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Research Article

Solubilization of the serotonin$_{1A}$ receptor monitored utilizing membrane dipole potential

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ABSTRACT

Solubilization of membrane proteins by amphiphilic detergents represents a crucial step in studies of membrane proteins in which proteins and lipids in natural membranes are dissociated giving rise to mixed clusters of proteins, lipids and detergents in the aqueous dispersion. Although solubilization is a popular method, physicochemical principles underlying solubilization are not well understood. In this work, we monitored solubilization of the bovine hippocampal serotonin$_{1A}$ receptor, a representative member of the GPCR family, using membrane dipole potential measured by a dual fluorescence ratiometric approach with a potential-sensitive fluorophore. Our results show that membrane dipole potential is a good indicator of solubilization and reflects the change in dipolar environment upon solubilization due to dipolar reorganization associated with solubilization. To the best of our knowledge, these results constitute the first report linking membrane dipole potential with solubilization. We envision that these results are potentially useful in providing a molecular mechanism for membrane protein solubilization.

1. Introduction

Solubilization of membrane proteins constitutes an important step in their purification (Kalipatnapu and Chattopadhyay, 2005; Duquesne and Sturgis, 2010; Kubicek et al., 2014; Chattopadhyay et al., 2015). Solubilization enables proteins and lipids in natural membranes to be dissociated by use of a suitable amphiphilic detergent. The dissociation of the natural membrane components results in the formation of small mixed clusters of proteins, lipids and detergents in aqueous dispersion. A hallmark of effective solubilization is that the function of a given membrane protein be retained to a considerable extent. This could prove to be tricky, since many detergents induce irreversible denaturation of membrane proteins (Garavito and Ferguson-Miller, 2001). In case of G protein-coupled receptors (GPCRs), an important class of membrane receptors that act as signaling hubs and major drug targets (Rosenbaum et al., 2009; Chattopadhyay, 2014; Jacobson, 2015), solubilization and purification from natural sources poses considerable challenge due to low amounts of receptor present in the native tissue.

Dipole potential represents an important electric potential in organized molecular assemblies (such as membranes or micelles) and it originates due to the nonrandom orientation of electric dipoles (lipids, detergents, proteins, water molecules) inside the assembly (Brockman, 1994; Clarke, 2001; O'Shea, 2005; Wang, 2012; Sarkar and Chattopadhyay, 2015). The increasing application of dipole potential to problems related to biological and model membranes is evident from a growing body of literature in this area. Membrane dipole potential has been shown to provide novel information on the nature of membrane lipids (Starke-Peterkovic et al., 2006; Starke-Peterkovic and Clarke, 2009; Haldar et al., 2012; Bandari et al., 2014), function and lipid interactions of membrane proteins (Cladera and O'Shea, 1998; Duffin et al., 2003; Starke-Peterkovic et al., 2005; Singh et al., 2013; Chaudhuri and Chattopadhyay, 2014; Clarke, 2015; Richens et al., 2015; Sarkar et al., 2017).

With an overall goal to explore lipid specificities in GPCR function, in our laboratory, we have focused on lipid interactions of the serotonin$_{1A}$ receptor. The serotonin$_{1A}$ receptor is a representative GPCR that serves as a crucial neurotransmitter receptor, and is implicated in behavior, learning, development and cognition (Pucadyil et al., 2005; Müller et al., 2007). Importantly, the serotonin$_{1A}$ receptor represents an important drug target for neuropsychiatric disorders such as anxiety and depression and in neuronal developmental defects (Fiorino et al., 2014). In our previous work, we demonstrated the requirement of

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; di-8-ANEPPS, 4-(2-(6-(dioctylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium inner salt; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; GPCR, G protein-coupled receptor; HLB, hydrophile-lipophile balance; PMSF, phenylmethylsulfonyl fluoride

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membrane cholesterol (Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Jafurulla and Chattopadhyay, 2013) and sphingolipids (Jafurulla and Chattopadhyay, 2015) in the function of the serotonin$_{1A}$ receptor. In the process, we utilized solubilization of the receptor and the lipid loss associated with it as a convenient strategy to explore lipid-receptor interaction (Chattopadhyay et al., 2015). The process of solubilization involves reorganization of membrane components and lipid-protein interaction (Valpuesta et al., 1986; de Foresta et al., 1989). Solubilized membranes are composed of heterogeneous complexes of detergent, lipid and protein (Kalipatnapu and Chattopadhyay, 2005; Singh et al., 2011; Chattopadhyay et al., 2015). They are more disordered (loosely packed) relative to native membranes, thereby inducing increased water penetration. The dielectric environment in solubilized membranes could therefore be considerably different from natural membranes. Keeping this in mind, we monitored membrane dipole potential along with solubilization (as monitored by specific ligand binding to the serotonin$_{1A}$ receptor) using a potential-sensitive fluorescent probe by a dual wavelength ratiometric approach. Our results show that membrane dipole potential is well correlated with the extent of solubilization.

2. Materials and methods

2.1. Materials

BSA, CHAPS, DMPC, EDTA, EGTA, iodoacetamide, $MgCl_2$, $MnCl_2$, NaCl, Na_2HPO_4, PEG, PMSF, polyethylenimine, serotonin, sucrose, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Di-8-ANEPPS was purchased from Molecular Probes/Invitrogen (Eugene, OR). BCA reagent for protein estimation was from Pierce (Rockford, IL). [^{3}H]8-OH-DPAT (sp. activity 141 Ci/mmol) was purchased from MP Biomedicals (Santa Ana, CA). GF/B glass micro fiber filters were from Whatman International (Kent, U.K.). All other chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. 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 −80 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously (Harikumar and Chattopadhyay, 1999). Bovine hippocampal tissue (~50 g) was homogenized as 10% (w/v) in a polytron homogenizer in 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 buffer. The homogenate was centrifuged at 900 × *g* for 10 min at 4 °C. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 50,000 × *g* for 20 min at 4 °C. The pellet obtained was suspended in 10 vols of 50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4 buffer 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 hippocampal membranes) was suspended in a minimum volume of 50 mM Tris, pH 7.4, homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −80 °C. The protein concentration was assayed using the BCA reagent (Smith et al., 1985).

2.2.2. Solubilization of native membranes

Hippocampal membranes were solubilized as described previously using the zwitterionic detergent CHAPS (Chattopadhyay and Harikumar, 1996; Chattopadhyay et al., 2002, 2005; Jafurulla et al., 2014). Native hippocampal membranes were incubated with 5 mM CHAPS and 1 M NaCl in buffer A (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) at a final protein concentration of ~2 mg/ml for 30 min at 4 °C with occasional shaking. 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 at the beginning and end of the incubation period. After incubation for 30 min at 4 °C, the contents were centrifuged at 100,000 × *g* for 1 h at 4 °C. The clear supernatant containing CHAPS-solubilized membrane was carefully removed from the pellet, and was reconstituted using PEG (termed solubilized membranes). PEG precipitation was performed to remove NaCl from the solubilized extract, since the agonist binding of the serotonin$_{1A}$ receptor is inhibited by NaCl (Harikumar and Chattopadhyay, 1998). This procedure is believed to remove detergent and salt (Gal et al., 1983; Kremenetzky and Atlas, 1984). PEG precipitation was carried out by diluting the extract with equal volume of 40% (w/w) PEG-8000 in buffer A. 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 A, resuspended in buffer A and used for radioligand binding assays or dipole potential measurements.

2.2.3. Radioligand binding assays

Receptor binding assays were carried out as described earlier (Pucadyil and Chattopadhyay, 2004) with some modifications. Tubes in duplicate with ~1 mg native hippocampal membrane protein (or ~0.5 mg for solubilized membranes) in a total volume of 1 ml of 50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4 buffer were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.5 nM) for 1 h at 25 °C. Nonspecific binding was determined by performing the assay in the presence of 10 μM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter glass micro fiber filters (1 μm pore size), which were presoaked in 0.3% polyethylenimine for 1 h (Bruns et al., 1983). Filters were then washed three times with 3 ml of cold water (4 °C) and dried. The retained radioactivity was measured in a Packard Tri-Carb 2900 liquid scintillation counter using 5 ml of scintillation fluid.

2.2.4. Estimation of cholesterol

Cholesterol content in native and solubilized membranes was estimated using Amplex Red cholesterol assay kit (Amundson and Zhou, 1999).

2.2.5. Estimation of inorganic phosphate

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid (McClare, 1971) using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.6. Sample preparation for dipole potential measurements

Di-8-ANEPPS was added from a methanolic stock solution to native or solubilized hippocampal membranes containing 100 nmol total phospholipid in 1.5 ml of 50 mM Tris, pH 7.4 buffer. The amount of di-8-ANEPPS added was such that the final probe concentration was ~1 mol% with respect to total phospholipid content. The concentration of the stock solution of di-8-ANEPPS in methanol was estimated from its molar absorption coefficient of 37,000 M^{-1} cm^{-1} at 498 nm (Le Goff et al., 2007). The final di-8-ANEPPS concentration was 0.66 μM in all cases and methanol content was always low (0.02%, v/v). This ensures optimal fluorescence intensity with negligible membrane perturbation. Control experiments showed that at this concentration of methanol, ligand binding properties of the receptor are not altered (Pucadyil and Chattopadhyay, 2004). Di-8-ANEPPS was added to membranes while being vortexed for 1 min at room temperature (~23 °C). Background