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# Solubilization of Serotonin$_{1A}$ Receptors Heterologously Expressed in Chinese Hamster Ovary Cells

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**SUMMARY**

1. 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 behavioral and cognitive functions.

2. We report here, for the first time, the solubilization of 5-HT$_{1A}$ receptors stably expressed in Chinese Hamster Ovary (CHO) cells using the zwitterionic detergent CHAPS in presence of NaCl followed by polyethylene glycol (PEG) precipitation. We show by ligand-binding assay that the 5-HT$_{1A}$ receptor solubilized this way is functionally active. We have optimized the efficiency of solubilization with respect to total protein and NaCl concentration.

3. Our results show that careful control of salt and protein concentration is crucial in optimal solubilization of membrane receptors heterologously expressed in cells in culture. The effective solubilization of important neurotransmitter receptors such as 5-HT$_{1A}$ receptors which are present in very low amounts in the native tissue may represent an important step in characterizing membrane receptors expressed in mammalian cells in culture.

**KEY WORDS:** 5-HT$_{1A}$ receptors; solubilization; 8-OH-DPAT; CHAPS; CHO cells; heterologous expression.

## INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT) receptors are members of a superfamily of seven transmembrane domain receptors (Strader *et al*., 1995) that couple to GTP-binding regulatory proteins (G-proteins). Serotonergic signaling appears to play a key role in the generation and modulation of various cognitive and behavioral functions including sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression, and learning (Artigas *et al*., 1996; Ramboz *et al*., 1998). 5-Hydroxytryptamine or 5-HT is an intrinsically fluorescent (Chattopadhyay *et al*., 1996), biogenic amine found in a wide variety of sites in the central and peripheral nervous systems. Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine,

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depression, suicidal behavior, infantile autism, eating disorders, and obsessive compulsive disorder (Heisler *et al*., 1998; Ramboz *et al*., 1998; Tecott *et al*., 1995).

Among the various types of serotonin receptors, the G-protein-coupled 5-$HT_{1A}$ receptor subtype 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-hydroxy-2-(di-*N*-propylamino) tetralin (8-OH-DPAT) that allows extensive biochemical, physiological, and pharmacological characterization of the receptor (Gozlan *et al*., 1983). The 5-$HT_{1A}$ receptor is the first among all the serotonin receptors to be cloned and sequenced (Albert *et al*., 1990; Fargin *et al*., 1988). Furthermore, it was the first serotonin receptor for which polyclonal antibodies were obtained (Fargin *et al*., 1988) allowing their visualization at the subcellular level in various regions of the brain. Certain 5-$HT_{1A}$ receptor agonists have been shown to be effective as anxiolytic agents (Julius, 1998). In addition, antagonists of the 5-$HT_{1A}$ receptor appear to accelerate and increase the antidepressant action of serotonin reuptake inhibitors such as Prozac (Julius, 1998). As a result, the 5-$HT_{1A}$ receptor has become an important target in the development of therapeutic agents to treat neuropsychiatric disorders such as anxiety and depression. The 5-$HT_{1A}$ receptor therefore represents an important member of the seven transmembrane domain G-protein-coupled receptor family. We have earlier partially purified and solubilized the 5-$HT_{1A}$ receptor from bovine hippocampus in a functionally active form (Chattopadhyay *et al*., 2002; Chattopadhyay and Harikumar, 1996) and have shown modulation of ligand binding by metal ions, guanine nucleotides, alcohols, and covalent modifications of the disulfides and sulfhydryl groups (Harikumar *et al*., 2000; Harikumar and Chattopadhyay, 1998a,b, 1999, 2000, 2001).

An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the membrane and individually dispersed in solution. This is most effectively accomplished by using amphiphilic detergents and the process is known as solubilization (Banerjee, 1999; Hjelmeland and Chrambach, 1984; Jones *et al*., 1987). Effective solubilization and purification of membrane receptors in a functionally active form represents 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 is a formidable challenge since many detergents irreversibly denature membrane proteins (Garavito and Ferguson-Miller, 2001).

The mild, nondenaturing, zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) is one of the most commonly used detergents in membrane biochemistry (Hjelmeland, 1980). CHAPS is a derivative of the naturally occurring bile salts and combines useful features of both the bile salt hydrophobic group and the N-alkyl sulfobetaine-type polar group (Hjelmeland, 1980; Hjelmeland *et al*., 1983). 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 (Banerjee *et al*., 1995; Ofri *et al*., 1992). We have previously used CHAPS at low concentration to solubilize 5-$HT_{1A}$ receptors from bovine hippocampus in a functional form (Chattopadhyay *et al*., 2002; Chattopadhyay and Harikumar, 1996).

Mammalian cells in culture heterologously expressing membrane receptors represent convenient systems to address problems in receptor biology (Fraser, 1990; Tate and Grisshammer, 1996). Since native tissues (of neuronal origin in particular) often have very low quantities of a specific type of receptor, solubilization and purification of neuronal receptors from native sources continue to be one of the most challenging issues in contemporary membrane biology. It is in this context that heterologously expressed membrane receptors assume significance. Although the 5-HT$_{1A}$ receptor has been heterologously and stably expressed in fibroblast cells earlier (Banerjee *et al*., 1993; Newman-Tancredi *et al.*, 1992; Singh *et al*., 1996), no attempts have so far been made to solubilize the heterologously expressed receptor in a functional form. In this paper, we report, for the first time, the solubilization of 5-HT$_{1A}$ receptors stably expressed in Chinese Hamster Ovary (CHO) cells using CHAPS in presence of NaCl followed by polyethylene glycol (PEG) precipitaton. We show by ligand-binding assay that the 5-HT$_{1A}$ receptor solubilized this way is functionally active. Further, we have optimized the efficiency of solubilization with respect to total protein and NaCl concentration.

## MATERIALS AND METHODS

### Materials

CHAPS, EDTA, EGTA, $MgCl_2$, $MnCl_2$, NaCl, phenylmethylsulfonyl fluoride (PMSF), Tris, PEG, polyethylenimine, penicillin, streptomycin, gentamycin sulphate, and serotonin were obtained from Sigma (St. Louis, MO). [$^3$H]8-OH-DPAT (specific activity 127.0 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Fetal calf serum was purchased from Sigma (St. Louis, MO). D-MEM/F-12 (Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) (1:1)) was from Life Technologies (Grand Island, NY). Geneticin (G 418) was obtained from Boehringer Mannheim (Germany). GF/B glass microfiber filters were from Whatman International (Kent, U.K.). Bicinchoninic acid (BCA) reagent kit for protein estimation was obtained from Pierce (Rockford,IL). All other chemicals used were of the highest available quality.

### Cell Culture

CHO cells stably transfected with the intronless human genomic clone G-21 (Fargin *et al.*, 1988) and heterologously expressing the human serotonin$_{1A}$ receptor (referred to as T-CHO, see Banerjee *et al*., 1993) were a generous gift from Dr Probal Banerjee (College of Staten Island, City University of New York). Cells were grown in D-MEM/F-12 (1:1) supplemented with 1.2 g/L of sodium bicarbonate, 10% fetal calf serum, 60 $\mu$g/mL penicillin, 50 $\mu$g/mL streptomycin, 50 $\mu$g/mL gentamycin sulphate and 200 $\mu$g/mL Geneticin in 5% $CO_2$ at 37°C.

### Preparation of Cell Membranes

Confluent T-CHO cells were harvested by treatment with ice-cold buffer containing 10 mM Tris, 5 mM EDTA, 0.1 mM PMSF, pH 7.4. Cells were then homogenized

(10 s) at 4°C at maximum speed using Polytron homogenizer. The cell lysate was centrifuged at 500 × *g* for 10 min at 4°C and the resulting postnuclear supernatant was centrifuged at 40,000 × *g* for 30 min at 4°C. The pellet thus obtained was suspended in 50 mM Tris buffer, pH 7.4, flash frozen in liquid nitrogen and stored at −70°C till further use. Total protein concentration in membranes thus isolated was determined using BCA reagent (Smith *et al.*, 1985).

## Solubilzation of Cell Membranes

Cell membranes were treated with 5 mM CHAPS and varying concentrations of NaCl or protein in buffer containing 50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4 for 30 min at 4°C with occasional shaking. Membranes were briefly sonicated (5 s) using a Branson model 250 sonifier fitted with a microtip and mildly homogenized using a hand-held Dounce homogenizer (five times) at the beginning of the incubation. The homogenization was repeated at 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 subjected to PEG precipitation. PEG precipitation of the CHAPS solubilized cell membranes was performed to remove NaCl from the solubilized extract since the agonist binding of the serotonin$_{1A}$ receptor was found to be inhibited by NaCl (Harikumar and Chattopadhyay, 1998a). PEG precipitation was carried out by diluting the solubilized extract with equal volume of 40% PEG-8000 prepared in buffer containing 50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4. Following vigorous vortexing and incubation on ice for 10 min, the samples were centrifuged at 15,000 × *g* for 10 min at 4°C. The pellet was carefully rinsed with 50 mM Tris, pH 7.4 buffer, and resuspended in the same buffer and used for radioligand-binding assays immediately.

## Agonist-Binding Assays

Agonist-binding assays of cell membranes and PEG-precipitated CHAPS solubilized cell membranes were performed as described earlier (Harikumar and Chattopadhyay, 1998a). Briefly, tubes in duplicate containing membranes were incubated in a total volume of 1 mL of buffer containing 50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4 in the presence of 0.29 nM [$^3$H]8-OH-DPAT for 1 h at room temperature. Nonspecific binding was determined by performing the assay in the presence of 10-$\mu$M unlabeled serotonin. The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B (1.0-$\mu$m pore size) 2.5-cm-diameter glass microfiber filters which were presoaked in 0.15% (w/v) polyethylenimine for 3 h (Bruns *et al.*, 1983). The filters were then washed three times with 3 mL each 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. The specific [$^3$H]8-OH-DPAT bindings (%) shown in Figs. 1 and 2 are normalized to the amount of total protein used in the assay. Although the total protein content varied in different assays, control experiments showed specific binding to be linear with protein content (not shown).

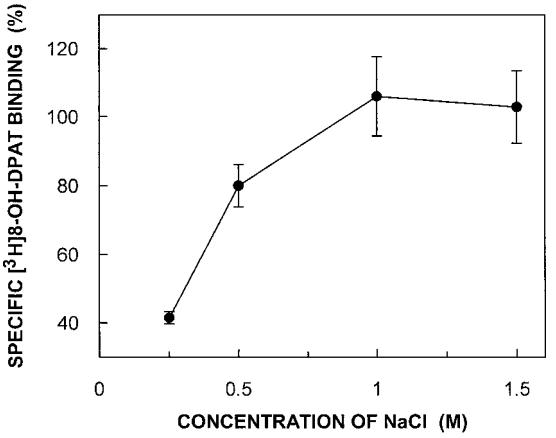


**Fig. 1.** Effect of NaCl concentration on the solubilization of the 5-HT$_{1A}$ receptor heterologously expressed in CHO cells. Values are expressed as percentage of specific binding of the agonist [$^3$H]8-OH-DPAT obtained for control membranes without solubilization. The concentration of CHAPS used was 5 mM and the total protein concentration was 2 mg/mL in all cases. The specific binding shown is normalized to the amount of total protein used in the assay. The data points are the mean ± SE from three independent experiments. See Materials and Methods Section for other details.

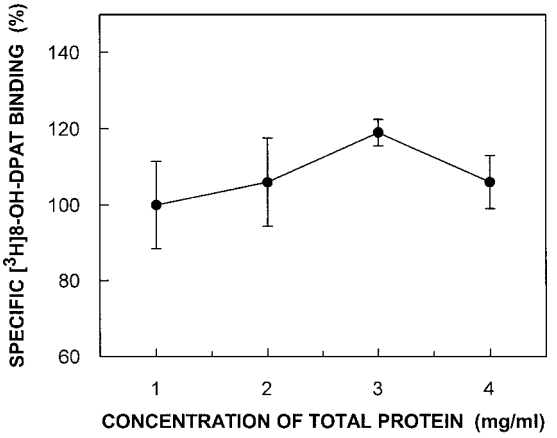


**Fig. 2.** Effect of total protein concentration on the solubilization of the 5-HT$_{1A}$ receptor heterologously expressed in CHO cells. Values are expressed as percentage of specific binding of the agonist [$^3$H]8-OH-DPAT obtained for control membranes without solubilization. The concentration of CHAPS used was 5 mM and NaCl concentration was 1 M in all cases. The specific binding shown is normalized to the amount of total protein used in the assay. The data points are the mean ± SE from three independent experiments. See Materials and Methods Section for other details.

## RESULTS AND DISCUSSION

We have previously shown that the critical micelle concentration (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. Thus, the CMC of CHAPS decreases from 6.41 mM in absence of any salt to 4.10 mM in presence of 1.5 M NaCl which amounts to a reduction of 36% (Chattopadhyay *et al*., 2002; Chattopadhyay and Harikumar, 1996).

The solubilization of T-CHO cell membranes containing the expressed 5-$HT_{1A}$ receptor, as monitored by the specific binding of the agonist [$^3$H]8-OH-DPAT as a function of NaCl concentration is shown in Fig. 1. CHAPS has been shown to be an efficient detergent for solubilization since it is effective in breaking protein–protein interactions. PEG precipitation of the 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 constitutes a method in which the detergent along with salt is rapidly removed (Gal *et al.*, 1983, Kremenetzky and Atlas, 1984; Ofri *et al*., 1992) thus overcoming the problem of long dialysis periods (Aguilar and Ochoa, 1986; Medrano *et al*., 1989). The solubilization efficiency is moderate when 0.25 M NaCl is used. The extent of solubilization shows marked increase when NaCl concentration is increased up to 1 M. The solubilization efficiency decreases slightly when 1.5 M NaCl is used. Optimal solubilization of agonist-binding sites is achieved using 5-mM CHAPS in presence of 1 M NaCl.

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. The change in solubilization efficiency of CHAPS with NaCl concentration, observed earlier by us in case of native hippocampal membranes (Chattopadhyay *et al*., 2002) and in our present results with T-CHO membranes, could be related to the dependence of its CMC with NaCl concentration.

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. Solubilization therefore involves multiple changes in organization of the membrane and is dependent on a host of membrane parameters. The solubilization efficiency of the 5-$HT_{1A}$ receptor in T-CHO cells was found to depend on the concentration of the protein (as determined by BCA assay) in the membrane sample taken. Figure 2 shows that changing the protein concentration (keeping the NaCl concentration fixed at 1 M) alters the solubilization of T-CHO cell membranes containing the 5-$HT_{1A}$ receptor. Under this condition, a total protein concentration of 3 mg/mL was found to be optimal. We conclude that careful control of salt and protein concentration is crucial in optimal solubilization of membrane receptors heterologously expressed in cells in culture. This may constitute an important step in the purification of important

neurotransmitter receptors such as 5-HT$_{1A}$ receptors which are present in very low amounts in the native tissue.

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