THE INFLUENCE OF THE HOFMEISTER SERIES OF ANIONS ON THE BSA-HYP COMPLEX INTERACTION WITH ARTIFICIAL LIPID MEMBRANES

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Abstract. Among the broad range of polyphenols, Hypericin (Hyp) is known as an antidepressant, a potent virucidal agent and a therapeutic agent in treatment/detection of cancer, showing also an anti-inflammatory activity. Using an electrical method, BLM (Black Lipid Membrane), we have studied the modifications induced by the simultaneous presence of Bovine Serum Albumin (BSA)-Hyp complex and of some lyotropic anions on the electric properties of artificial lipid membranes. We ascertained that the membrane capacitance increases for all the anions and the conductance decreases for chaotropes, therefore we conclude that the interaction of BSA–Hyp complex with the membrane leads to a protective effect against the disturbances at the level of the artificial bilayer induced by the most chaotrope anions. Because BSA-Hyp complex induces a smaller increase in the electric properties of the membrane we believe that this is due to the fact that Hyp binds not only to a specific subdomain to BSA, but also nonspecifically and these nonspecifically bound Hyp molecules are released in the presence of lyotropic anions and are able to interact with the membrane. As a consequence, when Hyp is specifically bound, its interaction with the cell membrane does not take place through the lipids.

Key words: Hypericin, BLM, BSA, lyotropic anions, Hofmeister series.

INTRODUCTION

In the context of the general tendency to find alternative, natural, with no side effects therapies for various diseases, the research on plant extracts effects at cellular and subcellular level is amplifying. One of the most numerous and widely spread groups of plant metabolites which are an integral part of both human and animal diets is the polyphenols group. Most of them have remarkable antioxidant capacities and, therefore, they are involved in the treatment and prevention of different human pathologies such as cancer and cardiovascular disease, offering

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protection against age-related deficits in cognitive and motor function [16]. The free-radical scavenging capacity of polyphenols is probably a result of cells response, mainly through direct interactions with receptors or enzymes involved in signal transduction and this may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [21].

Hypericin (Hyp) (Fig. 1), a polyphenol belonging to the class of phenanthroperylene 7–14 diones, is found in more than 300 species of the genus *Hypericum*, with *Hypericum perforatum* (St. John's wort) as the most representative species [10].

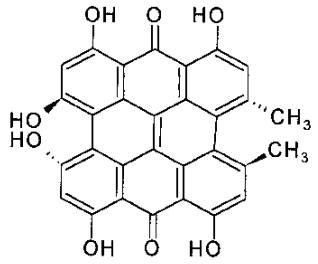

Fig. 1. Chemical structure of Hyp.

Hyp is a fluorescent red dye with an amphiphilic character. It is insoluble in water, oil, methylene chloride and most other nonpolar solvents, but soluble in alkaline aqueous solutions, organic bases and polar organic substances. It is also soluble in biological media and therefore it leads to complex formation with biological macromolecules such as DNA, membrane fragments and cellular compounds, human and bovine serum albumin and other plasma proteins [20]. Hyp blood transport implies the attachment to serum albumins, its binding site being located on IIA subdomain of human serum albumin (HSA) and bovine serum albumin (BSA) [11, 17], this interaction being mainly hydrophobic. It appears that some Hyp molecules bind nonspecifically to the surface of human serum albumin [8]. Hyp binding to serum albumins helps to overcome its difficulties in solubilization and dispersion in aqueous physiological solution [19].

Hyp is known due to its various properties: antidepressant, potent virucidal agent, anti-inflammatory activity, therapeutic agent in treatment/detection of cancer [1, 9]. As the most powerful naturally occurring photosensitizer, Hyp is under consideration for photodynamic therapy treatment of bladder cancer [4]. Normal human umbilical endothelial cells as well as cancer human glioma cell lines U-87 MG and U-373 MG, in in vitro conditions, are sensitive to photoactivated Hyp [23]. But, several biological effects of Hyp also seem to occur in dark such as

catalytic inhibition of human DNA topoisomerase II, as well as antiviral and antitumoral activities [3, 22].

In a previous work [15], we investigated the interaction of Hyp, in dark, with pure artificial lipid bilayers and we explained the large augmentation of the values of electric capacitance and conductance of the membrane by the Hyp incorporation mainly on the outer surface of the membrane, modifying the electric permittivity of the interface layer. We have also explored the effects of co-application of Hyp and some lyotropic anions on the properties of the lipid bilayer. Hyp modulates the lyotropic anions effects on lipid membrane and this modulation appeared to be mutual. Hyp presence results in protection of the membrane against the electrical and structural perturbances effects that Hofmeister series anions have on artificial lipid membranes, by altering the electric properties at the interface solution/lipid bilayer and/or the membrane dipole potential.

The Hofmeister series (lyotropic series) ranges anions and cations based on the amplitude of their effects, given in terms of the ability of the ions to stabilize the structure of proteins [13]. Anions are more likely to promote the less solubility of amphiphiles. Proteins tend to become more soluble in the presence of anions series as it follows [7, 12]: $SO_4^{2-} < HPO_4^{2-} < OH^- < F^- < HCOO^- < CH_3COO^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$.

The anions found on the right side of Cl^- (considered to be neutral) are known as chaotropes (strongly hydrated, “water structure makers”, with stabilizing and salting out effects on proteins and macromolecules), while the ones situated on the left side of Cl^- are known as kosmotropes (weakly hydrated, “water structure breakers”, with destabilizing and salting in effects on proteins and macromolecules) [24].

Lyotropic anions are incidentally present in human daily diet (nitrate, acetate), being able to affect kinetics of chemical reactions occurring in nutritional supplies [6] and they are also encountered in household products (for instance perchlorate).

Since Hyp blood transport necessitates its attachment to serum albumins, a more realistic approach would be to investigate the effects of BSA-Hyp (Bovine Serum Albumin-Hypericin) complex on the artificial lipid membrane.

In our research, we explored the effects of BSA-Hyp complex on the electric properties of the artificial bilayer in the presence of various lyotropic anions, in order to explain the interaction of the complex with the pure artificial lipid membrane. While Hyp might have protective and/or stabilizing effects on the membrane experimentally seen as a lower increase in the capacitance and conductance values as in the absence of anions, BSA-Hyp complex preserves this

protective effect on the membrane, the bilayer's conductance diminution suggests that the complex shields the lipid membrane against the perturbances induced by the strong chaotrope anions, even better than Hyp does.

MATERIALS AND METHODS

Hypericin, 1,3,4,6,8,13-Hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-opqra]perylene-7,14- dione (Fig. 1) was purchased from Santa Cruz Biotechnology USA and it had been prepared as 2 mM stock solution in 10% ethanol and 90% distilled water, subsequent dilutions being obtained in distilled water. Sodium salts ($NaCH_3COO$, NaCl, $NaClO_4$, $NaNO_3$) and HEPES (N-2-hydroxyethlypiperazine-N'-ethanesulfonic acid) were purchased from Sigma-Aldrich Germany (purity > 98%) and prepared as 4 M stock salts solutions and 1 M stock solution HEPES in distilled water. The membrane forming solution contained 1.5% (w/v) diphytanoyl-phosphatidylcholine (Avanti Polar Lipids, USA) and 0.025% (w/v) octadecylamine in n-decane (Fluka, Sigma-Aldrich Germany, >98%). To facilitate the membrane formation by impregnating with lipid the margins of the hole, a small amount of 0.5% (w/v) diphytanoyl-phosphatidylcholine in hexane (Fluka, Sigma-Aldrich Germany, >98%) was used. Bovine Serum Albumin (BSA) (Sigma-Aldrich Germany) was solubilized in distilled water to a 2 mM stock solution. For the preparation of BSA-Hyp complex, Hyp was prepared as a 500 μM stock solution in 10% ethanol and 90% distilled water. Before each experiment the BSA-Hyp complex was freshly prepared by incubating equal BSA and Hyp stock solution volumes at a constant temperature of 37 °C for six hours. The concentration ratio BSA:Hyp was 4:1.

In order to investigate the modifications of the electric properties of the artificial lipid membrane in the presence of Hyp and BSA-Hyp complex, we have used the Black Lipid Membrane method (BLM). The method consists in forming a very thin lipid membrane in a 1mm diameter hole situated in the wall that separates two 2 mL compartments of a Teflon cuvette. 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. The stirring of the solutions inside the cuvette was performed by a magnetic agitator operated by a small magnet placed under the cuvette, which is rotated with variable velocity. Using the function generator, one can measure the electric capacitance (by applying a triangular 10 mV pulse) and conductance (by applying a continuous

100 mV voltage jump) of the membrane, typical values for those electric characteristics of the black lipid membrane being 400 nF/cm^2 for the specific capacitance and 7 nS/cm^2 for the specific conductance [2].

The artificial lipid membranes were formed in the presence of 20 mM Hepes, at pH = 7, Hyp and BSA-Hyp complex were added on both sides of the membrane after its stabilization. After each addition, the electric capacitance and conductance of the membrane were measured three times in a 15 minutes interval. The final value was the average of the three recorded values, normalized to the values of the capacitance/conductance of the stabilized membrane before the polyphenols were added. All the experiments were performed at room temperature (22–24 ºC), in dark.

RESULTS

Figure 2 shows the evolution of two electric properties of the artificial lipid membrane, the capacitance and the conductance, in the conditions of increasing Hyp and BSA-Hyp complex concentrations. While the capacitance of the bilayer increases with Hyp concentration, this parameter of the bilayer remains constant for a BSA-Hyp complex concentration up to 50 μM, above this value the membrane became instable. The 50 μM Hyp concentration should constitute a reasonable upper limit for the investigations, given the fact that *in vivo* Hyp concentration used for phototherapy ranges up to 10 μM [3], while in dark, it reaches 50 μM in *in vitro* essays on bovine aorta endothelial cell growth [18].

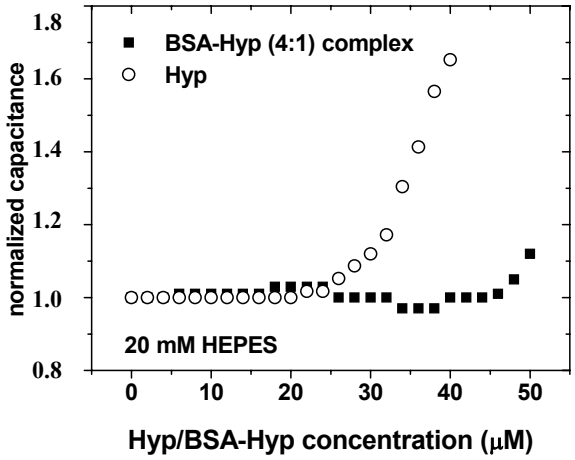

Fig. 2. a. Comparative evolution of membrane capacitance in the presence of 20 mM HEPES at pH = 7 and in the presence of Hyp or BSA-Hyp complex.

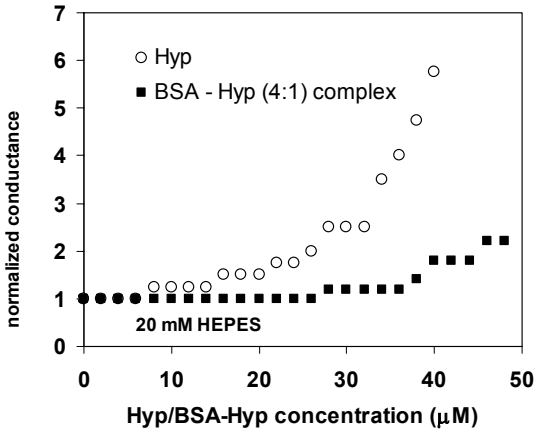

Fig. 2. b. Comparative evolution of membrane conductance in the presence of 20 mM HEPES at pH = 7 and in the presence of Hyp or BSA-Hyp complex.

The obvious different conduct of the bilayer's capacitance and conductance in the two cases suggests that Hyp preferentially binds to BSA in order to form the complex, rather than to lipid membrane.

Hyp presence changes both electric parameters of the artificial lipid membrane, starting at concentrations higher than 25 μM. The BSA-Hyp complex presence does not change the bilayer's capacitance, and determines only a slight increase of the bilayer's conductance.

Due to its unique molecular structure in which one-half of the molecule is hydrophilic while the other half is hydrophobic, Hyp might bond to the outer surface of the cell membrane, and at the same time could bond hydrogen to the aqueous media. If one accepts the idea of Hyp insertion at the surface of the membrane, leading to a modification of the electric permittivity of the membrane surface, this is not the case anymore when BSA-Hyp complex is present. Because the capacitance does not vary, we presume that BSA-Hyp complex does not insert into the surface of the membrane, and being soluble in the aqueous medium it energetically prefers the water surroundings. Since in the presence of BSA-Hyp complex the membrane becomes much less conductive than in the case of Hyp presence, this electric parameter exhibiting only a small increase, we suggest the possibility for the complex to orientate with its Hyp side towards the membrane, inducing slight modifications at the level of the membrane dipole moment. At the same time, if we take into consideration the hypothesis according to which Hyp binds to albumins also nonspecifically [8], our results would be in favor of this idea. We can imagine that the complex does not orient the Hyp molecules bound to IIA subdomain, which might be well shielded, but Hyp molecules nonspecifically bound to the albumins towards the membrane, hence inducing a slight effect on its dipole moment value. Probably, Hyp bound to albumins does not interact with the cell membrane through the lipid regions.

When increasing concentrations of lyotropic anions are added on both sides of the artificial lipid membrane, the capacitance is independent of the type or of the concentration of the lyotropic anion, while its conductance is increasing with the anions concentrations, showing a relationship between the conductance increase rate and the position of the anion in the Hofmeister series [14]. In order to see the manner in which the presence of the BSA-Hyp complex affects the interaction of lyotropic anions with the membrane, we have recorded the variations of the electrical properties of the artificial lipid membrane in the simultaneous presence of BSA-Hyp complex in increasing concentrations, and Hofmeister anions in 100 mM concentration, in 20 mM HEPES, pH = 7.

The capacitance of the bilayer increases only at high BSA-Hyp complex concentrations (> 30 μM), and the augmentation varies with the anion type (Fig. 3). All the salts increased the bilayer's capacitance, probably due to the fact that BSA became less soluble than in the absence of the anions and released Hyp molecules, allowing them to interact with the membrane. Since Hyp attaches not only on a specific subdomain of HSA and BSA, but also nonspecifically on HSA [8], our results show that this nonspecific attachment of Hyp might be also valid in the case of BSA-Hyp complex, and Hyp molecules which are bound nonspecifically to BSA are the ones that detach and become available to interact with the artificial lipid bilayer.

In Fig. 5 a) we plotted the ratio between the values of membrane capacitance in the presence of BSA-Hyp complex and Hyp, when different anions in 100 mM concentration were added to the electrolytes on both sides of the artificial lipid bilayer, while in Fig. 5 b) the ratio between the values of membrane conductance, appears in the same conditions. Except for perchlorate, the rest of the investigated anions led to a decrease in the capacitance value in the presence of the complex compared to the situation when Hyp alone was present. That shows that only a smaller amount of Hyp molecules were unbound and free to interact with the lipid bilayer when the experiment was conducted with BSA-Hyp complex compared to the situation with free Hyp. Because this ratio decreases as the concentration of BSA-Hyp complex / Hyp increases it means that the amount of available free Hyp molecules is less for BSA-Hyp complex than for Hyp only.

For perchlorate, the ratio between the capacitance of the artificial lipid bilayer in the two different conditions has an average of 1 and increases slowly with BSA-Hyp complex/Hyp concentration, becoming larger than 1 for a concentration of 40 μM. It means that in the presence of perchlorate, known to be strongly chaotrope, all Hyp molecules were released by the complex and are able to interact with the membrane.

While the other three studied anions induced an increase in the magnitude of the membrane capacitance in the same order (nitrate, acetate), perchlorate induced an augmentation with 70% in the presence of 40 μM BSA-Hyp complex, and a decrease with 30% of this parameter in the presence of Hyp compared to the control value. At 48 μM BSA-Hyp complex, the increase in capacitance induced

by the presence of perchlorate reaches a 2.6 times higher value than at the beginning of the experiment (Fig. 3).

The kosmotrope acetate induces an increase in capacitance in between two chaotrope anions (larger than nitrate, but smaller than perchlorate) and it appears there is no connection between the effect and the anion position in the Hofmeister series (Fig. 3).

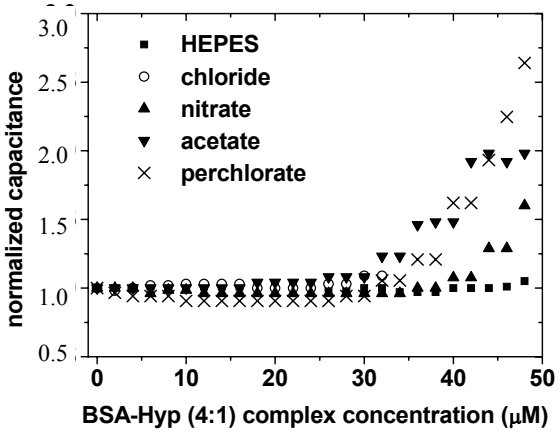

Fig. 3. The variation of the artificial lipid membrane capacitance in the presence of BSA-Hyp complex and some lyotropic anions in 100 mM concentration in 20 mM HEPES, pH = 7.

As for the increase in the conductance values, the most accentuated modification is induced by acetate followed by perchlorate and nitrate (Fig. 4). This might be correlated with anions position in Hofmeister series.

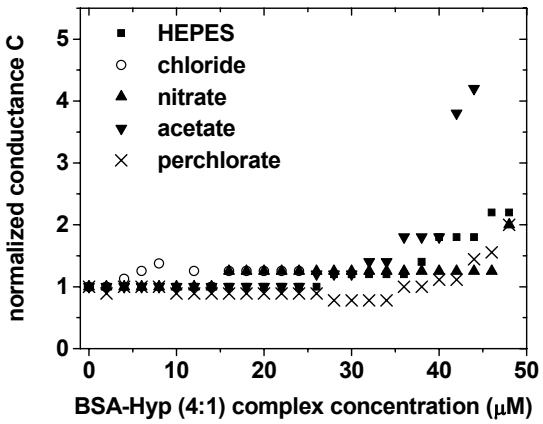

Fig. 4. The variation of the artificial lipid membrane conductance in the presence of BSA-Hyp complex and some lyotropic anions in 100 mM concentration in 20 mM HEPES, pH = 7.

The artificial lipid membrane became less conductive in the simultaneous occurrence of nitrate/perchlorate and Hyp/BSA-Hyp complex (Fig. 5 b). This fact suggests a protective effect of Hyp on lipid membrane displayed regarding the perturbances induced by the presence of a strong chaotrope anion, which is accentuated when BSA-Hyp complex is present.

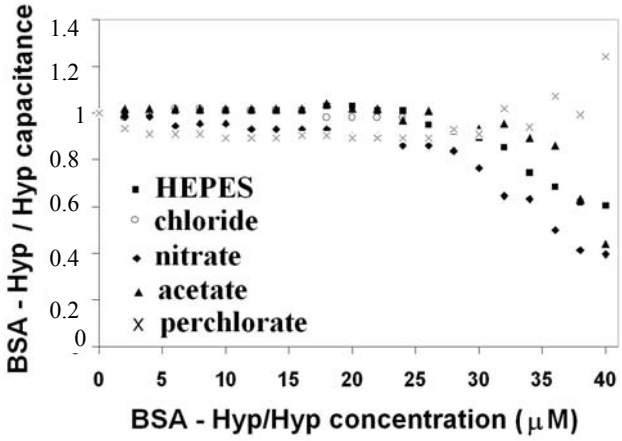

Fig. 5. a. BSA-Hyp complex and Hyp membrane capacitance ratio in the presence of 20 mM HEPES and 100 mM anions, pH = 7.

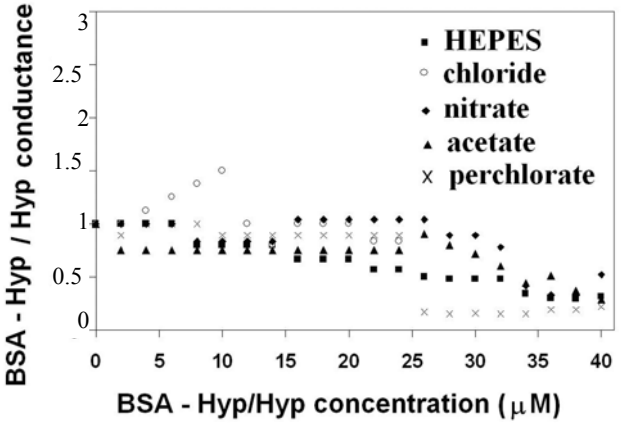

Fig. 5. b. BSA-Hyp complex and Hyp membrane conductance (b) ratio in the presence of 20 mM HEPES and 100 mM anions, pH = 7.

Acetate induces similar effects in the presence of 40 μM Hyp or BSA-Hyp complex idle an increase in capacitance larger for Hyp than for BSA-Hyp complex, and a small increase in relative conductance, for both substances, these augmentations accentuating as BSA-Hyp complex concentration enhances (Fig. 6).

Due to its kosmotrope nature, acetate is highly hydrated and it orders surrounding water molecules, therefore the effective albumin concentration increases favoring BSA precipitation and discharging of Hyp molecules, this effect becoming more explicit as BSA-Hyp concentration increases.

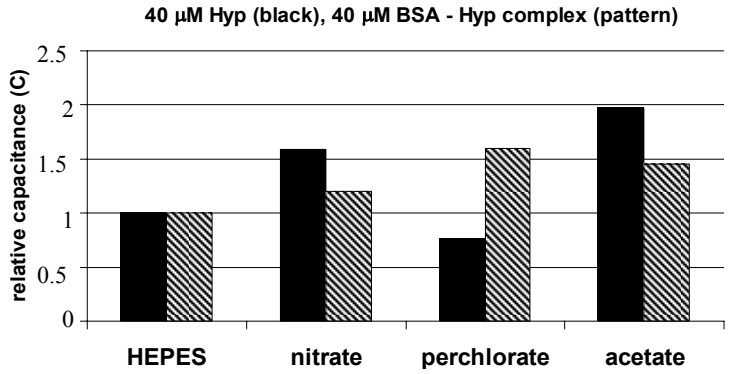

Fig. 6. a. Comparative membrane conductance at 40 μM BSA-Hyp complex or Hyp in the presence of 20 mM HEPES and 100 mM anions, pH = 7.

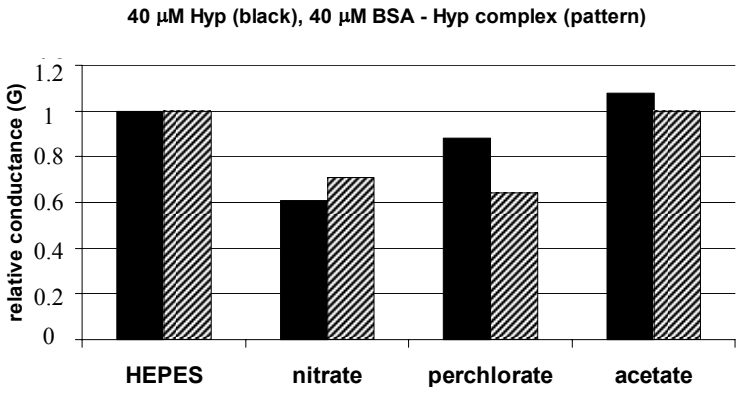

Fig. 6. b. Comparative membrane conductance at 40 μM BSA-Hyp complex or Hyp in the presence of 20 mM HEPES and 100 mM anions, pH = 7.

In conclusion, the behavior of the two investigated electric parameters of the membrane is different in the presence of increasing concentrations of BSA-Hyp complex, and also the interaction of the BSA-Hyp complex with the artificial lipid membrane is significantly different from the interaction of Hyp alone. While the electric capacitance of the lipid membrane increases dramatically with Hyp concentration, indicating a strong interaction at the level of the interface, BSA-Hyp complex keeps the capacitance values constant, while the conductance increases slowly. We imply that the complex does not interfere with the bilayer, modifying its thickness or its electric permeability, but it probably changes the membrane

dipole potential, enabling a slight augmentation of the artificial lipid membrane conductance, probably due to its preferential orientation nearby the membrane with Hyp molecules towards the artificial lipid bilayer. That means that Hyp rather binds to BSA than to the lipid membrane, and it appears that Hyp effect at the level of the membrane is not done through the lipid region when albumin is present.

When different anions are present in 100 mM concentration, on both sides of the membrane, the capacitance, as well the conductance of the bilayer, increase with the complex concentration, but in a slower manner than in the simultaneous presence of Hyp and anions. This behavior might have two different causes: (a) either BSA-Hyp complex incorporates into the membrane in the presence of anions slower than Hyp alone; (b) or there is no complex incorporation, but the complex releases Hyp molecules allowing them to interact with the membrane. The released Hyp molecules are probably nonspecifically bound to albumin, and this bond is weaker than the specific one. It appears that the release of Hyp molecules is more evident when kosmotrope anions, such as acetate, are present, the behavior of the electric conductance of the membrane being dependent on the anions position on Hofmeister series.

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REFERENCES

1. AGOSTINIS, P., A. VANTIEGHEM, W. MERLEVEDE, P.A. DE WITTE, Hypericin in cancer treatment: More light on the way, *The International Journal of Biochemistry and Cell Biology*, 2002, **34(**3**)**, 221–241.
2. BAMBERG, E., H.J. APELL, N.A. DENCHER, W. SPERLING, H. STIEVE, P. LÄUGER, Photocurrents generated by bacteriorhodopsin on planar bilayer membranes, *Biophysics of Structure and Mechanism*, 1979, **5**, 277–292.
3. BLANK, M., G. KOSTENICH, G. LAVIE, S. KIMEL, Y. KEISARI, A. ORENSTEIN, Wavelength-dependent properties of photodynamic therapy using hypericin *in vitro* and in an animal model, *Photochemistry and Photobiology*, 2002, **76(**3**)**, 335–340.
4. BUYTAERT, E., J.Y. MATROULE, S. DURINCK, P. CLOSE, S. KOCANOVA, J.R. VANDENHEEDE, P.A. DE WITTE, J. PIETTE, P. AGOSTINIS, Molecular effectors and modulators of hypericin-mediated cell death in bladder cancer cells, *Oncogene*, 2008, **27(13**), 1916–1929.
5. BLANK, M., M. MANDEL, S. HAZAN, Y. KEISARI, G. LAVIE, Anti-cancer activities of hypericin in the dark, *Photochemistry and Photobiology*, 2001, **74 (2)**, 120–125.
6. CALLIGARIS, S., M. C. NICOLI, Effect of Selected Ions from Lyotropic Series on Lipid Oxidation Rate, *Food Chemistry*, 2006, **94 (1)**, 130–134.
7. COHN, E.J., J.T. EDSALL, J. TILESTON, *Proteins, amino acids and peptides as ions and dipolar ions*, Reinhold Publishing, N.Y., 1943.
8. DAS, K., A. V. SMIRNOV, J. WEN, P. MISKOVSKY, J. W. PETRICH, Photophysics of hypericin and hypocrellin A in complex with subcellular components: Interactions with human serum albumin, *Photochemistry and Photobiology*, 1999, **69(6)**, 633–645.

9. D'HALLEWIN, M.A., A.R. KAMUHABWA, T. ROSKAMS, P. DE WITTE, L. BAERT, Hypericin-based fluorescence diagnosis of bladder carcinoma, *BJU International*, 2002, **89**, 760–763.
10. FALK, H., From the photosensibilisator hypericin to the photoreceptor stentorin – the chemistry of the phenanthroperylene quinones, *Angewandte Chemie International Edition*, 1999, **38**, 3134–3154.
11. Falk, H., J. Meyer, On the homo- and heteroassociation of hypericin, *Monatshefte für Chemie*, 1994, **125**, 753–762.
12. HODGKIN, A. L., P. HOROWICZ, The effect of nitrate and other anions on the mechanical response of single muscle fibres, *The Journal of Physiology (London),* 1960, **153**, 404–418.
13. HOFMEISTER, F., Zur Lehre von der Wirkung der Salze. II, *Arch. Exp. Pathol. Pharmakol.***,** 1888, **24**, 247–260.
14. IFTIME, I., M. DRAGUSIN, D. IONESCU, C. GANEA, The Hofmeister effect of anions on electrical parameters of lipid bilayers, *Revista Medico-Chirurgicală a Societăţii de Medici şi Naturalişti din Iaşi,* 2005, **109(**1**)**, 173
15. IONESCU, D., C. GANEA, The Hofmeister effect of anions in the insertion of hypericin in lipid bilayers, *International Biophysics Congress, European Biophysical Journal*, 2005, **34(**6**)**, 699
16. JOSEPH, J.A., B. SHUKITT-HALE, F.C. LAU, Fruit polyphenols and their effects on neuronal signaling and behavior in senescence, *Annals of the New York Academy of Sciences*, 2007, **1100(**1**)**, 470–485.
17. KÖHLER, M., J. GAFRET, J. FRIEDRICH, H. FALK, J. MEYER, Hole-burning spectroscopy of proteins in external fields: Human serum albumin complexed with the hypericinate ion, *Journal of Physical Chemistry*, 1996, **100**, 8567–8572.
18. MARTINEZ-POVEDA, B., A. R. QUESADA, M. A. MEDINA, Hypericin in the dark inhibits key steps of angiogenesis *in vitro*, *European Journal of Pharmacology,* 2005, **516(2)**, 97–103.
19. MISKOVSKY, P., J. HRITZ, S. SANCHEZ-CORTES, G. FABRICIOVA, J. ULICNY, L. CHINSKY, Interaction of hypericin with serum albumins: Surface-enhanced Raman spectroscopy, resonance Raman spectroscopy and molecular modeling study*, Photochemistry and Photobiology*, 2001, **74(2)**, 172–183.
20. MISKOVSKY, P., Hypericin – a new antiviral and antitumor photosensitizer: Mechanism of action and interaction with biological macromolecules*, Current Drug Targets*, 2002, **3(**1**)**, 55–84.
21. MOSKAUG, J.Ø., H. CARLSEN, M.C.W. MYHRSTAD, R. BLOMHOFF, Polyphenols and glutathione synthesis regulation, *The American Journal of Clinical Nutrition*, 2005, **81(**1**)**, 277S–283S.
22. PEEBLES K.A, R.K. BAKER, E.U. KURZ, B.J. SCHNEIDER, D.J. KROLL, Catalytic Inhibition of human DNA topoisomerase IIα by hypericin, a naphthodianthrone from St. John's Wort (*Hypericum perforatum*), *Biochemical Pharmacology*, 2001, **62**,1059–1070.
23. STUPAKOVA V., L. VARINSKA, A. MIROSSAY, M. SARISSKY, J. MOJZIS, R. DANKOVCIK, P. URDZIK, A. OSTRO, L. MIROSSAY, Photodynamic effect of hypericin in primary cultures of human umbilical endothelial cells and glioma cell lines, *Phytotherapy Research,* 2009, **23(**6**),** 827–832.
24. TIMASHEFF, S. N., The control of protein stability and association by weak interaction with water, *Annual Review of Biophysics and Biomolecular Structure*, 1993, **22**, 67–97.