Critical Review

Membrane Protein Solubilization: Recent Advances and Challenges in Solubilization of Serotonin$_{1A}$ Receptors

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Summary

Solubilization of integral membrane proteins is a process in which the proteins and lipids that are held together in native membranes are suitably dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in formation of small protein and lipid clusters that remain dissolved in the aqueous solution. Effective solubilization and purification of membrane proteins, especially heterologously-expressed proteins in mammalian cells in culture, in functionally active forms represent important steps in understanding structure-function relationship of membrane proteins. In this review, critical factors determining functional solubilization of membrane proteins are highlighted with the solubilization of the serotonin$_{1A}$ receptor taken as a specific example.

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INTRODUCTION

Biological membranes are complex assemblies of a diverse variety of lipids and proteins and represent important sites for several cellular signaling functions. In order to understand the functioning of the membrane, it is often necessary to dissociate the membrane into its components. Membrane protein purification represents an area of considerable challenge in contemporary molecular biology (*1*). Studies carried out on purified and reconstituted membrane receptors have considerably advanced our knowledge of the molecular aspects of receptor function (*2*). An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the native membrane and dispersed individually in solution. This is most effectively accomplished

using amphiphilic detergents and the process is known as solubilization (*3–7*). Solubilization of membrane proteins is a process in which the proteins and lipids that are held together in native membranes 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 a membrane protein in a functionally active form represent important steps in understanding the structure-function relationship of a given protein. However, solubilization of a membrane protein with retention of activity poses a formidable challenge since many detergents irreversibly denature membrane proteins (*8*). This is the main reason for the rather modest list of membrane proteins which have been solubilized with retention of function, although ∼30% of all cellular proteins are estimated to be integral membrane proteins (*9*) many of which possess tremendous therapeutic potential (*10*). This review will highlight critical factors for solubilization of membrane proteins in general, with the functional solubilization of the serotonin$_{1A}$ (5-HT$_{1A}$) receptor taken as a specific example.

The serotonin$_{1A}$ (5-HT$_{1A}$) receptors are important members of the superfamily of seven transmembrane domain G-protein coupled receptors (GPCR). They appear to be involved in generation and modulation of various behavioral, cognitive and developmental functions. Serotonin (5-hydroxytryptamine or 5-HT) is a biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems. Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups (at least 14 subtypes) on the basis of their pharmacological responses to specific ligands, sequence similarities at the gene and amino acid levels, gene organization, and second messenger coupling pathways (*11*). Most of the serotonin receptors, except the 5-HT$_3$ receptor, belong to the large family of seven transmembrane domain G-protein coupled receptors (*12*) that couple to and transduce signals via guanine nucleotide binding regulatory proteins (G-proteins)

(*13*). The G-protein-coupled receptor superfamily comprises the largest class of molecules involved in signal transduction across the plasma membrane, thus providing a mechanism of communication between the exterior and the interior of the cell (*12, 14*) and represents $\sim 1\%$ of the mammalian genome (*15*). It is estimated that up to 50% of clinically prescribed drugs act as either agonists or antagonists at GPCRs which points out their immense therapeutic potential (*16*). The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important representative of this large family of receptors and is the most extensively studied of the serotonin receptors for a number of reasons (*17, 18*). The 5-HT$_{1A}$ receptor is implicated in regulation of blood pressure, feeding, temperature and working memory (*18*). It has recently been shown to have a role in neural development (*19*) and protection of stressed neuronal cells undergoing degeneration and apoptosis (*20*). The 5-HT$_{1A}$ receptor agonists and antagonists represent a major class of molecules with potential therapeutic effects in anxiety- or stress-related disorders (*18*). Interestingly, mutant (knockout) mice lacking the 5-HT$_{1A}$ receptor generated a few years back exhibit enhanced anxiety-related behavior. The 5-HT$_{1A}$ receptor knockout mouse serves as an excellent model system to understand anxiety-related behavior in higher animals (*21*).

In spite of the significance of serotonergic signaling in several physiological processes, none of the G-protein coupled serotonin receptors have been purified to homogeneity yet from natural sources. Since solubilization is the first step toward purification of any integral membrane protein, it is important to identify factors crucial for achieving successful solubilization. In this review, we will describe such factors applicable to solubilization of membrane proteins with specific reference to recent advances in functional solubilization of the 5-HT$_{1A}$ receptor. Efficient solubilization of the receptor from the native source with retention of ligand binding function and signal transduction ability would constitute the first step in the molecular characterization of G-protein-coupled receptors.

CHOOSING A SUITABLE DETERGENT

Detergents are soluble amphiphiles (*3*) i.e., they possess both hydrophilic and hydrophobic groups, with a higher degree of hydrophilicity than most lipids in biological membranes. Detergents can be broadly classified based on their charge as: (i) anionic (e.g., sodium dodecyl sulphate (SDS), the bile salts such as cholate and deoxycholate), (ii) cationic (e.g., cetyltrimethylammonium bromide (CTAB)), (iii) zwitterionic (e.g., 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)), and (iv) nonionic (e.g., the polyoxyethylene series of detergents such as Triton X-100). Representative members of each of these classes of detergents are shown in Fig. 1. The ability of a detergent to solubilize membranes is believed to depend on the empirical parameter termed hydrophile-lipophile balance (HLB), especially for solubilization by nonionic detergents (*3, 22*). This principle has been utilized earlier in order to achieve optimum solubilization of membrane components (*23*). HLB is a measure of the hydrophilic character of a detergent and is based on the weight percentage of hydrophilic *vs.* lipophilic groups present in a detergent. Detergents with a relatively high HLB value of 12–20 are recommended in order to solubilize membrane proteins in a non-denatured condition (*24*).

Detergents belonging to the class of nonionic and zwitterionic detergents have become popular for their ability to solubilize membrane proteins with retention of function. For example, CHAPS, a mild, non-denaturing, zwitterionic detergent originally synthesized by Hjelmeland (*25*), is one of the most commonly used detergents in membrane biochemistry (*17*). It is a derivative of the naturally occurring bile salts. CHAPS combines useful features of both the bile salt hydrophobic group and the *N*-alkyl sulfobetaine-type polar group (see Fig. 1). 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-
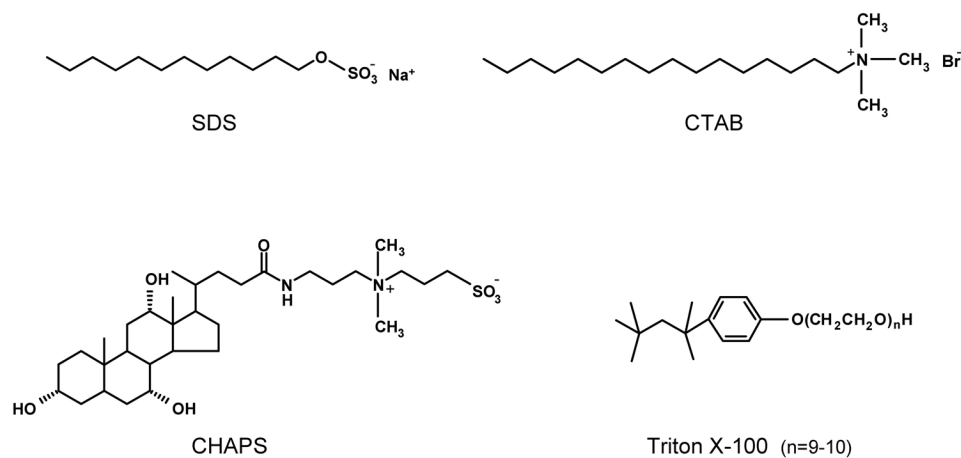

Figure 1. Chemical structures of representative detergents of various charge types.

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 the extensive use of CHAPS in solubilization of membrane proteins and receptors (17, 26 – 28). However, it must be emphasized here that in spite of these overall criteria, the choice of a suitable detergent for optimal solubilization of a given membrane protein still has to be worked out on a case-by-case basis. For example, efficient solubilization of the IgE receptor has been shown to occur with the anionic detergent cholate but not with the nonionic detergent octylglucoside (29). In addition, factors such as compatibility of the detergent in biochemical assays and reconstitution methods following solubilization should be kept in mind in choosing a detergent. Although Triton X-100 is a widely used nonionic detergent for solubilization of membrane proteins, it may not be an appropriate detergent during reconstitution due to its low CMC which makes its removal difficult by dialysis (6). In general, no single detergent can be stated to be the best detergent suitable for all situations. The choice of a specific detergent therefore depends more on precedent and empirical factors (see below) than scientific principles.

CRITICAL FACTORS FOR FUNCTIONAL SOLUBILIZATION

(i) Critical Micelle Concentration of Detergents

Detergent molecules exist as monomers at low concentrations in an aqueous solution. As the detergent monomer concentration is increased above a critical concentration (strictly speaking, a narrow concentration range), referred to as the critical micelle concentration (CMC), they self associate to form thermodynamically stable, non-covalent aggregates called micelles (30). The general principle underlying the formation of micelles (i.e., the hydrophobic effect) is common to other related assemblies such as lipid bilayers and biological membranes. Micelles are highly cooperative and are dynamic in nature (31) and have been used as membrane-mimetic systems to characterize membrane proteins and peptides (32).

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 (29). 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 such as 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 (4). Concentrations of detergents above the CMC invariably led to loss of protein function in these cases. The mechanism by which 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' (6, 29, 33) which is the concentration of detergent existing as monomers at a given condition. The effective CMC takes into account contributions from other components (such as lipids, proteins, ionic strength, pH, temperature) in the system under study. Thus, solubilization could occur below the CMC if the effective CMC is lower than literature CMC. Determination of the effective CMC could serve as a useful indicator in solubilization of membrane proteins under various experimental conditions (34). The phenomenon of reduction in CMC of a detergent upon addition of salt can be exploited to achieve functional solubilization of membrane proteins at low detergent concentrations. This is particularly relevant for bile acid-derived detergents such as CHAPS and cholate which have been reported to induce dissociation and depletion of the $\beta\gamma$ dimer of heterotrimeric G-proteins, when used in high concentrations (35 – 37). Addition of salt is known to drastically reduce the CMC of charged detergents such as SDS (38, 39) since salt would tend to reduce the repulsion between the charged headgroups. This helps in micelle formation to occur at lower concentrations of the detergent. The effect of salt on CMC of uncharged detergents is expected to be less pronounced because of the absence of charge interactions. Nonetheless, it has previously been shown that the CMC of the zwitterionic detergent CHAPS (34) and the neutral detergent octylglucoside (40) decrease with increasing salt concentrations. Interestingly, a low ('pre-micellar') concentration of CHAPS has been effectively used for solubilizing 5-HT$_{1A}$ receptors in presence of salt (17, 34, 41). The CMC of CHAPS ($\sim$6.4 mM in the absence of any salt) has been shown to reduce to $\sim$4.3 mM in presence of 1 M NaCl (34). Utilizing this phenomenon, efficient solubilization of 5-HT$_{1A}$ receptors with a high ligand binding affinity and ability to couple to G-proteins was achieved (17). A detergent (CHAPS) concentration of 5 mM in the presence of 1 M NaCl was found to be optimal in order to solubilize 5-HT$_{1A}$ receptors from bovine hippocampal membranes (17, 34, see Fig. 2). Higher concentrations of the detergent, such as 10 and 15 mM in presence of salt, were found not to be favorable for functional solubilization. Higher concentrations of CHAPS are not recommended for functional solubilization of G-protein-coupled receptors due to possible perturbation in receptor G-protein coupling due to dissociation and depletion of the $\beta\gamma$ dimer of the heterotrimeric G-proteins as mentioned above (35 – 37). This is particularly true for the hippocampal 5-HT$_{1A}$ receptor since it is negatively coupled to adenylate cyclase through G$_i$-proteins (42). Although 5-HT$_{1A}$ receptors have been solubilized using CHAPS earlier (27, 28, 43), the efficiency of solubilization in these cases was low and concentrations of detergent used were high. In this context, the use of salt to effectively lower the
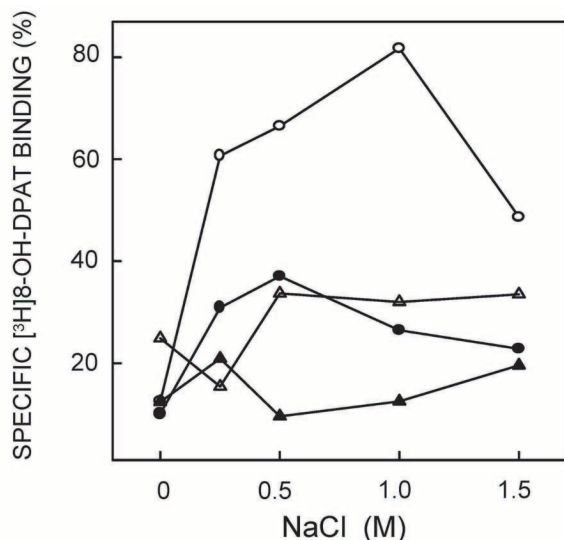
Figure 2. Demonstration of functional solubilization of membrane receptors in the presence of optimal effective ('pre-micellar') concentration of detergent. The plot shows solubilization of $5\text{-}HT_{1A}$ receptors from native hippocampal membranes at various concentrations of CHAPS in presence of increasing concentration of salt. 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 (O), 7.5 (●), 10 (△), and 15 (▲) mM. Notice that solubilization of $5\text{-}HT_{1A}$ receptors is modulated by various concentrations of salt. Maximum functional solubilization of $5\text{-}HT_{1A}$ receptors occurs at 5 mM CHAPS and 1 M NaCl. Data taken from Ref. 17.

detergent concentration required to achieve optimal solubilization of the $5\text{-}HT_{1A}$ receptor represents an elegant approach (17).

(ii) Detergent-Lipid-Protein Ratios

Membrane solubilization by detergents is a complex process and can be described as a three stage process (3, 4, 6, 44; see Fig. 3). The detergent-lipid-protein ratio is an important factor for the successful solubilization of membrane proteins. One could attain different stages of solubilization depending on the detergent-protein ratio used (2). This aspect would be evident upon examining various stages of the process of membrane solubilization as shown in the schematic representation in Fig. 3. At low concentrations of the detergent, the detergent monomers merely bind to the membrane with minimal perturbation of the membrane followed by penetration into the membrane (Fig. 3B). As the concentration of detergent increases, the membrane bilayer is disrupted leading to 'lysis' giving rise to lipid-protein-detergent mixed micelles (Fig. 3C). Any further increase in detergent concentration results in heterogeneous complexes of

detergent, lipid and protein and progressive delipidation of the lipid-protein-detergent mixed micelles forcing the lipids to distribute among the increasing concentrations of detergent micelles. This leads to formation of lipid/detergent and protein/detergent mixed micelles (Fig. 3D).

As the concentration of detergent is increased to solubilize membranes at a given protein or lipid concentration, a steady increase in the solubilized lipid (45) or protein (46) could be observed until saturation is reached, where the extent of solubilization no longer increases with higher detergent concentrations. However, it is often difficult to use such high concentrations of detergent which can provide maximum solubilization since the activity of the membrane protein in question may be compromised under such conditions. Higher detergent concentrations such as 15 mM CHAPS were found to affect functional solubilization of the $5\text{-}HT_{1A}$ receptor (17). It is therefore recommended to use a mild concentration of detergent which can strike a balance between these two aspects i.e., maximize solubilization yet preserve protein activity. However, there is no precise approach to arrive at an ideal ratio of detergent to lipid or protein. Such a condition is most often arrived at by a trial and error basis. The most effective way to achieve this is to monitor solubilization over a wide range of detergent-lipid ratios. A useful relationship combining some of these experimental parameters was developed by Rivnay and Metzger (29). According to this formalism, the parameter (ρ) was defined as the molar ratio of detergent to lipid optimal for functional solubilization (29).

$$\rho = \frac{[\text{Detergent}] - \text{CMC}_{\text{eff}}}{[\text{Phospholipid}]}$$

where CMC_{eff} represents the CMC determined under specific experimental conditions (as described in section (i) above). The CMC_{eff} is preferred over the literature CMC as the former often depends on lipids, proteins, pH and salt (as described above, see Ref. 34). An increase in solubilization is expected with increase in the value of the ρ parameter (29). Even though this parameter may vary for different proteins and could depend on other conditions during reconstitution, it provides an approximate range of favorable detergent to lipid ratios for a solubilization experiment. Application of the ρ factor for all types of solublization is limited due to the number of variables in a solubilization protocol and due to the fact that the ρ value does not take into consideration specific lipid-detergent interactions.

Alternatively, one can monitor optimal solubilization with varying detergent to protein ratios since concentrations of protein and lipid are related in a given membrane. For example, a protein concentration of 3 mg/ml appears to be preferred over lower protein concentrations in solubilizing heterologously expressed $5\text{-}HT_{1A}$ receptors from Chinese Hamster Ovary (CHO) cell membranes (41).
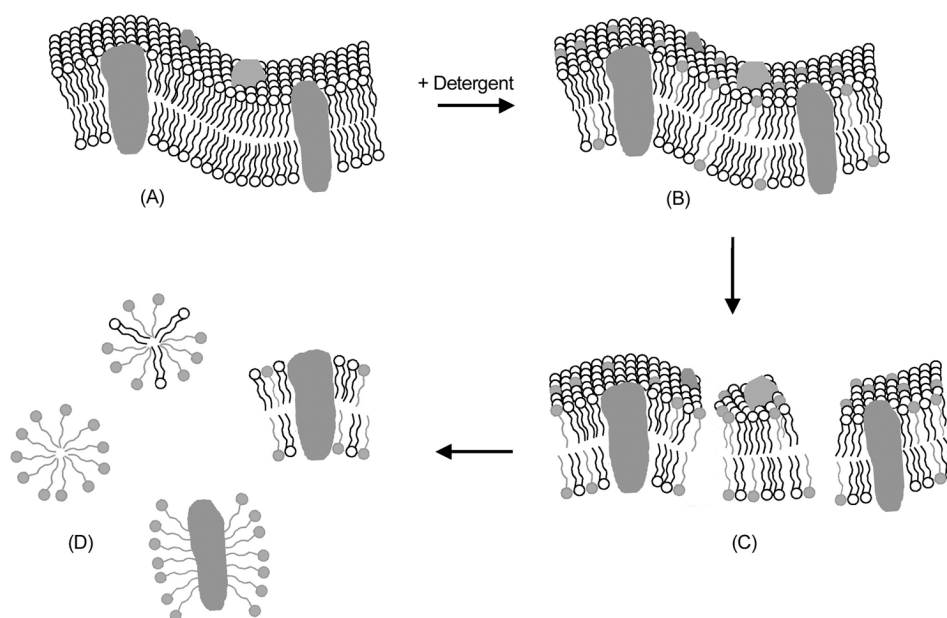
Figure 3. A schematic representation of various stages of solubilization of biological membranes by detergents. When low concentrations of a detergent are added to biological membranes (shown in A), the detergent monomers (shown in gray with single tails) merely bind to the membrane with minimal perturbation of the membrane followed by penetration into the membrane (B). As the concentration of detergent increases, the membrane bilayer is disrupted (C). At still higher detergent concentrations, heterogeneous complexes of detergent, lipid and protein begin to form which result in mixed micelles of lipid and detergent and that of protein and detergent (D).

(iii) Lipid Environment

A large portion of any integral membrane protein remains in contact with the membrane lipid environment. This raises the obvious possibility that membrane lipids could be important modulators of membrane protein structure and function. Considering the significance of lipid-protein interactions in maintaining the structure and function of biological membranes (47, 48), it is conceivable that replacement of a specific lipid environment with detergent or detergent-lipid during solubilization could affect the function of a membrane protein. For example, displacement of lipids from the receptor has been shown to be an integral feature of detergent-induced inactivation in case of the nicotinic acetylcholine receptor (49). The phenomenon of delipidation and its consequences on activity of solubilized membrane proteins have previously been utilized to gain insight into the specific lipid requirements of membrane proteins (49–51).

Solubilization of biological membranes is often accompanied by selective or differential solubilization of membrane lipids due to asymmetric extraction of membrane lipids by detergents (52). This means that certain lipids could be enriched at the expense of a few others in solubilized membranes. For example, cholesterol, sphingomyelin and glycolipids were found to be enriched when red blood cell membranes were extracted with Triton X-100 (53). Thus, the lipid microenvironment around a protein assumes significance since it could not only determine the extent of solubilization (due to differential solubilizing ability of detergents for different classes of membrane lipids) but also the function of the solubilized protein (owing to loss of specific lipid-protein interactions). Differential solubilization of membrane lipids could either be due to the intrinsic property of lipids themselves or due to their organization in the membrane (e.g., tight packing of fatty acyl chains) which can influence detergent extractability. The possibility of membranes being organized into domains consisting of certain class of lipids and proteins which are resistant to detergent extraction (solubilization) has generated a lot of interest in exploring organization of biological membranes by utilizing detergent insolubility as a biochemical tool to explore domain organization of membranes (45, 54–56).

In the case of 5-HT$_{1A}$ receptors, the choice of the detergent CHAPS and its ability to solubilize 5-HT$_{1A}$ receptors from bovine hippocampal membranes (17, 34) which is not achieved optimally using other detergents (Harikumar, K. G., and Chattopadhyay, A., unpublished observations), bring to light the potential role of membrane lipids in maintaining the function of membrane proteins. Several other detergents such as Brij 35, NP-40 produce weakly active soluble preparations (57), while Triton X-100 irreversibly affects the ligand binding of the 5-HT$_{1A}$ receptor (Kalipatnapu, S., and Chattopadhyay,

A., unpublished observations). It has been shown that solubilization of ovine brain membranes by CHAPS leads to differential extraction, with membrane lipids such as phosphatidylethanolamine and phosphatidylcholine being solubilized to a much greater extent over other lipids such as cholesterol (52). This is accompanied by an enrichment of saturated phospholipids (58). It is possible that the ability of a detergent to solubilize a membrane protein in its functional state depends on cosolubilization of certain membrane lipids. While CHAPS can efficiently solubilize 5-HT$_{1A}$ receptors from bovine hippocampus in a functionally active form (17, 34), a fraction of functional receptors is lost during solubilization. This could either be due to inability of the detergent to solubilize those receptors or could be a consequence of delipidation of the receptor. Solubilization of the hippocampal 5-HT$_{1A}$ receptors by CHAPS has previously been shown to be accompanied by loss of membrane cholesterol (59). Importantly, the role of cholesterol in modulation of ligand binding and G-protein coupling of the hippocampal 5-HT$_{1A}$ receptor has been demonstrated earlier (60−62). It is therefore possible that the apparent loss in activity of the solubilized receptor could be due to loss of cholesterol. This proposal has recently been tested by incorporating cholesterol in bovine hippocampal membranes solubilized in presence of CHAPS and NaCl. Interestingly, replenishment of membrane cholesterol to solubilized bovine hippocampal membranes resulted in an increase in ligand binding of the 5-HT$_{1A}$ receptor (59). This reinforces the importance of the membrane lipid environment in function of membrane proteins.

The observation that different classes of detergents used for solubilization of membrane receptors result in differential solubilization of lipids and proteins could be due to the possibility that some detergents extract even the 'annular' lipids necessary for preserving the function of the receptor (28, 49). This could result in solubilized but non-functional receptor. Therefore, the relevance of the immediate lipid environment of the membrane protein must be considered while choosing the appropriate detergent for optimal solubilization with retention of function.

CONCLUSION AND FUTURE PERSPECTIVES

Solubilization and purification of membrane proteins continue to be challenging tasks in contemporary membrane biology. Most of the membrane proteins, with exceptions such as bacteriorhodopsin and the nicotinic acetylcholine receptor, occur at very low levels in their native tissues. This makes solubilization and purification of membrane proteins even more daunting. In this context, mammalian cells in culture heterologously-expressing membrane receptors represent convenient systems (63, 64). A recent report describes effective solubilization of 5-HT$_{1A}$ receptors in a functionally active form from heterologously-expressed CHO cells in culture (41). This has been achieved using CHAPS by careful control of salt and protein concentration. Although the 5-HT$_{1A}$ receptor has been heterologously and stably expressed in fibroblast cells earlier (65−67), this is the first report of solubilization of this heterologously-expressed receptor in a functional form. This represents a significant step toward purification of this important G-protein coupled neurotransmitter receptor. The conditions optimized for functional solubilization of the 5-HT$_{1A}$ receptor could be useful in solubilizing other membrane proteins, especially G-protein coupled receptors expressed in heterologous systems.

Membrane organization of the 5-HT$_{1A}$ receptor and the role of lipids such as cholesterol in ligand binding and G-protein coupling of the receptor (60−62) are just beginning to be understood. Exploring membrane organization and dynamics of the 5-HT$_{1A}$ receptor fused to enhanced yellow fluorescent protein under a variety of conditions such as G-protein activation and cholesterol depletion has recently added a new dimension to this area of research (56, 68, 69). Advances toward purification of the 5-HT$_{1A}$ receptor could open up many more opportunities. The prospect of eventual determination of the structure, dynamics and molecular mechanism of functioning of membrane receptors in healthy and diseased states makes solubilization and purification of membrane proteins relevant in current membrane biology.

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