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On the mechanisms of galantamine interaction with artificial lipid bilayers

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Abstract

The influence of galantamine (GAL), a natural alkaloid, on the lipid bilayers was investigated using the solid supported membrane method. This method allows the investigation of artificial lipid membranes while one can rapidly change the concentration of various components in the buffer above the membrane. Concentration jumps of sodium salts solutions of Hofmeister series anions were applied to artificial membranes having various lipid compositions, in absence or presence of galantamine. New information about GAL interaction with lipid membranes was obtained. The amplitude of the capacitive signals increases as GAL concentration increases for all the Hofmeister anions studied in a manner that depends on the lipid bilayer composition. These results seem to confirm the hypothesis that GAL influences the electrical behavior of the membranes through binding (either insertion in the bilayer or attachment at its surface).

Keywords: galantamine; lipid membranes; SSM; Hofmeister anions; diphytanoyl phosphatidylcholine (DPPC); 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DOPG); cholesterol; 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP);

Introduction

Galantamine (GAL), an alkaloid from snowdrops is a natural substance that exhibits beneficial effects in the treatment of some neurodegenerative disorders that involve nicotinic neural pathways (WILCOCK [1], CUMMINGS [2], ROCKWOOD & al. [3], WOODRUFF-PACK & al. [4], SANTOS & al. [5], DAJAS-BAILADOR & al. [6], LOPES & al. [7]). Recently we have found that galantamine modulates the recovery from ACh-induced desensitization, an effect which is concentration dependent and non-linear (POPESCU & al. [8]). Nevertheless, the precise mechanisms of GAL effects at the level of nicotinic receptor are not elucidated so far, and neither was possible to establish the precise location of galantamine binding site [8]. On the other hand, previous researches (e.g. HODGKIN & HOROWICZ [9], RYCHKOV & al. [10], CLARKE & LUEPFERT [11], GANEA & al. [12]) have shown that the various charged species are able to influence the membrane-related physiological processes either by modifying the membrane dipole moment or by disorganizing the lipid bilayer in the vicinity of various proteins embedded in it. Thus, it would be possible that the action of GAL at the level of nervous system, and in particular on the nicotinic receptor, could be due not only to specific interactions with the receptor itself, but also to non-specific interactions with the lipid membrane, such as GAL attachment and/or insertion in the lipid bilayer, leading to modifications of various membrane parameters (e.g. membrane fluidity, dipole moment etc.) and influencing thus the functionality of membrane proteins. In a recent study, using BLM (Black Lipid Membrane) method (IFTIME & al. [13]) we have shown that the electrical parameters of the lipid bilayer are modified when GAL at various concentrations were added to it. The results of this study suggested that the mechanisms underlying the above mentioned effects imply not only the insertion of GAL in bilayer but other mechanisms could be involved as well, e.g.: interactions at the interface lipid

- GAL - containing solution, leading to modifications in membrane architecture and/or dipole moment; or the attachment of GAL molecules to the lipid bilayer, more important for the negatively charged lipids [13]. In the present paper we took a step farther in investigating the interaction of GAL with artificial lipid bilayers by means of another electrophysiological method, namely the SSM (*solid supported membrane*) method. In comparison to the BLM method, the SSM method has the advantage of a higher membrane stability and, moreover, allows a rapid exchange of solutions at membrane surface giving the possibility of studying not only the stationary phenomena but the transient ones as well (SEIFERT & al. [14], PINTSCHOVIUS & FENDLER [15]). Recently the SSM method has been used to study the ion binding to a lipid membrane (GARCIA-CELMA & al. [16]). The authors have shown that the ions belonging to Hofmeister series are attracted to the membrane independent on the membrane composition and that this general trend is modulated by electrostatic interactions of the ions with the lipid head group charge (GARCIA-CELMA & al. [16]). Taking into account their findings and our own results obtained by means of BLM method (IFTIME & al. [13]), the effects of increasing GAL concentrations on artificial lipid bilayers have been studied indirectly, by monitoring the modifications induced in the amplitude of electrical signals elicited by concentration jumps of Hofmeister anions against a NaCl solution taken as inactive.

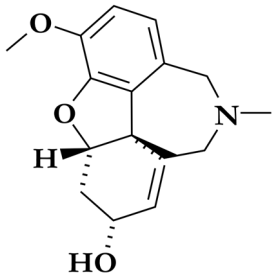

Figure 1. Galantamine structure

Materials and method

SSM method and measuring procedure

The SSM method is a relative novel electrophysiological procedure that allows the monitoring of electrical parameters of artificial lipid membranes and the study of membrane proteins either in natural membrane fragments or reconstituted in proteoliposomes (for recent reviews see TADINI-BUONINSEGNI & al. [17], GANEA & FENDLER, [18]). This technique allows a rapid exchange of solutions at membrane surface, such as the contact of solution with the bilayer takes place only in very short time interval (SEIFERT & al. [14], PINTSCHOVIUS & FENDLER [15]). The SSM consists of an alkanethiol monolayer covalently bound to a gold surface (150 nm thickness) deposited on a glass support (1 mm thickness) via the sulphydryl group, with a lipid monolayer on top of it in order to obtain a double layer. The hybrid alkanethiol / phospholipid bilayer is obtained in two sequential self-assembly steps, as described by PINTSCHOVIUS & FENDLER [15]. The lipid membrane formed has an area of 1-2 mm^2. The SSM is then fixed in a special plexiglas cuvette, which allows the activating and non-activating solution to flow through. The cuvette has an inner volume of 17□l. The electrical connection to the membrane electrode is made by a metal plate pressed on the gold surface of the SSM and connected to an amplifier. The counter-electrode is an Ag/AgCl electrode separated from the solution by a salt bridge. The electrodes are connected to an external electrical circuit. The substrates containing solutions are driven by means of a system of tubes and valves to the SSM under a constant pressure of ca. 0.6 bar.

Further details about the set-up can be found in PINTSCHOVIUS & FENDLER [15]. After the formation of SSM, the capacitance and the conductance values, which become constant after a waiting time of cca 90 min, are measured. The usual values range between 300-500 nF/cm^2 for capacitance and between 50-100 nS/cm^2, for the conductance. A typical solution exchange protocol consists of three steps: 1) washing the cuvette with the non-activating solution (2 s), 2) activation (concentration jump, 2 s) and 3) deactivation and cleaning (2 s) with the non-activating solution. The system is checked for artifacts generated by solution exchange when the buffer used in the experiment has been taken as both activating and non-activating solutions. If the artifacts are absent or negligible, only the baseline will appear on the monitor, indicating the absence of charge displacements. A concentration jump of the active solution yielding a capacitive signal will indicate an electrogenic activity at the membrane-electrode system.

Chemicals

The standard buffer solutions contained 10 mM TRIS/HEPES, pH 7. Four lipids were used for lipid membrane formation: diphytanoyl phosphatidylcholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)(DOPG),1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP) and cholesterol (chol). All lipids were purchased from Avanti Polar Lipids Inc., Pelham, AL. DPPC, DOPG and DOTAP have been deposited as stock solutions in chloroform, in concentrations of 20 mg/ml (DPPC) and 10 mg/ml (DOPG and DOTAP). Cholesterol was deposited as anhydrous substance. The lipid forming solutions were prepared in n-decan (after evaporating the chloroform under a flow of nitrogen) with final concentrations of 1.5g/100ml DPPC, DOPG, DOTAP or DPPC+chol(10%). DPPC solution contained also 0.025% octadecylamine (Riedel-de-Haen, Hannover, Germany). Galantamine (Galantamine hydrobromide, Sigma) was prepared in ultrapure water (Millipore) to desired concentrations. The solutions containing the sodium salts of Hofmeister anions (NaF, $NaCH_3COO$, NaBr, $NaNO_3$, NaI, NaSCN şi $NaClO_4$) and the reference NaCl solution have been prepared in concentration of 100 mM in TRIS/HEPES buffer, pH 7. All salts were purchased from Sigma. The NaCl solution was taken as reference (inactive) due to the fact that Hofmeister effects show a sign inversion at Na^+ and Cl^-. The measurements have been performed at room temperature (22^0C).

Protocols

After lipid bilayer formation and stabilization, concentration jumps of active solutions (containing the salts of interest) against the reference NaCl solution were realized, in the absence of GAL and the amplitude of the capacitive signal was measured. The electrical signal obtained reflects the capacitive coupling characteristic to the presence of lipid bilayers which are impermeable to ions. After performing the control experiment in the absence of GAL, the SSM was incubated for 30 min. with 40 µl GAL at the desired concentration and successive sets of measurements for progressive GAL concentrations have been carried out. After each incubation the SSM was washed with NaCl solution. The studied GAL concentrations have been: 0.5 µM, 1 µM, 5 µM, 10 µM and 20 µM.

Each set of measurements has been repeated on 3-4 different membranes having the same composition.

Data analysis

The recorded data for each anion have been normalized to the values of capacitive currents amplitudes in the absence of GAL. The normalized values have been averaged for 3-4 different membranes. In order to compare the profiles of GAL insertion in the membrane, a second normalization of averaged values was performed with the view to obtain values between 0 and 1. The formula according to which the normalization has been done was:

$$i_{norm} = (i - i_{min})/(i_{max} - i_{min})$$

where i_{norm} represents the normalized current, i the averaged current after first normalization,

i_{max} and i_{min} represent the maximal and minimal values respectively of the capacitive currents obtained for a given anion into a group including all tested GAL concentrations. The statistical data analysis has been done with the aid of Origin 7.5 software.

Results and discussions

The interaction of GAL with artificial lipid bilayers has been evidenced indirectly by studying the effects of its presence on the amplitudes of capacitive electrical signals elicited by a rapid exchange of active (containing Hofmeister anions)/inactive (reference) solution at the level of lipid membrane. We have studied the effects of galantamine on four types of lipid membranes: DPPC, DPPC + cholesterol 10% (DPPC-chol), DOPC and DOTAP. In a previous work (IFTIME & al. [13]) we have shown, by means of BLM method, that galantamine induces changes in the electrical parameters of artificial lipid membranes that depend both on GAL concentration and the charge of the lipids used to form the bilayer. The present paper brings additional data concerning the mechanisms of GAL interaction with lipid bilayers by studying its interference with Hofmeister effects of the anions. From the measurements performed with the relative novel electrophysiological method SSM we have been able to compare the amplitudes of the capacitive signals elicited by concentration jumps of Hofmeister anions in the absence of GAL with those obtained in its presence. This comparison allowed us to obtain new information about GAL interaction with lipid membranes.

On imposing on the lipid bilayer concentration jumps of sodium salts of Hofmeister anions, electrical capacitive signals, having an amplitude that depends on the position of the given anion in the series, can be recorded (GARCIA-CELMA & al. [16]; present work) (fig. 2A) On incubating the lipid membrane with GAL, the amplitude of the signals increases (fig. 2B). Figure 2 depicts the evolution of capacitive signals recorded in the absence and in the presence of GAL 20 μM when the lipid forming the bilayer was DPPC.

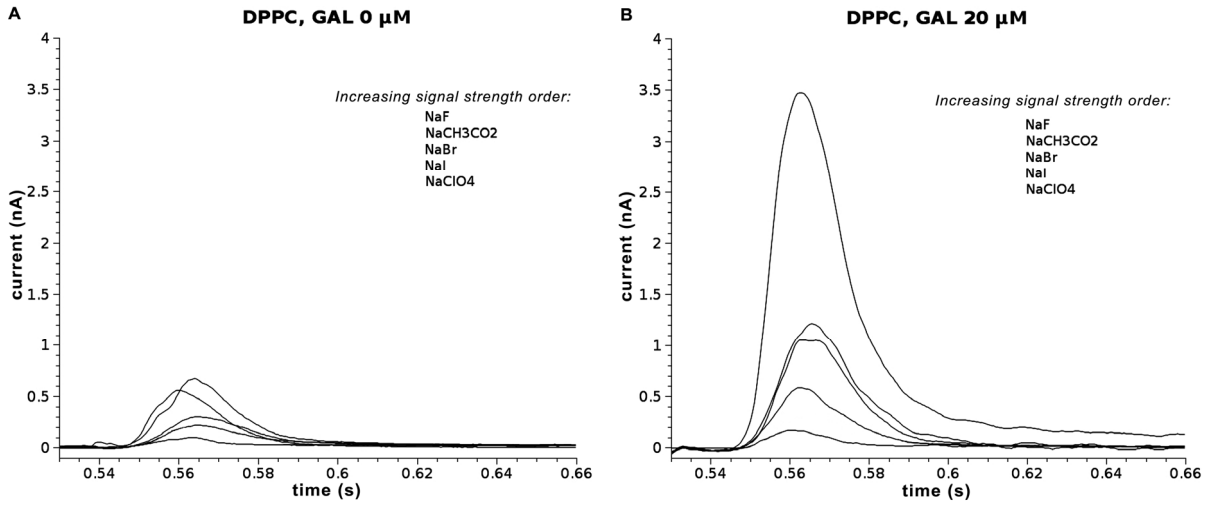

Figure 2. Electrical capacitive signals obtained after imposing concentration jumps of sodium salts of Hofmeister anions, without (A) and with (B) galantamine 20 μM. The lipid used for membrane formation was diphytanoyl phosphatidylcholine (DPPC)

Figure 3 depicts the dependence of the peak current on GAL concentration for the four types of lipids used in the experiment. Each point in the graphical representation is the average of the measured signals for 3-4 different lipid membranes. The amplitudes of the capacitive signals depend both on the lipid used for membrane formation and the GAL concentration (Fig. 3). As a general trend it can be noticed that, for all types of lipids used in the hybrid bilayer, the amplitude of the capacitive signals increases as GAL concentration increases.

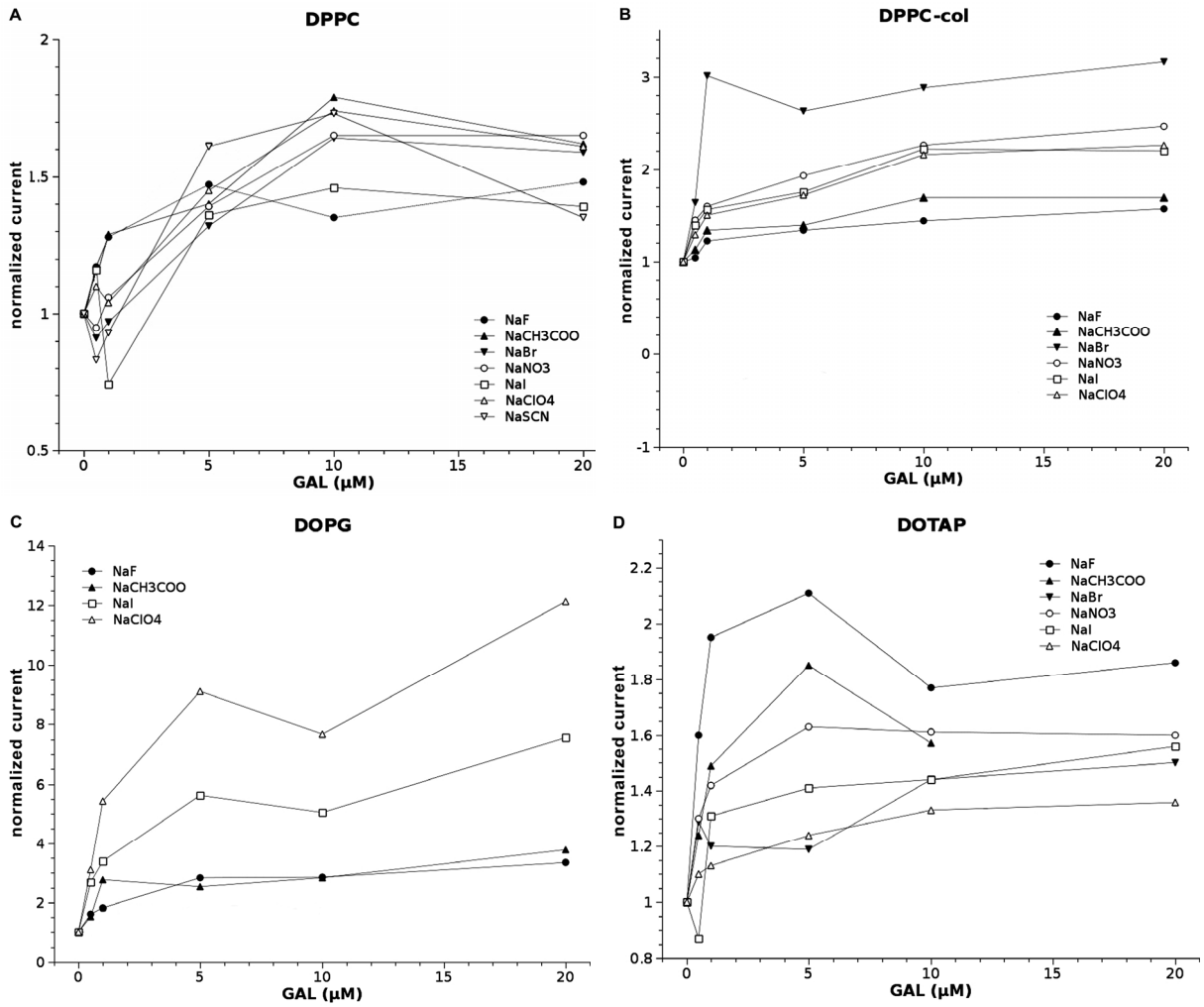

Figure 3. Variation of capacitive signals in relation with concentration jumps of Hofmeister anions and with galantamine (GAL) concentration, for different lipid membranes: A) diphytanoyl phosphatidylcholine (DPPC); B) diphytanoyl phosphatidylcholine and cholesterol (DPPC-col); C) 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG); D) 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP). In order to simplify the graphs the error bars were omitted.

In the case of DPPC membranes the amplitude of the signals elicited by concentration jumps of the anions located in the chaotropic domain (NaBr, NaNO3, NaI, NaClO4 and NaSCN) decreases at GAL concentrations of 0.5 – 1 μM. At higher concentrations the amplitude of the signals increases again as compared to control values (i.e. in absence of GAL). It is possible that at small concentrations GAL does not bind yet to the membrane but can interfere either with the chaotropic anions ability to bind to the lipid or with the water structure in the vicinity of the lipid membrane. As it was already pointed out (GARCIA-CELMA & al. [16]) the geometry of the water molecules around the ions and lipid head groups could play an important role in the observed Hofmeister effects. It seems, thus, that at concentrations below 1 μM galantamine has a stabilizing effect on the bilayer, diminishing the disorganizing effect of the chaotropic anions. At higher concentrations GAL has an opposite effect on the membrane and the amplitude of the signals starts to increase again. Taken together, these results suggest a bell-shaped dose-effect curve having a maximum at 0.5 – 1 μM GAL. A similar effect of GAL has been reported in the literature for the effect of allosteric potentiation of (SANTOS & al. [5], DAJAS-BAILADOR et al. [6], LOPES & al. [7]) and for the influence of GAL on nicotinic Ach receptors desensitization (POPESCU & al. [8]). Our

results suggest, thus, that the above mentioned effects of GAL on nicotinic receptors might be partially due also to a non-specific interference with the mechanisms involved in the action on the lipid bilayer. The presence of cholesterol in DPPC lipid bilayer modifies the profiles of GAL concentration dependency of the capacitive signals. As compared to DPPC membranes, the amplitudes increase more significantly and the initial decrease of the signals al low concentrations of GAL are not observed any more. It is possible that the presence of cholesterol favors the attachment of GAL to the bilayer and consequently its effect of electrical destabilization of the membrane is dominant even at low concentrations of galantamine.

When the lipid bilayer was constituted of DOTAP, which is positively charged, the amplitude of the signals increases as GAL concentration increases. It is interesting to note that while for DPPC membranes the effect of GAL is approximately the same for all anions, for DOTAP ones the effect is more pronounced for kosmotropic anions. This finding might point to a tendency of positively charged GAL to interact also with anions in solution not only the lipid bilayer.

The highest increase of signals amplitudes as compared to the control is obtained for the negatively charged DOPG membranes, especially for the salts of strong chaotropic anions (NaI, $NaClO_4$). This suggests a maximal structural and electrical disorganization of DOPG membranes under the influence of chaotropic anions that is amplified by the presence of GAL. The positive charge of GAL bound to DOPG membrane might lead to a screening of the negative charge of the lipid enhancing thus the interaction of chaotropic anions with the complex GAL-lipid bilayer. These results are in agreement with our previous results obtained with the aid of BLM method [13]. Another interesting observation can be done when our results are compared to those of GARCIA-CELMA & al. [16]. Unlike in the results presented above, i.e. in the presence of GAL, in their experiments the Hofmeister effect of anions was more pronounced for positively charged membranes (DOTAP), and less important in the case of negatively charges ones (DOPG). This difference could be explained by taking into account that the common feature of all the concentration dependence profiles of capacitive signals amplitudes is the presence of positively charged GAL, suggesting thus that the modulatory influence of GAL of the Hofmeister effect of anions might be due to the binding of GAL to the lipid bilayer having as consequence the modification of its structure and electrical properties. At the same time an effect on the water structure in the vicinity of the lipid membrane cannot be excluded.

The normalization of the data showing the dependency of capacitive signals on GAL concentration to values between 0 and 1 (i.e. by dividing them to the values corresponding to the highest GAL concentration) reveals a very small variability of the curves for the same type of lipid (Fig. 4). The normalized values seem to be practically independent of the anion whose concentration jump was performed. Figure 4 depicts the averaged data corresponding to normalized curves representing the results obtained for each of the four lipids used to form the bilayer. The shape of the curves confirms the hypothesis according to which at the basis of the described phenomena lies the binding (either insertion in the bilayer or attachment at its surface) of GAL to the lipid membrane. It becomes possible thus to extract information about the affinity of GAL to the different types of studied lipids. The concentration dependence of the signals amplitudes is sigmoidal, suggesting a kinetic modeled by a Hill equation:

$$i = k_1[GAL]^n / (k_2 + [GAL]^n)$$

i being the amplitude of the capacitive signals, k_1 and k_2 constants of the kinetic model (k_2 providing information concerning the binding affinity of GAL) and n the Hill coefficient, that indicates the cooperativity of GAL binding to the bilayer. The values of the parameters of the fit with a Hill equation are given in the Table 1.

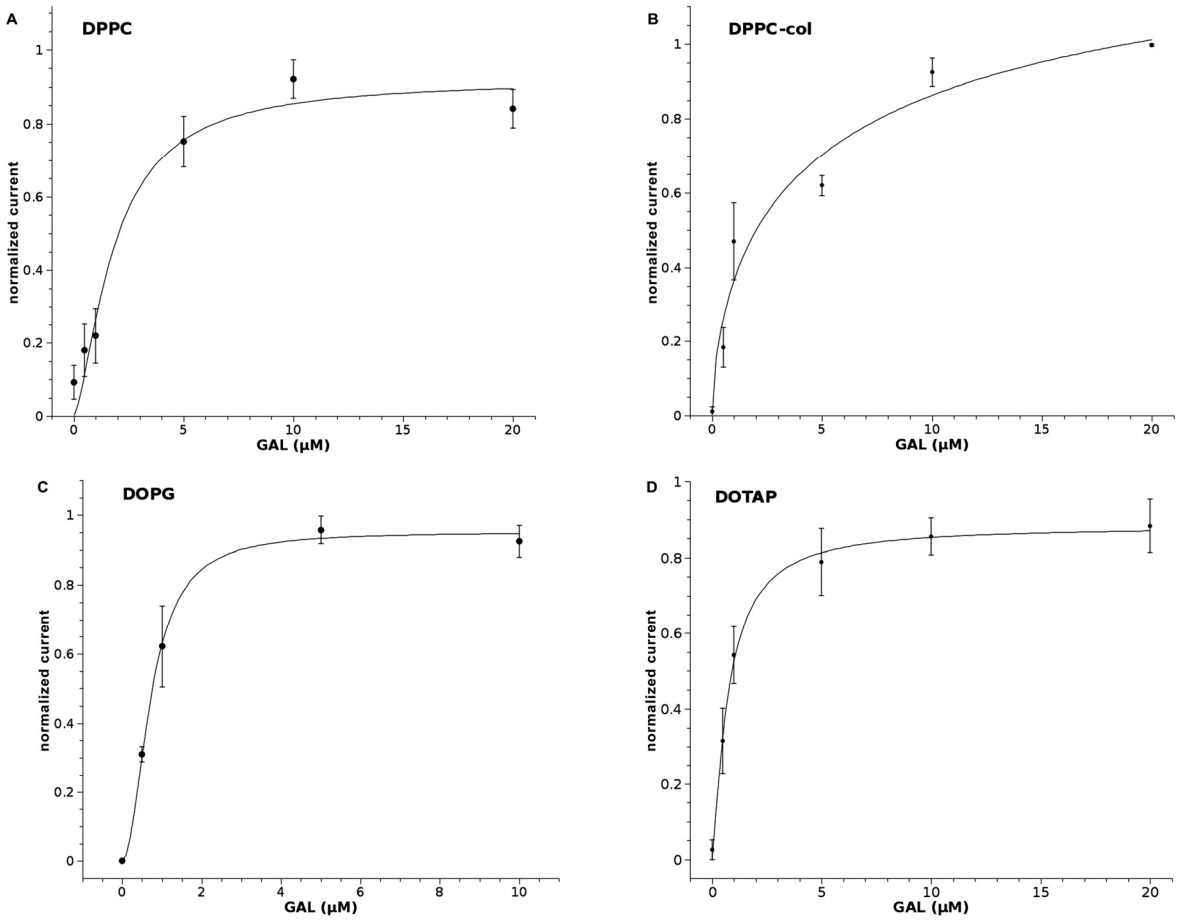

Fig. 4. The dependency of capacitive signals on galantamine (GAL) concentration. The points represents the averaged experimental data, and the curves the fitting modeled by a Hill equation. The membrane forming lipids are: A) diphytanoyl phosphatidylcholine (DPPC); B) diphytanoyl phosphatidylcholine and cholesterol (DPPC-col); C) 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG); D) 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP).

The presence of cholesterol in DPPC membranes does not influence significantly the affinity of GAL (reflected by the coefficient k_2), but has effect on the stoechiometry and the cooperativity of GAL binding. A Hill coefficient over 1 (n = 1.5), for DPPC membranes, suggests a certain degree of cooperativity of GAL binding to DPPC. In the presence of cholesterol the Hill coefficient lowers to about 0.5 suggesting thus the binding of GAL to a supramolecular complex including probably both DPPC and cholesterol molecules.

GAL has similar affinities for DOPG and DOTAP membranes and the affinities are significantly higher than for DPPC membranes. This could explain the bigger amplitudes of the signals for these two lipids. If in the case of DOTAP the binding does not seem to be cooperative, the Hill coefficient of n = 2 for DOPG membranes suggests clearly the cooperativity for GAL binding.

Table 1.

	k_1	k_2	n
DPPC	0.92 +/- 0.11	2.47 +/- 1.04	1.5 +/- 0.5
DPPC-col	1.47 +/- 0.94	3.01 +/- 2.6	0.6 +/- 0.3
DOPG	0.95 +/- 0.02	0.52 +/- 0.07	2 +/- 0.2
DOTAP	0.88 +/- 0.03	0.69 +/- 0.1	1.3 +/- 0.2

Conclusions

Our experiments show that increasing GAL concentrations lead to the increase of the amplitude of capacitive signals elicited by concentration jumps of Hofmeister anions on artificial lipid membranes. The chaotropic anions induce lower amplitude signals in DPPC membranes in the presence of GAL concentrations of 0.5 – 1 μM than in its absence, suggesting a stabilizing effect of GAL at these concentrations. The maximal stabilizing effect of GAL on DPPC membranes appears at the same concentrations where its effect of allosteric potentiation and on nicotinic receptors desensitization is maximal (POPESCU & al. [8]) suggesting that these last effects could be also due to non-specific interaction of GAL with the lipid bilayer. GAL induces a bigger structural disorganization of negatively charged DOPG membranes, reflected by much higher amplitudes of the capacitive signals elicited by concentrations jumps of Hofmeister anions as GAL concentration increases. The binding of GAL to DPPC and DOPG membranes is a cooperative process with Hill coefficients of 1.5 respectively 2. In DPPC-cholesterol membranes GAL binds itself probably to a supramolecular complex (GAL interacts both with a DPPC and a cholesterol molecule) which is also suggested by a Hill coefficient below 1. The affinity of GAL for DPPC is not significantly influenced by the presence of cholesterol and the affinities for DOPG and DOTAP are similar and significantly higher than for DPPC.

Acknowledgments

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