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Solubilization of high affinity G-protein-coupled serotonin$_{1A}$ receptors from bovine hippocampus using pre-micellar CHAPS at low concentration

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Summary

The serotonin$_{1A}$ (5-HT$_{1A}$) receptors are members of a superfamily of seven transmembrane domain receptors that couple to G-proteins. They appear to be involved in various behavioural and cognitive functions. This paper reports an efficient strategy to solubilize 5-HT$_{1A}$ receptors from bovine hippocampal membranes using the zwitterionic detergent CHAPS which is mild and non-denaturing. Since high concentration of CHAPS has earlier been shown to induce dissociation and depletion of G-protein sub-units, a low (pre-micellar) concentration of CHAPS was used for solubilizing 5-HT$_{1A}$ receptors in the presence of NaCl followed by PEG precipitation. This results in solubilization of 5-HT$_{1A}$ receptors with a high degree of efficiency and gives rise to high affinity, functionally active G-protein-sensitive solubilized receptors. Optimal solubilization of the receptor from the native source with high ligand binding affinity and intact signal transduction components may constitute the first step in the molecular characterization of the 5-HT$_{1A}$ receptor in particular, and G-protein-coupled receptors in general.

Keywords: 5-HT$_{1A}$ receptor, CHAPS, solubilization, 8-OH-DPAT, *p*-MPPF.

Abbreviations: BCA, bicinchoninic acid; 5-CT, carboxamido tryptamine maleate; CHAPS, 3-[(3-cholamidopropyl)-dimethyl ammonio]-1-propanesulphonate; GTP-γ-S, guanosine-5′-*O*-(3-thio triphosphate); 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-N-propylamino)tetralin; PMSF, phenylmethylsulphonyl fluoride; *p*-MPPF, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; *p*-MPPI, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido] ethyl-piperazine; PEG, polyethylene glycol.

Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent (Chattopadhyay *et al.* 1996, Maiti *et al.* 1997), biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems (Jacobs and Azmitia 1992). It mediates a variety of physiological responses in distinct cell types. Serotonergic signalling appears to play a key role in the generation and modulation of various cognitive and behavioural functions including sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression, learning and memory (Artigas *et al.* 1996, Ramboz *et al.* 1998, Rocha *et al.* 1998, Casadio *et al.* 1999, Bailey *et al.* 2000). Disruptions in serotonergic systems have been implicated in the aetiology of mental disorders such as schizophrenia, migraine, depression, suicidal behaviour, infantile autism, eating disorders, and obsessive compulsive disorder (Heisler *et al.* 1998, Parks *et al.* 1998, Ramboz *et al.* 1998).

Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups (Zifa and Fillion 1992, Peroutka 1993, Hoyer *et al.* 2002). Serotonin receptors are members of a superfamily of seven transmembrane domain receptors (Strader *et al.* 1995) that couple to GTP-binding regulatory proteins (G-proteins). Among the various types of serotonin receptors, the G-protein-coupled 5-HT$_{1A}$ receptor sub-type has been the most extensively studied for a number of reasons (Harikumar and Chattopadhyay 1998a, Harikumar *et al.* 2000). One of the main reasons for this is the availability of a selective ligand (8-OH-DPAT) that allows extensive biochemical, physiological and pharmacological characterization of the receptor (Gozlan *et al.* 1983). The 5-HT$_{1A}$ receptor was the first among all the serotonin receptors to be cloned and sequenced (Kobilka *et al.* 1987, Fargin *et al.* 1988, Albert *et al.* 1990). The human, rat and mouse 5-HT$_{1A}$ receptors have been cloned, and their amino acid sequences deduced (Fargin *et al.* 1988, Albert *et al.* 1990, Charest *et al.* 1993). The cloning of the 5-HT$_{1A}$ receptor gene has shown that it belongs to the superfamily of G-protein-coupled receptors, with 50% amino acid homology with the β_2-adrenergic receptor in the transmembrane domain. Furthermore, it was the first serotonin receptor for which polyclonal antibodies were obtained (Fargin *et al*. 1988, El Mestikawy *et al.* 1990), allowing their visualization at the sub-cellular level in various regions of the brain. The 5-HT$_{1A}$ receptor has recently been shown to have a role in neural development (Olmo *et al.* 1998) and protection of stressed neuronal cells undergoing degeneration and apoptosis (Singh *et al.* 1996). The 5-HT$_{1A}$ receptor antagonists represent a major class of molecules with potential therapeutic effects in anxiety- or stress-related disorders (Griebel 1999). The 5-HT$_{1A}$ receptor gene has also been implicated in Tourette's syndrome, a common hereditary motor and vocal tic disorder (Lam *et al.* 1996). The 5-HT$_{1A}$ receptor was earlier partially purified from bovine hippocampus in a functionally active form (Chattopadhyay and Harikumar 1996) and modulation of ligand binding was shown by metal ions, guanine nucleotides, alcohols, and covalent modifications of the disulphides and sulphydryl groups (Harikumar and Chattopadhyay 1998a, b, 1999, 2000, 2001, Harikumar *et al.* 2000).

An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the native membrane and individually dispersed in solution. This is most effectively accomplished using amphiphilic detergents and the process is known as

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solubilization (Helenius and Simons 1975, Lichtenberg *et al.* 1983, Hjelmeland and Chrambach 1984, Madden 1986, Jones *et al.* 1987, Paternostre *et al.* 1987, Silvius 1992, Banerjee 1999). Solubilization of a membrane protein is a process in which the proteins and lipids that are held together in the native membrane are suitably dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in the formation of small protein and lipid clusters that remain dissolved in the aqueous solution. Effective solubilization and purification of G-protein-coupled receptors in functionally active form represent an important step in understanding structure-function relationship and pharmacological characterization of a specific receptor. Yet, solubilization of a membrane protein with retention of activity poses a formidable challenge since many detergents irreversibly denature membrane proteins (Garavito and Ferguson-Miller 2001).

One of the most commonly used detergents in membrane biochemistry is CHAPS (Hjelmeland 1980, Stark *et al.* 1984) which is a mild, non-denaturing, zwitterionic detergent and is a derivative of the naturally occurring bile salts (see figure 1). It has a critical micelle concentration (CMC) of 6.41 mM at 23°C in water in absence of any added salt (Chattopadhyay and Harikumar 1996). CHAPS combines useful features of both the bile salt hydrophobic group and the N-alkyl sulphobetaine-type polar group (Hjelmeland 1980, Hjelmeland *et al.* 1983). It is more efficient in solubilizing membrane proteins than the structurally related carboxylic acid anions such as cholate and is much more effective in breaking protein–protein interactions than either sodium cholate or Triton X-100. In addition, CHAPS has very low absorbance at 280 nm (unlike Triton X-100) and does not have circular dichroic activity in the far UV region, making it ideal for optical studies of proteins. These factors have led to extensive use of CHAPS in solubilization of membrane proteins and receptors (Klausner *et al.* 1982, Gozlan *et al.* 1987, El Mestikawy *et al.* 1988, Kline *et al.* 1989, Ofri *et al.* 1992, Banerjee *et al.* 1995, Chattopadhyay and Harikumar 1996, Cladera *et al.* 1997). CHAPS has previously been used to solubilize the 5-HT_{1A} receptor (Gozlan *et al.* 1987, El Mestikawy *et al.* 1988, Kline *et al.* 1989, Banerjee *et al.* 1995). However, the efficiency of solubilization in these cases was found to be low (<50%) and the concentration of CHAPS used in these studies was high and ranged between 10–30 mM.

It has recently been reported that treatment of membranes with high concentration of CHAPS (or similar detergent such as cholate) results in dissociation and depletion of the $\beta\gamma$ dimer of trimeric G-proteins (Jones and Garrison 1999, Waldhoer *et al.* 1999, Bayewitch *et al.* 2000). For example, it has been shown that 16 mM CHAPS can extract >90% of the $\beta\gamma$ dimers from the membrane (Waldhoer *et al.* 1999). Use of CHAPS at high concentration may, therefore, be detrimental for solubilizing G-protein-coupled receptors in a functionally active form. Since the 5-HT_{1A} receptor is negatively coupled to the adenylate cyclase system through G-proteins (Emerit *et al.* 1990), high concentration of CHAPS may lead to inefficient solubilization due to depletion of $\beta\gamma$ dimers of G-proteins from the membrane. This paper reports an efficient strategy to solubilize high affinity, functionally active G-protein-sensitive 5-HT_{1A} receptors from bovine hippocampal membranes with a high degree of efficiency (>80%) using CHAPS at a low (pre-micellar) concentration in presence of NaCl followed by PEG precipitation. PEG precipitation represents a useful approach to efficiently remove detergent and NaCl from solubilized membranes (Gal *et al.* 1983, Kremenetzky and Atlas 1984, Aguilar and Ochoa 1986, Aguilar *et al.* 1987, Medrano *et al.* 1989), which leads to the formation of liposomes from endogenous lipids (Pellegrino de Iraldi *et al.* 1986).

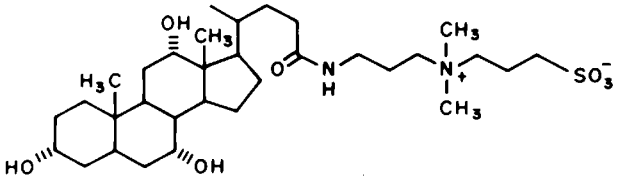

Figure 1. Chemical structure of the zwitterionic detergent CHAPS.

Results

Solubilization of high affinity 5-HT_{1A} receptors from bovine hippocampal membrane with CHAPS and NaCl

The authors previously showed that the CMC of CHAPS, which is zwitterionic and carries no net charge, is dependent on the salt concentration of the medium and there is significant reduction in CMC with increasing salt concentration (Chattopadhyay and Harikumar 1996). The steady decrease in CMC

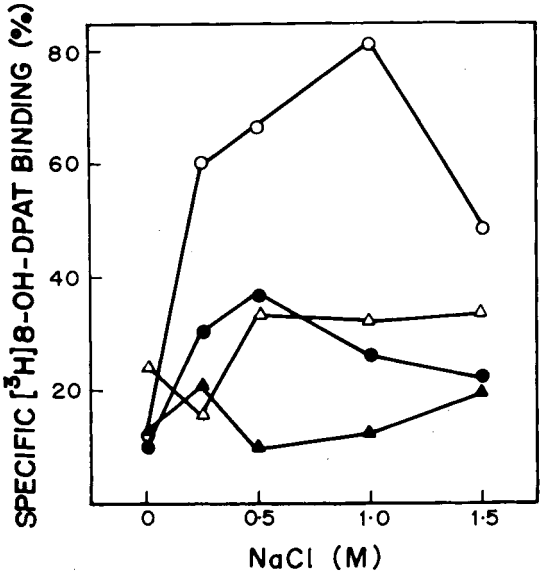

Figure 2. Solubilization of native hippocampal membranes containing the 5-HT_{1A} receptor with different concentrations of CHAPS and NaCl. Values are expressed as percentage of specific binding of the agonist [^{3}H]8-OH-DPAT obtained for native membranes without solubilization. The concentrations of CHAPS used were 5 (○), 7.5 (●), 10 (◇), and 15 (◆) mM. The data points are the means of duplicate points from three independent experiments. See Experimental Procedures for other details.

of CHAPS with increasing salt concentration is shown in table 1. Thus, the CMC of CHAPS decreases from 6.41 mM in the absence of any salt to 4.10 mM in a presence of 1.5 M NaCl, which amounts to a reduction of 36%.

The solubilization of native hippocampal membranes containing the 5-HT$_{1A}$ receptor, as monitored by the specific agonist [^{3}H]8-OH-DPAT binding, as a function of CHAPS and NaCl concentration is shown in figure 2. PEG precipitation of the crude CHAPS-solubilized membrane was performed to remove NaCl from the solubilized extract, since the agonist binding of the 5-HT$_{1A}$ receptor is inhibited by NaCl (Harikumar and Chattopadhyay 1998a). This procedure is believed to remove salt (Gal *et al.* 1983, Kremenetzky and Atlas 1984) and constitutes a method in which the detergent (CHAPS) is also rapidly removed, thus overcoming the problem of long dialysis periods (Kremenetzky and Atlas 1984, Aguilar and Ochoa 1986, Aguilar *et al.* 1987, Medrano *et al.* 1989). The solubilization efficiency is low (<20% in most cases) without NaCl and changes with concentration of CHAPS used even when the concentration of NaCl is kept constant. For a given CHAPS concentration, solubilization efficiency shows an overall increase with increase in salt concentration up to 1 M NaCl. Optimal solubilization of agonist binding sites (>80%) is achieved using 5 mM CHAPS in the presence of 1 M NaCl. Figure 2 also shows that higher concentrations of CHAPS (>5 mM) or NaCl (>1 M) reduce the efficiency of solubilization as measured by specific [^{3}H]8-OH-DPAT binding. The terms CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes will be used for membranes solubilized using 5 mM CHAPS (without NaCl) and 5 mM CHAPS in the presence of 1 M NaCl, respectively, throughout this paper.

Although selective 5-HT$_{1A}$ agonists such as 8-OH-DPAT were discovered more than a decade back (Gozlan *et al.* 1983), the development of selective 5-HT$_{1A}$ antagonists has been relatively slow and less successful. Recently, two specific antagonists for the 5-HT$_{1A}$ receptor, *p*-MPPI and *p*-MPPF, have been introduced (Kung *et al.* 1994a, b, 1995, Zhuang *et al.* 1994, Thielen and Frazer 1995, Allen *et al.* 1997). These compounds bind specifically to the 5-HT$_{1A}$ receptor with high affinity. The solubilization of native hippocampal membranes containing the 5-HT$_{1A}$ receptor measured by the specific antagonist [^{3}H]*p*-MPPF binding as a function of CHAPS and NaCl concentration is shown in figure 3. In this case also, the solubilization efficiency is low (<20%) without NaCl and varies with concentration of CHAPS used. As observed with agonist binding, the solubilization efficiency is increased with increase in salt concentration for a given CHAPS concentration. Optimal solubilization of antagonist binding sites (~70%) is achieved using 5 mM CHAPS in the presence of 1 M NaCl. Higher concentrations of CHAPS (>5 mM) or NaCl (>1 M) reduces the efficiency of solubilization similarly to what was observed in figure 2.

It should be mentioned that, even when PEG was used without NaCl, good recovery of specific activity was obtained (with respect to control membranes), possibly due to the ability of PEG to reconstitute the receptor in an environment enriched with native lipids. However, NaCl was still used along with CHAPS in the experiments prior to PEG precipitation, because the overall amount of active receptor solubilized was found to be more when NaCl was used. This is due to a more efficient solubilization achieved in the presence of NaCl, a result of lowering the CMC of CHAPS, as mentioned above. Lower concentration (2.5 mM) of CHAPS generally resulted in poor solubilization efficiency even in presence of NaCl (data not shown).

Table 1. CMC of CHAPS as a function of salt concentration[a]

NaCl concentration (mM)	CMC (%)	CMC (mM)
0	0.394	6.41
100	0.382	6.21
200	0.365	5.94
500	0.347	5.64
1000	0.267	4.34
1500	0.252	4.10

[a]Taken from (Chattopadhyay and Harikumar 1996).

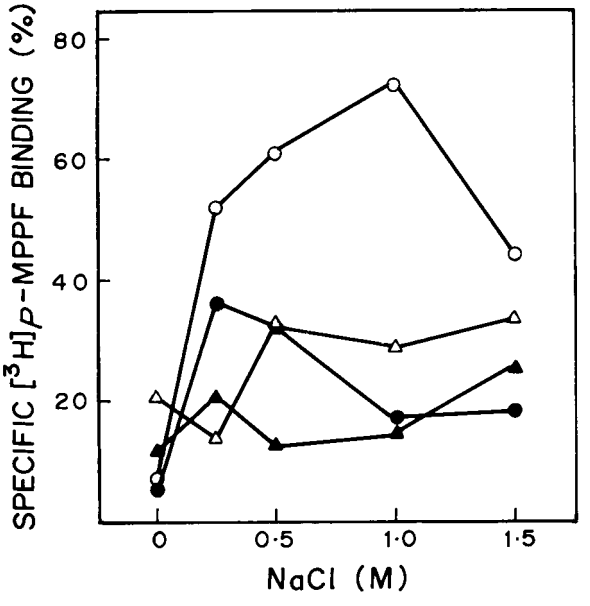

Figure 3. Solubilization of native hippocampal membranes containing the 5-HT$_{1A}$ receptor with different concentrations of CHAPS and NaCl. Values are expressed as percentage of specific binding of the antagonist [^{3}H]*p*-MPPF obtained for native membranes without solubilization. The concentrations of CHAPS used were 5 (○), 7.5 (●), 10 (◇), and 15 (◆) mM. The data points are the means of duplicate points from three independent experiments. See Experimental Procedures for other details.

Guanine nucleotide sensitivity of [^{3}H]8-OH-DPAT binding in CHAPS-solubilized membranes and PEG-precipitated CHAPS-solubilized membranes

Since most of the transmembrane domain receptors are coupled to G-proteins (Clapham 1996), guanine nucleotides are known to regulate agonist binding. The 5-HT$_{1A}$ receptor is negatively coupled to the adenylate cyclase system through pertussis toxin-sensitive G-proteins (G_i/G_o) (Emerit *et al.* 1990). It has previously been shown that the specific agonist [^{3}H]8-OH-DPAT binding to bovine hippocampal 5-HT$_{1A}$ receptors is sensitive to guanine nucleotides and the

specific binding is inhibited with increasing concentrations of GTP-γ-S, a non-hydrolyzable GTP analogue (Harikumar and Chattopadhyay 1999). Figure 4 shows the inhibition of specific [^{3}H]8-OH-DPAT binding in presence of GTP-γ-S to the 5-HT$_{1A}$ receptor in CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes in a characteristic concentration-dependert manner. It has earlier been shown that GTP-γ-S induces transition of the receptor from a high affinity to a low affinity state (Harikumar and Chattopadhyay 1999). Figure 4 shows that the half maximal inhibition concentration (IC_{50}) value for inhibition of specific [^{3}H]8-OH-DPAT binding by GTP-γ-S is 63 nM for native membranes similar to that reported earlier (Harikumar and Chattopadhyay 1999). The inhibition curve for the CHAPS-solubilized membranes, however, exhibits a large shift toward higher concentration of GTP-γ-S, with an IC_{50} value of 944 nM. This implies that the G-protein coupling of the CHAPS-solubilized receptors is drastically reduced by this treatment. Interestingly, PEG-precipitated CHAPS-solubilized receptors display efficient G-protein coupling (as apparent from a lower IC_{50} value of 119 nM) similar to what is observed with the native membrane. PEG precipitation after solubilization using CHAPS and NaCl, therefore, induces G-protein coupling.

Characterization of [^{3}H]8-OH-DPAT binding affinity and sites in CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes by saturation binding analysis

Figures 5 and 6 show that the Scatchard analysis of the specific binding of [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor in CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes in the absence and presence of 50 μM GTP-γ-S, respectively. The binding parameters under these conditions are summarized in tables 2 and 3. Table 2 shows that the binding affinity of [^{3}H]8-OH-DPAT shows no significant change either in CHAPS-solubilized or PEG-precipitated CHAPS-solubilized membranes. However, there is a significant reduction ($\sim$70% when compared to the native membrane) in the maximum number of binding sites (B_{max}) for the CHAPS-solubilized receptors. It is interesting to note that the maximum number of binding sites is retained in the case of PEG-precipitated CHAPS-solubilized receptors. Thus, PEG precipitation helps to reconstitute the receptor into an active, high affinity state, which retains most of the binding sites. Table 3 shows that the binding affinity of [^{3}H]8-OH-DPAT shows a considerable reduction at high

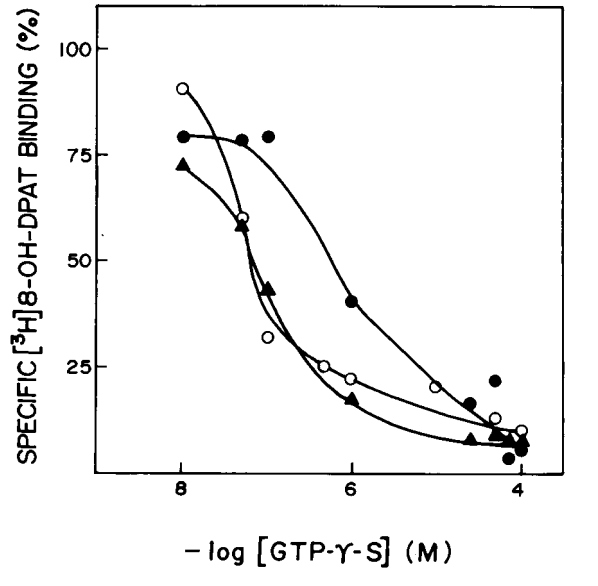

Figure 4. Effect of increasing concentrations of GTP-γ-S on the specific binding of the agonist [^{3}H]8-OH-DPAT to the 5-HT$_{1A}$ receptor in bovine hippocampal native (○), CHAPS-solubilized (●), and PEG-precipitated CHAPS-solubilized membranes (◆). Values are expressed as percentage of the specific binding obtained with native membranes without solubilization and in the absence of GTP-γ-S. The data points are the means of duplicate points from three independent experiments. See Experimental Procedures for other details.

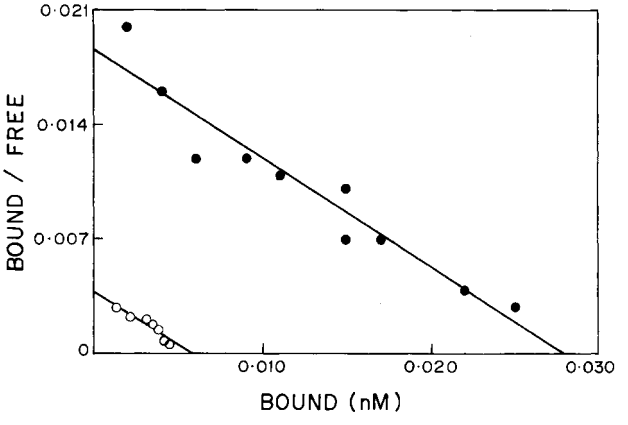

Figure 5. Scatchard analysis of specific binding of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ receptors in CHAPS-solubilized (○) and PEG-precipitated CHAPS-solubilized (●) membranes obtained from bovine hippocampus. The concentration of [^{3}H]8-OH-DPAT ranged from 0.1–7.5 nM. Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Experimental Procedures for other details.

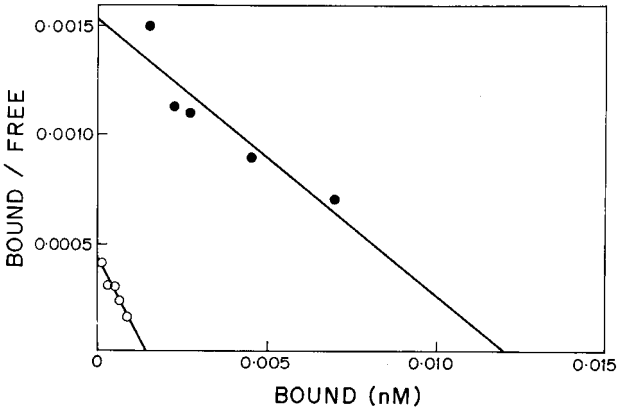

Figure 6. Scatchard analysis of specific binding of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ receptors in the presence of 50 μM GTP-γ-S in CHAPS-solubilized (○) and PEG-precipitated CHAPS-solubilized (●) membranes obtained from bovine hippocampus. The concentration of [^{3}H]8-OH-DPAT ranged from 0.1–7.5 nM. Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Experimental Procedures for other details.

Table 2. Affinity and B_{max} values of [^{3}H]8-OH-DPAT binding to 5-HT$_{1A}$ receptors under various conditions[a]

Condition	K_d (nM)	B_{max} (fmol/mg/protein)
Native membrane	1.68±0.16	101.20±2.55
CHAPS-solubilized membrane PEG-precipitated	1.26±0.21	31.80±1.31
CHAPS-solubilized membrane	1.34±0.14	98.50±1.75

[a]The binding parameters shown in this table represent the means ±SEM of duplicate points from three independent experiments, while saturation binding data shown in figure 5 are from representative experiments. See Experimental Procedures for other details.

Table 3. Affinity and B_{max} values of [^{3}H]8-OH-DPAT binding to 5-HT$_{1A}$ receptors in the presence of GTP-γ-S under various conditions[a]

Condition	K_d (nM)	B_{max} (fmol/mg/protein)
Native membrane	4.31±0.53	73.7±1.34
CHAPS-solubilized membrane PEG-precipitated	3.24±0.36	5.30±0.80
CHAPS-solubilized membrane	6.50±0.71	28.20±4.70

[a]The binding parameters shown in this table represent the means ±SEM of duplicate points from three independent experiments, while saturation binding data shown in figure 6 are from representative experiments. The saturation binding assay was carried out in the presence of 50 μM GTP-γ-S. See Experimental Procedures for other details.

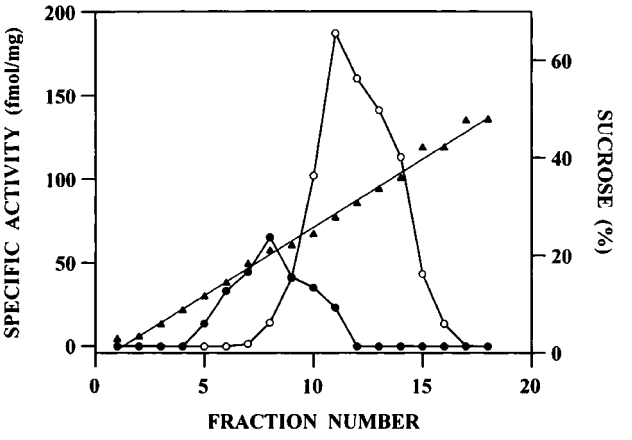

Figure 7. Sucrose density gradient analysis of CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes. Percent sucrose (◆) was determined by the refractive index and ranged from 10–50% at the end points. The specific activity for CHAPS-solubilized (●) and PEG-precipitated CHAPS-solubilized (○) membranes was obtained by normalizing the specific [^{3}H]8-OH-DPAT binding with respect to total protein concentration measured using BCA reagent. Data shown are from a representative experiment of three independent experiments and each point is the mean of duplicate determinations. See Experimental Procedures for other details.

concentration (50 μM) of GTP-γ-S, in all cases confirming that the receptor is in a low affinity state in the presence of GTP-γ-S. The reduction in affinity was found to be more in the case of PEG-precipitated CHAPS-solubilized membranes, which ensures that this procedure is appropriate for preserving G-protein coupling.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was carried out to determine the effect of PEG precipitation on the CHAPS-solubilized membranes using the property of [^{3}H]8-OH-DPAT binding as a marker. Figure 7 shows the sucrose density gradient pattern of the distribution of specific [^{3}H]8-OH-DPAT binding to both CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes. In the case of CHAPS-solubilized membranes, the gradient displays majority of the specific [^{3}H]8-OH-DPAT binding toward the top of the gradient. However, following PEG precipitation of the CHAPS-solubilized membranes, the specific [^{3}H]8-OH-DPAT binding peak gets shifted to the bottom of the gradient. This data suggests that PEG precipitation resulted in the inclusion of the solubilized protein in liposomes and gives rise to a conformation for effective specific [^{3}H]8-OH-DPAT binding. The liposomes are formed presumably from residual endogenous lipids extracted out from the native membranes during solubilization using CHAPS. Earlier studies have shown that membrane receptors such as the muscarinic acetylcholine receptor from bovine heart atrial tissue (Aguilar and Ochoa 1986, Pellegrino de Iraldi *et al.* 1986, Aguilar *et al.* 1987), the nicotinic acetylcholine receptor from *Torpedinidae* electroplax (Medrano *et al.* 1989), and opioid receptors from bovine striatum (Ofri *et al.* 1992) can be reconstituted into liposomes following solubilization and PEG precipitation, which lead to formation of liposomes from endogenous lipids.

Competition binding analysis

Competition binding analysis using unlabelled ligands such as 5-HT, 8-OH-DPAT and 5-CT was performed by determining their ability to compete for binding to both CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes labelled with the 5-HT$_{1A}$ receptor specific agonist [^{3}H]8-OH-DPAT. Figures 8 and 9 show the competitive displacement curves of [^{3}H]8-OH-DPAT by the competing ligands 5-HT, 8-OH-DPAT and 5-CT for CHAPS-solubilized (figure 8) and PEG-precipitated CHAPS-solubilized membranes (figure 9). The half maximal inhibition concentrations (IC_{50}) and K_i values for the competing ligands are reported in table 4. As is shown in table 4, the K_i and IC_{50} values for all the competing ligands are lower in the case of PEG-precipitated CHAPS-solubilized membranes when compared to the corresponding values obtained with CHAPS-solubilized membranes. This suggests that solubilization of 5-HT$_{1A}$ receptors from bovine hippocampus using CHAPS/NaCl and subsequent PEG precipitation lead to preservation of the high affinity agonist binding sites.

Discussion

The concept of micelle formation is relevant to solubilization and reconstitution studies of membrane proteins since it appears that there is some correlation between the ability to form micelles and the concentration of detergent required for solubilization (Rivnay and Metzger 1982). The CMC is an

Table 4. IC_{50} and K_i values of competitive ligands for binding to 5-HT_{1A} receptors in CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes of bovine hippocampal membranes[a]

	CHAPS-solubilized membranes		PEG-precipitated CHAPS-solubilized membranes	
Ligands	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)
5-HT	6.00±2.00	4.90±1.60	1.12±0.24	0.92±0.19
8-OH-DPAT	3.20±0.90	2.60±0.70	1.33±0.33	1.09±0.27
5-CT	1.60±1.20	1.30±0.90	0.19±0.06	0.16±0.05

[a]The IC_{50} values and the apparent dissociation constants (K_i) shown in the table represent the means±SEM of duplicate points from three independent experiments. The apparent dissociation constants (K_i) for the competing ligands were calculated by using the following relation: $K_2=IC_{50}/(1+[L]/K_d)$, where IC_{50} is the concentration of the ligand leading to 50% inhibition of specific total binding and [L] and K_d are the concentration and dissociation constant of the radiolabelled agonist [^{3}H]8-OH-DPAT (see Cheng and Prusoff 1973). See Experimental Procedures for other details.

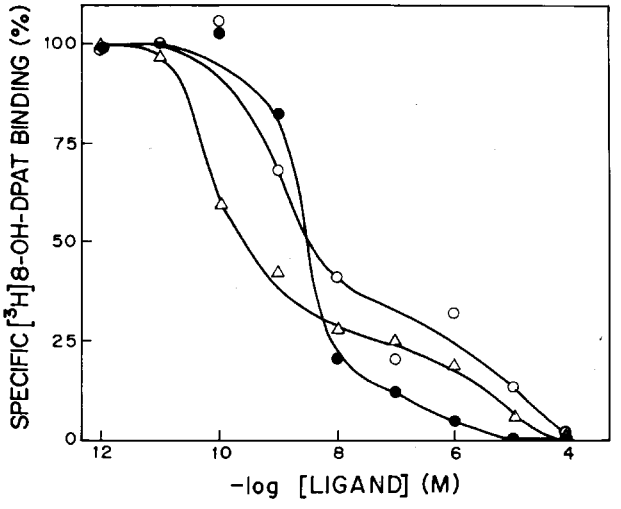

Figure 8. Competition binding analysis of various ligands to 5-HT_{1A} receptors in CHAPS-solubilized membranes. The concentration range used was from 10^{-12} to 10^{-4} M for serotonin (○), 8-OH-DPAT (●), and 5-CT (◇). Values of specific binding measured in the presence of the competitive ligands are expressed as percentage of total binding. The data points are the means of duplicate points from three independent experiments. Non-specific binding was determined in the presence of 10^{-4} M unlabelled serotonin. K_i values were determined according to Cheng and Prusoff (1973) and average values are listed in table 4. See Experimental Procedures for other details.

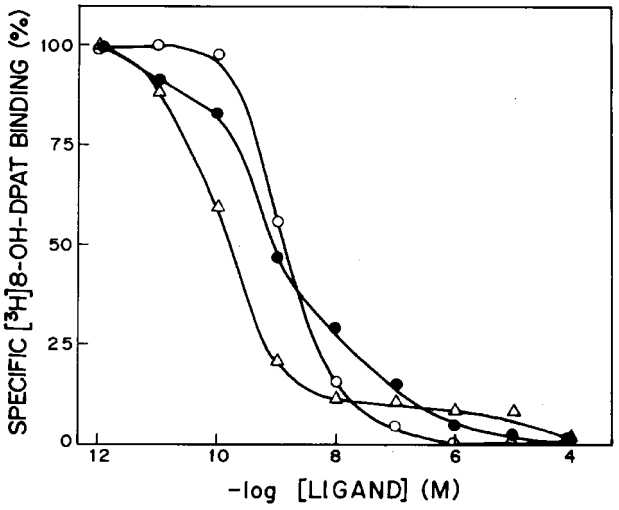

Figure 9. Competition binding analysis of different ligands to 5-HT_{1A} receptors in PEG-precipitated CHAPS-solubilized membranes. The concentration range used was from 10^{-12} to 10^{-4} M for 5-HT (○), 8-OH-DPAT (●), and 5-CT (◇). All other conditions are as in figure 8. See Experimental Procedures for other details.

important parameter for a given detergent, since at this concentration the detergent starts to accumulate in the membrane. Studies on several receptors including the insulin receptor, opioid receptor and angiotensin II receptor indicate that successful solubilization is achieved only with high (>1 mM) CMC detergents such as CHAPS and octyl glucoside at concentrations below the CMC (Hjelmeland and Chrambach 1984). Concentrations of detergents above the CMC invariably led to loss of function in these cases. The mechanism by which these detergents solubilize membranes at concentrations below the CMC, and the related loss of function above the CMC remain largely unexplored. This has given rise to the concept of 'effective CMC' (the concentration of detergent existing as monomers at any given condition) (Rivnay and Metzger 1982, Klausner *et al.* 1984, Schurholz 1996) which takes into account contributions from all the components (lipids, proteins, pH, temperature and ionic strength) in the specific system under study. Determination of the effective CMC could serve as a useful indicator in solubilization of membrane proteins under various experimental conditions. Thus, solubilization could occur below the CMC if the effective CMC is low.

It has previously been shown that the CMC of CHAPS, which is zwitterionic and carries no net charge, is dependent on the salt concentration of the medium and there is significant reduction in CMC with increasing salt concentration (Chattopadhyay and Harikumar 1996). It is interesting to note that the CMC of the neutral detergent octylglucoside has also been shown to decrease with increasing salt concentration (Paternostre *et al.* 1987). In addition, it was shown that optimal solubilization of the 5-HT_{1A} receptor can be achieved with pre-micellar concentration of CHAPS under high salt concentration (Chattopadhyay and Harikumar 1996). This paper reports an efficient strategy to solubilize high affinity, functionally active G-protein-sensitive 5-HT_{1A} receptors from bovine hippocampal membranes with a high degree of efficiency (>80%) using CHAPS at a low (pre-micellar) concentration in the presence of 1 M NaCl followed by PEG precipitation. It has been previously reported that NaCl improves the

solubilization yield of G-protein coupled receptors (Ofri *et al.* 1992). The results assume significance in the light of the recent observation that treatment of membranes with high concentration of CHAPS results in removal and loss of $\beta\gamma$ dimer of trimeric G-proteins (Jones and Garrison 1999, Waldhoer *et al.* 1999, Bayewitch *et al.* 2000), which would affect G-protein coupling of the receptor in question. As the data show (figure 4), solubilization using a low concentration of CHAPS followed by PEG precipitation preserves the guanine nucleotide sensitivity of the 5-HT$_{1A}$ receptor. In general, it is shown here that efficient solubilization of membrane proteins can be achieved by using detergents at pre-micellar concentration in the presence of a high concentration of salt.

These results clearly show that PEG precipitation following CHAPS solubilization enhances the agonist and antagonist binding and G-protein coupling to the receptor and restores it back to a native-like state. PEG precipitation has previously been shown to be a useful approach to functionally reconstitute membrane receptors by efficiently removing detergent from solubilized membranes (Kremenetzky and Atlas 1984, Aguilar and Ochoa 1986, Aguilar *et al.* 1987, Medrano *et al.* 1989), which leads to the formation of liposomes from endogenous lipids (Pellegrino de Iraldi *et al.* 1986).

In summary, the successful solubilization of high affinity, functionally active G-protein-sensitive 5-HT$_{1A}$ receptors from bovine hippocampus using pre-micellar CHAPS in combination with NaCl followed by PEG precipitation is reported here. Efficient solubilization of the receptor from the native source with high ligand binding affinity and intact signal transduction components may constitute the first step in the molecular characterization of the G-protein-coupled receptor.

Experimental procedures

Materials

CHAPS, EDTA, EGTA, $MgCl_2$, iodoacetamide, PMSF, polyethyleneglycol, polyethylenimine, serotonin, sodium azide, sucrose and Tris were obtained from Sigma Chemical Co. (St. Louis, MO, USA). GTP-γ-S was from Boehringer Mannheim (Germany). 8-OH-DPAT and *p*-MPPI were obtained from Research Biochemicals International (Natick, MA, USA). 5-CT was obtained from Research Biochemicals International (Natick, MA, USA) and was a kind gift from Dr V. Bakthavachalam (National Institute of Mental Health Chemical Synthesis Program, Natick, MA, USA). [^{3}H]8-OH-DPAT (sp. activity 127.0 Ci/mmol) and [^{3}H]*p*-MPPF (sp. activity 78.3 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). All other chemicals used were of the highest available quality. GF/B glass microfibre filters were from Whatman International (Kent, UK). BCA reagent kit for protein estimation was obtained from Pierce (Rockford, IL, USA). Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at -70°C until further use.

Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described earlier by Harikumar and Chattopadhyay (1998a). In short, bovine hippocampal tissue ($\sim$100 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide pH 7.4). The homogenate was centrifuged at 900 g for 10 min at 4°C. The supernatant was filtered through four layers of cheese-cloth and the pellet was discarded. The supernatant was further centrifuged at 50 000 g for 20 min at 4°C. The resulting pellet was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50 000 g for 20 min at 4°C. This procedure was repeated until the supernatant was clear. The final pellet (native membrane) was resuspended in a minimum volume of 50 mM Tris buffer (pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at -70°C and used for radioligand binding assays or for solubilization studies.

Solubilization of native membranes

Native hippocampal membranes were incubated with varying concentrations of CHAPS and NaCl in buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) at a final protein concentration of $\sim$2 mg/ml for 30 min at 4°C with occasional shaking. For antagonist binding studies, buffer D (50 mM Tris, 1 mM EDTA, pH 7.4) was used for incubation of native membranes. The membranes were briefly sonicated (5 s) using a Branson model 250 sonifier at the beginning of the incubation period, and mildly homogenized using a hand-held Dounce homogenizer (5 times) at the beginning and the end of the incubation period. After incubation for 30 min, the contents were centrifuged at 100 000 g for 1 h at 4°C. The clear supernatant was carefully removed from the pellet, and either used immediately for binding assay or for PEG precipitation.

PEG precipitation of the crude CHAPS-solubilized membrane was performed to remove NaCl from the solubilized extract, since the agonist binding of the 5-HT$_{1A}$ receptor is inhibited by NaCl (Harikumar and Chattopadhyay 1998a). This procedure is believed to remove the salt (Gal *et al.* 1983, Kremenstzky and Atlas 1984). PEG precipitation was carried out by diluting the extract with equal volume of 40% PEG-8000 in buffer C or buffer D (50 mM Tris, 1 mM EDTA, pH 7.4). Following vigorous vortexing and incubation for 10 min on ice, the samples were centrifuged at 15 000 g for 10 min at 4°C. The pellet was carefully rinsed twice with buffer C (or buffer D in the case of antagonist binding assay) and finally resuspended in buffer C (or D) and used for radioligand binding assays.

Radioligand binding assays

Agonist binding assays for both CHAPS-solubilized (using 5 mM CHAPS) and PEG-precipitated CHAPS-solubilized membranes (using 5 mM CHAPS in the presence of 1 M NaCl) were performed as follows: Tubes in duplicate containing 500 μl of either CHAPS-solubilized or PEG-precipitated CHAPS-solubilized membranes were incubated in a total volume of 1 ml of buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) in the presence of 0.29 nM [^{3}H]8-OH-DPAT (sp. activity 127.0 Ci/mmol) for 1 h at room temperature. Non-specific binding was determined by performing the assay in the presence of 10 μM unlabelled serotonin. The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B (1.0 μm pore size) 2.5 cm diameter glass microfibre filters which were pre-soaked in 0.3% polyethylenimine for 3 h (Bruns *et al.* 1983). The filters were then washed three times with 3 ml of ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid. Antagonist binding assays for both CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes were performed as above using [^{3}H]*p*-MPPF (sp. activity 78.3 Ci/mmol) as the radioligand. The assay tubes contained 0.5 nM [^{3}H]*p*-MPPF in a total volume of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, pH 7.4). Non-specific binding was determined by performing the assay in the presence of 10 μM unlabelled *p*-MPPI.

Agonist binding assays in presence of GTP-γ-S, a non-hydrolyzable analogue of GTP, for both CHAPS-solubilized and PEG-precipitated CHAPS-solubilized membranes were performed as

described earlier (Harikumar and Chattopadhyay 1999). Tubes in duplicate containing native hippocampal membrane (1 mg of total protein) or 500 μl CHAPS-solubilized or PEG-precipated CHAPS-solubilized membranes were incubated in the presence or absence of varying concentrations of GTP-γ-S and agonist binding assay was carried out as mentioned earlier.

Saturation binding assays

Saturation binding assays were carried out using varying concentrations (0.1–7.5 nM) of radiolabelled agonist [^{3}H]8-OH-DPAT using either native membranes containing 1 mg of total protein or 500 μl CHAPS-solubilized or PEG-precipitated CHAPS-solubilized membranes in the presence and absence of 50 μM GTP-γ-S. Non-specific binding was measured in the presence of 10 μM unlabelled serotonin. Binding assays were carried out at room temperature as mentioned above (see radioligand binding assay). Binding data were analysed as described by Hulme (1990). The concentration of bound ligand was calculated from the equation:

$$[RL^*] = 10^{-9}xB/(VxSAx2220)\ M$$

where *B*=bound radioactivity in disintegrations per minute (dpm) (i.e. total dpm non-specific dpm), *V* is the assay volume in ml, and SA is the specific activity of the radioligand. Scatchard plots (i.e. plots of $[RL^*]/[L^*]$ vs. $[RL^*]$) were analysed using Sigma-Plot (version 3.1) in an IBM PC. The dissociation constants (K_d) were obtained from the negative inverse of the slopes, determined by linear regression analysis of the plots (r=0.90–0.99). The B_{max} values were obtained from the intercept on the abscissa. The B_{max} values reported in tables 2 and 3 have been normalized with respect to the amount of native membrane used. The binding parameters shown in tables 2 and 3 were obtained by averaging the results of duplicate points from three independent experiments, while the saturation binding data shown in figures 5 and 6 are from representative experiments. Protein concentration was determined using BCA reagent (Smith *et al.* 1985).

Sucrose density gradient centrifugation

Continuous sucrose gradients (10–50%) were prepared in buffer C and 5 ml gradients were poured in 24 × 89 mm tubes and equilibriated overnight at 4°C. Five millilitres of either CHAPS-solubilized or PEG-precipitated CHAPS-solubilized membranes were carefully layered on the top of the gradients and centrifuged at 75 000 g for 12 h at 4°C in a Beckman SW-28 rotor. After centrifugation, 1 ml fractions were collected from the top of the gradients manually and agonist binding assays were performed as mentioned above. Protein assays of the collected fractions were carried out using a BCA reagent kit. The fraction densities and sucrose concentration were determined by measurement of the refractive index using a Schmidt and Haensch refractometer.

Competition binding assays

The competition binding assays were carried out as follows. Tubes in duplicate containing 500 μl of either CHAPS-solubilized or PEG-precipitated CHAPS-solubilized membranes were incubated in the presence of 0.29 nM [^{3}H]8-OH-DPAT in a total volume of 1 ml of buffer C. Non-specific binding was determined by performing the assay in the presence of 10 μM of unlabelled serotonin. The final concentrations of the competitive ligands in the assay tubes ranged from 10^{-12} to 10^{-4} M. The radioligand binding assay was carried out at room temperature for 1 h, as mentioned above. The affinity values of the displacing ligands are expressed as the apparent dissociation constants (K_i) for the competing ligands, where K_i value is calculated from IC_{50} (concentration of ligand which inhibits 50% of binding) value using the Cheng-Prusoff (1973) equation:

$$K_i = IC_{50}/(1 + [L]/Kd)M$$

where IC_{50} is the concentration of the competing ligand leading to 50% inhibition of specific binding and [L] and K_d are the concentration and dissociation constant of the labeled ligand. K_d value for [^{3}H]8-OH-DPAT binding to the 5-HT_{1A} receptor was taken from table 2. The average of the K_i values for the competitive ligands are shown in table 4.

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