely different σ_2 to σ_1 ratio. The amount of σ_2 decreased dramatically relative to σ_1. Similarly, the σ_2 to σ_1 ratio of Extract 2 indicated an even larger percentage of solubilized σ_1 sites relative to σ_2. This indicates that σ_2 sites were more resistant to solubilization compared to σ_1 sites.

An important point to note along with the changing ratios in the soluble fractions is that the particulate fractions demonstrated no significant change in the B_{max} ratio until after the second extraction, where the σ_2 to σ_1 ratio was 3.8 compared to the original σ_2 to σ_1 ratio of 1.7 in the P_2 membranes. Furthermore, not only did the *ratio* of σ_2 to σ_1 receptor increase, but the σ_2 B_{max} value (specific activity of receptor) increased nearly 2-fold upon repeated extraction of the particulate fraction. This shows a slight enrichment in σ_2 activity which most likely results from the preferential removal of other membrane proteins. Conversely, the specific activity of σ_1 receptors in the CHAPS-treated particulate fraction did not change significantly, indicating that it was extracted as readily as most of the other membrane proteins. These observations with the particulate fractions are again consistent with preferential solubilization of σ_1 sites relative to σ_2 receptors.

Decreased extraction of σ_2 sites was also seen when the total yield of receptors was taken into account. Table 5 shows that only 14% of the of the original P_2 membrane σ_2 receptors is present in the soluble fractions (Extract 1 + Extract 2), compared to 29% of σ_1 sites. Furthermore, after two CHAPS extractions, a larger percentage of the original σ_2 receptors remained particulate compared to σ_1. In addition, the actual relative decrease in σ_2 extraction may be even greater than is apparent, since the total percent recovery of σ_1 sites across all fractions was lower than that of σ_2 sites, possibly masking some of the difference.

Decreased σ_2 activity in the CHAPS extracts was not due to an artifact of the use of polyethyleneimine-coated filters to electrostatically retain receptors, since similar results were observed when soluble proteins were precipitated with polyethylene glycol and physically trapped on filters. A possibility which cannot be completely ruled out at present is that the decreased σ_2 extraction is due to differential inactivation of a portion of σ_2 sites upon treatment with CHAPS. However, this seems unlikely since the pharmacological properties (K_d of [^{3}H]DTG and K_i of competing compounds) of the soluble σ_2 sites were very similar to those of native P_2 membranes, and the particulate fractions extracted with CHAPS had an *increased* specific activity of σ_2 sites, observations inconsistent with receptor inactivation due to exposure to CHAPS. Furthermore, the overall recovery of σ_2 sites across all fractions is higher for σ_2 sites compared to σ_1 (Table 5), suggesting that σ_2 sites may be less labile than σ_1 sites. Protease activity did not appear to play a role since similar results were obtained when solubilization and incubations were carried out in the presence of protease inhibitors.

Solubilization with 1% Triton X-100 or 15 mM sodium cholate gave similar results as solubilization with CHAPS, indicating that the differential solubilization is not a detergent-specific effect and probably does not depend on the ionic nature of the detergent. Failure to extract σ binding activity with 0.5 M NaCl or dimethyl maleic anhydride suggests that neither σ_1 nor σ_2 receptors are peripheral membrane proteins. Furthermore, σ binding activity was not extractable with chloroform-methanol, suggesting that σ receptors are not related to proteolipids. However, other possibilities such as interaction of σ receptors (particularly σ_2 receptors) with cytoskeletal membrane proteins, will need to be investigated further.

In summary, we have solubilized rat liver σ_1 binding sites with relative ease in 7 mM CHAPS, while retaining binding characteristics as determined using the highly selective probe [^{3}H](+)-pentazocine. These data are consistent with a previous report of CHAPS-solubilized rat liver σ sites labeled using [^{3}H](+)-SKF 10047 (McCann and Su, 1991) and the reported purification of rat liver σ_1-like receptors labeled with [^{3}H]haloperidol (Schuster et al., 1994). Furthermore, this is the first demonstration and characterization of solubilized σ_2 receptors, as determined using [^{3}H]DTG in the presence of a masking concentration of dextrallorphan. The binding characteristics of the membrane-bound form of σ_2 receptors were again retained in the soluble preparation. However, the σ_2 activity was not as readily solubilized with detergent, as much of the binding activity tended to remain in the particulate fractions. The differential solubilization of σ_1 and σ_2 receptors provides further support for the notion that σ_1 and σ_2 sites are distinct macromolecules. This also suggests that they are associated with the membrane in different ways and thus may play distinct roles in membrane function.

This information will aid in the purification and subsequent molecular characterization of σ_1 and σ_2 receptors.

References

Booth, R.G., S.D. Wyrick, R.J. Baldessarini, N.S. Kula, A.M. Myers and R.B. Mailman, 1993, New σ-like receptor recognized by novel phenylaminotetralins: Ligand binding and functional studies, Mol. Pharmacol. 44, 1232.

Bowen, W.D., 1993, Biochemical pharmacology of sigma receptors, in: Aspects of Synaptic Transmission, Vol. II: Acetylcholine, Sigma Receptors, CCK and Eicosanoids, Neurotoxins, ed. T.W. Stone (Taylor and Francis, London) p. 113.

Bowen, W.D., 1994, Interaction of sigma receptors with signal transduction pathways and effects on second messengers, in: The Sigma Receptors, Neuroscience Perspectives Series, ed. Y. Itzhak (Academic Press, London), p. 139.

Bowen, W.D. and S.B. Hellewell, 1988, Characterization of sigma receptors on PC12 cells: Soc. Neurosci. Abstr. 14, 703.

Bowen, W.D., B.R. De Costa, S.B. Hellewell, J.M. Walker and K.C. Rice, 1993, [^{3}H](+)-Pentazocine: a potent and highly selective benzomorphan based probe for sigma-1 receptors, Mol. Neuropharmacol. 3, 117.

Bowen, W.D., B.J. Vilner, K.S. Lee, X.-S. He, B.R. De Costa and D.R. Weinberger, 1995, A haloperidol-insensitive binding site for sigma-active aryl ethylene diamine-related compounds: A novel sigma receptor subtype?, Soc. Neurosci. Abstr. 21 (no. 219.5), 526.

Bruce, A.E., S.B. Hellewell and W.D. Bowen, 1990, Characterization of 'sigma-like' sites in rat liver membranes: Further evidence for sigma-1 and sigma-2 sites, Soc. Neurosci. Abstr. 16, 370.

Bruns, R.F., K. Lawson-Wending and T.A. Pugsley, 1983, A rapid filtration assay for soluble receptors using polyethyleneimine-treated filters, Anal. Biochem. 132, 74.

Cheng, Y.-C. and W. H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of the inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Cuatrecasas, P., 1972, Isolation of the insulin receptor of liver and fat cell membranes, Proc. Natl. Acad. Sci. USA 69, 318.

Debonnel, G., 1993, Current hypotheses on sigma receptors and their physiological role: Possible implications in psychiatry, J. Psychiatr. Neurosci. 18, 157.

De Costa, B.R., W.D. Bowen, S.B. Hellewell, J.M. Walker, A. Thurkauf, A.E. Jacobson and K.C. Rice, 1989, Synthesis and evaluation of optically pure [^{3}H](+)-pentazocine, a highly potent and selective radioligand for sigma receptors, FEBS Lett. 251, 53.

Di Paolo, L., F.I. Carroll, P. Abraham, X. Bai, K. Parham, S.W. Mascarella, X. Zhang, P. Wallace, J.M. Walker and W. D. Bowen, 1991, *N*-substituted derivatives of normetazocine: differentiation of sigma-1 and sigma-2 receptors, Soc. Neurosci. Abstr. 17, 814.

Ferris, C.D., D.J. Hirsch, B.P. Brooks and S.H. Snyder, 1991, Sigma receptors: From molecule to man, J. Neurochem. 57, 729.

Hampson, D.R. and S.E. Poduslo, 1986, Purification of proteolipid protein and production of specific antiserum, J. Neuroimmunol. 11, 177.

Hellewell, S.B. and W.D. Bowen, 1988, Photolabelling of sigma receptors in guinea pig brain, rat brain, and PC12 cells using [^{3}H]azido-ditolylguanidine: evidence for receptor heterogeneity, Soc. Neurosci. Abstr. 14, 703.

Hellewell, S.B. and W.D. Bowen, 1990, A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that in guinea pig brain, Brain Res. 527, 244.

Hellewell, S.B., A.E. Bruce and W.D. Bowen, 1990, Characterization of 'sigma-like' binding sites in rat liver membranes: Further evidence for σ_1 and σ_2 sites, in: New Leads in Opiod Research: Proceedings of the International Narcotics Research Conference, ICS 914, eds. J.M. van Ree, A.H. Mulder, V.M. Wiegant and T.B. van Wimersma Greidanus (Excerpta Medica/Elsevier, Amsterdam) p. 270.

Hellewell, S.B., A. Bruce, G. Feinstein, J. Orringer, W. Williams and W.D. Bowen, 1994, Rat liver and kidney contain high densities of σ_1 and σ_2 receptors: characterization by ligand binding and photoaffinity labeling, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 268, 9.

Itzhak, Y., 1994, Multiple sigma binding sites in the brain, in: The Sigma Receptors, Neuroscience Perspectives Series, ed. Y. Itzhak (Academic Press, London) p. 113.

Itzhak, Y. and I. Stein, 1990, Sigma binding sites in the brain: An emerging concept for multiple sites and their relevance for psychiatric disorders, Life Sci. 47, 1073.

Kavanaugh, M.P., B.C. Tester, M.W. Scherz, J.F.W. Keana and E. Weber, 1988, Identification of the binding subunit of the sigma-type opiate receptor by photoaffinity labeling with 1-(4-azido-2-methyl[6-^{3}H]phenyl)-3-(2-methyl[4,6-^{3}H]phenyl)guanidine, Proc. Natl. Acad. Sci. USA 85, 2844.

Kavanaugh, M.P., J. Parker, D.H. Bobker, J.F. Keana and E. Weber, 1989, Solubilization and characterization of sigma receptors from guinea pig brain membranes, J. Neurochem. 53, 1575.

Largent, B.L., A.L. Gundlach and S.H. Synder, 1986, Sigma receptors on NCB-20 hybrid neurotumor cell labeled with (+)-[^{3}H]SKF10047 and (+)-[^{3}H]3-PPP, Eur. J. Pharmacol. 135, 261.

Martin, W.R., C.G. Eades, J.A.Thompson, R.E. Huppler and P.E. Gilbert, 1976, The effects of morphine and nalorphine-like drugs in the non-dependent and morphine-dependent chronic spinal dog. J. Pharmacol. Exp. Ther. 197, 517.

McCann, D.J. and T.-P. Su, 1991, Solubilization and charaterization of haloperidol sensitive (+)-[^{3}H]SKF10047 binding sites (sigma sites) from rat liver membranes., J. Pharmacol. Ther. 257, 547.

Musacchio, J.M., M. Klein and L.J. Santiago, 1988, High affinity dextrallorphan binding sites in guinea pig brain: Further characterization and allosteric interactions. J. Pharmacol. Exp. Ther. 247, 424.

Musacchio, J.M., M. Klein and P.D. Canoll, 1989, Dextromethorphan and sigma ligands: Common sites but diverse effects, Life Sci. 45, 1721.

Quirion, R., W.D. Bowen, Y. Itzhak, J.L. Junien, J.M. Musacchio, R.B. Rothman, T.-P. Su., S.W. Tam and D.P. Taylor, 1992, A proposal for the classification of sigma binding sites, Trends Pharmacol. Sci. 13, 85.

Ross, S.B., 1991, Heterogeneous binding of sigma radioligands in the rat brain and liver: Possible relationship to subforms of cytochrome P-450, Pharmacol. Toxicol. 68, 293.

Samovilova, N.N., K.N. Yarygin, and V.H. Vinogradov, 1985, Specific binding of *N*-allynormetazocine (SKF10047), a ligand of sigma-opioid receptors, to rat liver membranes., Biorg. Khim. 11, 1380.

Samovilova, N.N., L.V. Nagornaya, and V.H. Vinogradov, 1988, (+)-[^{3}H]SKF10047 binding sites in rat liver, Eur. J. Pharmacol. 147, 259.

Schuster, D.I., G.K. Ehrlich and R.B. Murphy, 1994, Purification and partial amino acid sequence of a 28 kDa cyclophilin-like component of the rat liver sigma receptor, Life Sci. 55, PL 151.

Schuster, D.I., F.J. Arnold and R.B. Murphy, 1995, Purification, pharmacological characterization and photoaffinity labeling of sigma receptors from rat and bovine brain, Brain Res. 670, 14.

Shanahan, M.F. and M.P. Czech, 1977, Partial purification of the D-glucose transport system in rat adipocyte plasma membranes, J. Biol. Chem. 252, 6554.

Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk, 1985, Anal. Biochem. 150, 76.

Su, T.-P., 1982, Evidence for sigma opioid receptor: Binding of [^{3}H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. J. Pharmacol. Exp. Ther. 223, 284.

Su, T.P., 1991, Sigma receptors: Putative links between nervous, endocrine, and immune systems, Eur. J. Biochem. 200, 633.

Su, T.-P., 1993, Delineating biochemical and functional properties of sigma receptors: Emerging concepts, Crit. Rev. Neurobiol. 7, 187.

Tam, S.W., 1983, Naloxone-inaccessible σ receptor in rat central nervous system. Proc. Natl. Acad. Sci. USA 80, 6703.

Tam, J.W. and L. Cook, 1984, Sigma opiates and certain antipsychotic drugs mutually inhibit (+)-[^{3}H]SKF10047 and [^{3}H]haloperidol binding in guinea pig brain membranes, Proc. Natl. Acad. Sci. USA 81, 5618.

Vilner, B.J., and W.D. Bowen, 1992, Characterization of sigma-like binding sites of NB41A3, S-20Y, and N1E-115 neuroblastomas, C6 glioma, and NG108-15 neuroblastoma-glioma hybrid cells: further evidence for sigma-2 receptors, in: Multiple Sigma and PCP Receptor Ligands: Mechanisms for Neuromodulation and Neuroprotection?, eds. J.-M. Kamenka and E.F. Domino (NPP Books, Ann Arbor, MI) p. 341.

Vilner, B.J., C.S. John and W.D. Bowen, 1995, Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines, Cancer Res. 55, 408.

Walker, J.M, W.D. Bowen, F.O. Walker, R.R. Matsumoto, B.R. De Costa and K.C. Rice, 1990, Sigma receptors: Biology and function, Pharmacol. Rev. 42, 355.

Walker, J.M., A.G. Hohmann, M.K. Hemstreet, W.J. Martin, M. Beierlein, J.S. Roth, S.L. Patrick, F.I. Carroll and R.L. Patrick, 1993, Functional role of sigma receptors in the nervous system. in: Aspects of Synaptic Transmission Vol. II: Acetylcholine, Sigma Receptors, CCK and Eicosanoids, Neurotoxins, ed. T.W. Stone (Taylor and Francis, London, UK) p. 91.

Weber, E., M. Sonders, M. Quarum, S. McLean, S. Pou and J.F.W. Keana, 1986, 1,3-di(2-[5-^{3}H]tolyl)guanidine: a selective ligand that labels sigma type receptors for psychomimetic opiates and antipsychotic drugs, Proc. Natl. Acad. USA 81, 5618.

Wolfe, Jr., S.A., C. Kulsakdimun, G. Battaglia, J.H. Jaffe, and J.B. DeSouza, 1988, Initial identification and characterization of sigma receptors on human peripheral blood leukocytes., J. Pharmacol. Exp. Ther. 247, 1114.

Wolfe, Jr., S.A., J.G. Culp, and E.B. DeSouza, 1989, Sigma receptors in endocrine organs: identification, characterization, and autoradiographic localization in rat pituitary, adrenal, testis, and ovary, Endocrinology 124, 1160.