MODULATION BY QUERCETIN OF THE EFFECT OF CERTAIN HOFMEISTER ANIONS ON ARTIFICIAL LIPID BILAYERS

DIANA IONESCU, ANCA POPESCU, MAGDA DRĂGUŞIN, MIHAI DIMA, ADRIAN IFTIME, CONSTANŢA GANEA

Department of Biophysics, "Carol Davila" University of Medicine and Pharmaceutics, 8, Eroii Sanitari Blvd., 050474-Bucharest, Romania

Abstract. Our paper investigates, by means of the black lipid membranes (BLM) technique, the effect of the natural flavonoid quercetin (QCT) on the electrical parameters of artificial phospholipidic membranes subjected to the action of Hofmeister anions (chloride, nitrate, acetate) in different concentrations. Quercetin shows a modulator effect on the anion-induced electrical parameters changes of lipid bilayers. As suggested by our results, quercetin acts by insertion in the artificial membrane, as well as by influencing the anion-lipid interactions. Our results indicate that the effects of quercetin and Hofmeister anions are not summative, which points out complex interactions in the membrane-anions-quercetin system.

Key words: quercetin, Hofmeister anions, BLM.

INTRODUCTION

Quercetin is a natural flavonoid, contained in vegetables and fruits which are part of our daily diet (apples, onions, etc.). Quercetin is known to act as an antioxidant substance, protecting the living cells against the damage induced by free radicals [1, 2] and opposing lipid peroxidation. *In vivo*, the antioxidant effect of quercetin is probably facilitated by its ability to insert into lipid membranes, due to its planar structure [19]. On artificial lipid bilayers, quercetin insertion is influenced by concentration and pH [16, 17], and results in increased conductance and capacitance of the artificial membranes [13]. Suggested mechanisms are changes in the thickness and surface of the lipid membrane and possible pore formations [13, 16, 17].

The Hofmeister ions (a series of ions classically described in 1889 by Hofmeister, based on their ability to influence protein stability and degradation) can stabilize or destabilize conformational transitions of macromolecules, for example the helix-coil transition [3, 11]. Although it is now acknowledged that the

Received April 2007.

ROMANIAN J. BIOPHYS., Vol. 17, No. 2, P. 85–95, BUCHAREST, 2007

documented Hofmeister phenomena are based on solvent effects, there is no satisfying explanation regarding the mechanism of action of different ions on the structure and function of biomolecules. Suggested explanations involved solvation water, water structure, ion-dipole interactions and direct interactions with amidic groups of model molecules [5]. Collins and Washabaugh [9] showed that anions are much more efficient than cations, in what concerns Hofmeister effects, and classify the ions in cosmotropic ions (strong hydrophilic ions, able to break the hydrogen bounds in the water structure and reorganize the water molecules around them) and chaotropic ions (which can not break the hydrogen bonds between the water molecules and, as a result, are surrounded by clatrat-forming molecules). The effects of certain specific anions, corresponding to their adsorbability [12] increase in the following order: $SO_4^{2-} < F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$ [8].

The effect of Hofmeister ions is not restricted to proteins. Lipid membranes and vesicles also seem to be susceptible to changes of lipid phase behavior or surface potential and dipolar moment alterations, induced by these ions [6, 7, 14, 15, 18]. In the close neighborhood of lipid membranes, the chaotropic ions show the tendency to insert into the hydrophobic membrane (salting-in effect), while the cosmotropic ions distribute in the surrounding water (salting-out effect).

Since many of the Hofmeister anions are dietary additives, it is important to search for a method to diminish their effects on cell membranes. The purpose of our study is to investigate whether or not quercetin could prevent or diminish the structural and electrical changes induced by a few commonly encountered Hofmeister anions: chloride, nitrate and acetate.

MATERIAL AND METHOD

The electrical properties (capacitance and conductance) of artificial membranes have been measured by means of the BLM (black lipid membrane) technique (for details about the technique, see Bamberg *et al*., 1979 [4]). The lipid membrane has been formed on a 10^{-2} cm^2 orifice on the separating wall between the two compartments of a teflon cuvette, each filled with 1.5 ml electrolyte solution. The lipid stock solution contained 1.5 mg / 100 ml diphytanoylphosphatidylcholine (Avanti Chemicals, Birmingham, AL) and 0.025 mg / 100 ml octadecylamine (Riedel-de-Haen, Hannover, Germany) dissolved in n-decane. The presence of octadecylamine in the lipid bilayer provided a slight positive surface charge of the artificial membrane. The membrane formation has been observed through a magnifying glass.

Quercetin (Sigma) has been prepared as 10 mM stock solution in DMSO and added in the cuvette solution to achieve the required concentrations. The DMSO concentration in the cuvette did not exceed 1/1000.

Sodium chloride NaCl, sodium nitrate $NaNO_3$ (Merck) and sodium acetate $NaCH_3COO$ (Sigma) salts have been prepared as 4 M stock solutions in 20 mM HEPES. Successive dilutions have been made in 20 mM HEPES (Merck), at pH 7 (adjusted with NaOH (Merck)).

We have performed two types of experiments. First, we studied the effect of increasing concentrations of anions (8 mM, 16 mM, 32 mM, 52 mM, 72 mM, 92 mM, 112 mM, 132 mM, 152 mM, 172 mM) added bilaterally in the cuvette solution, in the absence (control experiments) and the presence of quercetin 30 μM added single-sided in the cuvette (CIS position). The CIS position refers to the side of the separating wall (and of the orifice) on which the lipid membrane was formed. The electrical parameters of the membrane (conductance and capacitance) have been measured three times, at 5 min intervals, for each of the tested anionic concentrations, and averaged for a final result. The results have been normalized relatively to the membrane capacitance and conductance in the absence of anions, in a 20 mM HEPES solution containing 30 μM quercetin.

The second type of experiments studied the effect of increasing concentrations of quercetin (10 μM, 20 μM, 30 μM and 40 μM) added CIS in the BLM cuvette, in the presence of a constant anionic concentration. The same measuring protocol has been used, but we considered the last value of the series of measurements, accounting for the necessary time for quercetin to insert in the membranes. The normalization has been made relatively to the values measured for 10 μM quercetin. The initial solution in the cuvettes was 100 mM NaCl + 20 mM HEPES, at pH 7.

Each experiment has been repeated on 3–4 different membranes, and the results have been averaged. Fitting of the experimental data has been performed using the software OriginPro.

RESULTS AND DISCUSSION

We have studied the effect of quercetin 30 μM on the concentration-dependent changes of membrane capacitance and conductance induced by chloride, nitrate and acetate. The artificial membrane has been formed in 100 mM NaCl + HEPES 20 mM, and quercetin has been added unilaterally (CIS) from the 10 mM stock solution in DMSO, to achieve a 30 μM concentration in the cuvette solution. After reaching the stability of the membrane (reflected by stable electrical parameters of the membrane), the anion solutions have been applied bilaterally in the cuvette, at progressively higher concentrations, as described in *Material and Method.*

Quercetin 30 μM induces an anion concentration-dependent decrease of membrane capacitance, compared to the control experiments, when chloride is applied in the cuvette in increasingly bigger concentrations, but shows lesser effect on membrane capacitance in the presence of nitrate or acetate (Fig. 1).

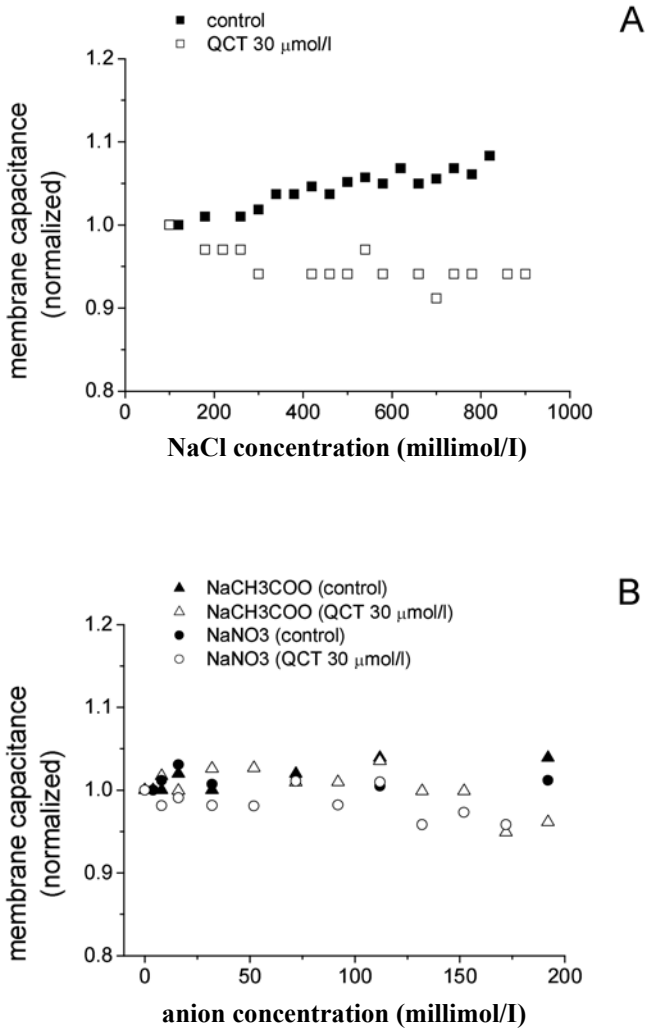

Fig. 1. Dependence of membrane capacitance on the anion concentrations, at constant quercetin concentrations. A. Concentration dependency of membrane capacitance for NaCl, in the presence of quercetin 30 μM compared to control (no quercetin) B. Concentration dependency of membrane capacitance for $NaNO_3$ and $NaCH_3COO$, in the presence of quercetin 30 μM compared to control (no quercetin).

Quercetin insertion in the lipid bilayers is better reflected by the changes in membrane conductance, as shown in figure 2. Our experiments indicate an increase of membrane conductance in the presence of chloride, acetate and nitrate (in the absence of quercetin), while the presence of quercetin induces an anion concentration–dependent decrease of membrane conductance.

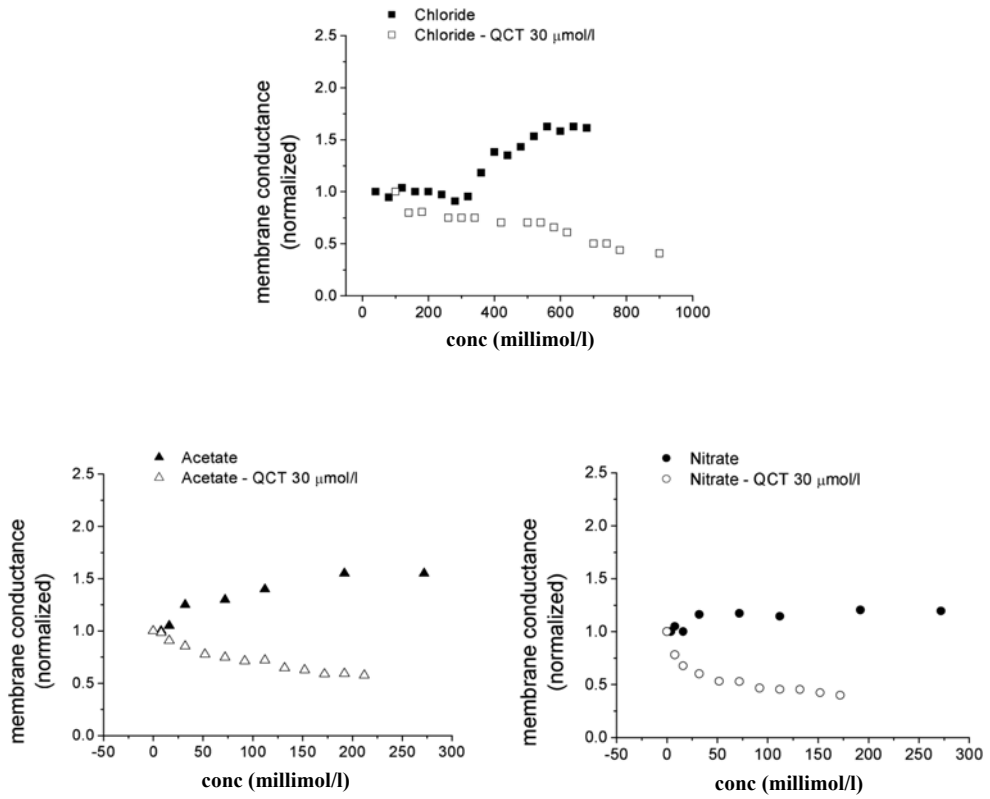

Fig. 2. Dependence of BLM measured conductance on anion concentrations, at constant concentrations of quercetin.

In the absence of quercetin, the most significant increase is recorded for chloride. One possible explanation accounts for the ionic mobility of the anions and cations dissolved in the bathing solution and on the dissociation constant of the salts. These parameters influence the amount of ionic electrical charge accumulated at the membrane-solution interface, which result in changes of measured membrane conductance. Both the dissociation constant and the ionic mobility are bigger for NaCl than for $NaCH_3COO$ or $NaNO_3$, which could explain the more important overall increase of measured conductance for NaCl than for the other salts.

When quercetin is added in the cuvette solution, the substance has the tendency to insert into the lipid bilayer [16, 17], which results in decreased conductance of the lipid bilayer due to increased membrane thinckness. Our results indicate that the process is influenced by the presence of chloride, nitrate and acetate. The most important effect (associated to a more significant decrease in membrane conductance) is demonstrated by nitrate, while the presence of chloride shows the smallest influence on quercetin insertion (Fig. 3).

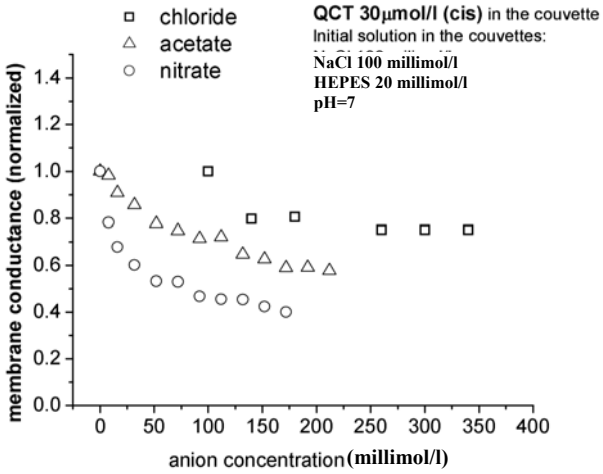

Fig. 3. Quercetin-induced changes of membrane conductance. Quercetin insertion is facilitated by the presence of chloride, acetate and nitrate. Nitrate shows the most important effect on quercetin insertion, while the influence of chloride is the mildest.

The insertion of the quercetin in the lipid bilayers, in the presence of anions, seems to be a biphasic kinetic process. The insertion is more important at smaller concentration of anions. The first binding phase (the fast binding phase) resulted from the quercetin-chloride and quercetin-acetate experiments is a two-exponential process, suggesting a more complex insertion kinetics at a smaller anion concentration (Fig. 6.). The second binding phase, occurring at higher anionic concentrations, is governed by a single decay constant. When acetate is added to the cuvette solution, although we can clearly differentiate two binding stages, a single exponential best fits each stage.

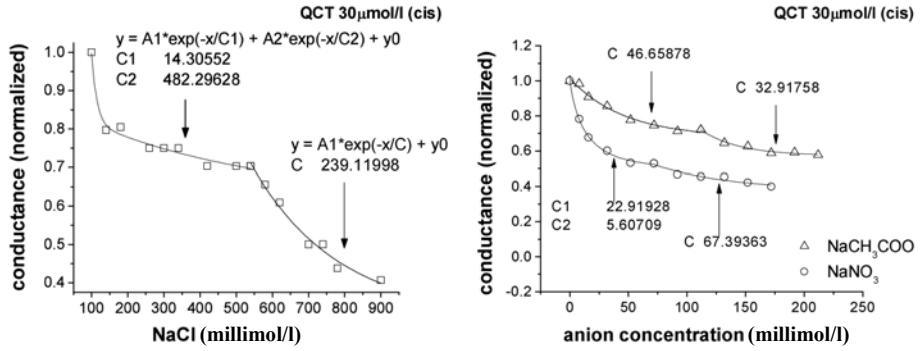

Fig. 4. Kinetic parameters of quercetin insertion into the lipid bilayer. The first insertion stage is fitted by a two-exponential equation for both the quercetin-NaCl system and the quercetin-$NaNO_3$ system. A single exponential is the best fit for the second insertion stage in all the studied systems (quercetin-NaCl, quercetin-$NaNO_3$, quercetin-$NaCH_3COO$), but also for the first stage of the quercetin-$NaCH_3COO$ system.

In order to obtain more information about quercetin insertion kinetics in the presence of chloride, nitrate and acetate we performed a second group of experiments. The lipid membrane has been formed in NaCl 100 mM + HEPES 20 mM, at pH 7, and anion solutions have been added to reach the desired concentration. Anion concentration has been maintained constant in the cuvettes, while quercetin has been added in increasingly higher concentrations. For each quercetin concentration, capacitance and conductance have been measured at 10 minutes intervals, until the parameters became stable.

The membrane capacitance has been stable for different quercetin concentrations, regardless of the amount of anions present in the cuvette (Fig. 5). These findings (consistent with the results for the first type of experiments we performed) imply that the conductance and not the capacitance changes are best correlated with quercetin insertion in the lipid bilayer.

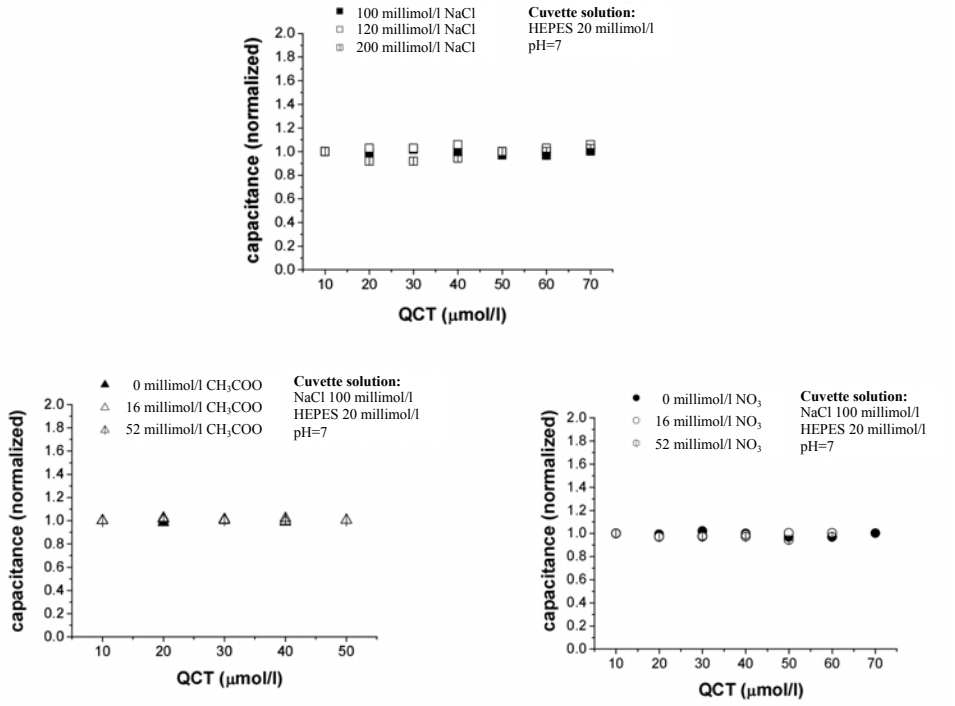

Fig. 5. Dependence of membrane capacitance on the quercetin concentrations, at constant anion concentrations. Except for low-level stochastic fluctuations in the presence of NaCl, the membrane capacitance remains constant at different concentrations of quercetin for cuvette solutions containing different concentrations of NaCl, $NaCH_3COO$ and $NaNO_3$.

There are obvious differences between the (quercetin concentration – system conductance) dependency profiles at different constant anion concentrations (Fig. 6).

The control curve has been measured with NaCl 100 mM + HEPES 20 mM in the cuvettes. Consistent with the previous results, quercetin insertion is facilitated by anions.

The exponential decrease of membrane conductance accounts for quercetin insertion in the lipid bilayer. Anions influence both the rate of quercetin insertion and the maximum amount of quercetin inserted in the membrane. The first parameter is reflected by the slope of the exponential (the exponential decrease constant), while the steady-state conductance (the plateau) offers information about the amount of quercetin inserted in the membrane.

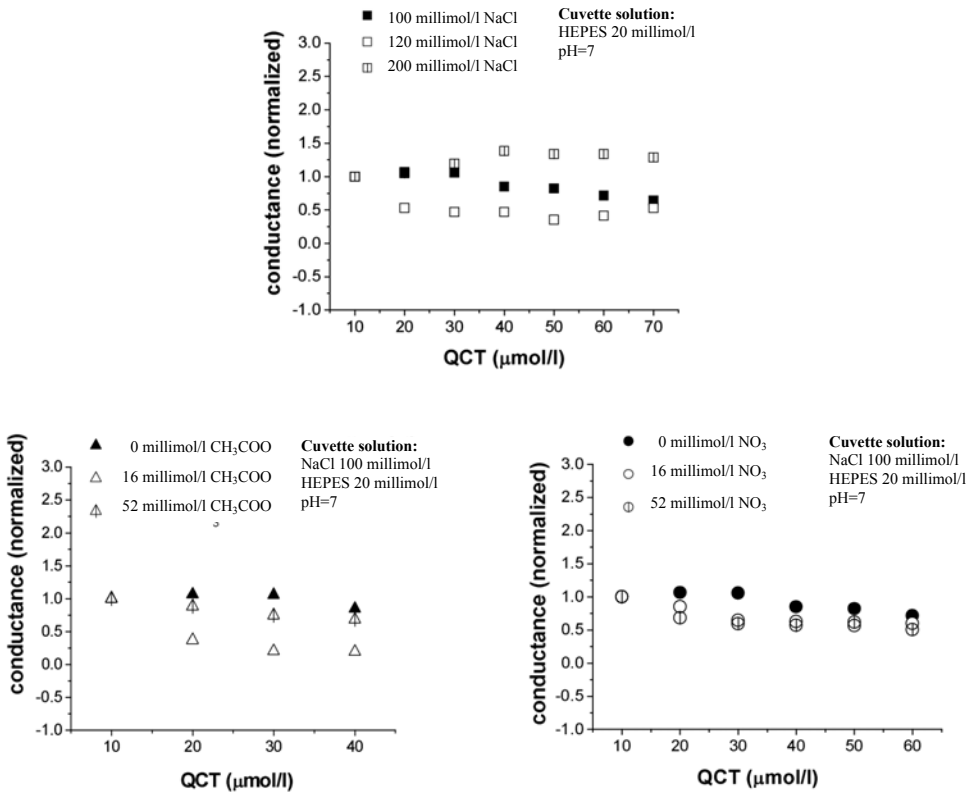

Fig. 6. Dependence of measured BLM conductance on the quercetin concentration. Results presented for NaCl 100 mM, 120 mM and 200 mM (upper row), $NaCH_3COO$ 0 mM, 16 mM and 52 mM (lower row, left) and $NaNO_3$ 0 mM, 16 mM and 52 mM (lower row, right).

Table 1 shows the values of the exponential decrease constant and of the plateau conductance obtained by fitting the experimental data for all the tested cases.

Table 1

Kinetic parameters for quercetin insertion in the lipid bilayer

	Exponential decrease constant k_d (mol/l)	**Steady-state conductance G_{ss} (normalized)**
NaCl 100 mM	28.72752	0.63
NaCl 120 mM	5.53575	0.41
$NaCH_3COO$ 16 mM	6.83649	0.19
$NaCH_3COO$ 52 mM	42.29525	0.68
$NaNO_3$ 16 mM	16.90025	0.59
$NaNO_3$ 52 mM	8.10046	0.59

A lower steady-state conductance G_{ss} is associated with a larger amount of quercetin inserted in the lipid bilayer. As our results show, $NaCH_3COO$ proves the most significant effect on quercetin insertion. Insertion is the fastest for NaCl 120 mM (the smallest decrease constant). Since 120 mM is very close to the NaCl concentration of the *in vivo* extracellular space, the fact that quercetin attaches more efficiently to the membranes at this concentration is of high importance in what regards the use of quercetin as dietary supplement. Moreover, the quercetin concentration in the membrane reached in the presence of NaCl 120 mM is one of the highest, as reflected by the low steady-state conductance (0.41). A higher concentration of quercetin in the membrane is achieved only for $NaCH_3COO$ 16 mM. Considering that the normal acetate values for the human blood are approx. 0.1 mM and rise up to 0.75 mM in pathological conditions (ethanol intoxication, diabetes mellitus) [10], our findings are not of clinical relevance. Further studies have to be performed in order to establish if the acetate facilitator effect on quercetin membrane insertion is also present at lower concentrations. An interesting aspect observed is that there is no clear concentration dependence between the kinetics of quercetin insertion and the concentration of anions. In fact, quercetin insertion is more important at 16 mM than at 52 mM, both as insertion speed and maximum concentration in the membrane. At the same concentrations of nitrate, the steady-state concentration of quercetin in the membrane is the same, what differs is the rate of insertion.

CONCLUSIONS

Artificial lipid membranes subjected to the effect of Hofmeister anions – precisely chloride, nitrate and acetate – show changes of their electrical parameters, which is important in regard to their function. The protective effect of quercetin is related to its ability to insert in the lipid bilayers. Our data show that there is a reciprocal interaction between the anion effects on the membranes and quercetin insertion kinetics.

At a constant quercetin concentration (30 μM), the membrane capacitance decreases as a function of the anion concentration. The effect is more significant for NaCl than for acetate or nitrate. The membrane capacitance decrease can be explained by changes of the membrane geometry (surface, thickness) – since the artificial lipid bilayers can be modeled by a planar non-ideal capacitor – but can also be due to modifications by the quercetin-anion complex of the electrical permittivity of the membrane. More information is obtained from the conductance profiles of the membrane subjected to increasingly higher concentrations of anions. Our paper suggests that the membrane conductance in the presence of anions could be influenced by the ionic concentration, the dissociation constant and the ionic mobility of the dissolved species.

By adding quercetin at a constant concentration (30 μM) in the bath solution, membrane conductance decreases as a result of quercetin insertion in the membrane. Under these circumstances (constant quercetin concentration and increasing anion concentration), the conductance decreases exponentially in a two-step fashion. The first decrease phase is best fitted by a double exponential for chloride and nitrate and by a single exponential for acetate, while the second (slower) phase is a single exponential.

Experiments performed at constant anion concentrations while quercetin concentration was progressively increased offered more insights on the quercetin insertion kinetics. The anions showed a facilitator effect on quercetin insertion, but the effect was not dose-dependent. The process was evaluated based on two parameters, determined by fitting the experimental data: the exponential decrease rate for the conductance profile (accounting for the speed of the process) and the steady-state conductance (accounting for the maximum concentration of quercetin inserted in the lipid bilayer). Nitrate showed the smallest potentiating effect on quercetin insertion. The process was the fastest in the presence of NaCl 120 mM, a finding with important clinical implications, since this chloride concentration is near the biological value for the human extracellular fluid. The highest concentration of inserted quercetin was computed in the presence of acetate 16 mM. Although the blood acetate concentration is much lower under normal circumstances (0.1 mM) and rarely exceeds 0.5 mM (in case of diabetic keto-acidosis or acute ethanol intoxication), this information could be of great clinical importance. One of the major problems in diabetes mellitus is the oxidative stress leading to cellular functional and structural abnormalities, and quercetin is known for its antioxidative effects, which could be increased by the increased concentrations of acetate in diabetes. Further studies are, though, required, in order to verify if the potentiating effects of acetate on quercetin insertion are maintained at lower concentrations of acetate (in the range of the biological blood levels).

Acknowledgments. This work was supported by the Romanian Education and Research Ministry grants CEEX 74/2006, CNCSIS 152/2004 and CNCSIS 614/2006.

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