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Membrane order and ionic strength modulation of the inhibition of the membrane-bound acetylcholinesterase by epigallocatechin-3-gallate

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Abstract

In the present work, we analyzed how external factors can modulate the efficiency of epigallocatechin-3-O-gallate (EGCG) inhibition of a membrane-bound isoform of the acetylcholinesterase. Increasing the ionic strength but not the osmolarity of the bulk medium proved to be an important factor. In addition, we verified a clear correlation between the inhibitory activity with the order degree of the membranes by using cholesterol-partially depleted red blood cell ghosts. These two factors i.e. high salt concentration in the bulk medium and less viscous membranes, allow a deeper insertion of the EGCG into the lipid bilayer, thus leading to a greater inhibition of AChE. As a corollary, we propose that any treatment or process that leads to a slight decrease in cholesterol content in the membranes can efficiently enhance the inhibitory activity of EGCG, which can have important consequences in all the pathologies where the inhibition of AChE is recommended.

Keywords: ionic strength, membrane interaction, cholesterol

1. Introduction

Acetylcholinesterase (AChE) is a serine hydrolase involved in the termination of cholinergic neurotransmission. The three main variants of AChE formed by alternative splicing share a common core of 543 amino acids and differ in their C-terminal sequences as well as in their cellular localization and subunit structures. AChE-S, the “synaptic” form, is the principal variant present in brain and muscle tissues. It may be present as four AChE-S monomers that are covalently linked to a three-stranded collagen-like tail (ColQ) to form a basal lamina-anchored tetramer at the neuromuscular junction. Even more, three of these tetramers could hybridize to make “asymmetric” dodecamers bound to membranes. Besides ColQ, the protein called PRiMA can also interact with AChE. Indeed, it attaches the S-derived tetramers to plasma membranes in cells [1]. In addition to AchE-S, the “readthrough” monomeric form (AchE-R), is expressed in embryonic and tumor cells and induced in response to psychological stress and AChE inhibition. Finally, the “erythrocytic” variant that is referred as AChE-E, forms dimers and is linked to red blood cells (RBC) membranes by a glycosylphosphatidylinositol anchor (GPI) [2].

Acetylcholinesterase inhibitors (AChEI) are clinically used to counteract various pathologies including myasthenia gravis, autoimmune autonomic ganglionopathy, postural tachycardia syndrome and the most notorious one, Alzheimer’s disease. Treatment with these compounds may alleviate symptoms by enhancing cholinergic functions and increasing the amount of acetylcholine present in cholinergic synapses. In addition to the classical role in modulating nerve impulse, non-cholinergic functions have been proposed, some of which may involve protein–protein interactions [3]. In Alzheimer’s disease, the hypothesis that AChE might bind β-amyloid and promote its deposition has been suggested [4]. Several compounds have been approved for the symptomatic treatment of these diseases. However, synthetic AChE inhibitors such as physostigmine, tacrine and donepezil have been reported to have adverse effects. Actually, tacrine that was the first inhibitor of its kind was discontinued some years ago [5,6]. Parkinson’s disease patients may also benefit from AChE inhibitors [7]. Last but not least, AChEI were proposed as a tool for combating leishmaniasis, a disease that globally affects millions of people. *Leishmania braziliensis*, the major etiologic agent cannot synthesize choline, which is essential for the synthesis of phosphatidylcholine. Therefore, growth and development

of the parasite may be affected by inhibiting AChE activity [8–10]. Thus, there is a big interest in the development of new AChE inhibitors and among other compounds, polyphenols have emerged as promising candidates [11].

Polyphenols are plant metabolites with phenolic groups in their structures. A number of health benefits have been described for polyphenols so far. Many of these compounds are well-known as potent antioxidants and strong metal chelators. However, they are not antioxidants just because they scavenge reactive oxygen species but instead they can protect from oxidative stress because they may activate signaling pathways and key protective enzymes [12]. Antimicrobial properties as well as antiallergic and anti-hypertensive activities have also been described. In addition, due to their abilities to inhibit ABC transporters associated to the efflux of xenobiotic compounds, anti-cancer properties were also reported [13,14].

Polyphenols were shown to interact not only with proteins but also with membrane lipids [15,16], which in turn may be associated with some of the activities mentioned above. For instance, we demonstrated that the stilbene resveratrol enhanced ABCG1 ATPase activity in a non-transcriptional way by interacting with the lipid bilayer where ABCG1 was inserted [17]. In the same trend, we were able to show that epigallocatechin-3-gallate (EGCG), a flavan-3-ol that is the major constituent of the green tea, interacted with the surface of both liposomes and human erythrocytes, thus enhancing its inhibition of AChE-E. Actually, resveratrol and EGCG had already been described as AChE reversible inhibitors and at the same time leishmanicidal agents [8,18]. Our main contribution was to show that EGCG was more efficient as AChE inhibitor when the enzyme was bound to membranes as compared to its soluble form. In fact, AChE-E has proved to be a good model for studying the enzymatic activity of membrane-bound AChE isoforms [19], hence this variant was chosen to carry out the present work.

The activity of AChE as well as the activities of a number of membrane-bound enzymes have been shown to depend on the membrane lipid composition and membrane order [20–23]. That is to say, membrane lipid alterations may induce changes in the activities of membrane-bound enzymes. The cholesterol content of RBC membranes is in equilibrium with unesterified plasma cholesterol, which is regulated by the enzyme lecithin-cholesterol acyltransferase [6,24]. Hence, unbalanced nutrition, hormonal disorders and some pathologies such as hypercholesterolemia,

may lead to alterations in plasma cholesterol levels and subsequently to alterations in cholesterol content of erythrocyte plasma membranes [25].

As commented above, we recently demonstrated that EGCG did inhibit AChE-E and it was more active on the membrane-bound enzyme. In our hands, EGCG did not get inserted into the lipid bilayer under the conditions tested. On the contrary, Cyboran et al. showed that EGCG may enter the hydrophobic core of membranes when isotonic buffers were used, unlike our experiments that were carried out in hypotonic buffer [26]. On this point, Kajiya et al. demonstrated that the incorporation of green tea catechins into lipid bilayers is stimulated by the increase of salt concentration and inhibited by the increase of negative charge of lipid bilayers [27]. Therefore, in the present work we studied the influence of the ionic strength on the AChE inhibition by EGCG. In other words, we aimed to know if the localization of EGCG in the membrane had influence on the degree of AChE inhibition. At the same time, since EGCG interacts with model membranes in a way dependent on the cholesterol content, i.e. the lower the cholesterol content in the membrane, the deeper it gets inserted, we analyzed the inhibition of AChE upon addition of EGCG when cholesterol level in erythrocyte membranes was reduced.

2. Materials and Methods

2.1. Reagents

Acetylthiocholine iodide (ATC), epigallocatechin-3- gallate (EGCG), quercetin, Triton X-100 and methyl-β-cyclodextrin (MβCD) were purchased from Sigma-Aldrich, Argentina. EGCG was dissolved in methanol, kept under nitrogen at -20ºC and its concentration was routinely checked by the Folin method, with quercetin as standard [28]. 5, 5’ -dithiobis-2- nitrobenzoic acid (DTNB) was purchased from ICN, octadecyl rhodamine B chloride (R18), N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Life Technologies, Argentina. Fluorescent probes were prepared as methanolic solutions and stored at -20ºC. All other reagents were of the highest purity grade commercially available.

2.2. Membrane preparation, solubilization and depletion of cholesterol content

Human blood was obtained by venous puncture using Na_2EDTA as anticoagulant (1 mg/mL blood) from healthy volunteers, residents of San Miguel de Tucuman (Argentina), after giving informed consent and signing consent documents according to our Institutional Ethics Committee. Blood samples were centrifuged (2,000 x *g*, 10 min), plasma and white cells were discarded and erythrocytes were washed with 10 volumes of 0.9% NaCl three times. Afterwards, erythrocytes were lysed with 30 volumes of hemolysis buffer (5 mM phosphate buffer, pH 7.4, containing 1 mM Na_2EDTA). Hemoglobin-free membranes or "ghosts" were obtained by repeated centrifugations at 10,000 x *g*, 20 min each cycle. All steps were performed at 0 - 4ºC. Even though ghosts can preserve full acetylcholinesterase activity for nearly 2 months when stored at 4ºC [20] , they were used within a week.

The solubilization of AChE from erythrocyte membranes was carried out by diluting membrane suspension 20 times with the same buffer containing the non-ionic detergent Triton X-100 at a final concentration of 0.04%. After gently mixing for 15 min at room temperature, the sample was centrifuged for 1 h at 10,000 x *g* at 4ºC and the pellet was discarded [29]. Cyclodextrins, oligomers of glucose, are widely known for the ability to solubilize lipids through the formation of molecular inclusion complexes. Particularly, methyl-beta-cyclodextrin (MβCD) is able to extract and solubilize cholesterol, among other molecules from biological and synthetic membranes [30,31]. The extraction of cholesterol from RBC membranes was performed by incubating equal volumes of ghost suspension and different concentrations of MβCD solution (3 mM and 0.75 mM of MβCD in 5 mM Tris-HCl buffer, pH 7.4) for 15 minutes at 37ºC with gently mixing. After this, cholesterol-depleted ghosts were obtained by repeated and exhaustive washing with 5 mM Tris-HCl buffer, pH 7.4 and centrifugation at 10,000 *x g* 20 min. All steps were performed at 0 - 4ºC.

2.3. Acetylcholinesterase inhibition by EGCG

The inhibition of AChE-E activity by EGCG was determined by the Ellman's method, using ATC as a substrate [32]. Briefly, ghost membranes, cholesterol-depleted ghosts and solubilized samples were diluted in different buffers (hemolysis buffer, hemolysis buffer containing 150 mM NaCl, hemolysis buffer containing 300 mM NaCl and hemolysis buffer containing 300 mM mannitol). All buffers also contained 0.33 mM DTNB. Then, 100 µM EGCG was added and

preincubated for 15 minutes at 37ºC. For control reactions, the addition of EGCG was replaced with the same volume of methanol. ATC was then added to the mixture and absorbance of the 5-thio 2-nitrobenzoate that was being produced was measured at 412 nm with a 10-sec intervals for 3 min at 37°C in an automated microplate reader (SpectraMax Plus 384). Parallel reactions performed without enzyme and/or without substrate were used to correct absorbance readings.

2.4. Fluorescence spectroscopy. Analysis of EGCG interaction with membranes

Possible changes in membrane order or fluidity induced by EGCG were estimated by steady-state fluorescence anisotropy using the fluorescent probe DPH. In this case, membrane fluidity refers to the degree in which phospholipid acyl chains conformation vary during the lifetime of the excited state of the probe i.e. DPH inserted into the hydrophobic core of the lipid bilayer [33]. Any interaction of EGCG with the hydrophobic core of the membrane bilayer would translate into changes in the membrane order that in turn might be detected as variations in the anisotropy values of DPH.

Fluorescent probes were added under constant vortexing to membrane suspensions in hemolysis buffer, in a 1:800 probe:phospholipid molar ratio. Samples were incubated at room temperature for 30 min in the dark. Fluorescence measurements of control conditions and treated samples with 100 µM EGCG were carried out using an ISS PC1 spectrofluorometer, L-format equipped with a thermostatized cuvette holder The steady-state DPH fluorescence anisotropy was determined adjusting the excitation and emission wavelengths at 350 and 450 nm, respectively. Steady-state anisotropy (r) was measured by using excitation and emission polarizers, and r was automatically calculated by the Vinci software (ISS). In addition to anisotropy values, total fluorescence intensities were also recorded, looking for possible quenching of the DPH fluorescence as another way to detect interactions of EGCG with red blood cell membranes [34,35]. Each measurement was done in triplicate.

Besides, for assessing the possible binding of EGCG to the phospholipid head groups at the erythrocyte plasma membrane, quenching of the R18 fluorescence was measured upon addition of EGCG [19,36]. R18 was added to membrane suspensions under constant vortexing,

at a 1:1,000 probe:phospholipid final molar ratio, in which high fluorescence intensity was achieved. R18 fluorescence emission spectra were recorded upon addition of EGCG from a 1 mM stock solution. Fluorescence measurements were carried out in an ISS PC1 spectrofluorometer equipped with a thermostatic cuvette holder set at 37ºC. Excitation wavelength was set at 520 nm (16 nm slit), while the emission spectra were taken from 550 nm to 650 nm (16nm slit).

2.5. Fourier-transform infrared spectroscopy analysis (FTIR)

As a first step, ghost membrane preparations were lyophilized in order to remove the water and then resuspended in deuterated water. Afterwards, FTIR absorption spectra of ghosts membranes, before and after the treatment with MβCD were collected in transmission mode, using a demountable liquid cell (Harrick Sci, NJ, USA) equipped with calcium fluoride windows and 56 μm PTFE spacers. The temperature of the cell was controlled by means of a circulating water bath and a custom made thermocouple for measuring the temperature of the cell. Measurements were undertaken in a Nicolet 5700 spectrometer (Thermo Nicolet, Madison, WI) equipped with DTGS detector, operated by the software provided (OMNIC) at 2 cm^{-1} spectral resolution and averaging 64 scans. For our experiments, approximately 20 μl of sample was deposited on the cell, equivalent to 20 mM of phosphate in the sample. Spectra of the buffer were obtained using the same conditions as the samples, in order to eliminate any signal attributable to buffer components by spectra subtraction. The spectra were analyzed by second derivative methods using NRC software in order to obtain the component bands. Band fitting was routinely carried out with SpectraCalc. The order of the hydrophobic core was assessed by following the evolution of the wavenumber of symmetric stretching of methylene (circa 2850 cm^{-1}) with temperature. The hydration level of the interfacial region of the membranes was studied by fitting the contribution of hydrogen and non hydrogen-bounded carbonyl groups to the stretching vibrational

mode (circa 1740 cm^{-1}). To verify the reproducibility and reliability of the spectral results, three independent ghosts preparations were analyzed.

2.6. Miscellaneous

Phospholipid concentration in membranes was estimated by the Ames method [37]. Protein was determined by the Bradford assay [38]. Cholesterol concentration in membranes was determined using a commercially available kit (Colestat enzimatico, WienerLab, Argentina).

3. Results

3.1. Acetylcholinesterase inhibition by EGCG.

EGCG inhibited the activity of the membrane-bound form of AChE-E at the concentrations tested. As can be seen in Fig. 1, a greater inhibition was observed upon increasing salt concentration, attributable to ionic strength because no significant change in inhibition was detected with the increase in osmolarity obtained with mannitol. However, the solubilized AChE-E displayed a different pattern since no correlation between the inhibition of soluble AChE-E and the ionic strength was observed. This result may be related to the greater membrane insertion of EGCG when buffer contains more salt as it was proposed by Cyboran et al. (see discussion and below) [26].

As it was demonstrated by our group in a previous work [15], cholesterol content of model membranes also influences EGCG localization in the lipid bilayer. The lower the cholesterol content the deeper localization in the membrane of the polyphenols. The different penetration of EGCG in membrane ghost may affect the way it interacts with the AChE-E. Thus, the cholesterol content of ghosts was decreased by treating them with increasing concentrations of MβCD. Cholesterol content was routinely checked after each treatment by a colorimetric assay based on cholesterol oxidase method (Table 1). The inhibition of AChE-E bound to ghost membranes containing different cholesterol levels is shown in Fig. 2. The reduction in cholesterol content did enhance the inhibition of the enzymatic activity by EGCG. This result led us to propose that the reduction of membrane cholesterol content might allow EGCG to have a stronger association with membranes.

3.2. Modulation of membrane order degree in the presence of EGCG

The fluorescent probe DPH was used to label the hydrophobic core of the lipid bilayer allowing the detection of any change in the order degree of this region by measuring the fluorescence anisotropy. As a first step, the ionic strength of the bulk medium was modified by adding NaCl to the working buffer. When ghosts were suspended in a hypotonic NaCl-free buffer, EGCG slightly changed the ordering of membranes. However, if ghosts were suspended in 150 mM NaCl, a reduction of the anisotropy of control membranes was observed. The addition of EGCG reversed this reduction, inducing an increase of the membrane order, strongly suggesting an important interaction between this polyphenol and the lipid bilayer. Since the enhancement of EGCG effect had been found to be more evident at 300 mM NaCl, this concentration of salt was also tested. Even though there was no change in the membrane order in this condition in the absence of polyphenol as compared to the control, a more pronounced increase of the anisotropy was detected upon addition of EGCG (Fig. 3A and 3B). As expected, MβCD treatment did lower the membrane order as compared to untreated membranes. The addition of EGCG to cholesterol-partially depleted ghosts increased the DPH fluorescence anisotropy values (see Fig. 4A and 4B). The cholesterol extraction from ghosts allowed a deeper penetration of EGCG beyond the interphase and well into the hydrophobic core, as discussed below. Probably, due to its structure, EGCG displayed an ordering/ rigidifying effect on these membranes.

3.3. Association of EGCG to erythrocyte membrane surface

For evaluating the possible binding of EGCG to the phospholipid head groups at the erythrocyte plasma membrane, quenching of the R18 fluorescence was measured upon addition of EGCG. The localization of the probe is such that the rhodamine head group situates at the interfacial region while the hydrocarbon moiety penetrates into the hydrophobic core of the membrane [39].

The addition of NaCl was an important factor that allowed a more efficient quenching of the R18 fluorescence upon addition of EGCG. In this way, it is clear that this catechin was more readily associated to the membrane and to the interface when the ionic

strength was higher (Fig 5A). As a control, R18 fluorescence quenching was also tested with an AChE-solubilized form. No changes in fluorescence was detected in the whole range of EGCG concentrations studied (data not shown).

The R18 measurements were repeated with membranes containing lower concentrations of cholesterol. As it can be observed from Fig. 5B, the degree of quenching was related to the cholesterol content, i.e. the lower the cholesterol content, the higher the quenching of R18 fluorescence. Once again, it can be proposed that a lower cholesterol content in membranes may allow an enhanced penetration of EGCG in the lipid bilayer. In this way, the R18 dye becomes more accessible to EGCG which acts as the quencher, resulting in higher values of K_{SV}.

The incubation with either NaCl or MβCD might induce a differential release of proteins from ghosts, which in turn could potentially affect the extent of binding and insertion of EGCG. Therefore, the possible release of proteins was studied. Even though some proteins were solubilized from the membranes upon incubation during the time frame of the experiment, the bands observed in the SDS-PAGE were the same in all cases and the percentage of protein released from the ghosts was similar under all the conditions tested (approximately 5% of total ghost protein). Besides, no correlation between the released protein concentration and the EGCG effect on AChE was found (Supplementary information, Fig. S1 and Table S1). More importantly, the incubation with MβCD and/or NaCl did not released AChE from the ghosts. In fact, no AChE activity was found in the filtrates since TNB^{-2} anion was not generated from DTNB even after incubation times as long as 2 h. This result indicates that the greater binding and insertion of EGCG to erythrocyte membranes in the presence of increasing concentrations of NaCl or upon partial depletion of cholesterol are not due to significant loss of proteins from cytoskeleton or other membrane-associated proteins.

3.4. FTIR analysis of red blood cell membranes

We studied the IR spectra of ghosts before and after cholesterol extraction with MβCD and before and after the treatment and pre-incubation with EGCG. As shown in Fig. 6 A, the FTIR absorption spectra of both ghosts are complex since samples include a variety of molecules such as mix of different lipids, proteins, carbohydrates and, when added, the catechin itself. As indicated in Fig. 6A, the stretching vibrations of methylene and methyl groups of phospholipid hydrocarbon tails dominate the spectral range of 3,050–2,800 cm^{-1}, whereas ester carbonyl absorption of lipids is detected around 1,740 cm^{-1} [40,41], and the Amide I band of proteins dominates the spectrum between 1,700 and 1,600 cm^{-1} (C=O stretching and the NH bending of the peptide bond) [42,43]. Even though the Amide I band is sensitive to the protein secondary structure, no interpretation was made in the present work because of the wide range and number of different membrane proteins present in the membrane of erythrocytes. The analysis of the infrared spectra revealed that EGCG perturbs membrane properties in ghosts with low cholesterol content. The detection of these changes became evident after the analysis of the second derivative spectra, which allowed the resolution of the overlapping components of the IR absorption bands. As lipid absorption in the mid IR was very complex since many different lipid moieties were also involved, we focused on the absorption of methylene and methyl stretching (at ~2,921, 2,851 cm^{-1}) and carbonyl stretching (at ~1,740 cm^{-1}) vibrational modes.

Table 2 shows that the CH_2 symmetric stretching vibration significantly changed after the treatment with EGCG. In particular, the down-shift in ghosts treated with MβCD, from 2,851.4 to 2,850.93 cm^{-1}, indicates that a higher degree of acyl chain packaging is induced by the presence of EGCG. This effect was lower in the case of untreated ghosts, i.e. with normal level of cholesterol. These results are in agreement with the fluorescence anisotropy measurements commented above. At first sight, the ordering of the low cholesterol ghosts by EGCG does not seem to be an important change in the packaging of hydrocarbon tails of lipids based on the small change in wavenumbers.

However, this change is in the same order of magnitude than those observed in control ghosts and in ghosts treated with MβCD in the absence of EGCG, when temperature increases from 15 to 45ºC (see Supplementary information, Fig. S2). Thus, EGCG exerts a greater perturbation on the degree of packaging of phospholipid tails when cholesterol level is lower. Surprisingly, despite the fact that EGCG perturbs the hydrophobic core of the lipid bilayer, only minor changes on the hydration level of the interfacial region of ghosts membranes was observed, as slight increase in the contribution of hydrogen compared to non hydrogen-bounded carbonyl groups to the stretching vibrational mode (see Supplementary Information, Table S2) was obtained. It can be proposed that hydroxyl groups from the catechin may replace water molecules as hydrogen donors, as the highest increase is in the presence of EGCG in cholesterol depleted ghosts. The detection of these changes became evident after the analysis of the second derivative spectra, since it allowed the resolution of the overlapping components of the IR absorption bands by undertaking curve fitting.

Apart from that, what was even more interesting was the finding of a band in the spectra attributable to the EGCG. This band, placed at approximately 1,700 cm^{-1} was assigned as the carbonyl stretching vibration mode of the EGCG (see Fig. 6C and Fig. 6E) [44]. The position of such band in samples containing EGCG and membranes is shifted towards higher wavenumbers from its position when compared to EGCG alone, either dried or hydrated with D_2O (see Fig. 6F). Therefore, the catechin carbonyl groups are non-hydrogen bonded in the presence of red cell ghosts, confirming that EGCG readily partition into membranes.

4. Discussion

In the present work we demonstrated that the inhibition of the AChE activity by EGCG could be modulated by the ionic strength. In fact, not only the degree of inhibition by EGCG but also the enzymatic activity itself changed when varying the ionic strength.

As a matter of fact, this latter finding is in agreement with several reports in the literature that described how salts can enhance the activity of AChE from various sources [45, 46]. The substrates acetylcholine and acetylthiocholine are cationic compounds, while the active site of AChE has several anionic residues. Therefore, salts can modulate the interaction of them and ultimately the kinetics of the reaction. Besides the considerations of the charges in the enzyme itself, it is tempting to speculate that salt bridges that are formed between some membrane components and the enzyme may reduce the activity when working at low salt concentrations. On the contrary, increasing the NaCl to physiological levels may disrupt those interactions and let the enzyme work in full activity. Actually, it had been reported that AChE can form self-aggregates in media with low salt concentrations [45]. Furthermore, Wille et al. have found that the buffer system used for measuring AChE activity can be a major factor that determines the efficiency by which this enzyme hydrolyzes its substrate [47]. EGCG is not a charged molecule, thus the direct association of this polyphenol with AChE would not be influenced by the ionic strength. However, the stronger interaction of EGCG with membranes when salt concentration is raised could allow this flavonoid to approach more efficiently to AchE-E. When the enzyme was solubilized upon addition of Triton X-100 a higher activity was also seen, but no greater inhibition was observed in the presence of EGCG. Therefore, these two events are independent from each other.

The addition of EGCG slightly increased the order of the membranes in control ghosts and this effect was more pronounced when NaCl was present. It may be reasonable to speculate that a better membrane insertion can help EGCG to interact more efficiently with the enzyme. On the contrary, an indirect inhibition of AChE because of the changes in the membrane order induced by EGCG could be ruled out. In fact, it has been previously reported that membrane composition, i.e. double bond index, does not

affect the activity itself but only the allosteric properties of non competitive inhibition [23].

In addition, since cholesterol is a well-known modulator of the membrane order, we decided to study the possible inhibition of AChE by EGCG in membranes containing lower concentrations of cholesterol. We found that the lower the membrane order the greater the inhibition measured, which was correlated with a greater insertion of EGCG in membranes, as it could be inferred from the increased fluorescence anisotropy that was induced by EGCG in partially-depleted cholesterol membranes. We hypothesize that any change that leads to efficient interaction/insertion of EGCG with membranes will enhance the inhibition of AChE. It has been previously reported that cholesterol content of red blood cells is in equilibrium with free cholesterol in plasma [6,24,48], thus any therapy or regime that lowers cholesterol may have an impact on the inhibitory activity of EGCG. The central nervous system has an independent pool of cholesterol that does not depend on the diet but it may be altered by some drugs like the statins, which inhibit the endogenous synthesis of cholesterol [48]. Even though it has been demonstrated that statins do not alter AChE activity [49], the results presented in this manuscript may suggest that a combination of statins and polyphenols such as EGCG can be of interest in the palliative treatment of Alzheimer's disease, because only little changes in cholesterol would be needed for enhancing the EGCG inhibitory activity. However, *in vivo* and clinical studies will be needed in order to corroborate the efficacy of such combinations.

Conflict of interest

The authors declare that they do not have conflicts of interest.

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Table 1. Cholesterol depletion by MβCD

	protein (μg/μL)	phosphate (nmol/μL)	cholesterol (nmol/μL)	nmol chol/μg protein	nmol chol/nmol phosphate
control	4.80 ± 0.19	5.00 ± 0.20	6.70 ± 0.33	1.40 ± 0.12	1.34 ± 0.12
MβCD 0.75 mM	4.40 ± 0.22	4.00 ± 0.16	5.30 ± 0.18	1.20 ± 0.10	1.32 ± 0.10
MβCD 3 mM	4.30 ± 0.26	4.00 ± 0.21	2.80 ± 0.17	0.65 ± 0.08	0.70 ± 0.08

Table 2. Position of the symmetric and asymmetric stretching vibrational mode of ghost membranes with different cholesterol content and treated with EGCG

	Wavenumber [cm^{-1}]	
Sample	**v symmetric CH2**	**v asymmetric CH2**
Ghost Control	2851.7	2922.9
Ghost Control + EGCG	2851.5	2922.4
Ghost MβCD	2851.4	2922.4
Ghost MβCD + EGCG	2850.9	2921.4

Figure legends

Fig. 1. EGCG inhibition of acetylcholinesterase is enhanced by ionic strength. The membrane-bound form (A) and the solubilized form (B) of AChE-E were diluted in working buffer containing either 0 mM, 150 mM, 300 mM NaCl or 300 mM mannitol, as described in Materials and Methods and erythrocyte acety-cholinesterase activity was measured by Ellman´s method in the presence (gray bars) or the absence (black bars) of 100 µM EGCG. Experiments were performed at least three times and in triplicate; values are expressed as mean ± standard deviation.

Fig. 2. Inhibitory activity of EGCG is also influenced by membrane cholesterol content. RBC ghosts were treated with either 0, 0.75 mM or 3 mM MβCD, as described in Materials and Methods. After that, cholesterol content was measured in each sample (table S1). Finally, AChE-E activity was assessed by Ellman's method in the presence (gray bars) or the absence (black bars) of 100 µM EGCG. The shown values are mean ± standard deviation and were determined at least three times and in triplicate.

Fig. 3. Membrane order is increased upon interaction with EGCG with ghost membranes. Erythrocyte ghosts were labeled with the fluorescent probe DPH and diluted in working buffer containing either 0 mM, 150 mM and 300 mM NaCl (plain bars), then EGCG was added to a final concentration of 100 µM (stripped bars) and DPH fluorescence anisotropy (r) was measured at 37ºC (A). For clarity, the Δpolarization (Δp) is also shown (B), where $\Delta p = p_{EGCG} - p_{control}$. The results shown are representative of at least five independent measurements.

Fig. 4. Membrane packaging induced by EGCG was enhanced in membranes with lower cholesterol content. RBC membranes were pre-treated with 3 mM MβCD during 15 min at 37ºC and DPH anisotropy was measured at 37ºC in the presence (stripped bars) and the absence

(plain bars) of EGCG (A). ΔPolarization (Δp) for this system is also shown (B). The results shown are representative of at least five independent measurements.

Fig. 5. Ionic strength and cholesterol content regulates the binding of EGCG to membrane. R18 was added to membrane suspensions at a 1:1000 probe: phospholipid molar ratio. R18 fluorescence emission was recorded upon addition of different concentrations of EGCG and fluorescence in each sample was expressed as relative to the R18 fluorescence in the absence of EGCG. A) Membranes diluted in NaCl-free buffer (●), membranes diluted in 150 mM NaCl (■). Besides, membranes were treated with different concentrations of MβCD and R18 fluorescence was determined after the addition of EGCG. In this case, Stern-Volmer plots are presented (B). Regression analysis was perform using GraphPad Prism software ($P < 0.001$) and KSV values were calculated from linear regression: control without dextrin (●), KSV value 0.0842±0.0053; membranes treated with 0.375 mM MβCD (■), KSV value 0.0996±0.0041; membranes treated with 0.75 mM MβCD (▲), KSV value 0.1206±0.0048; membranes treated with 1.5 mM MβCD (▼), KSV value 0.1206±0.0048; membranes treated with 3 mM MβCD (◆), KSV value 0.1474±0.0065 and membranes treated with 6 mM MβCD (⬣), KSV value 0.1938±0.0035. Excitation wavelength was set at 540 nm, whereas the emission spectra were taken from 560 nm to 650 nm.

Fig. 6. Infrared spectra of ghosts with different cholesterol content in the presence and the absence of ECGC. Main vibrational modes are shown in A) in a buffer subtracted spectrum. Carbonyl and amide I' regions from ~1,800 to 1,500 cm^{-1} (C-F) were analyzed by curve fitting due to band overlapping of different vibrational modes. Relative contribution of hydrogen and non-hydrogen bonded (red band and blue band) carbonyl groups of control ghosts (C) and MβCD-treated ghosts (E) alone and upon incubation with EGCG (D and F, respectively). The maximum of the EGCG carbonyl is shown in solution (B) and in the presence of membranes containing low (D) or high (F) cholesterol content.

Highlights

- Ionic strength not only increases AChE activity but also enhances the inhibitory effect of EGCG
- Partial depletion of cholesterol also favors the inhibition of AChE mediated by EGCG
- Both the increase of the ionic strength and the reduction of membrane cholesterol induce a deeper interaction of EGCG with red blood cell ghosts

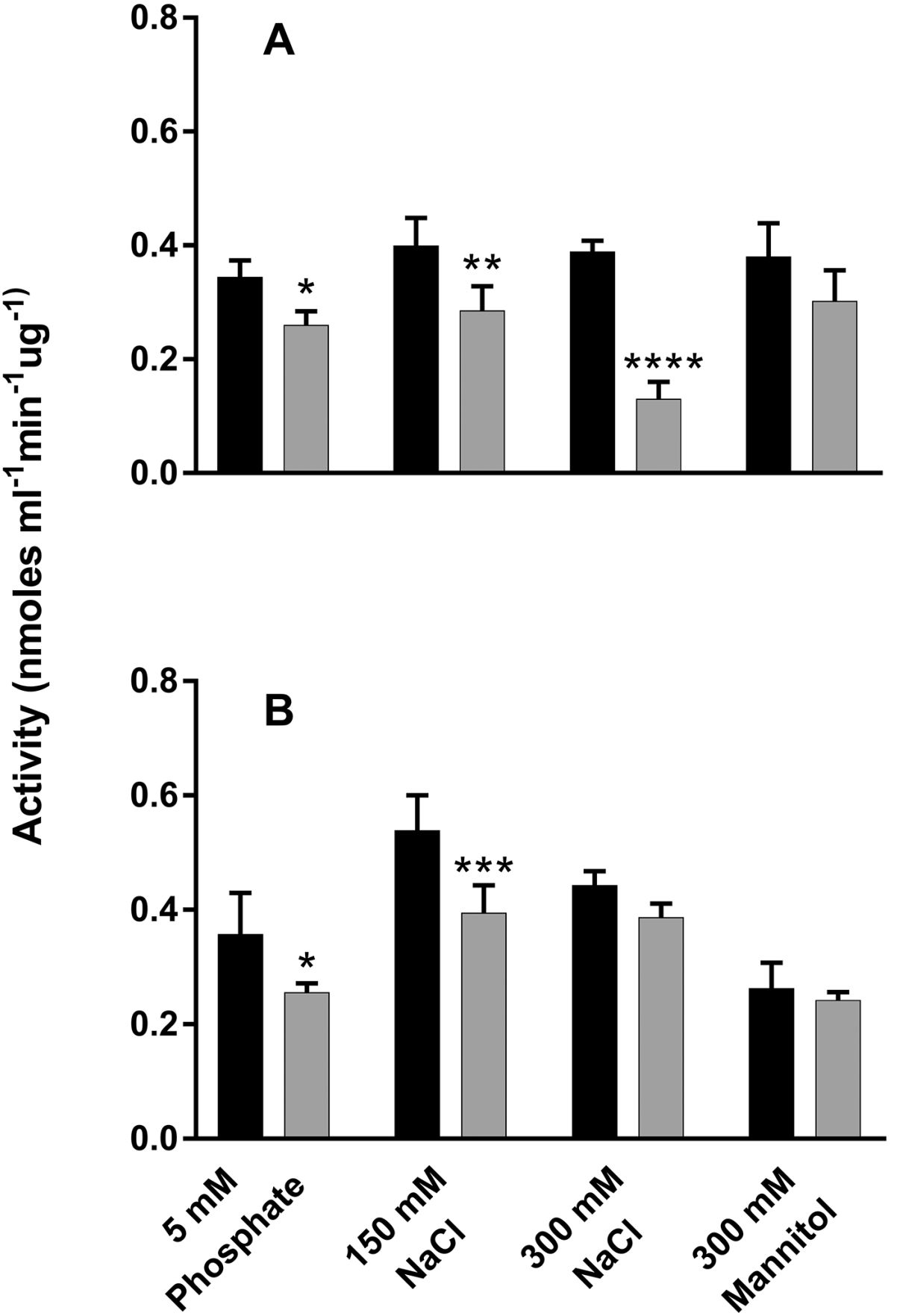

A
B
0.8
0.6
0.4
0.2
0.0
*
**
****
***
Activity (nmoles ml-1min-1ug-1)
5 mM Phosphate
150 mM NaCl
300 mM NaCl
300 mM Mannitol

Figure 1

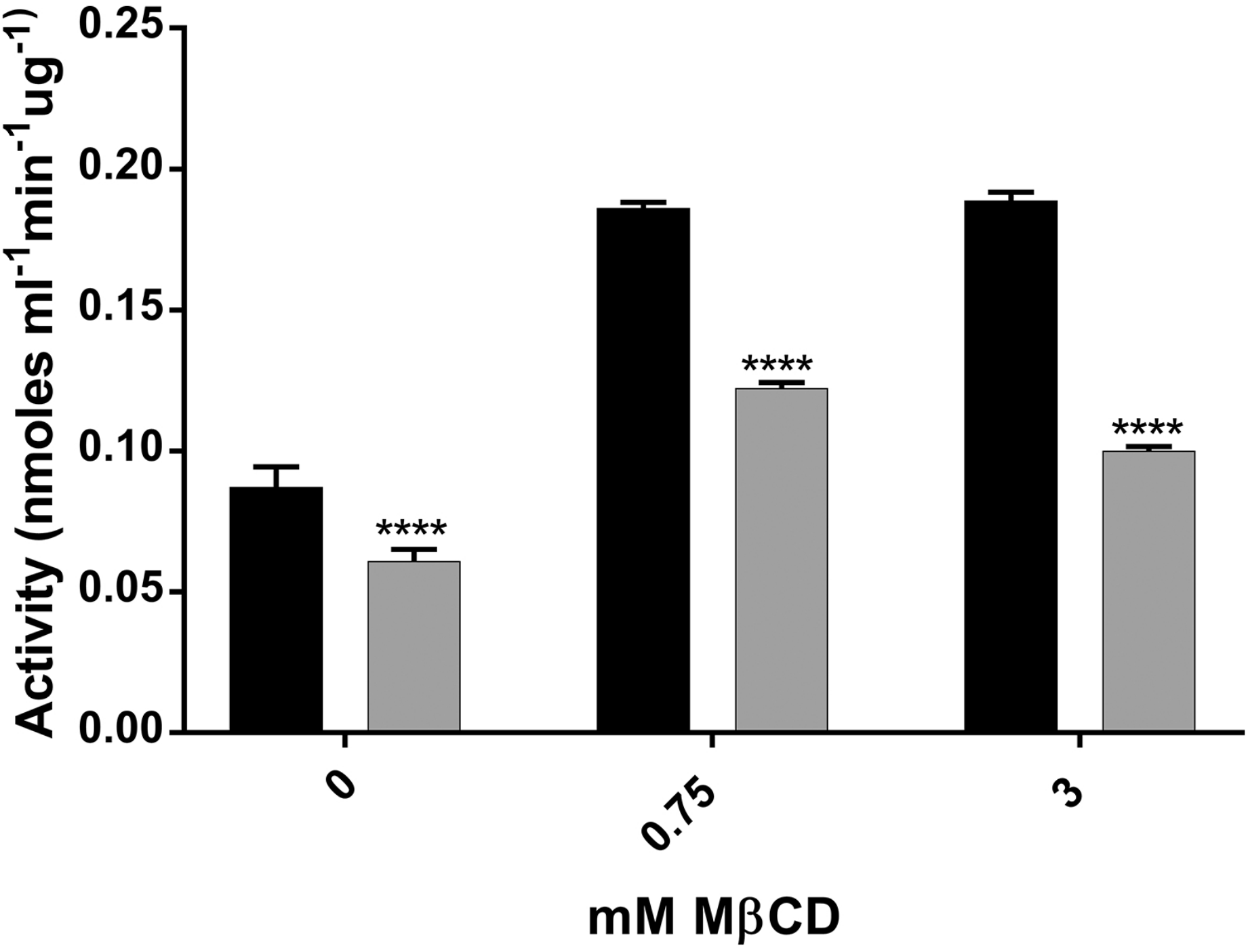

Activity (nmoles ml-1min-1ug-1)
0.25
0.20
0.15
0.10
0.05
0.00
****
****
****
0
0.75
3
mM MβCD

Figure 2

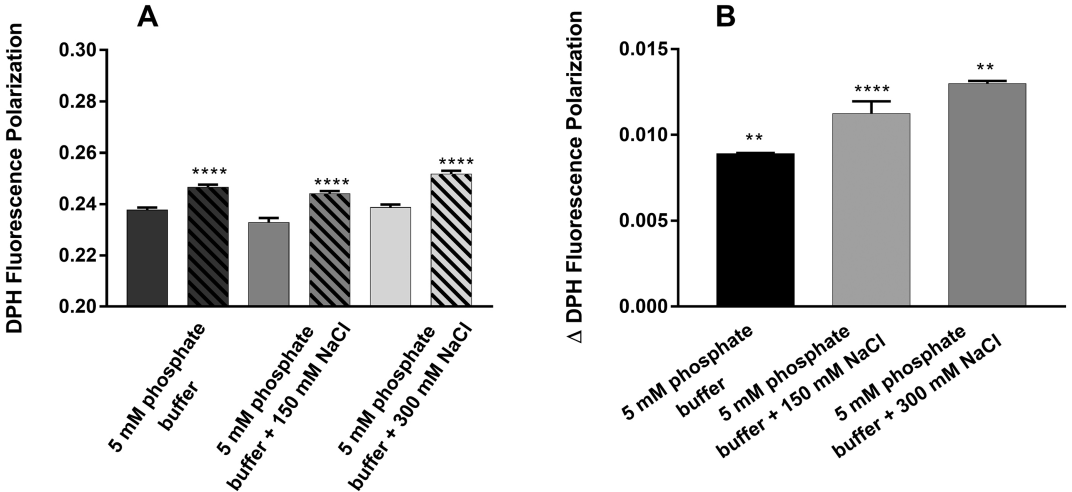

A
DPH Fluorescence Polarization
0.30
0.28
0.26
0.24
0.22
0.20
****
****
****
5 mM phosphate buffer
5 mM phosphate buffer + 150 mM NaCl
5 mM phosphate buffer + 300 mM NaCl
B
Δ DPH Fluorescence Polarization
0.015
0.010
0.005
0.000
**
****
**
5 mM phosphate buffer
5 mM phosphate buffer + 150 mM NaCl
5 mM phosphate buffer + 300 mM NaCl

Figure 3

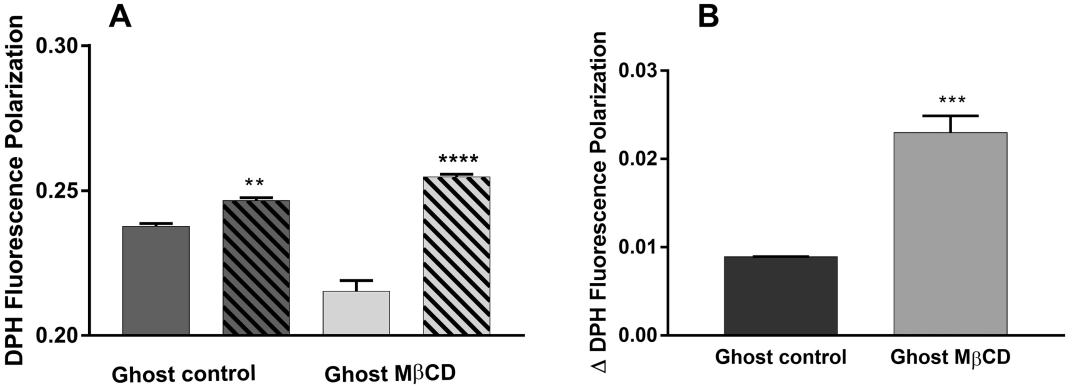
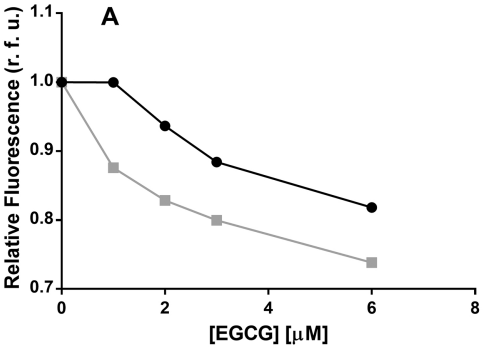

A
DPH Fluorescence Polarization
0.30
0.25
0.20
**
****
Ghost control
Ghost MβCD
B
Δ DPH Fluorescence Polarization
0.03
0.02
0.01
0.00
***
Ghost control
Ghost MβCD

Figure 4


A
Relative Fluorescence (r. f. u.)
1.1
1.0
0.9
0.8
0.7
0
2
4
6
8
[EGCG] [μM]

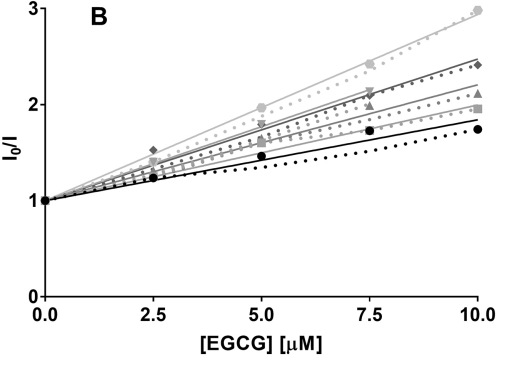

B
I0/I
3
2
1
0
0.0
2.5
5.0
7.5
10.0
[EGCG] [μM]

Figure 5

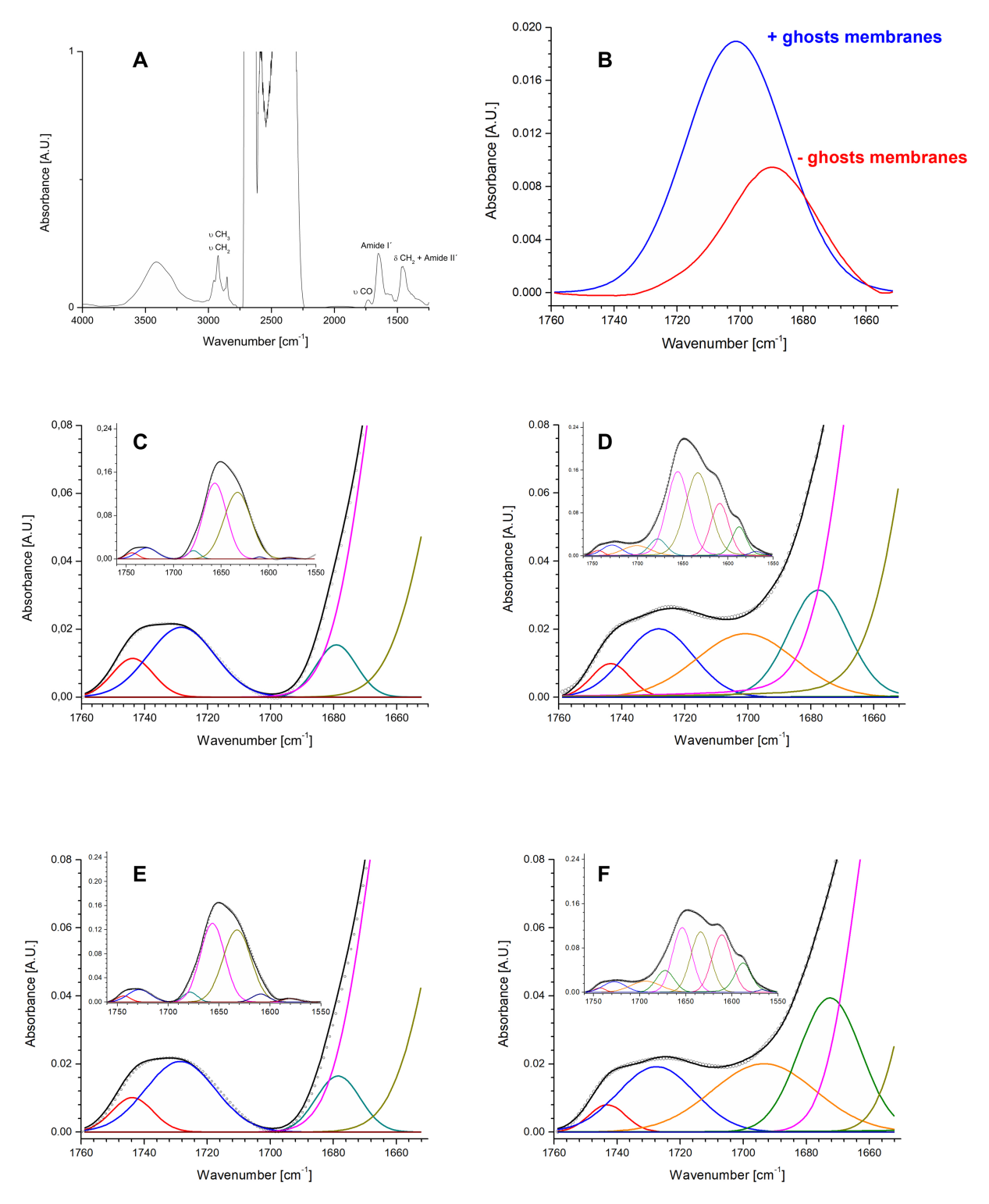

A
Absorbance [A.U.]
Wavenumber [cm-1]
υ CH3
υ CH2
Amide I´
δ CH2 + Amide II´
υ CO
B
+ ghosts membranes
- ghosts membranes
Absorbance [A.U.]
Wavenumber [cm-1]
C
D
E
F

Figure 6