GINKGOLIDE B EFFECTS ON ARTIFICIAL LIPID MEMBRANES

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Abstract. Ginkgolide B (GKB), a natural substance belonging to the family of ginkgolides from *Ginkgo biloba* extracts, is acknowledged as a therapeutic drug having beneficial effects in neurological disorders or as a blood platelets antiaggregant. Because of the increasing interest in the mechanisms of interaction of the substance with various structures of living systems, we have proposed ourselves to follow up its effects on the artificial lipid membranes by studying the modifications induced by increasing concentrations of GKB in the electric properties of lipid membranes formed with diphytanoyl-phosphatidylcholine (DPPC) in the absence and in the presence of cholesterol. With this view two electrophysiological techniques have been used, the Black Lipid Membrane (BLM) and the Solid Supported Membrane (SSM) methods. Our results indicate that GKB binds to the lipid bilayers and that the presence of cholesterol does not influence significantly the affinity and the cooperativity of GKB binding, but leads to a decrease in the conductance as compared to the values in its absence.

Key words: GKB, BLM, SSM, capacitance, conductance, DPPC, cholesterol.

INTRODUCTION

Ginkgolide B (GKB) is a terpenoid obtained from *Ginkgo biloba* extracts. Standardized *Ginkgo biloba* extracts are already employed as therapeutic drugs and it has been shown that they enhance cognition, improve blood rheology and tissue metabolism, and oppose the detrimental effects of ischaemia [3]. They contain two important categories of substances: ginkgolides and bilobalides and various representatives of both families are currently used in clinical medicine. Among ginkgolides the most biological active one is the Ginkgolide B (Fig.1). In the treatment of Alzheimer disease, clinical studies show that the administration of GKB prevents beta-amyloid toxicity to brain cells [10] and has moderate effects (less significant than in the case of cholinesterase inhibitors) on the cognitive function impairment [4, 13]. GKB also enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer's disease.

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[16]. *Ginkgo biloba* extracts are also employed as neuroprotectant drugs [1, 4] and exhibit beneficial effects to patients with Alzheimer's disease (reviewed in [1]).

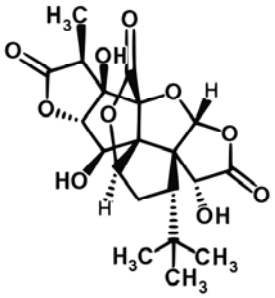

Fig. 1. Ginkgolide B structure. (1R,3R,7S,8S,10R,11R,12R,13S,16S,17R)-8-tert-Butyl-6,12,17-trihydroxy-16-methyl-2,4,14,19 tetraoxahexacyclo [8.7.2.0~1,11~.0~3,7~.0~7,11~.0~13,17~] nonadecane-5,15,18-trione.

Many of GKB effects involve the interaction with various membrane structures, such as receptors or membrane proteins, e.g. the nicotinic receptor of acetylcholine. Previous researches [e.g. 5, 7, 14] have shown that various species are able to influence the membrane-related physiological processes by acting on the lipid bilayer in the vicinity of proteins embedded in it. In a previous work [12] we have shown that another drug used in the prevention and treatment of some neurodegenerative diseases, namely galantamine, acts specifically at the level of nicotinic receptors for acetylcholine, but that these specific effects might be also accompanied by non-specific ones at the level of lipid membranes where the receptors are embedded [8, 9]. Similarly, when examining the effects of GKB on membrane proteins involved in various metabolic pathways it is recommended to take into account also such non-specific interactions that could be the effect of GKB 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. The subject of the present paper is the effect of GKB on artificial lipid membranes followed up by monitoring the electrical parameters of lipid membranes consisting either of diphytanoyl phosphatidylcholine (DPPC) or diphytanoyl phosphatidylcholine + cholesterol (10%) (DPPC-col) by means of two electrophysiological methods, namely the BLM (black lipid membrane) and SSM (solid supported membrane). We have found that GKB binds itself to the DPPC lipid membranes and that the presence of cholesterol does not influence significantly the affinity and the cooperativity of GKB binding to the lipid bilayer, but leads to a decrease in the conductance as compared to its values in DPPC membranes.

MATERIALS AND METHODS

CHEMICALS

The standard buffer solutions contained 10 mM TRIS/HEPES, pH 7. The lipids used for lipid membrane formation were diphytanoyl phosphatidylcholine (DPPC) and diphytanoyl phosphatidylcholine + cholesterol (10%) (DPPC-col). All lipids were purchased from Avanti Polar Lipids Inc., Pelham, AL. DPPC has been deposited as stock solution in chloroform in a concentration of 20 mg/ml. Cholesterol was deposited as anhydrous substance. The lipid forming solutions were prepared in n-decane (after evaporating the chloroform under a nitrogen flow, in order to avoid peroxidation) with final concentrations of 1.5 g/100ml. DPPC solution contained also 0.025% octadecylamine (Riedel-de-Haen, Hannover, Germany). For SSM experiments the solutions containing the sodium salts of Hofmeister anions (Na_2SO_4, $NaCH_3COO$, $NaNO_3$, 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 ºC). GKB (Ginkgolide B from *Ginkgo biloba* leaves, purity 90%, Sigma) was prepared in ethanol as 100 mM stock solution and the desired concentrations have been prepared in ultrapure water (Millipore). For BLM experiments the lipid film forming solution contained the same lipids as in SSM measurements. Before painting the lipid film the cuvette hole was impregnated with a solution of 0.5% (w/v) corresponding lipid in hexane (Fluka, Sigma-Aldrich Germany, >98%). The artificial lipid membranes were formed in the presence of 20 mM HEPES (Sigma) and 100 mM NaCl (Merck, 99%) in both compartments of the cuvette. All the experiments were carried out at pH = 7 and at room temperature (22–24 ºC). The pH was adjusted with TRIS (Sigma) and before use the solutions were filtered through a cellulose-acetate filter 0.2 μm (Sartorius).

THE SSM METHOD AND MEASURING PROCEDURE

The electrophysiological SSM method 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 [6, 15]). The solid supported membrane (SSM) consists of an alkanethiol monolayer covalently bound to a gold surface deposited on a glass support via the sulphydryl group, with a lipid monolayer on top of it in order to obtain a hybrid bilayer [11]. The lipid membrane formed has an area of 1–2 mm^2. The SSM is then fixed in a special plexiglas cuvette, having an inner volume of 17 μL, which allows the solutions of interest to flow through. 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 [11]. After the formation of SSM, the capacitance and the conductance values, which become constant after a waiting time of ca. 90 min, are measured. The usual values range between 300–500 nF/cm^2 for capacitance and between 50–100 nS/cm^2, for 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. A concentration jump of the active solution yielding a capacitive signal will indicate an electrogenic activity at the membrane-electrode system.

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 GKB 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 GKB, the SSM was incubated for 30 min. with 40 μL GKB at the desired concentration and successive sets of measurements for progressive GKB concentrations have been carried out. After each incubation, the SSM was washed with NaCl solution. The studied GKB 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 GKB. The normalized values have been averaged for 3–4 different membranes. In order to compare the profiles of GKB 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:

$$i_{norm} = (i - i_{min})/(i_{max} - i_{min}) \quad (1)$$

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 GKB concentrations. The statistical data analysis has been done with the aid of Origin 7.5 software.

THE BLM METHOD AND MEASURING PROCEDURE

The black lipid membranes (BLM), having an area of *ca.* 10^{-2} cm^2, are formed in a Teflon cuvette, consisting of two compartments, each with a volume of 1.5 ml. The compartments are filled with an appropriate electrolyte solution and, via agar bridges and Ag/AgCl electrodes, each compartment is connected to an external electric circuit that contains a current amplifier, an oscilloscope, a function generator, an electric filter, and a computer for data acquisition. The whole set-up is placed inside a compact Faraday cage, with the possibility of optional light inside. By means of a function generator the electrical capacitance and conductance of the membrane can be measured. Typical values for those electric characteristics of the black lipid membrane are *ca.* 400 nF/cm^2 for the specific capacitance and ca. 7 nS/cm^2 for the specific conductance (for further details see [2]).

The desired Ginkgolide B (GKB) concentrations in the cuvette were obtained by adding bilaterally (on both sides of the lipid membrane) the appropriate amount from the stock solutions after checking lipid membrane stability, i.e. the electrical capacitance C and conductance G measured three times in a 15 minutes interval remained unchanged. The same protocol was used throughout the whole experiment after each addition of GKB. The concentrations of GKB varied in the range from 1 nM to 100 μM. For each measurement the final value of the electrical parameters was the average of the three recorded values, normalized to the values of capacitance and the conductance of the lipid bilayer, C_0 and G_0 respectively, recorded before the addition of GKB. All the experiments were performed at room temperature (22 – 24 ºC). The statistical data processing was performed by using the OriginPro 7.5 software.

RESULTS AND DISCUSSION

We have followed up the interaction of GKB with artificial lipid membranes, formed either with DPPC or with DPPC-cholesterol, by means of two electrophysiological methods, SSM (solid supported membrane) and BLM (black lipid membrane) techniques.

SSM EXPERIMENTS

As previously done by studying galantamine interaction with lipid membranes [9], the interaction of GKB with artificial lipid bilayers, in the case of SSM measurements, 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 found that, similarly to galantamine [9],

GKB enhances the Hofmeister effects on the lipid membranes as it can be seen from the increase of the amplitudes of capacitive electrical signals elicited by concentration jumps of lyotropic anions solutions (Fig. 2).

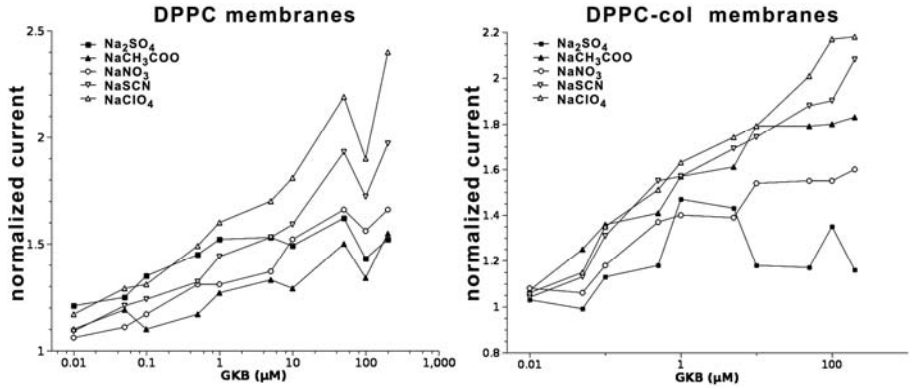

Fig. 2. Variation of capacitive signals in relation with concentration jumps of Hofmeister anions and with Ginkgolide B (GKB) concentration, for diphytanoyl phosphatidylcholine (DPPC) and diphytanoyl phosphatidylcholine + cholesterol (DPPC-col) lipid membranes. In order to simplify the graphs the error bars were omitted. Each point in the graphical representation is the average of the measured signals for 3–4 different lipid membranes.

Nevertheless, the normalization of the data showing the dependency of capacitive signals on GKB concentration to values between 0 and 1 (i.e. by dividing them to the values corresponding to the highest GKB concentration, as described in Materials and Methods) reveals a very small variability of the curves for the same type of lipid. The normalized values seem to be practically independent of the anion whose concentration jump was performed. Figure 3 depicts the averaged data corresponding to normalized curves representing the results obtained for each of the two types of lipid membranes. From the shape of the curves we inferred that at the basis of the described phenomena lies the binding of GKB to the lipid membrane. The concentration dependence of the normalized signals amplitudes is sigmoidal and it was modeled by a Hill equation:

$$i = k_1[\mathrm{GKB}]^n / (k_2 + [\mathrm{GKB}]^n) \quad (2)$$

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 GKB) and n the Hill coefficient, that indicates the cooperativity of GKB binding to the bilayer. The values of the parameters of the fit with a Hill equation are given in Table 1. It becomes possible thus to extract information about the affinity and cooperativity of GKB binding to the studied lipid membranes. It can be observed (Fig. 3) that the presence of cholesterol does not influence significantly either the GKB affinity for

the lipid membrane or the cooperativity of the binding as compared to DPPC membranes in the absence of cholesterol suggesting thus that GKB binds preponderantly to DPPC and that the interaction with cholesterol is minimal.

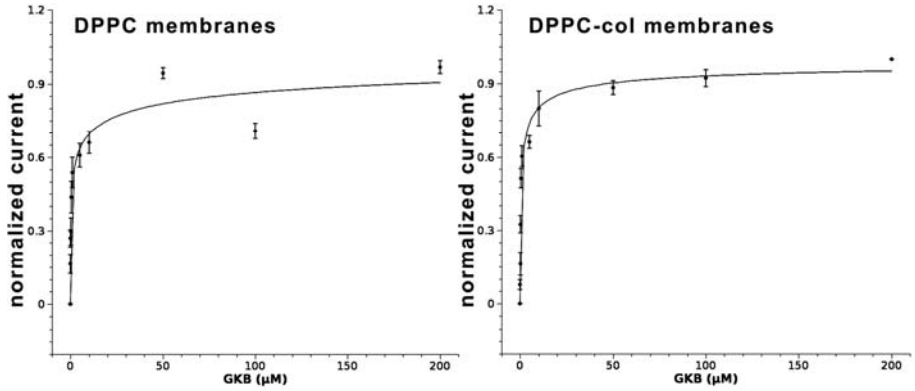

Fig. 3. The dependency of capacitive signals on GinkgolideB (GKB) concentration. The points represent the averaged experimental data, and the curves the fitting modeled by a Hill equation. The membrane forming lipids are diphytanoyl phosphatidylcholine (DPPC, left) and diphytanoyl phosphatidylcholine and cholesterol (DPPC-col, right).

Table 1

The fitting parameters for SSM experimental data

	k_1	k_2	n
DPPC	1.12 ± 0.28	1.27 ± 0.68	0.3 ± 0.1
DPPC-col	1 ± 0.06	0.8 ± 0.16	0.5 ± 0.07

Subunitary Hill coefficients suggest the binding of GKB to more lipid molecules, a result to be expected taking into account that GKB is a large molecule having a complex spatial structure (Fig. 1).

BLM EXPERIMENTS

GKB was found to modify both the electrical capacitance and conductance of the artificial lipid membranes formed either with DPPC or with DPPC-col in a concentration dependent manner. Figure 4A depicts the variation of electrical capacitance as a function of GKB concentration. As this variation was sigmoidal we have fitted the curves with a Hill equation and could thus estimate the parameters of GKB/lipid bilayer interaction. The parameters resulting from the fit according to the formula given above are presented in Table 2. The table shows

that the differences between the affinities of GKB binding to the DPPC lipid bilayer in the absence or in the presence of cholesterol are not significant confirming thus the results obtained in SSM experiments. Neither the Hill coefficients show statistically significant differences between the values obtained with the two types of membranes, though a slight smaller coefficient can be noticed in DPPC-col bilayers. The subunitary values of Hill coefficients suggest again the binding of GKB to more phospholipid molecules.

Table 2

The fitting parameters for BLM experimental data

	k_1	k_2	n
DPPC	0.399 ± 0.018	0.424 ± 0.106	0.58 ± 0.077
DPPC-col	0.112 ± 0.016	0.335 ± 0.254	0.372 ± 0.134

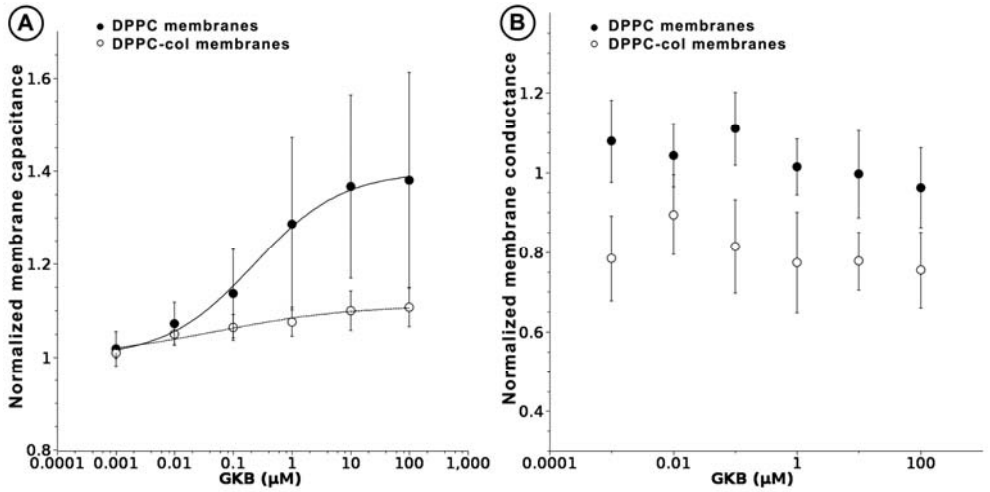

Fig. 4. A. Dependency of lipid membrane capacitance on GKB concentration. The points represent the experimental data and the solid lines represent the fit with a Hill equation. B. Dependency of lipid bilayer conductance on GKB concentration.

The bilayer conductance, on the other hand, undergoes less significant changes on applying GKB at various concentrations (Fig. 4B). The concentration dependence profiles are pretty similar in DPPC in the absence and in the presence of cholesterol only translated for DPPC-col membranes toward a lower conductance. This decrease in conductance is due to the insertion of cholesterol that might rigidify the bilayer.

COMPARATIVE ANALYSIS OF THE RESULTS OBTAINED IN SSM AND BLM EXPERIMENTS

It is interesting to note that in the case of solid supported membranes the cooperativity of GKB binding to the lipid membrane is different for DPPC and DPPC-col. A possible explanation might reside in the structural differences between the two types of model membranes. While the black lipid membrane (BLM) consists of a DPPC bilayer, the solid supported membrane (SSM) consists of a hybrid bilayer, a DPPC monolayer and an alkanethiol monolayer. Moreover, in BLM experiments the membranes are a much longer time exposed to GKB, usually for hours, while in SSM ones a 20 minutes incubation period is followed by wash-out. Thus, in BLM experiments it is possible to reveal phenomena that take place on a longer span of time.

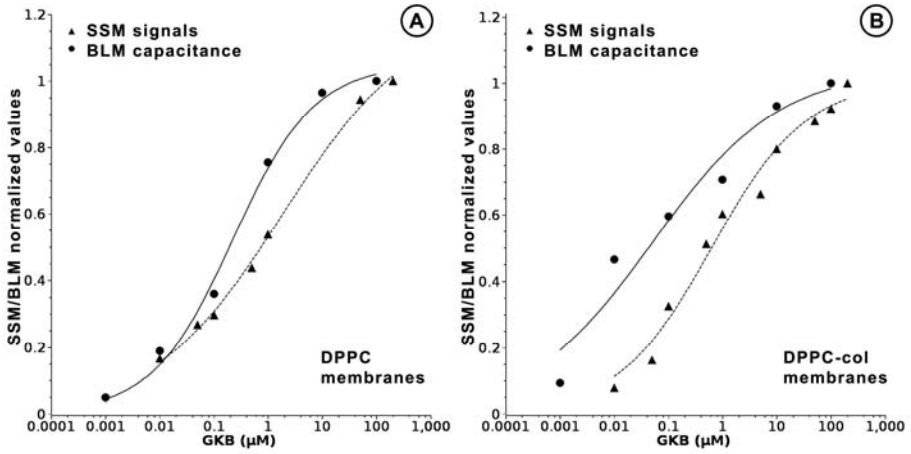

Fig. 5. Comparative representation of GKB insertion curves in the lipid bilayers obtained in BLM and SSM experiments; A – DPPC membranes; B – DPPC and cholesterol membranes.

Figure 5 depicts comparatively the curves showing GKB insertion in the lipid membranes in BLM and SSM experiments. It can be easily observed that while in DPPC membranes the differences between the curves obtained by the two methods are not so important, the presence of cholesterol induces more significant differences (note the logarithmic scale of representation) pointing again to the structural-architectural differences of the BLMs and SSMs.

CONCLUSIONS

The data obtained in both SSM and BLM experiments confirm the interaction of GKB with the artificial lipid membranes and show that GKB insertion respects a Hill equation. At the same time the subunitary values of Hill coefficients, as

calculated from the values obtained with both techniques, suggest the binding of GKB to more phospholipid molecules. The presence of cholesterol does not influence significantly the affinity and the cooperativity of GKB binding to the lipid bilayer, but leads to a decrease in the conductance as compared to its values in DPPC membranes. The profiles of GKB/DPPC interaction are similar in both types of experiments, i.e. SSM or BLM, the slight difference being probably due to the cooperativity of binding and it might be interpreted as a consequence of structural-architectural differences in the two types of membranes. The results presented in this paper point out to a possible non-specific effect of GKB when interactions with membrane proteins involved in various diseases are taken into account.

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