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Membrane Dipole Potential is Sensitive to Cholesterol Stereospecificity: Implications for Receptor Function

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Abstract

Dipole potential is the potential difference within the membrane bilayer, which originates due to the nonrandom arrangement of lipid dipoles and water molecules at the membrane interface. Cholesterol, an essential lipid in higher eukaryotic membranes, has previously been shown to increase membrane dipole potential. In this work, we explored the effect of stereoisomers of cholesterol, *ent*-cholesterol and *epi*-cholesterol, on membrane dipole potential, monitored by the dual wavelength ratiometric approach utilizing the probe di-8-ANEPPS. Our results show that cholesterol and *ent*-cholesterol share comparable ability in increasing membrane dipole potential. In contrast, *epi*-cholesterol displays a slight reduction in membrane dipole potential. Our results constitute the first report on the effect of stereoisomers of cholesterol on membrane dipole potential, and imply that an extremely subtle change in sterol structure can significantly alter the dipolar field at the membrane interface. These results assume relevance in the context of differential abilities of these stereoisomers of cholesterol in supporting the activity of the serotonin$_{1A}$ receptor, a representative G protein-coupled receptor. The close correlation between membrane dipole potential and receptor activity provides new insight in receptor-cholesterol interaction in terms of stereospecificity. We envision that membrane dipole potential could prove to be a sensitive indicator of lipid-protein interactions in biological membranes.

Keywords

Cholesterol; di-8-ANEPPS; dipole potential; *ent*-cholesterol; *epi*-cholesterol; serotonin$_{1A}$ receptor

1. Introduction

Dipole potential represents the potential difference within the membrane bilayer. The origin of membrane dipole potential is the nonrandom orientation of electric dipoles of lipid and

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water molecules at the membrane interface (Brockman, 1994; Clarke, 2001; O'Shea, 2005; Wang, 2012). The magnitude of dipole potential varies between 200–1000 mV, depending on membrane composition. Because dipole potential is operative over a relatively small distance in the membrane, the electric field generated due to dipole potential is enormous in magnitude and is in the range of 10^8–10^9 Vm^{-1} (Clarke, 2001; Wang, 2012). An important implication of membrane dipole potential is that it influences the function of membrane proteins and peptides such as Na^+/K^+-ATPase (Starke-Peterkovic et al., 2005) and the ion channel gramicidin (Duffin et al., 2003). We recently used membrane dipole potential as a useful parameter to monitor the binding of α-lactalbumin to membranes (Chaudhuri and Chattopadhyay, 2014). Importantly, it has been proposed that the dipole potential may play a crucial role in the structure and function of proteins associated with cholesterol-rich domains in the membrane (O'Shea, 2005).

Cholesterol is a crucial membrane lipid in higher eukaryotes and plays a vital role in membrane organization, dynamics, function, and sorting (Simons and Ikonen, 2000; Mouritsen and Zuckermann, 2004; Chaudhuri and Chattopadhyay, 2011). An important and emerging area is the role of cholesterol in the function and organization of membrane proteins and receptors (Burger et al., 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Oates and Watts, 2011; Jafurulla and Chattopadhyay, 2013). The mechanism underlying the effect of membrane cholesterol on the structure and function of membrane proteins and receptors appears complex (Paila and Chattopadhyay, 2009, 2010; Paila et al., 2009; Lee, 2011). A possible mechanism by which membrane cholesterol has been proposed to influence the function of membrane receptors is by a direct (specific) interaction that induces subtle conformational changes in the receptor. An alternative mechanism envisages change in membrane physical properties in which the receptor is embedded. These mechanisms need not be mutually exclusive, *i.e.*, another possibility could be a combination of both. Membrane cholesterol has been shown to modulate the function of a number of G protein-coupled receptors (GPCRs) in general (Burger et al., 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Oates and Watts, 2011; Jafurulla and Chattopadhyay, 2013), and the serotonin$_{1A}$ receptor in particular (Pucadyil and Chattopadhyay, 2004, 2005; Paila et al., 2008; Shrivastava et al., 2010; Jafurulla et al., 2014).

It has been reported earlier that membrane cholesterol increases dipole potential in model (Starke-Peterkovic et al., 2006; Haldar et al., 2012) and natural (Singh et al., 2013) membranes. However, the ability of a sterol to modulate membrane dipole potential is varied and was shown to depend on its exact molecular structure (Starke-Peterkovic et al., 2006; Haldar et al., 2012). For example, immediate biosynthetic precursors of cholesterol (7-dehydrocholesterol and desmosterol), differing with cholesterol *merely* in a double bond, lack the ability to increase membrane dipole potential. In other words, even a subtle difference in molecular structure (such as a double bond) can give rise to drastic difference in the ability to influence membrane dipole potential. With an overall goal to have a comprehensive understanding of finer structural details of the interaction of membrane cholesterol with membrane proteins and receptors, in this work, we explored the degree of structural (stereospecific) stringency in sterols in modulating membrane dipole potential.

Toward this goal, we monitored the effect of two stereoisomers of cholesterol, *ent*-cholesterol and *epi*-cholesterol, on membrane dipole potential. The enantiomer of cholesterol (*ent*-cholesterol) is the non-superimposable mirror image of native (natural) cholesterol (see Fig. 1a,b). Enantiomers have identical physicochemical properties, except for the direction of rotation of plane-polarized light. As a result, membrane biophysical properties (such as compressibility and phase behavior) are same for native cholesterol and *ent*-cholesterol (Mannock et al., 2003; Westover et al., 2003; Westover and Covey, 2004; Covey 2009). In addition, both native cholesterol and *ent*-cholesterol support normal growth of a mutant mammalian cell line (Xu et al., 2005). An interesting use of *ent*-cholesterol is to distinguish specific interaction of cholesterol from nonspecific effects (Mickus et al., 1992; Covey, 2009; D'Avanzo et al., 2011; Kristiana et al., 2012). On the other hand, *epi*-cholesterol is a diastereomer of cholesterol in which *only* the orientation of the hydroxyl group at carbon-3 is inverted relative to native cholesterol and is not a mirror image of cholesterol (Fig. 1c). While *ent*-cholesterol shares identical physicochemical properties with cholesterol, previous studies have shown that the biophysical properties of *epi*-cholesterol and native cholesterol are different (Westover and Covey, 2004; Covey, 2009). *epi*-Cholesterol has been reported to differ in its tilt angles, condensing ability, and phase transition properties from cholesterol in membranes (Demel et al., 1972; Dufourc et al., 1984; Murari et al., 1986; Cheetham et al., 1989). We show here that cholesterol and *ent*-cholesterol share comparable ability in increasing membrane dipole potential. In contrast to this, *epi*-cholesterol does not exhibit any increase in membrane dipole potential. Rather, there is a slight decrease in membrane dipole potential with increasing concentration of *epi*-cholesterol. We further discuss the implications of these results in terms of relative abilities of these stereoisomers of cholesterol in supporting the activity of the serotonin$_{1A}$ receptor, previously reported by us (Jafurulla et al., 2014). These results provide novel insight into the subtle structural requirements of cholesterol in its interaction with membrane proteins.

2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cholesterol, EDTA, NaCl and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). 3-Epicholesterol (5-cholesten-3α-ol), to be denoted as *epi*-cholesterol, was obtained from Steraloids (Newport, RI). The enantiomer of cholesterol (*ent*-cholesterol) was synthesized as previously described (Jiang and Covey, 2002; Westover and Covey, 2004). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 4-(2-(6-(Dioctylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium inner salt (di-8-ANEPPS) was purchased from Molecular Probes (Eugene, OR). Pre-coated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). The purity of lipids was checked by thin layer chromatography on silica gel precoated plates in chloroform/methanol/water (65:35:5, v/v/v) and was found to give only one spot with a phosphate-sensitive spray and on subsequent charring (Baron and Coburn, 1984). Solvents used were of analytical grade. All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Estimation of phospholipids—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.

2.2.2. Sample preparation—Experiments were performed using large unilamellar vesicles (LUVs) of 100 nm diameter of POPC containing increasing concentrations (0–40 mol%) of a given sterol (any one of the following sterols: cholesterol/*epi*-cholesterol/*ent*-cholesterol). All samples contained 1 mol% di-8-ANEPPS. In general, 640 nmol of total lipid (phospholipid and sterol) and 6.4 nmol of di-8-ANEPPS were mixed well and dried under a stream of nitrogen while being warmed gently (~35 °C). After further drying under a high vacuum for at least 3 h, the lipid mixture was hydrated (swelled) by addition of 1.5 ml of 30 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 7.2 buffer, and each sample was vortexed for 3 min to uniformly disperse the lipids and form homogeneous multilamellar vesicles. LUVs of 100 nm diameter were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada) as previously described (MacDonald et al., 1991). Briefly, multilamellar vesicles were freeze-thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions and then extruded through polycarbonate filters (pore diameter of 100 nm) mounted in an extruder fitted with Hamilton syringes (Hamilton Company, Reno, NV). Samples were subjected to 11 passes through the polycarbonate filters to give the final LUV suspension. Background samples were prepared in the same way except that di-8-ANEPPS was not added to them. The optical density of the samples measured at 420 and 510 nm were less than 0.15 in all cases, which rules out any possibility of scattering artifacts. Samples were incubated in dark for 12 h at room temperature (~23 °C) for equilibration before measuring fluorescence. Experiments were performed with multiple sets of samples at room temperature (~23 °C).

2.2.3. Measurement of membrane dipole potential—Membrane dipole potential measurements were carried out by dual wavelength ratiometric approach using the voltage sensitive fluorescence probe di-8-ANEPPS (Gross et al., 1994; Clarke and Kane, 1997; Starke-Peterkovic et al., 2005, 2006; Haldar et al., 2012). Steady state fluorescence measurements were performed with a Hitachi F-7000 (Tokyo, Japan) spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (~23 °C). Excitation and emission slits with a nominal bandpass of 3 nm were used for all measurements. Background intensities of samples were subtracted from each sample to cancel any contribution due to the solvent Raman peak and other scattering artifacts. Fluorescence intensities were recorded at two excitation wavelengths (420 and 510 nm). Emission wavelength was fixed at 670 nm. The fluorescence ratio (R), defined as the ratio of fluorescence intensities at an excitation wavelength of 420 nm to that at 510 nm (emission at 670 nm in both cases) was calculated (Starke-Peterkovic et al., 2006). The choice of the emission wavelength (670 nm) at the red edge of the fluorescence spectrum has previously been shown to rule out membrane fluidity effects (Clarke and Kane, 1997). Dipole potential (ψ_d) in mV was calculated from R using the linear relationship (Starke-Peterkovic et al., 2005, 2006):

$$\psi_d = (R + 0.3)/(4.3 \times 10^{-3})$$

R values remained invariant after dilution of membrane samples, indicating the absence of any scattering artifacts (Lentz et al., 1979).

3. Results and discussion

We carried out dipole potential measurements in POPC membranes in the presence of cholesterol and its stereoisomers by a dual wavelength ratiometric approach using the voltage-sensitive styrylpyridinium probe, di-8-ANEPPS (Gross et al., 1994; Clarke and Kane, 1997; Starke-Peterkovic et al., 2005, 2006). The dual wavelength ratiometric technique using di-8-ANEPPS represents a popular approach to monitor membrane dipole potential (Gross et al., 1994; Clarke and Kane, 1997; Starke-Peterkovic et al., 2006). Since membrane dipole potential has its origin in nonrandom orientation of dipolar residues and the majority of these residues are localized in the membrane interfacial region, the ideal location of any probe reporting dipole potential should be interfacial. We previously showed, using the parallax method (Chattopadhyay and London, 1987), that the fluorescent styrylpyridinium group in di-8-ANEPPS is localized at the membrane interface, at a distance of ~12 Å from the center of the bilayer (Haldar et al., 2012). The fluorescence ratio (R) of di-8-ANEPPS is sensitive to any change in the dipolar field at the membrane interface where the probe is localized. This is believed to be due to an electrochromic mechanism. According to this mechanism, the spectral shift displayed by di-8-ANEPPS is related to the electric field strength. It should be mentioned that the fluorescence ratio (R) of di-8-ANEPPS has been shown to be sensitive to *only* dipole potential and is independent of specific molecular interactions (Gross et al., 1994; Robinson et al., 2011).

The effect of cholesterol and its stereoisomers on the dipole potential of POPC membranes is shown in Fig. 2. The figure shows that the dipole potential of POPC membranes is ~369 mV. The membrane dipole potential exhibits progressive increase with increasing concentration of cholesterol and reaches a value of ~521 mV (*i.e.*, increases by ~41%) in presence of 40 mol% cholesterol. This is in agreement with previous work by us (Haldar et al., 2012; Singh et al., 2013) and others (Starke-Peterkovic et al., 2006) in which it was shown that cholesterol increases dipole potential in membranes. In order to explore the extent of structural stringency of cholesterol in its ability to modulate membrane dipole potential, we monitored the effect of stereoisomers of cholesterol, *ent*-cholesterol and *epi*-cholesterol, on membrane dipole potential. The change in membrane dipole potential is drastically different for *ent*-cholesterol and *epi*-cholesterol (see Fig. 2). The membrane dipole potential increased up to ~480 mV (~30% increase) when 40 mol% of *ent*-cholesterol was used. The increase in membrane dipole potential is therefore comparable in cases of cholesterol and *ent*-cholesterol, although not exactly same. This is in overall agreement with the fact that *ent*-cholesterol shares identical physicochemical properties with cholesterol. In contrast to this, the membrane dipole potential reduces to ~338 mV in presence of 40 mol% *epi*-cholesterol, thereby exhibiting a modest (~8%) decrease in dipole potential. This drastic difference in the pattern of change of membrane dipole potential in case of *epi*-cholesterol

reinforces the different physicochemical properties of *epi*-cholesterol relative to cholesterol. Membrane dipole potential depends on a number of factors (Haldar et al., 2012). Although the molecular details underlying this difference in dipole potential (for cholesterol and *epi*-cholesterol) is not clear, it could be due to difference in sterol headgroup orientation (membrane tilt angle) along the bilayer normal.

Our overall goal in the measurement of dipole potential in membranes containing cholesterol and its stereoisomers was to explore the role of dipole potential in the mechanism of receptor-cholesterol interaction, and to assess its functional implication. Fig. 3 brings out the relevance of membrane dipole potential in the context of the activity of the serotonin$_{1A}$ receptor, a representative GPCR (Pucadyil et al., 2005), as measured by specific agonist ([^{3}H]8-OH-DPAT) binding. Fig. 3a shows that while *ent*-cholesterol could replace cholesterol in supporting the function of the serotonin$_{1A}$ receptor, *epi*-cholesterol could not (Jafurulla et al., 2014). These results imply that the requirement of membrane cholesterol for the serotonin$_{1A}$ receptor function is diastereospecific, yet not enantiospecific. Fig. 3b shows the correlation of membrane dipole potential with activity of serotonin$_{1A}$ receptors. A linear correlation was observed between these parameters with a correlation coefficient (r) ~0.99. The close correlation between membrane dipole potential and receptor activity is rather interesting. We conclude that membrane dipole potential could be a sensitive determinant of lipid-protein interactions in biological membranes.

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Abbreviations

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
HM	hippocampal membranes
LUV	large unilamellar vesicle
MβCD	methyl-β-cyclodextrin
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SM	solubilized membranes

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Highlights

- Cholesterol and ent-cholesterol increase dipole potential to comparable extent.
- In contrast, epi-cholesterol reduces membrane dipole potential.
- Membrane dipole potential and serotonin1A receptor activity are closely correlated.

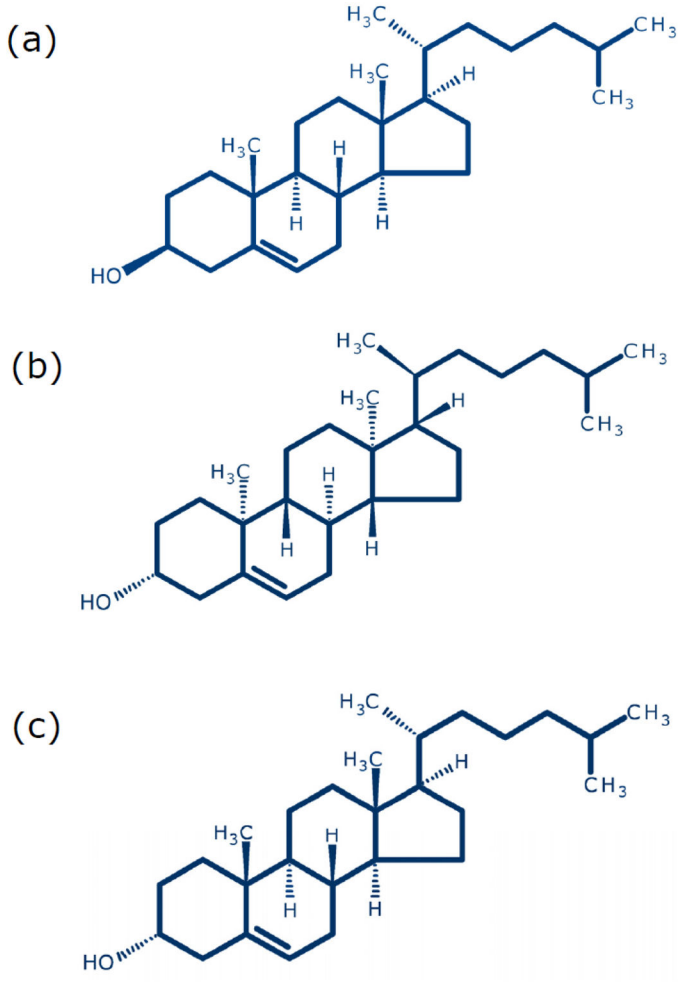

Fig. 1. Chemical structures of sterols used: (a) cholesterol, (b) *ent*-cholesterol and (c) *epi*-cholesterol

Both *ent*-cholesterol and *epi*-cholesterol are stereoisomers of cholesterol. *ent*-Cholesterol is the enantiomer of cholesterol. Enantiomers are non-superimposable mirror images of one another. *epi*-Cholesterol, on the other hand, is a diastereomer and is not a mirror image of cholesterol. *ent*-Cholesterol (but not *epi*-cholesterol) shares identical physicochemical properties with cholesterol. See text (section 1) for more details.

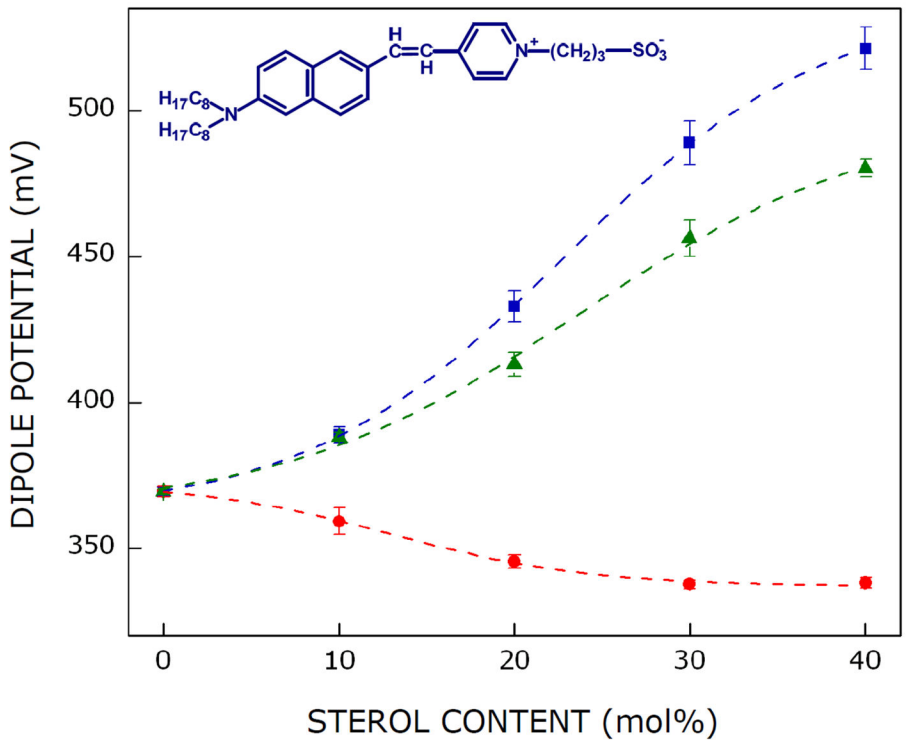

Fig. 2. Effect of stereoisomers of cholesterol on dipole potential of membranes

Dipole potential in POPC membranes plotted with increasing concentrations of cholesterol (■), *ent*-cholesterol (▲), and *epi*-cholesterol (●). Data points shown are means ± S.E. of at least three independent measurements. The ratio of di-8-ANEPPS to total lipid was 1:100 (mol/mol) and total lipid concentration was 0.43 mM. Measurements were carried out at room temperature (~23°C). Lines joining the data points are provided merely as viewing guides. The structure of voltage-sensitive probe di-8-ANEPPS is shown in the upper left side. See section 2 for details.

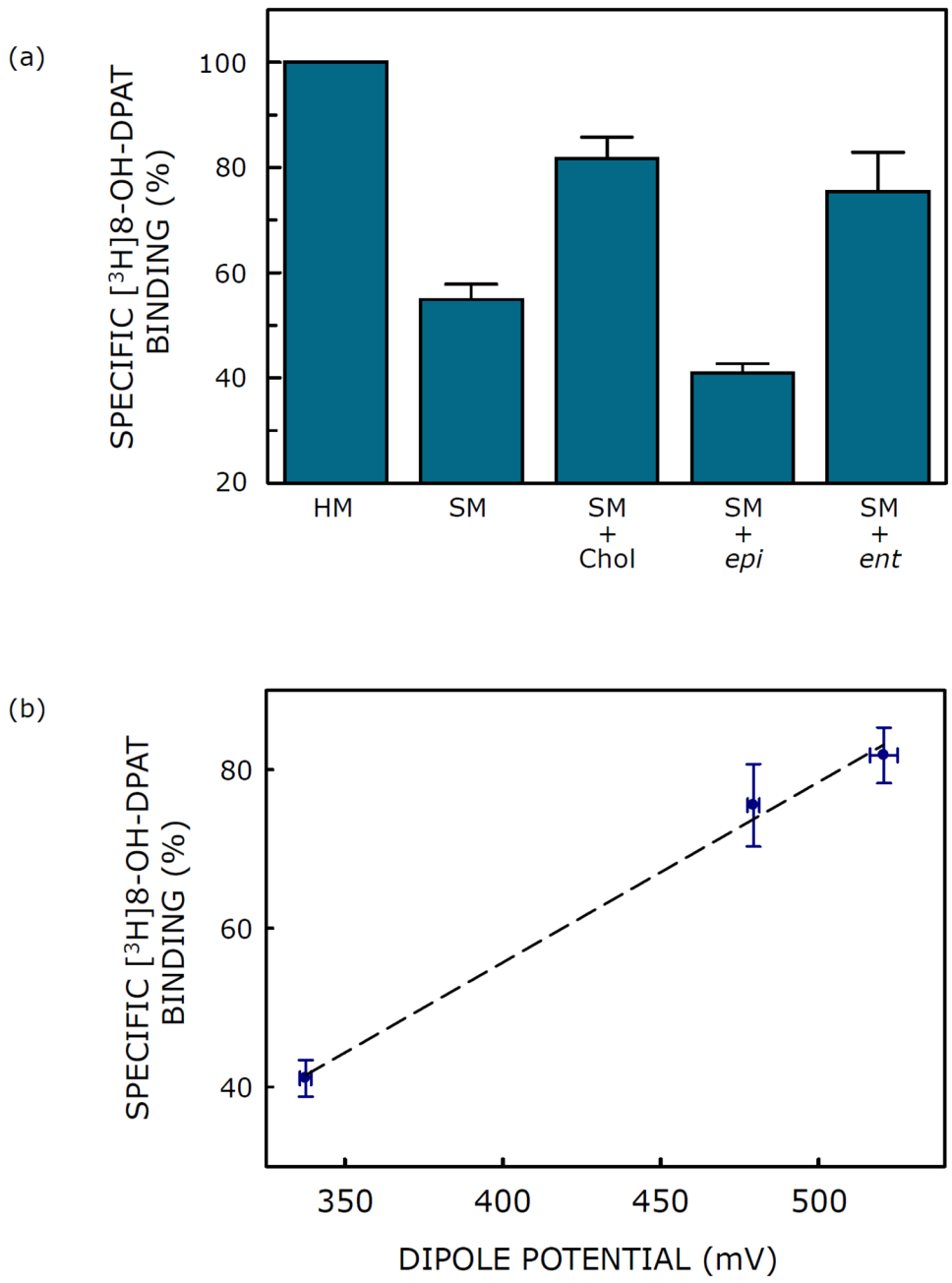

Fig. 3. Correlation of receptor activity with membrane dipole potential

(a) Effect of replenishment of cholesterol, *epi*-cholesterol (*epi*) and *ent*-cholesterol (*ent*) into solubilized membranes (SM) on specific binding of the agonist [^{3}H]8-OH-DPAT to the serotonin$_{1A}$ receptor. Solubilized hippocampal membranes were replenished with cholesterol, *epi*-cholesterol or *ent*-cholesterol using sterol:MβCD complex. Values are expressed as percentages of specific binding obtained in native hippocampal membranes (HM). Data shown are means ± S.E. of at least four independent experiments (taken from Jafurulla et al., 2014). (b) Correlation of membrane dipole potential with activity of serotonin$_{1A}$ receptors. Specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors (values

taken from Fig. 3a) and corresponding values of membrane dipole potential containing 40 mol% sterol (from Fig. 2) are shown. Linear regression analysis yielded a correlation coefficient (*r*) ~0.99. The tight correlation between membrane dipole potential and receptor activity is noteworthy. See sections 2 and 3 for more details.