THE JOURNAL OF PHYSICAL CHEMISTRY B LETTERS

2008, *112,* 15263–15266
Published on Web 11/07/2008

Second Harmonic Studies of Ions Crossing Liposome Membranes in Real Time

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Received: July 28, 2008; Revised Manuscript Received: October 17, 2008

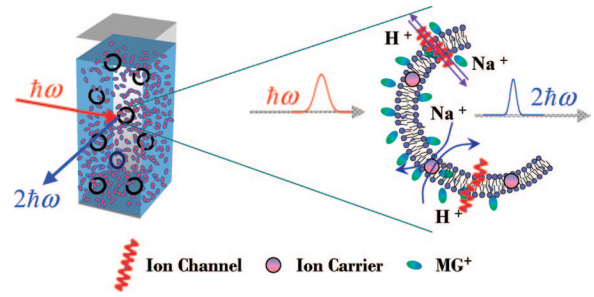
The transport kinetics of the positively charged triphenylmethane dye, malachite green (MG^+), across liposome bilayers effects the transport of monovalent inorganic cations when ionophores are present in the membrane. Three different types of ionophores characterized by different transport mechanisms have been studied. The ionophores are gramicidin A (gA) (a channel former), valinomycin (VAL) (a lipophilic cyclopeptide that encloses an alkali ion), and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) (a weak acid that functions as a protonophore). The effects of these ionophores on the kinetics and extent of MG^+ crossing into the liposome, investigated using the interface selective second harmonic generation method, were found to be markedly different.

Transmembrane movement of ions is an important cellular process that plays a vital role in transforming energy and processing information in living cells. Although the nonpolar core of biological membranes is highly impermeable to most inorganic ions, there are compounds, commonly called ionophores, that can facilitate the transfer of inorganic ions across cell membranes. There exist many different types of ionophores in biological systems for which the chemical mechanisms as well as the thermodynamic and kinetic properties are diverse.[1−3] These ionophores are of particular interest in the areas of biological and medical sciences and have been the subject of numerous studies.[4−7] In this paper, we report the use of the interface selective nonlinear optical process of second harmonic generation (SHG) to the study of ion transport across a phospholipid liposome bilayer membrane in real time. We present and compare the effect of three different types of ionophores—valinomycin (VAL), carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), and gramicidin A (gA)—on the transport kinetics of a probe molecule, the cationic form of the triphenylmethane dye malachite green, MG^+, and the associated transport of monovalent inorganic cations across negatively charged dioleoylphosphatidylglycerol (DOPG) liposome bilayers. It should be noted that most biological cells are also negatively charged.

The principal idea in this study is the SHG detection of MG^+ crossing the bilayer, which in turn induces inorganic ion transport when ionophores are present in the membrane. The inorganic ion and MG^+ transport are coupled processes, which are manifested by the change in the kinetics and extent of MG^+ crossing into the liposome (Scheme 1).

A Ti:sapphire femtosecond laser at 840 nm was used to generate the SHG signal in the sample. In brief, when MG^+ is rapidly mixed (<1 s) with an aqueous solution of liposomes, MG^+ adsorbs to the outer surface of the negatively charged liposome and generates a strong SHG signal.[8−10] The MG^+ in the bulk solution cannot generate a coherent SHG signal because

SCHEME 1: SHG Detection of Ions Crossing Liposome Bilayers



of symmetry restrictions. The diffusion and adsorption of MG^+ takes place in less than a second. Thereafter, due to the driving force of the concentration gradient across the bilayer, MG^+ crosses the bilayer membrane toward the interior of the liposome and adsorbs to the inner surface of the bilayer. By symmetry, these adsorbed molecules at the inner and outer layers are oppositely oriented, and since the thickness of the membrane (~5 nm) is much smaller than the SHG coherence length, the induced second order polarization from these oppositely oriented molecules cancels.[8,11] Thus, the initial SHG signal decays with time as more MG^+ permeates into the liposome. The magnitude of the SHG field, $E_{2\omega}$, is linearly proportional to the difference in populations of MG^+ on the outer surface and inner surface at time t after mixing. The MG^+ counterions, which in these experiments are Cl^- ions, do not cross the liposome bilayer in the time frame of the observed kinetics.[2,12]

It has been established that, as the positively charged malachite green crosses the bilayer, a positive potential develops inside the liposome.[13] This opposes more MG^+ from going into the liposome. Thus, the second harmonic signal approaches a steady state value at long times that is determined by the balance between the electrostatic forces and the chemical concentration gradient. It is possible to disrupt this balance by introducing an ionophore into the system. The basic idea is that the presence of the ionophore in the bilayer membrane will allow inorganic

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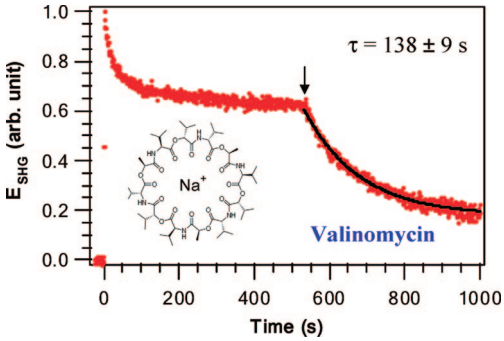

Figure 1. SHG electric field, E_{SHG}, as a function of time before and after addition of VAL. MG^+ is added to the DOPG solution at $t = 0$ s. The arrow indicates the time at which VAL is introduced into the MG^+−liposome solution. The solid black lines represent a single exponential fit to the data.

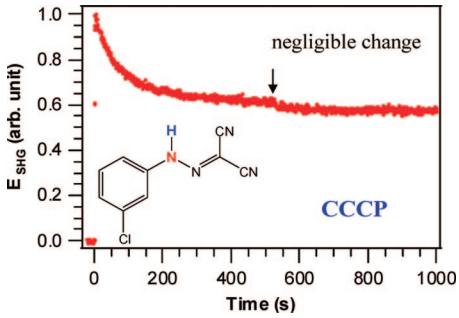

Figure 2. SHG electric field, E_{SHG}, as a function of time before and after addition of CCCP. MG^+ is added to the DOPG solution at $t =$ 0 s. The arrow indicates the time at which CCCP is introduced into the MG^+−liposome solution.

cations to be transported out of the liposome and thus relieve the electrostatic barrier created by the MG^+ that has crossed into the liposome. In this way, the concentration gradient will lead to more MG^+ being transported into the liposome, resulting in a decay of the second harmonic signal, and thereby revealing the effect of ionophores on MG^+ transport in real time.

We have investigated the effect of VAL (an alkali ion carrier), CCCP (a weak acid that serves as a proton transporter), and gA (an ion channel across which alkali and hydronium ions can be transported). Our experimental system consists of 8×10^{10} DOPG ($pK_a \sim 3$) liposomes/cm^3, with a diameter of 110 ± 5 nm obtained from dynamic light scattering measurement. The liposomes and MG^+ solutions were prepared with a 10 mM NaCl salt solution, and at pH 4 to favor the malachite green (pK_a 7) in its cationic form. The setup for the SHG measurements and the liposome preparation are described in detail elsewhere.[10,13] Figures 1−3 show the effect of the addition of VAL, CCCP, and gA with a final concentration of 1.25×10^{-8} M, 1.25×10^{-7} M, and 6.25×10^{-10} M, respectively, on MG^+ movement across the DOPG bilayer. The initial rise in signal at $t = 0$ s is due to MG^+ adsorption onto the outer surface of the DOPG liposome upon rapid mixing. The SHG signal decreases as MG^+ crosses the bilayer. After 550 s, the SHG signal levels off, indicating that there is no further net transport of MG^+ into the liposome. At this time, 550 s, the ionophore is injected, in an aliquot of 20 μL, into the stirred liposome solution.

Valinomycin is a naturally occurring macrocyclic ionophore that forms complexes with alkali cations.[14,15] It facilitates ion transport across the membrane by diffusing back and forth across the lipid membrane.[16] Figure 1 shows that when VAL is added to the liposome−MG^+ solution, a decrease in the SHG signal

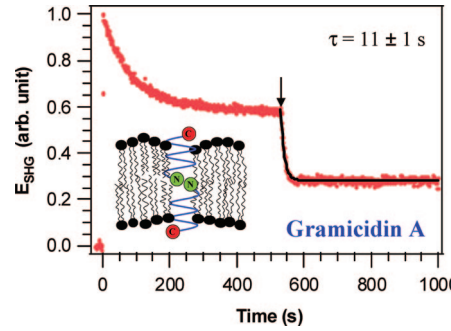

Figure 3. SHG electric field, E_{SHG}, as a function of time before and after addition of gA. MG^+ is added to the DOPG solution at $t = 0$ s. The arrow indicates the time at which gA is introduced into the MG^+−liposome solution. The solid black lines represent a single exponential fit to the data.

occurs, which we attribute to more MG^+ going into the liposome as VAL transports Na^+ ions out of the liposome. By fitting the data with a single exponential function, we obtain a time constant of $\tau = 138 \pm 9$ s. At long time, the N_i/N_o ratio (the number of MG^+ adsorbed on the inner vs the outer membrane) for the VAL assisted transport of MG^+ is 0.85 ± 0.02, which is equal to the ratio of the area of the inner leaflet to the outer leaflet for the DOPG liposomes, with a bilayer thickness of 5 nm, used in these experiments.

This marked increase in MG^+ transport across the bilayer using VAL is not the case when CCCP is introduced into the liposome−MG^+ solution (Figure 2). CCCP, a weak organic acid that only transports protons across membranes in its neutral acidic form and crosses back in its anionic form, is one of the most effective uncouplers of oxidative phosphorylation in living cells.[17−19] Figure 2 shows that there is a small decrease in the SHG signal upon addition of CCCP which, at long time, reaches a N_i/N_o value of 0.45 ± 0.02. In the absence of ionophores, the N_i/N_o value obtained from SHG measurements is 0.41 ± 0.03, which shows that CCCP has a negligible effect on MG^+ crossing the liposome bilayer. Our result demonstrates that CCCP is ineffective in transporting protons across the negatively charged DOPG liposome bilayer, which is in agreement with the effects of CCCP on transbilayer conductivity.[19−21] Briefly, this result is expected because the deprotonated form of CCCP is negatively charged and as a consequence its transport against the negative potential at the outer liposome surface is energetically unfavorable.

Gramicidin A is a linear peptide containing 15 alternating D and L amino acids. In lipid membranes, two gA monomers in opposing leaflets come together and are hydrogen bonded to form a β-helical dimer. The noncovalent head-to-head (formyl-NH-terminal-to-formyl-NH-terminal) association of monomers forms the single stranded antiparallel dimer which results in an open channel.[2,22,23] The adoption of the gA channel conformation in the lipid bilayer is dependent on the organic solvent from which it is added because gA can exhibit different conformations in organic solvents.[24] In methanol, cosolubilizing solvent used in our experiments, gA predominantly exists as β-helical monomer and, as a result, incorporates into the membrane directly as a single stranded β-helix.[24−26] In order to form a channel, the helical monomers in both leaflets, one in the outer leaflet and the other in the inner leaflet, of the bilayer must move to the same location in the bilayer. At this geometry, a channel across the bilayer can be formed.

In addition to alkali metals, gA channels are permeable to protons.[27] Experimental results obtained upon adding gA (Figure

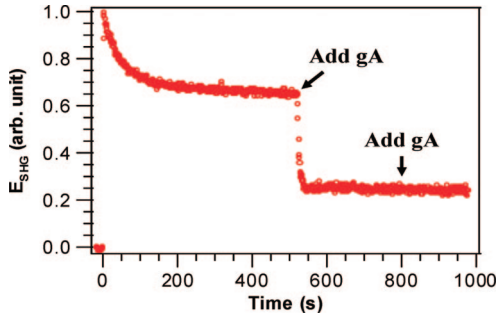

Figure 4. SHG electric field, E_{SHG}, as a function of time. In this experiment, gA was added twice to the same liposome−MG^+ solution (once at ∼550 s and then at 800 s).

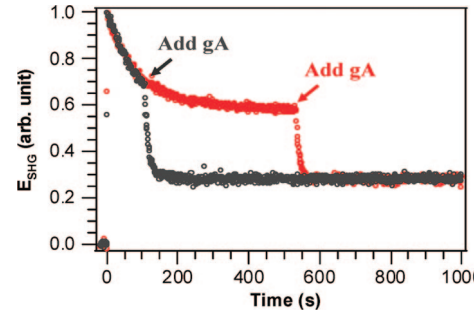

Figure 5. SHG electric field, E_{SHG}, as a function of time. Comparison of two different gA-mediated kinetics experiments. In one experiment, gA was introduced at ∼100 s (black), and in another, at ∼550 s (red).

3) indicate that the combined processes of gA insertion into the membrane, flipping of several monomers onto the opposite leaflet of the bilayer, formation of the ion channel, followed by the transport of H_3O^+ and Na^+ ions across the membrane occur in 11 ± 1 s. This is more than 10 times faster than that of the VAL facilitated ion transport. The marked difference in transport kinetics reflects the channel versus the carrier nature of these two different ionophores. We have also found that when the liposome is prepared with gA, i.e., gA monomers already present in the liposome bilayer, inorganic cations and MG^+ cross the bilayer in less than 1 s, which is faster than our time resolution. On the basis of the diffusion rate constant of $\sim 10^8$ M^{-1} s^{-1} for Na^+ ion through a single gA channel,[28] a fast ion transport is expected. In the experiments reported here, the number of gA monomers/cm^3 added to the solution divided by the number of liposomes/cm^3 is 8; i.e., on average, there are 8 gA monomers per liposome. Because gA is insoluble in water, methanol was used to bring the gA monomers into the liposome solution. We infer from the short time constant of less than 1 s, for the experiments where gA is already present in the bilayer, that the motion of gA in the leaflets is very fast and thereby forms gA channels very rapidly. We therefore attribute the 11 ± 1 s, for the experiments where gA is added after MG^+ has been mixed with the liposome solution, to the time of gA monomer insertion into the bilayer and the time for the gA monomers to flip from the outer leaflet, which is the initial location of the inserted monomers, to the inner leaflet. The rates of phospholipid translocation from one leaflet to the other are usually very slow, on the order of hours.[29,30]

An interesting aspect of gA-mediated ion transport is that gA fails to fully remove the charge buildup as MG^+ crosses the membrane, $N_i/N_o = 0.75 \pm 0.03$. To understand this, we first confirmed that MG^+ does not block the gA channel. This confirmation was obtained by adding gA twice, once at 550 s and then at 800 s, to the same liposome−MG^+ solution. Figure 4 shows that there is no change in the final N_i/N_o value upon addition of gA at $t = 800$ s. This indicates that addition of gA does not lead to further transport of MG^+ and thus verifies that MG^+ does not hinder inorganic ion transport via gA. Moreover, when gA is introduced to the MG^+−liposome solution at an earlier time, approximately at 100 s, the same N_i/N_o ratio is observed (Figure 5). The final SHG level is the same value for both cases; i.e., the addition of gA at a time, 100 s, when fewer MG^+ ions have crossed the bilayer, compared with gA added at a later time, 550 s, when more MG^+ ions have crossed the bilayer, does not affect the final N_i/N_o value. In other words, the different MG^+ concentration gradients at the time when gA is added, i.e., 100 vs 550 s, does not affect the final steady state values of MG^+ inside vs outside the liposome.

Because gA transports both Na^+ and H_3O^+ ions, the steady state reached involves these two ions and MG^+, which therefore differs from the steady state achieved with VAL. In the gA steady state, fewer MG^+ ions enter the liposome compared to when MG^+ is transported into the liposome in the presence of VAL. Unlike gA, valinomycin transports only Na^+ ions; thus, in its presence, the exchange of MG^+ entering the liposome and Na^+ leaving the liposome can continue until the inner surface is fully occupied by MG^+ ions. The maximum population of MG^+ inside the liposome is determined by the inside surface area. This maximum can be achieved in the presence of VAL because there is a sufficient number of Na^+ ions that can leave the interior via the VAL ionophore, which balances the number of MG^+ ions that cross into the liposome. On the other hand, the steady state reached with gA is one in which the number of MG^+ ions that enter the liposome is not equal to the maximum value that is reached with VAL. Thus, the decay in the SHG signal is incomplete compared with VAL.

In summary, we have used the surface selective method of SHG to monitor in real time the effects of ionophores on the transport kinetics of an organic cation and the effects of the organic ion transport on the transport of inorganic ions across a phospholipid liposome membrane. Two of the antibiotic ionophores, valinomycin and CCCP, are ion carriers, and the third one, gramicidin A, creates an ion channel that spans the liposome bilayer. Their effects on the kinetics and extent of MG^+ crossing into the liposome are markedly different. CCCP is found to be ineffective in transporting protons in the presence of the negative DOPG liposome surface potential. The differences in gA- and valinomycin-mediated transport are attributed to the balance of concentration gradients of MG^+, Na^+, and H_3O^+ across the bilayer, together with the electrostatic effects that arise when the interior population of the charged species is increased.

Acknowledgment. The authors wish to thank the National Science Foundation, the Chemical Sciences, Geosciences and Bioscience Division, Office of Basic Energy Sciences, Office of Science of the U.S. Department of Energy, and DTRA − W911NF-07-1-0116 for their support. We also acknowledge Dr. Yi Rao for fruitful discussions.

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JP806690Z