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Sigma-2 receptors are specifically localized to lipid rafts in rat liver membranes

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Abstract

We have previously shown that sigma-2 receptors are relatively difficult to solubilize (Eur. J. Pharmacol. 304 (1996) 201), suggesting possible localization in detergent-resistant lipid raft domains. Rat liver membranes were treated on ice with 1% Triton X-100 or 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and the extract subjected to centrifugation on a discontinuous gradient of 5%, 38%, and 40% sucrose. Gradient fractions were analyzed for sigma-1 receptors using [^{3}H](+)-pentazocine and for sigma-2 receptors using [^{3}H]1,3-di-*o*-tolylguanidine ([^{3}H]DTG), in the presence of dextrallorphan. Flotillin-2 was assessed by immunoblotting as a marker for lipid rafts. Sigma-2 receptors were found to discretely co-localize with flotillin-2 in lipid raft fractions. However, sigma-1 receptors were found throughout the gradient. Rafts prepared in CHAPS had sigma-2 receptors with normal pharmacological characteristics, whereas those in Triton X-100-prepared rafts had about seven-fold lower affinity for [^{3}H]DTG and other ligands. Thus, sigma-2 receptors are resident in membrane lipid rafts, whereas sigma-1 receptors appear in both raft and non-raft membrane domains. Lipid rafts may play an important role in the mechanism of sigma-2 receptor-induced apoptosis.

Keywords: Sigma receptor; Lipid raft; Sphingolipid; Apoptosis; Liver, rat

1. Introduction

Sigma receptors comprise a novel, pharmacologically defined family of drug-binding receptors, which recognize a diverse array of centrally acting compounds including some opiates, antipsychotics, and antidepressants (Walker et al., 1990; Bowen, 2000). Two pharmacologically distinct subtypes are currently known, termed sigma-1 and sigma-2 (Quirion et al., 1992; Hellewell et al., 1994). Both subtypes are expressed in high densities on a variety of tumor cell lines, derived from various tissues (Vilner et al., 1995b). Furthermore, sigma-2 receptors are upregulated when cells are in a state of rapid proliferation (Mach et al., 1997).

We have recently shown that sigma-2 receptor agonists cause a transient release of Ca^{2+} stores from the endoplasmic reticulum via an IP_3-independent mechanism (Vilner and Bowen, 2000). We have further demonstrated the ability of sigma-2 receptors to induce apoptosis in primary neurons and in various neuronal and non-neuronal tumor cell lines (Vilner et al., 1995a; Vilner and Bowen, 1997; Crawford and Bowen, 2002). The apoptotic mechanism is independent of p53 and caspases (Crawford and Bowen, 2002). Sphingolipid metabolism has been shown to be involved in regulation of cell growth (Kolesnick and Kronke, 1998). We have demonstrated that treatment of breast tumor cells (human MCF-7/Adr, T47D, and SKBr3) and neuroblastoma cells (human SK–N–SH) with sigma-2 receptor agonists leads to increases in ceramide and sphingosylphosphorylcholine, with concomitant decreases in sphingomyelin (Bowen et al., 2001; Crawford et al., 2002). This suggests activation of a sphingolipid-ceramide *N*-deacylase (SCDase)-like enzyme (Kita et al., 2001). Together with the high density in tumor cell lines, these findings suggest that sigma-2 receptors play a role in cell growth and proliferation, and can induce a novel form of sphingolipid-dependent apoptosis.

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Lipid rafts are organized microdomains in cell membranes and are enriched with cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)-linked proteins (Brown and London, 1998, 2000; Dobrowsky, 2000). These domains form in the plasma membrane and possibly in membranes of some organelles of the secretory and endocytic pathways of eukaryotic cells (Brown and London, 1998). Lipid rafts can form specialized structures termed caveolae upon incorporation of the cholesterol binding protein, caveolin (Brown and London, 1998; Dobrowsky, 2000). Caveolae usually appear as non-clathrin-coated, flask-like invaginations of the plasma membrane, but may also appear as flattened structures. Lipid raft domains and the proteins they contain are resistant to solubilization by detergents such as Triton X-100 and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), have low buoyant density, and can be isolated using sucrose density gradient centrifugation techniques. Lipid rafts and caveolae are believed to play a central role in cell signaling, membrane trafficking, and molecular sorting by sequestering cytoskeletal proteins and proteins involved in signal transduction such as receptors, G-proteins, kinases, and effector enzymes (Brown and Rose, 1992; Fiedler et al., 1993; Rodgers and Rose, 1996; Oliferenko et al., 1999; Dobrowsky, 2000; Nguyen and Hildreth, 2000; Palestini et al., 2000; Ikonen, 2001; Veri et al., 2001; von Haller et al., 2001). While some proteins are recruited into lipid rafts and may be assembled into functional complexes, other proteins may be selectively excluded from raft microdomains. We have shown that sigma-2 receptors are relatively resistant to solubilization by both CHAPS and Triton X-100 (Torrence-Campbell and Bowen, 1996). Furthermore, sigma-2 receptors can stimulate lysosphingolipid and ceramide formation in cell-free Triton X-100 extracts of breast tumor cells (Bowen et al., 2001). Several enzymes of sphingolipid metabolism have been localized to membrane lipid rafts or caveolae, and ceramide generation is known to occur in rafts (Liu and Anderson, 1995; Kalka et al., 2001; Romiti et al., 2001). This suggests that sigma-2 receptors and their targets could be components of lipid rafts. We thus investigated the possible presence of sigma receptors in lipid rafts of rat liver membranes, a rich source of sigma-1 and sigma-2 receptors (Hellewell et al., 1994). Portions of this work have been published previously in abstract form (Gebreselassie and Bowen, 2002).

2. Materials and methods

2.1. Membrane preparation

Male Sprague–Dawley rats (150–200 g) were sacrificed by guillotine. Livers were removed, immediately frozen on dry ice, and stored at −80 °C until use. Preparation of membranes (crude P_2 plasma membrane/mitochondrial fraction) was carried out as previously described (Hellewell et al., 1994; Torrence-Campbell and Bowen, 1996) with minor modifications. Livers were homogenized using a Potter-Elvehjem Teflon-glass homogenizer in 10 ml/g tissue wet weight of ice-cold 10 mM Tris–HCl/0.32 M sucrose, pH 7.4, containing protease inhibitor mixture (Roche Applied Science, Penzberg, Germany). The homogenate was centrifuged for 10 min at 1000 × *g* and the pellet discarded. The supernatant was centrifuged at 31,000 × *g* for 30 min. The pellet was resuspended in 3 ml/g of 10 mM Tris–HCl, pH 7.4 and allowed to incubate at room temperature for 25 min. After centrifugation at 31,000 × *g* for 30 min, the resulting P_2 pellet was resuspended in 1.5 ml/g original wet weight of 10 mM Tris–HCl, pH 7.4 with gentle homogenization. The preparation was stored in aliquots at −80 °C until use. Protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Use of animals was carried out in accordance to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

2.2. Membrane solubilization and equilibrium centrifugation

Membrane solubilization was performed using Triton X-100 or CHAPS according to procedures previously described, with modifications (Ilangumaran et al., 1996; Nguyen and Hildreth, 2000). Use of CHAPS was adapted from Fiedler et al. (1993). P_2 membrane suspensions were re-pelleted by centrifugation at 31,000 × *g* for 10 min. The packed pellets were weighed, and 600 mg of pellet was solubilized in 3 ml of TKM buffer (50 mM Tris–HCl pH 8.0, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA) containing either 1% Triton X-100 or 20 mM CHAPS, at 4 °C under gentle rotation for 1 h. The sample was then divided into microfuge tubes and centrifuged at 10,000 rpm for 10 min at 4 °C in a microfuge (Beckman Microfuge 12) to remove large particulate material. The resulting detergent extracts (supernatants) were collected, combined, and sufficient solid sucrose was added to adjust the final sucrose concentration in the extract to 40%. The sucrose was dissolved by gentle rotation. A volume of 500 μl of the sucrose-adjusted extract was applied to the bottoms of SW41 rotor tubes. The tubes (six tubes per run) were then overlaid by 6 ml of 38% sucrose/TKM (without detergent) followed by 4.5 ml of 5% sucrose/TKM. The tubes were then subjected to centrifugation at 200,000 × *g* for 18 h at 4 °C. Eleven 1-ml fractions were collected from the bottom of the tubes by needle puncture and peristaltic pump and assayed immediately for sigma receptor binding activity, flotillin-2, and protein concentration as described below.

2.3. Flotillin-2 dot- and immunoblotting

Fractions were assayed for the lipid raft marker, flotillin-2 (Bickel et al., 1997; Dermine et al., 2001; Salzer and Prohaska, 2001) using a dot-blot method described by

Ilangumaran et al. (1996), with minor modifications. The protein concentrations of the fractions collected were analyzed using the BCA-protein assay kit (Pierce). To detect raft-associated flotillin-2 protein in the fractions, 100 μl of the fraction was diluted to 1 ml with phosphate-buffered saline and adsorbed onto a nitrocellulose filter using a dot-blot apparatus (Schleicher and Schuell, Keene, NH). Blocking buffer (5% non-fat dry milk, 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) was used to reduce nonspecific antibody binding by soaking filters overnight at 4 °C. Immunoblots were routinely performed by incubating with the mouse monoclonal anti-flotillin-2 antibody (Lampire Biological Laboratories, Pipersville, PA; 1:2500 dilution in blocking buffer) for 1 h at room temperature. Blots were washed six times for 5 min each in wash buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20), and then incubated for 1 h at room temperature with peroxidase-conjugated goat anti-mouse immunoglobulin G (BD-Biosciences, San Diego, CA) (1:2000 dilution in the blocking buffer). After washing six times for 5 min, the immunoblots were incubated with the Luminol reagent for 1–2 min, and enhanced chemiluminescence (ECL, Santa Cruz Biotechnology, Santa Cruz, CA) was detected by Kodak Scientific Imaging Film. The image of the dot-blot strip from the exposed film was acquired using a Kodak Digital Science Image Station 440CF (Perkin-Elmer Life Sciences, Boston, MA). Flotillin-2 was quantified by measuring the intensity of the individual spots after outlining the area. Background measurements were taken from unexposed areas of the film and subtracted from flotillin spot intensities. Flotillin specific activity values in each fraction were determined by dividing the intensity value by the total amount of protein in the fraction.

For Western blotting, the membrane proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels after solubilization in loading buffer. The proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane and flotillin-2 was detected as above.

2.4. Sigma receptor binding assays

The fractions collected above were immediately examined for sigma-1 and sigma-2 receptor binding activity using a previously described method for soluble and membrane-bound receptors, but with modification of the incubation buffer (Hellewell et al., 1994; Torrence-Campbell and Bowen, 1996). Sigma-1 receptor activity was determined using 3 nM [^{3}H](+)-pentazocine, a selective probe for sigma-1 receptors. Sigma-2 receptor activity was determined using 3 nM or 6 nM [^{3}H]1,3-di-*o*-tolylguanidine ([^{3}H]DTG), in the presence of 1 μM dextrallorphan to mask sigma-1 sites. Assays were performed with radioligand using 300 μg of total protein in a final volume of 1 ml of 50 mM Tris–HCl pH 8.0, containing 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA. Samples were incubated for 120 min at 25 °C. Nonspecific binding was determined in the presence of 10 μM haloperidol. Assays were terminated by addition of 5 ml of ice-cold 10 mM Tris–HCl pH 7.4 and vacuum filtration through glass fiber filters using a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were then washed twice with 5 ml ice-cold 10 mM Tris–HCl, pH 7.4. Filters were soaked in 0.5% polyethyleneimine for 1 h at 25 °C prior to use. Filtration through polyethyleneimine-coated filters has been previously shown to efficiently retain soluble proteins as well as those in membranous and particulate fractions due to charge interactions which occur on the filters and to size retention, respectively (Bruns et al., 1983; Torrence-Campbell and Bowen, 1996). Radioligands were purchased from Perkin-Elmer Life Sciences.

3. Results

Flotillin-1 and flotillin-2 are 45-kDa integral membrane proteins which are related to the epidermal surface antigen family (Bickel et al., 1997). Flotillins have been found to be resident components of lipid rafts in several tissues and cell types, including erythrocytes, lymphocytes, skeletal muscle, neurons, retinal ganglion cells, macrophages, A498 kidney cells, and malignant melanoma cells (cf. Salzer and Prohaska, 2001; Bickel et al., 1997; Dermine et al., 2001; Solomon et al., 2002). Fig. 1 shows Western blot analysis of flotillin-2 from a human endothelial cell lysate and the rat liver membranes used in the present study after SDS-PAGE.

Figs. 2 and 3 show the distribution of sigma-1 and sigma-2 receptors in sucrose density gradients after equilibrium centrifugation of membrane extracts prepared with Triton X-100 (Fig. 2) and CHAPS (Fig. 3), compared to that of flotillin-2. Flotillin-2 was detected with the antibody used in Fig. 1 by dot-blot analysis. Quantitation of flotillin-2 content in each fraction relative to the total protein present (specific activity) is shown in Fig. 4. Lipid rafts were located in fraction numbers 6–8, as indicated by the presence of flotillin-2 at high specific activity (Fig. 4), and were visibly identified as a white-tan cloudy layer of material just above the middle of the gradient tube. Flotillin-2 present at the bottom of the gradient (fractions 1 and 2) is due to some residual crude membranous material not

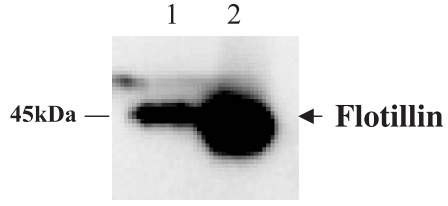

Fig. 1. Detection of flotillin-2, a 45-kDa marker protein for lipid rafts, in crude membrane preparations. Crude membrane preparations from human endothelial cell lysate (lane 1; 5 μg protein) and rat liver P_2 (lane 2; 20 μg protein) were subjected to SDS-PAGE and Western blotting as described in Materials and methods.

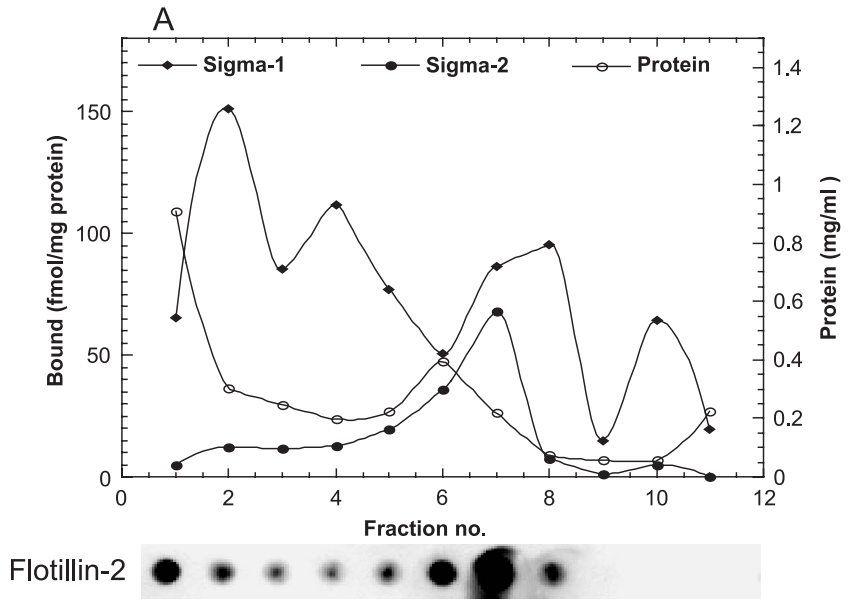

Fig. 2. Localization of sigma-1 and sigma-2 receptors after equilibrium centrifugation of Triton X-100 extracts of rat liver P_2 membranes in discontinuous sucrose density gradients. Rat liver P_2 membranes were treated with 1% Triton X-100 at 4 °C and the extracts subjected to centrifugation on discontinuous sucrose gradients as described in Materials and methods. Eleven 1-ml fractions were taken and sigma-1 and sigma-2 receptors were assayed using [^{3}H](+)-pentazocine and [^{3}H]DTG (in presence of 1 μM dextrallorphan), respectively. The lipid raft marker flotillin-2 was determined in each fraction by immunoblot detection. Protein was determined by the BCA method. Sigma-1 receptor (closed diamonds) and sigma-2 receptor (closed ovals) binding activity in each fraction is expressed as specific activity (fmol/mg protein) and shown on the left axis. Protein (open ovals) is shown on the right axis (mg/ml). The specific activity of flotillin-2 in each fraction was calculated and shown in Fig. 4. The profiles shown are the averages of two separate experiments, each carried out in duplicate. The flotillin-2 dot-blot strip is from a representative experiment. Fraction 1 = bottom (high density); Fraction 11 = top (low density). Flotillin-2 present at the bottom of the gradient is indicative of pelleted residual membranous material remaining in the detergent extract prior to placement on the gradient.

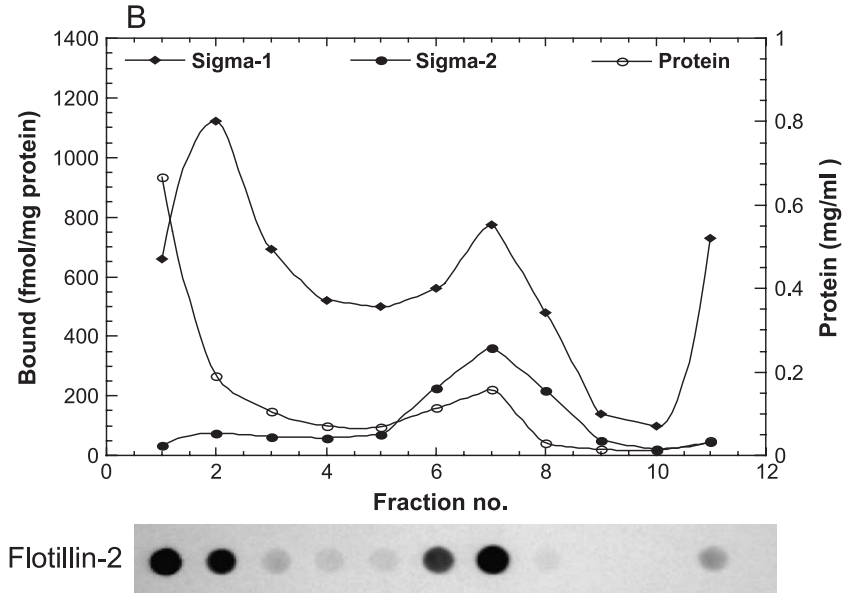

Fig. 3. Localization of sigma-1 and sigma-2 receptors after equilibrium centrifugation of CHAPS extracts of rat liver P_2 membranes in discontinuous sucrose density gradients. Rat liver P_2 membranes were treated with 20 mM CHAPS at 4 °C and the extracts subjected to centrifugation on discontinuous sucrose gradients as described in Materials and methods. Other procedures and symbol labeling are as stated in the legend to Fig. 2. The profiles shown are the averages of two separate experiments, each carried out in duplicate. The flotillin-2 dot-blot strip is from a representative experiment, and specific activity values across the gradient are shown in Fig. 4. Fraction 1 = bottom (high density); Fraction 11 = top (low density). Flotillin-2 present at the bottom of the gradient is indicative of pelleted residual membranous material remaining in the detergent extract prior to placement on the gradient.

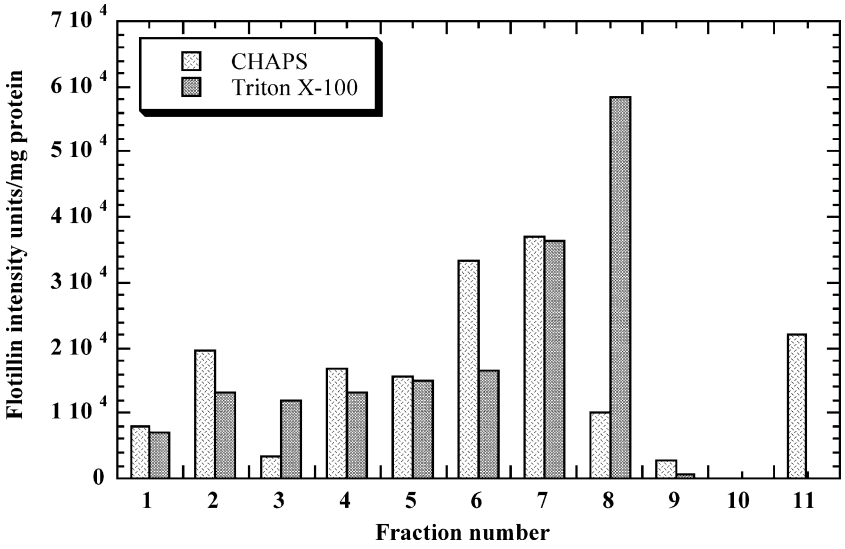

Fig. 4. Distribution of flotillin-2 in sucrose density gradient fractions relative to protein content. The representative dot-blots shown in Figs. 2 and 3 (derived from 100 μl of each fraction) were quantified using a digital imaging system as described in Materials and methods. Background intensity readings were subtracted from measurements taken of the spots, and the resulting relative intensity values were divided by the protein content in 100 μl of fraction. Specific activity values are expressed as relative intensity per mg protein. Triton X-100 extract, cross-hatched bars; CHAPS extract, stippled bars.

removed by low speed centrifugation prior to placement on the gradient. This was indicated by a brownish pellet at the bottom of the tube (fraction #1 with slight mixing into fraction #2), by the high protein content, and by the relatively low flotillin-2 specific activity (see Fig. 4). The localization of flotillin-2 in fractions 6–8 corresponded to the position of the raft components Thy-1 (a GPI-linked protein), heat-stable antigen glycoprotein, and ganglioside GM1 in gradients prepared from membranes of murine T lymphoma cell line P1798 and to GM1 in membranes from mouse intestinal tissue as reported by Ilangumaran et al. (1996). Also, Nguyen and Hildreth (2000) localized Thy-1 to fraction numbers 3–5, representing glycolipid-rich membranes from Jurkat cells. Note that fractions #6 and #7 here (Figs. 2 and 3), numbering from the bottom to the top of the gradient (high density to low density), correspond to the

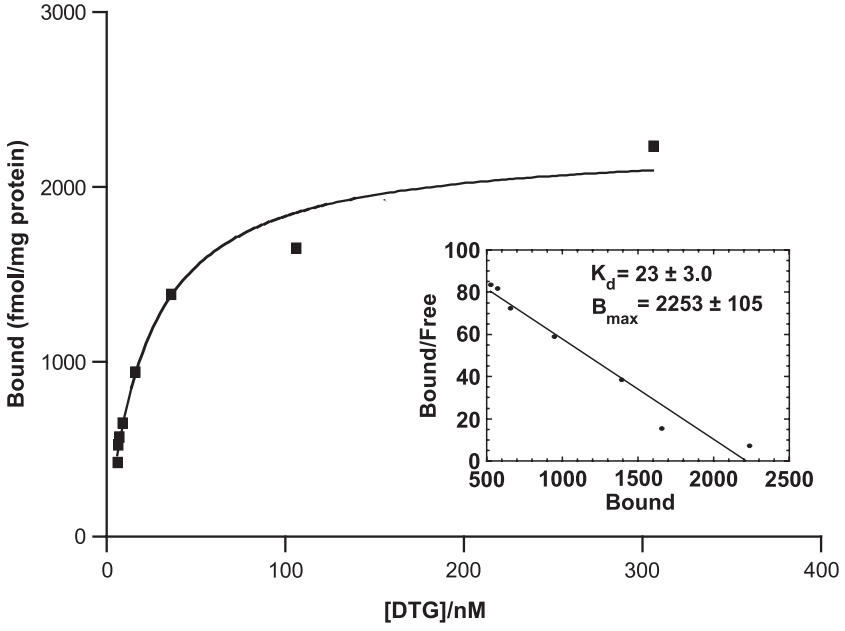

Fig. 5. Saturation (Scatchard) analysis of binding of [^{3}H]DTG to sigma-2 receptors in isolated lipid raft fractions of CHAPS-solubilized membranes. The raft-containing fractions (#6 and #7) were pooled and incubated with 6 nM [^{3}H]DTG and 1 μM dextrallorphan in presence of eight concentrations of unlabeled DTG ranging from 6 to 300 nM. K_d (nM) and B_{max} (fmol/mg protein) were determined by Scatchard analysis (inset) of the saturation curve using the iterative curve-fitting program GraphPAD Prism (San Diego, CA). Values are averages ± S.E.M. of two experiments, each carried out in duplicate. Points shown are the averages of the two experiments.

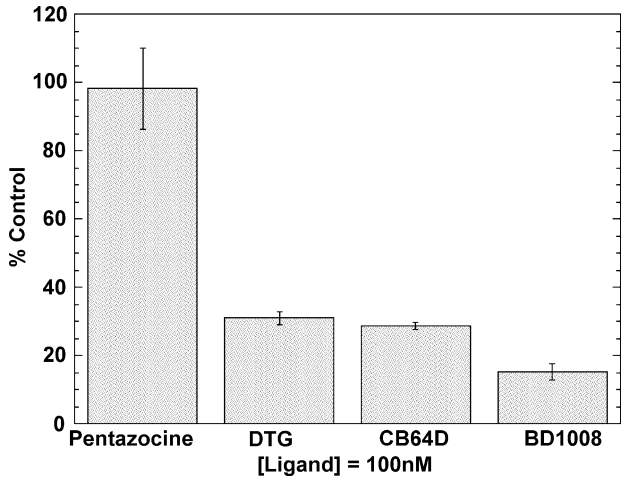

Fig. 6. Pharmacological profile of sigma-2 receptors in rafts extracted with CHAPS. Raft protein and 6 nM [^{3}H]DTG (with 1 μM dextrallorphan) were incubated with 100 nM of the indicated sigma ligands. Competition curves to determine K_i values were not performed due to scarcity of material. Values are expressed as percentage of binding in the absence of competing ligand. Values for each bar are averages ± S.E.M. of two experiments, each carried out in duplicate.

raft-containing fractions #4 and #5 of Ilangumaran et al. (1996) and Nguyen and Hildreth (2000), who numbered fractions from top to bottom of the gradient (low density to high density).

Sigma-1 receptors (labeled with [^{3}H](+)-pentazocine) and sigma-2 receptors (labeled with [^{3}H]DTG in presence of dextrallorphan) showed differential distribution across the density gradient. The distributions were generally similar in Triton X-100 and CHAPS extracts. Sigma-2 receptors migrated to fraction numbers 6–8, co-localizing with flotillin-2, with peak binding activity (specific activity) occurring in fraction #7. Sigma-1 receptors were localized throughout the gradient, with highest binding activity found in fraction #2 where the flotillin-2 specific activity is relatively low (Fig. 4). The discrete co-localization of [^{3}H]DTG binding with flotillin-2 indicates that sigma-2 receptors are resident components of lipid rafts. Sigma-1 receptors are also present in lipid rafts, but were more widely distributed outside of lipid raft domains.

The level of sigma receptor binding in CHAPS-extracted rafts was considerably higher compared to that in rafts prepared in Triton X-100. Fig. 5 shows results of Scatchard analysis of [^{3}H]DTG binding to sigma-2 receptors of lipid raft fractions prepared with CHAPS. Binding of [^{3}H]DTG was saturable, with $K_d = 23.0 \pm 3.0$ nM and $B_{max} = 2253 \pm 105$ fmol/mg protein. These receptors are of comparable affinity to those of native rat liver membranes, where $K_d = 19$ nM (Torrence-Campbell and Bowen, 1996). Fig. 6 shows the pharmacological profile of these sites. (+)-1*R*,5*R*-*E*-8-benzylidene-5-(3-hydroxyphenyl)-2-methylmorphan-7-one (CB-64D) and ibogaine are sigma-2-selective ligands, (+)-pentazocine is sigma-1-selective, while DTG and *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine (BD1008) bind to both sigma sites (Quirion et al., 1992; Hellewell et al., 1994; Vilner and Bowen, 2000). The binding to radiolabeled raft receptors prepared in CHAPS showed a profile consistent with sigma-2 sites, with BD1008, DTG, and CB-64D giving >50% inhibition of control binding at 100 nM. (+)-Pentazocine was inactive. The inhibitory poten-

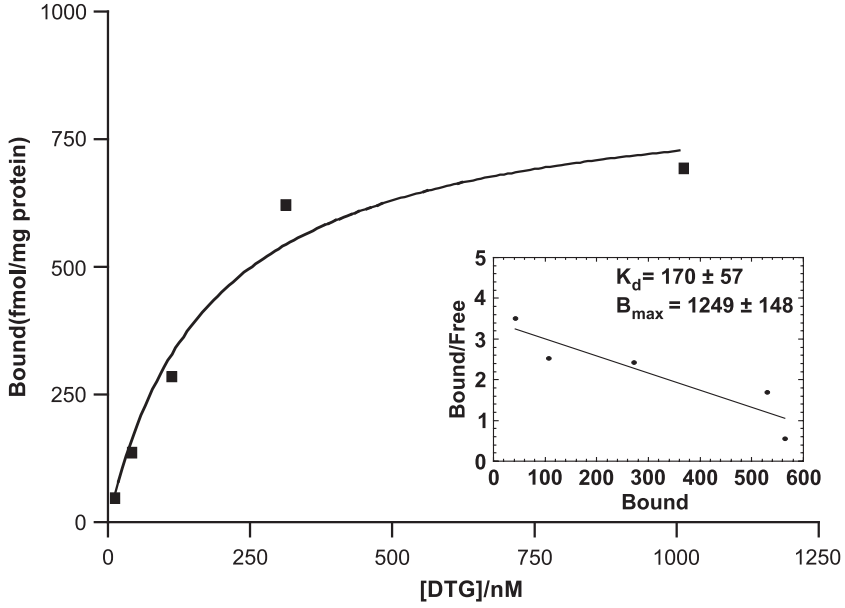

Fig. 7. Saturation (Scatchard) analysis of binding of [^{3}H]DTG to sigma-2 receptors in isolated lipid raft fractions of Triton X-100-solubilized membranes. The raft-containing fractions (#6 and #7) were pooled and incubated with 6 nM [^{3}H]DTG and 1 μM dextrallorphan in presence of five concentrations of unlabeled DTG ranging from 6 to 1000 nM. K_d (nM) and B_{max} (fmol/mg protein) were determined by Scatchard analysis (inset) of the saturation curve using the iterative curve-fitting program GraphPAD Prism. Values are averages ± S.E.M. of two experiments, each carried out in duplicate. Points shown are the averages of the two experiments.

cy demonstrated by the ligands at 100 nM is consistent with sigma-2 receptors of normal affinity.

Fig. 7 shows results of Scatchard analysis of [^{3}H]DTG binding to sigma-2 receptors of lipid raft fractions in Triton X-100 extracts. Binding of [^{3}H]DTG was saturable. Lipid rafts extracted into Triton X-100 yielded sigma-2 receptors with $K_d = 170 \pm 57$ nM and $B_{max} = 1249 \pm 148$ fmol/mg protein. These receptors are of significantly lower affinity compared to sigma-2 receptors in native rat liver membranes and sigma-2 receptors present in rafts prepared in CHAPS (Fig. 5). The pharmacological profile of these [^{3}H]DTG binding sites in Triton X-100 rafts is shown in Fig. 8. The binding to radiolabeled receptors showed the known profile of sigma-2 sites, with BD1008>CB-64D>DTG>ibogaine≫(+)-pentazocine. CB-64D produced displacement at all concentrations tested and ibogaine produced 60% inhibition at 10,000 nM, whereas (+)-pentazocine failed to displace at concentrations up to 10,000 nM. BD1008 was the most potent ligand. However, consistent with lower affinity for [^{3}H]DTG, the ligands all showed lower inhibitory potency compared to CHAPS-extracted sigma-2 receptors and to what might be expected for native membranes. For example, the level of inhibition produced by 500 nM ligand in Triton X-100 preparations is less than or equal to that produced by 100 nM ligand in CHAPS preparations (Fig. 6). This accounts for the apparent low potency of the sigma-2 selective ibogaine in the Triton X-100 preparations, which displaced only at 10,000 nM. Since ibogaine has only moderate affinity for sigma-2 sites in native rat liver membranes ($K_i = 201$ nM), the shift in potency of this ligand is not surprising.

Thus, while lipid rafts prepared in CHAPS contain sigma-2 receptors with normal characteristics, preparing rafts with Triton X-100 yields receptors which maintain the pharmacological characteristics of sigma-2 sites but which have lower affinity for ligands. The binding parameters (K_d and B_{max}) of sigma-1 receptors labeled with [^{3}H](+)-pentazocine were not determined. However, the ~8-fold lower level of [^{3}H](+)-pentazocine binding in Triton X-100 rafts compared to CHAPS rafts (Figs. 2 and 3) indicates that the same may hold true for sigma-1 receptors.

4. Discussion

We have previously shown that sigma-2 receptors are more difficult to solubilize compared to sigma-1 receptors (Torrence-Campbell and Bowen, 1996). When rat liver membranes were repeatedly extracted with CHAPS, the overall yield of soluble receptors in the 105,000 × *g* supernatant was 16% for sigma-2 receptors compared to about 37% for sigma-1 receptors. Furthermore, the specific activity of sigma-2 binding increased nearly two-fold in the particulate fractions sedimented after repeated CHAPS extraction, while that of sigma-1 receptors remained constant. This indicated that, compared to sigma-2 receptors, other membrane proteins were preferentially removed by the detergent, resulting in an apparent increase in B_{max} of sigma-2 receptors in the remaining membranes. Similar results were obtained using cholate and Triton X-100. Based on these data, we proposed that sigma-1 and sigma-2

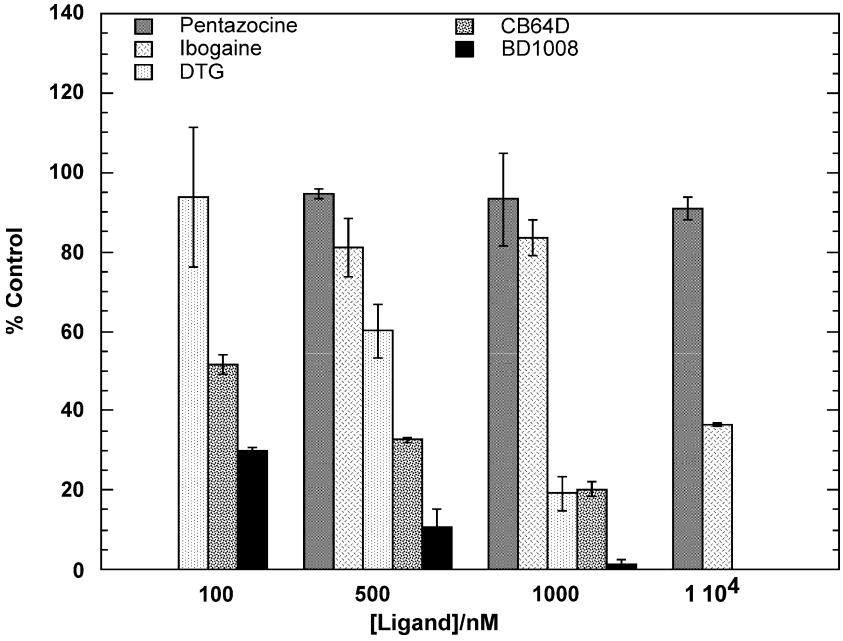

Fig. 8. Pharmacological profile of sigma-2 receptors in rafts extracted with Triton X-100. Raft protein and 6 nM [^{3}H]DTG (with 1 μM dextrallorphan) were incubated with 100, 500, 1000, and 10,000 nM of the indicated sigma ligands ("pentazocine" is (+)-pentazocine). Full competition curves to determine K_i values were not performed due to scarcity of material. Values are expressed as percentage of binding in the absence of competing ligand. Values for each bar are averages ± S.E.M. of two experiments, each carried out in duplicate.

receptors might be associated with the cell membrane in a different manner. This relative resistance to detergent solubilization suggested a possible association of sigma-2 receptors with lipid rafts.

Using procedures shown to generate lipid raft fractions from various cell types and tissues (Fiedler et al., 1993; Ilangumaran et al., 1996; Nguyen and Hildreth, 2000; Dermine et al., 2001; Salzer and Prohaska, 2001), we show that sigma-2 receptors are discretely localized in lipid rafts as identified using the raft marker protein, flotillin-2. By contrast, although there were sigma-1 receptors found in the raft fractions, they exhibited no discrete association with rafts since sigma-1 receptor binding activity was found throughout the gradient. Sigma-1 receptor specific activity was highest in fraction #2, where flotillin-2 specific activity was relatively low. In fact fraction #2 in particular, and this higher density region in general, exhibited the widest separation of sigma-1 and sigma-2 binding activities, giving clear indication that the two receptors are differentially associated with rat liver membranes.

Caveolae are considered to be specialized lipid rafts which specifically contain caveolin, and are isolated in the same way as non-caveolar lipid rafts (Brown and London, 1998; Dobrowsky, 2000). Flotillins have been shown to be components of lipid rafts which contain caveolin (Bickel et al., 1997), as well as those that do not (Solomon et al., 2002). In the current study, we have made no attempt to distinguish whether sigma receptors are localized to caveolae vs. non-caveolar lipid rafts. However, we were not able to reliably detect caveolin-1 in our rat liver P_2 membranes, suggesting that the subsequently derived raft preparations contain largely non-caveolar lipid rafts (data not shown).

Although sigma-2 receptors localized to rafts in both Triton X-100 and CHAPS extracts, receptors found in Triton X-100-prepared rafts were altered. The levels of [^{3}H]DTG binding in Triton X-100 extracted rafts were about six-fold lower compared to those prepared in CHAPS. Rafts prepared in CHAPS contained sigma-2 receptors which exhibited a K_d for [^{3}H]DTG which was nearly identical to that of native rat liver membranes. These receptors also showed the typical sigma-2 receptor profile, with potent displacement by competing ligands. However, sigma-2 receptors in rafts prepared with Triton X-100 had seven-fold lower affinity for [^{3}H]DTG, compared to those of native membranes and CHAPS-prepared rafts, and exhibited reduced affinity for other ligands.

The reason for the difference in sigma-2 receptor affinity between Triton X-100- and CHAPS-derived rafts is not clear. One possibility is that Triton X-100 has a direct adverse effect on sigma receptor binding. However, this is not likely since we have observed that truly soluble sigma-2 receptors (in 105,000 × *g* supernatants) have similar activity whether solubilized in CHAPS or Triton X-100 (Torrence-Campbell and Bowen, 1996). Fiedler et al. (1993) have compared the properties of rafts prepared in CHAPS to those prepared in a Triton-based detergent. Although the overall composition of proteins in CHAPS-derived rafts and rafts derived in Triton X-114 was qualitatively similar in Madin-Darby canine kidney (MDCK) cells, there were quantitative differences noted. CHAPS-derived rafts contained less GPI-linked protein compared to Triton X-114, indicating that more GPI-linked proteins may be extracted from membranes in soluble form by CHAPS. Also, the Triton-insoluble membrane fraction from MDCK cells was found to contain a higher percentage of lipid (>95% w/w) compared to the CHAPS-insoluble fraction, which was about 40% w/w lipid (Fiedler et al., 1993). Despite the lower percentage of lipid, the lipid composition of the CHAPS-insoluble fraction was similar to that of the Triton-insoluble fraction, with the exception of the Forssman antigen and lactosylceramide which were depleted in the CHAPS-derived raft material. In light of this, one possible explanation for the difference in sigma-2 receptor affinity could be that potentially inhibitory lipids or proteins may be absent or reduced in the CHAPS-derived rafts. This will need further investigation.

It should be noted that a small but significant amount of sigma-2 receptor activity is readily extractable from rat liver membranes. About 12% of activity is solubilized (remains in 105,000 × *g* supernatant) upon a single extraction of membranes with 7 mM CHAPS (Torrence-Campbell and Bowen, 1996). This low level of activity is likely not detectable in the current study. It is not known whether this represents a separate, non-raft population of sigma-2 receptors or whether sigma-2 receptors can leave the rafts depending on functional state or other conditions.

The localization of sigma-2 receptors in lipid rafts is consistent with observations regarding the possible apoptotic signaling mechanisms utilized by sigma-2 receptors. We have shown that sigma-2 receptor activation results in an increase in ceramide levels in breast tumor cells with a concomitant decrease in the levels of sphingomyelin (Crawford et al., 2002). The mechanism appears to involve activation of a putative sphingolipid-ceramide *N*-deacylase (SCDase)-like enzyme which hydrolyzes sphingomyelin to form sphingosylphosphorylcholine and which acylates sphingosine to form ceramide (Kita et al., 2001; Bowen et al., 2001). Several enzymes of sphingolipid metabolism have been found to be present in lipid rafts, including sphingomyelinase, ceramidase, and ganglioside sialidase (Liu and Anderson, 1995; Kalka et al., 2001; Romiti et al., 2001). Also, ceramide production can be localized to rafts (Liu and Anderson, 1995). Sigma-2 receptor-mediated stimulation of SCDase-like activity remains intact in 1% Triton X-100 extracts of breast tumor cells since both the acylation of sphingosine with [^{3}H]palmitic acid and deacylation of [^{3}H]sphingomyelin can be demonstrated in diluted Triton X-100 extracts of cells (Bowen et al., 2001; submitted for publication). This shows that the coupling of sigma-2 receptors to this activity is not disrupted by Triton X-100 and suggests that the components might be present in rafts.

The localization of sigma-2 receptors in sphingomyelin-rich rafts would place it in close proximity to substrate for its putative target enzyme. This possibility is under further investigation.

The potencies of sigma-2 agonists to produce effects on Ca^{2+} and to induce apoptosis are much lower than predicted by their sigma-2 receptor binding affinities (K_i values) in isolated cell membrane preparations (Vilner et al., 1995a; Vilner and Bowen, 1997, 2000). Instead, the potency of ligands appears to be determined more by Log*P* value, with the more hydrophobic ligands being the more potent (Bowen et al., 1999). Furthermore, raising the extracellular pH from 7.2 to 8.2 greatly increases the potency of hydrophilic ligands, indicating that the deprotonated, more hydrophobic, form of the compound is the active species (Vilner et al., 1995a; Bowen et al., 1999). These observations suggest that the ligands are not accessible to receptors from the outer cell surface and that the compounds must cross the cell membrane to access sigma-2 receptors in an intracellular locale. Since lipid rafts are presumed to be largely plasma membrane microdomains, the results might be consistent with association of sigma-2 receptors with rafts on the inner leaflet of the plasma membrane. This mode of membrane association with rafts would be analogous to that of several acyl lipid-anchored proteins, such as H-ras and G-protein α-subunits (von Haller et al., 2001; El-Husseini and Bredt, 2002). Thus, ligands would either have to gain access to the cell cytoplasm or penetrate into the lipid raft in order to activate sigma-2 receptors located on the cytoplasmic face of the membrane. The development of molecular tools such as receptor antibodies and fluorescence tagging will be necessary before further details of the association of sigma-2 receptors with the cell membrane can be fully elucidated.

Although sigma-1 receptors were not discretely localized to lipid rafts as were sigma-2 receptors, raft fractions were not devoid of sigma-1 binding activity. Thus, they are not selectively excluded from rafts as are some other proteins such as CD45 glycoprotein and immunoglobulin binding protein (BiP) chaperone (Ilangumaran et al., 1996; Rodgers and Rose, 1996; Nguyen and Hildreth, 2000). Similar to sigma-2 receptors, the level of [^{3}H](+)-pentazocine binding in CHAPS-derived rafts was eight-fold higher compared to Triton X-100 rafts, suggesting that components in Triton X-100-derived rafts might also alter sigma-1 receptors.

Sigma-1 receptors have been shown to be located in endoplasmic reticulum, mitochondria, perinuclear, and plasmalemmal regions of cells (Alonso et al., 2000; Hayashi and Su, 2001) and evidence suggests that they can translocate in the presence of agonist (Morin-Surun et al., 1999; Hayashi and Su, 2001). Sigma-1 agonists triggered translocation of sigma-1 receptors from the endoplasmic reticulum to the plasma membrane, regulating neuronal activity via a phospholipase C/protein kinase C cascade (Morin-Surun et al., 1999). Sigma-1 agonists enhanced IP_3 receptor function in the endoplasmic reticulum by causing dissociation and transport of a sigma-1 receptor/ankyrin B complex from the IP_3 receptor (Hayashi and Su, 2001). In a recent study, it was shown that sigma-1 receptors of NG108-15 cells are associated with endoplasmic reticulum lipid droplets, suggesting that they may be involved in lipid compartmentalizaton and export (Hayashi and Su, 2003). Interestingly, the sigma-1 receptors were found in detergent-resistant microdomains which differed from those of classical, glycosphingolipid-containing lipid rafts. Sigma-1 receptor immunoreactivity was detected in almost all fractions with higher buoyant density than lipid rafts. These higher density fractions contained cholesterol, but no GM1 ganglioside or Src, which are markers for classical lipid rafts. Furthermore, the sigma-1 receptor was co-localized with ankyrin in these fractions, and a portion of these receptors could be shifted into the lower density, classical lipid raft fraction when the sigma-1 receptor-ankyrin association was disrupted (Hayashi and Su, 2003). Thus, the sigma-1 results reported here with rat liver membranes are consistent with those found for NG108-15 cells, with the exception that there appears to be relatively more sigma-1 receptor in the classical lipid raft fraction in rat liver membranes.

The functional significance of the differential localization between sigma-1 and sigma-2 receptors is not clear at present. The mode of receptor signaling for sigma-1 receptors appears to require a highly mobile protein which can readily translocate between organelles (Morin-Surun et al., 1999; Hayashi and Su, 2001; Hayashi and Su, 2003). Thus, it could be proposed that restricted sequestration to lipid rafts would not be suited for this mode of signaling. The converse could be true for sigma-2 receptors. It is possible that signaling does not involve such receptor trafficking, with all events occurring within the lipid raft environment as suggested by the continued ability to signal in a detergent extract as described above (Bowen et al., 2001; submitted for publication). More work will be needed to define this issue. Future studies will investigate the effects of agonist binding on the distribution of sigma-2 receptors in lipid rafts and the effect of raft disruption on sigma-2 receptor signaling.

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