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.

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 $\beta_2$-adrenergic receptor in the transmembrane domain. Furthermore, it was the first serotonin receptor for which polymodal 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 sulphhydril 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...

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, Offi et al. 1992, Banerjee et al. 1995, Chattopadhyay and Harikumar 1996, Cladera et al. 1997). CHAPS has previously been used to solubilize the 5-HT1A 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 βγ 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 βγ 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-HT1A 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 βγ dimers of G-proteins from the membrane. This paper reports an efficient strategy to solubilize high affinity, functionally active G-protein-sensitive 5-HT1A 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).

**Results**

**Solubilization of high affinity 5-HT1A 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 has been shown that 16 mM CHAPS can extract >90% of the βγ 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-HT1A 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 βγ dimers of G-proteins from the membrane. This paper reports an efficient strategy to solubilize high affinity, functionally active G-protein-sensitive 5-HT1A 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 2.** Solubilization of native hippocampal membranes containing the 5-HT1A receptor with different concentrations of CHAPS and NaCl. Values are expressed as percentage of specific binding of the agonist [3H]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 mM NaCl, which amounts to a reduction of 36%.

The solubilization of native hippocampal membranes containing the 5-HT1A receptor, as monitored by the specific agonist [3H]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-HT1A 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 mM NaCl. Optimal solubilization of agonist binding sites (>80%) is achieved using 5 mM CHAPS in the presence of 1 mM NaCl. Figure 2 also shows that higher concentrations of CHAPS (>5 mM) or NaCl (>1 mM) reduce the efficiency of solubilization as measured by specific [3H]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 mM NaCl, respectively, throughout this paper.

Although selective 5-HT1A agonists such as 8-OH-DPAT were discovered more than a decade back (Gozlan et al. 1983), the development of selective 5-HT1A antagonists has been relatively slow and less successful. Recently, two specific antagonists for the 5-HT1A receptor, p-MPPI and p-MPPP, 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-HT1A receptor with high affinity. The solubilization of native hippocampal membranes containing the 5-HT1A receptor measured by the specific antagonist [3H]p-MPPP 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 mM NaCl. Higher concentrations of CHAPS (>5 mM) or NaCl (>1 mM) 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

<table>
<thead>
<tr>
<th>NaCl concentration (mM)</th>
<th>CMC (%)</th>
<th>CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.394</td>
<td>6.41</td>
</tr>
<tr>
<td>100</td>
<td>0.382</td>
<td>6.21</td>
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<tr>
<td>200</td>
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<tr>
<td>500</td>
<td>0.347</td>
<td>5.64</td>
</tr>
<tr>
<td>1000</td>
<td>0.267</td>
<td>4.34</td>
</tr>
<tr>
<td>1500</td>
<td>0.252</td>
<td>4.10</td>
</tr>
</tbody>
</table>

*Taken from (Chattopadhyay and Harikumar 1996).*

Guanine nucleotide sensitivity of [3H]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-HT1A receptor is negatively coupled to the adenylate cyclase system through pertussis toxin-sensitive G-proteins (Gi/Go) (Emerit et al. 1990). It has previously been shown that the specific agonist [3H]8-OH-DPAT binding to bovine hippocampal 5-HT1A 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-dependent 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 \(\mu M\) 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 (~70% when compared to the native membrane) in the maximum number of binding sites (B\(_{\text{max}}\)) 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 concentrations of GTP-γ-S. 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 \(\mu M\) 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.

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**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.
concentration (50 \, \mu M) of GTP-\gamma-S, in all cases confirming that the receptor is in a low affinity state in the presence of GTP-\gamma-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 [\textsuperscript{3}H]8-OH-DPAT binding as a marker. Figure 7 shows the sucrose density gradient pattern of the distribution of specific [\textsuperscript{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 [\textsuperscript{3}H]8-OH-DPAT binding toward the top of the gradient. However, following PEG precipitation of the CHAPS-solubilized membranes, the specific [\textsuperscript{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 [\textsuperscript{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\textsubscript{1A} receptor specific agonist [\textsuperscript{3}H]8-OH-DPAT. Figures 8 and 9 show the competitive displacement curves of [\textsuperscript{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\textsubscript{50}) and K\textsubscript{i} values for the competing ligands are reported in table 4. As is shown in table 4, the K\textsubscript{i} and IC\textsubscript{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\textsubscript{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
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 (41mM) CM C detergents such as CHAPS and octylglucoside 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-HT1A 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-HT1A 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

<table>
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<th>Ligands</th>
<th>IC50 (nM)</th>
<th>Ki (nM)</th>
<th>IC50 (nM)</th>
<th>Ki (nM)</th>
</tr>
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<tbody>
<tr>
<td>5-HT</td>
<td>6.00 ± 2.00</td>
<td>4.90 ± 1.60</td>
<td>1.12 ± 0.24</td>
<td>0.92 ± 0.19</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>3.20 ± 0.90</td>
<td>2.60 ± 0.70</td>
<td>1.33 ± 0.33</td>
<td>1.09 ± 0.27</td>
</tr>
<tr>
<td>5-CT</td>
<td>1.60 ± 1.20</td>
<td>1.30 ± 0.90</td>
<td>0.19 ± 0.06</td>
<td>0.16 ± 0.05</td>
</tr>
</tbody>
</table>

a The IC50 values and the apparent dissociation constants (Ki) shown in the table represent the means ± SEM of duplicate points from three independent experiments. The apparent dissociation constants (Ki) for the competing ligands were calculated by using the following relation: Ki = IC50/(1+[L]/Kd), where IC50 is the concentration of the ligand leading to 50% inhibition of specific total binding and [L] and Kd are the concentration and dissociation constant of the radiolabelled agonist [3H]8-OH-DPAT (see Cheng and Prusoff 1973). See Experimental Procedures for other details.
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 βγ 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-HT1A 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-HT1A 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, MgCl2, iodoacetamide, PMSF, polyethylene-neglycol, polyethyleneimine, serotonin, sodium azide, sucrose and Tris were obtained from Sigma Chemical Co. (St. Louis, MO, USA). GTPγS 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. Baktihvachalam (National Institute of Mental Health Chemical Synthesis Program, Natick, MA, USA). [3H]8-OH-DPAT (sp. activity 127.0 Ci/mmol) and [3H]p-MPPI (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 microfilter cures 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 (~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 20,000 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 MgCl2, pH 7.4) at a final protein concentration of ~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-HT1A receptor is inhibited by NaCl (Harikumar and Chattopadhyay 1998a). This procedure is believed to remove the salt (Gal et al. 1983, Kremenetzky 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 mM 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 MgCl2, 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) and used for radioligand binding assays.
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-precipitated CHAPS-solubilized membranes were incubated in the presence or absence of varying concentrations of GTPγS 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 [3H]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 GTPγS. 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:

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

where \(\text{B= bound radioactivity in disintegrations per minute (dpm)}\) (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 x 89 mm tubes and equilibrated overnight at 4°C. Five milliliters 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 [3H]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 varied from 10^{-11} 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:

\[ K_i = IC_{50}/(1 + [L]/K_d)M \]

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 [3H]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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