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Calcium modulates the mechanical properties of anionic phospholipid membranes

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Abstract

Using micropipette aspiration and fluorescence techniques, we have studied the material properties of charged lipid vesicles in calcium solutions. Vesicles were composed of phosphatidylglycerol (PG)/phosphatidylcholine (PC) or phosphatidic acid (PA)/PC mixtures. For the case of PG/PC membranes, we measure no effect of anionic lipid fraction on elasticity but a monotonic decrease up to 20% for tension required to induce membrane failure. Both of these observations are rationalized by a model we have developed to describe membrane electrostatic interactions in a two-component salt solution and the resulting changes in membrane properties. Critical tensions measured for PA/PC membranes, on the other hand, did not depend on anionic lipid fraction and were uniformly ∼35% lower than PG/PC vesicles. This is likely due to a lateral phase separation in the membrane. By combining mechanical properties with fluorescence observations we propose that the PA-rich phase separates into small unconnected domains.

Keywords: Micropipette aspiration; Gouy–Chapman–Stern theory; Phase separation; Cochleate phase

1. Introduction

Although present in modest concentrations in typical biological solutions, calcium plays a key role in cellular membrane function. Calcium binds strongly to the anionic lipids that make up a small but essential fraction of almost every biological membrane [1]. This binding has been shown to be a key in processes such as nerve excitation, muscle contraction, and membrane fusion [2–4]. Like many examples of ion binding in biology, calcium:lipid binding is highly substrate-specific. Thus, the interaction between calcium and a host of anionic model biological membranes has been studied.

Calcium binding to membranes of phosphatidylglycerol (PG) lipid results in significant neutralization of membrane surface charge [5]. The pair bind in a 1:1 stoichiometric ratio with a measured binding constant between 8.5 and 19.5 M^{-1} [5,6]. These binding constants illustrate the strength of the PG:Ca^{2+} interaction, as they are 1–2 orders of magnitude higher than binding constants reported for PG and typical monovalent ions [7]. Calcium also induces a thermotropic change in PG behavior, as the gel transition temperature increases markedly [8]. This calcium-induced phase transition is abolished by including small amounts of neutral lipids such as phosphatidylcholine (PC) in the membrane [8]. The resulting membrane is a fully miscible lipid mixture [6,8,9]. As a result, PG/PC membranes may be described using standard Gouy–Chapman–Stern theory both in the absence and the presence of calcium [5,6].

Although a close structural relative to PG, phosphatidic acid (PA) possesses surprisingly different properties. Due to lower steric hindrance, PA lipids bind ions from solution with higher efficacy than do PG lipids [1]. While binding between PA and calcium similarly occurs on a 1:1 basis, the resulting complex has different charge properties. PA, which like PG bears a single negative charge at neutral pH, is a doubly ionizable molecule. Calcium binding displaces the second proton, leaving a neutral PA:Ca^{2+} complex [10], unlike PG:Ca^{2+}, which has a net positive charge [5]. The adsorption of calcium induces a phase change in liquid crystalline PA membranes through the creation of a tightly packed and well-ordered structure [10]. However, unlike the case with PG lipids, the addition of neutral PC to the membrane does not abolish this phase transition. Instead, experimental results suggest a lateral phase separation between a PC-rich phase and a phase enriched in PA:Ca^{2+} complex, commonly referred to as the cochleate phase [3,11–13].

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The micropipette aspiration technique is a powerful tool for examining the properties of lipid membranes. It has been used to elucidate the effects that lipid chain unsaturation [14,15], lysolipid adsorption [16,17], and cholesterol inclusion [18] have on lipid membranes. In an earlier publication, we used the technique to show the effects of electrostatic interactions on the mechanical properties of model membranes in monovalent electrolyte solutions [19]. In particular, we demonstrated that the tension required to rupture a vesicle decreased with increasing surface charge and modeled the effect using the concept of electrostatically induced tension.

In this paper, we utilize the micropipette aspiration technique to study the effects of calcium adsorption on model membranes made of PG/PC and PA/PC mixtures. First, we extend the electrostatic destabilization model to predict the reduction in rupture tension for anionic vesicles in two-electrolyte solutions and show the large effect of even low amounts of calcium in solution. Next, we use this model to analyze the mechanical properties of PG/PC/Ca^{2+} membranes and show that the model describes the data well, thus reaffirming the concept that electrostatic interactions destabilize lipid membranes. Finally, we show that the properties of PA/PC/Ca^{2+} membranes display an obvious deviation from the electrostatic destabilization model. These membranes rupture at tensions that are ∼35% lower than for PG/PC/Ca^{2+}. We interpret this in terms of a lateral phase separation. By combining micropipette observations with fluorescence microscopy, we propose that the addition of calcium to PA/PC membranes causes the phase separation of PA:Ca^{2+} complexes into small, isolated domains.

2. Methods and materials

2.1. Micropipette aspiration measurements

We used the micropipette aspiration technique to measure the mechanical properties of anionic lipid membranes in the presence of calcium. A description of the experimental technique was given earlier [19]. We give a brief summary along with a few key changes in procedure here.

Vesicles were manufactured using a variation of the electroformation technique [20,21]. Neutral palmitoyloleoylphosphatidylcholine (POPC) was combined with anionic lipids palmitoyloleoylphosphatidylglycerol (POPG) or palmitoyloleoylphosphatidic acid (POPA) in the desired ratios and then deposited on platinum electrodes. Vesicles, commonly 30–40 μm in diameter, formed under the action of an applied alternating voltage.

The interior solution used to manufacture vesicles was 150 mM sucrose, 1 mM KCl, 20 μM Hepes, and 10 μM $CaCl_2$, titrated to pH 7.4 with KOH. This concentration of calcium results in anionic lipid:[Ca^{2+}] ratios that range from 1:30 to 1:3. Prior to manipulation, vesicles were mixed with an equal volume of exterior solution, which was identical to the interior solution except that 170 mM glucose was substituted for the sucrose. Divalent ions are commonly found as trace impurities in even the purest commercially available sucrose and glucose. We attempted to quantify the concentration of divalent impurities in our sugar solutions by spectrophotometric assay [22]. Signals from sugar solutions were all below the assay detection limit and we consequently conclude that impurity levels were less than 0.5 μM. We therefore assume that the Ca^{2+} we add to the solution swamps any impurities and the divalent concentration on both the inside and outside of the vesicles is 10 μM.

For mechanical characterization, suction pressures were applied to individual lipid vesicles via a small glass capillary. Vesicles were first prestressed to take up all available membrane area by applying 3″ of water suction pressure then returned to a less than 0.2″ of water. A kdScientific syringe pump (New Hope, PA), previously calibrated with Validyne pressure transducers (Advanced Controls, Warminster, PA), then increased the applied suction pressure at a constant rate of 0.015 mN/m s. The resulting vesicle deformation was recorded using the apparatus described earlier [19]. By optically measuring the vesicle and pipette features and monitoring the applied suction pressure, one can calculate vesicle tension, τ, and areal strain, α, of the test subject [23]. These two properties were used to find K, the membrane areal elasticity, and τ_c^{mech}, the critical tension required for vesicle rupture. K was determined as the slope of τ vs α for tensions greater than 0.5 mN/m. Critical tension was defined as the last tension recorded before vesicle rupture. Values reported here are the average of approximately 20 vesicles manipulated by at least two different micropipettes.

2.2. Fluorescence imaging of lipid vesicles

Vesicles were imaged with fluorescence optics in an attempt to visualize phase coexistence. Vesicles were made at the desired PA/PC ratio and included 1 mol% of either 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl] phosphocholine (NBD-PC) or 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidic acid (NBD-PA). Except for the inclusion of the fluorescent label, vesicles were identical to those examined by micropipette analysis. The same microscope, camera, and software package described above were used in the collection and analysis of images. Filter cubes with excitation wavelengths of 465–495 nm and emission cutoff of 535 nm were used and the focus plane was either the top or the bottom of the vesicle under observation. Other experimental details were identical to those described above.

2.3. Materials

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and stored at −8 °C as ∼0.5 mg/ml chloroform solutions. Exact lipid concentration was determined by spectrophotometric assay. Water used was produced by a Milli-Q

UF unit (Millipore, Bedford, MA) and had a resistivity of 18.2 MΩ cm. Unless otherwise stated, all other chemicals were from Sigma (St. Louis, MO), of the highest grade available, and used as received.

3. Results and discussion

3.1. Modeling mechanical properties of anionic membranes in two-electrolyte solutions

The following is a phenomenological model designed to predict the mechanical properties of anionic lipid vesicles in solutions that contain both a 1:1 and 1:2 salt. We showed in a previous publication that lipid membranes that bear a surface charge have negligibly altered membrane stretch elasticities but fail at lower applied tensions [19]. This critical tension reduction reached up to 75% and was shown to scale with fraction of charged (unbound) lipid in the membrane. We presented a model for this occurrence based on an electrostatically induced tension that was able to successfully describe micropipette data from PG/PC and PA/PC vesicles in monovalent solutions (chelated KCl and TMACl solutions). The present development extends the model to the case of an aqueous solution of monovalent and divalent ions. Although this development is based on earlier concepts, we give a full derivation here for completeness.

There are two key assumptions of the model. The first is that regardless of their cause, bilayer tensions are simply additive quantities. Thus the total tension of a lipid membrane is simply the sum of the mechanical tension, such as arises from micropipette aspiration, and an effective tension that arises from electrostatic interactions (as we will see below, this is equivalent to assuming the free energies of mechanical deformation and electrostatics are additive). This electrostatically induced tension results from the membrane dilation that follows the introduction of surface charges onto a lipid membrane [24,25]. The second assumption is that membranes rupture when a certain critical tension is reached and this critical value is unaffected by membrane surface charge. Together, these two assumptions essentially state that a charged lipid membrane behaves exactly as a neutral membrane (i.e., ruptures at a well-defined total critical tension) that has an additional electrostatic tension applied. Thus increasing the electrostatically induced tension, denoted as τ^{el}, reduces the mechanical tension, τ_c^{mech}, that can be applied to the membrane before the total critical tension to rupture is reached. This is embodied in the equation

$$\tau_c^{\mathrm{mech}} = \tau_c^{\mathrm{total}} - \tau^{\mathrm{el}}, \tag{1}$$

where τ_c^{total} is the total tension required to rupture the membrane, equivalent to the critical tension of a neutral membrane.

Recalling that tension is simply the derivative of free energy with respect to membrane area, the value of τ^{el} can be calculated if the electrostatic contribution to membrane free energy, f^{el}, is known [26]. This value is commonly approximated using Gouy–Chapman theory. We use the derivation of May [27] that was found following the method of Lekkerkerker [28], giving f^{el} of an anionic lipid monolayer as

$$f^{\mathrm{el}} = 2kT\lambda^{\mathrm{eff}}\left[\frac{1-q}{p} + \ln(p+q)\right] \tag{2}$$

with $p = \lambda^{\mathrm{eff}} e^2/(2\kappa a\varepsilon\varepsilon_0 kT)$ and $q = \sqrt{p^2+1}$, where λ^{eff} is the lipid fraction that is effectively charged (i.e., not neutralized by ion binding), e is the elementary charge, a is the area per lipid molecule, κ is the inverse Debye length, ε is the dielectric constant, and ε_0 is the permittivity of vacuum. We note that this approximation for f^{el} is chosen strictly for convenience; any of the Gouy–Chapman solutions will give similar results. In fact, if we examine May's formulation for f^{el} in the Debye–Hückel limit (that is, $p \ll q \sim 1$), we see $f^{\mathrm{el}} \simeq \lambda^{\mathrm{eff}^2} e^2/(\kappa a\varepsilon\varepsilon_0)$, which is the commonly quoted "capacitor approximation" (e.g., see [29]) for electrostatic membrane free energy.

With f^{el} in hand, the electrostatically induced membrane tension can be found through differentiation as

$$\tau^{\mathrm{el}} = -\frac{df^{\mathrm{el}}}{da} = 2\frac{2kT\lambda^{\mathrm{eff}}}{a}\left(\frac{q-1}{p}\right), \tag{3}$$

where the additional factor of 2 is to account for the two monolayers in a bilayer. In Eq. (3) we see that the parameter that sets τ^{el}(and therefore the decrease in τ_c^{mech} from Eq. (1)) is λ^{eff}, the effectively charged lipid fraction in the membrane. Because anionic lipids are partially neutralized by ion binding, this quantity is a function of both λ, the anionic lipid fraction in the membrane, and the choice and concentration of electrolyte in solution.

The relationship between λ and λ^{eff} is found by considering the chemical equilibrium between lipid molecules and ions in solution. Thus for the simplest case of a single monovalent cation C^+ that binds 1:1 to singly charged lipid, the charged fraction is found as

$$\lambda^{\mathrm{eff}} = \frac{\lambda}{1 + B^+[C^+]\exp(-e\psi_0/kT)}, \tag{4}$$

where B^+ is the first-order binding constant, the brackets indicate bulk solution concentration, and the exponential function accounts for the accumulation of cations at the interface due to electrostatic attraction. Note that in this work, we neglect both cation binding to neutral lipids and anion binding to neutral and charged lipids.

It is straightforward to extend this concept to multicomponent solutions if the binding characteristics of each individual ion are known. Binding between PG and calcium occurs on a 1:1 basis, leaving the resulting complex with a single positive charge [5]. As a result, the effective charged fraction for a PG/PC membrane in a solution of 1:1 salt (denoted as C^+:C^-) and 1:2 salt such as $CaCl_2$ (denoted as C^{2+}:$2C^-$) is described by the equation

$$\lambda^{\mathrm{eff}} = \frac{\lambda(1-B^{2+}[C^{2+}]\exp(-2e\psi_0/kT))}{1+B^+[C^+]\exp(-e\psi_0/kT)+B^{2+}[C^{2+}]\exp(-2e\psi_0/kT)}, \tag{5}$$

where B^+ and B^{2+} are the binding constants to anionic lipid of C^+ and C^{2+}, respectively, and we assume the two ions to bind competitively. Note the factor of 2 in the exponential attraction function for C^{2+}; this partially accounts for the large impact that divalents such as calcium have on charged interfaces.

For the case of calcium binding to PA membranes, binding also occurs 1:1 [10]. However, PA, though singly charged at neutral pH, is doubly ionizable. Calcium binding rejects the second proton, leaving a neutrally charged PA:Ca^{2+} complex [10]. The resulting binding equation for PA in a solution of a 1:1 and 1:2 salt such as $CaCl_2$ is thus

$$\lambda^{\text{eff}} = \frac{\lambda}{1+B^+[C^+]\exp(-e\psi_0/kT)+B^{2+}[C^{2+}]\exp(-2e\psi_0/kT)}. \quad (6)$$

Because binding is modulated by the surface potential ψ_0, Eqs. (4)–(6) must be solved simultaneously with the Gouy equation,

$$\sinh\left(\frac{e\psi_0}{2kT}\right) = \frac{-e\lambda^{\text{eff}}/a}{\sqrt{8N\varepsilon\varepsilon_0 kT[C^+]}}, \quad (7)$$

where N is Avogadro's number and we have assumed $[C^+] \gg [C^{2+}]$. For calculations we assume negligible depletion of both C^+ and C^{2+} (calculations show that under experimental conditions, a maximum of 7% of the calcium in solution is bound to the interface). Once the value of λ^{eff} is determined, it is input into Eq. (3), which predicts the electrostatically induced tension in the membrane and thus the reduction in the mechanical tension that may be supported before rupture.

Equations (4)–(7) can be used to illustrate the tremendous impact that even a small amount of calcium has on the charge characteristics of anionic membranes. Figure 1 shows a simulation of the binding characteristics of a PG/PC vesicle in a 1 mM KCl solution with varying levels of calcium. Although the effect saturates at large calcium concentrations, even trace levels of calcium drastically decrease membrane charge, as the effectively charged lipid fraction decreases 20% when 1 μM Ca^{2+} is added to solution. Because the decrease in τ_c^{mech} scales with λ^{eff}, this suggests that the presence of calcium should greatly affect the mechanical properties of anionic lipid vesicles.

3.2. Mechanical properties of PG/PC vesicles in calcium solutions

We used the micropipette aspiration technique to measure the areal stretch elastic modulus, K, and the applied mechanical tension required to rupture, τ_c^{mech}, of PG/PC vesicles in 1 mM KCl, 10 μM $CaCl_2$ solutions. Across all PG fractions tested (0.04–0.4), vesicle appearance was consistent with a single-phase liquid crystalline membrane. Vesicles were fluid (i.e., could not support surface shear), had linear stress/strain relationships, and showed negligible hysteresis between increasing and decreasing applied pressures (data not shown).

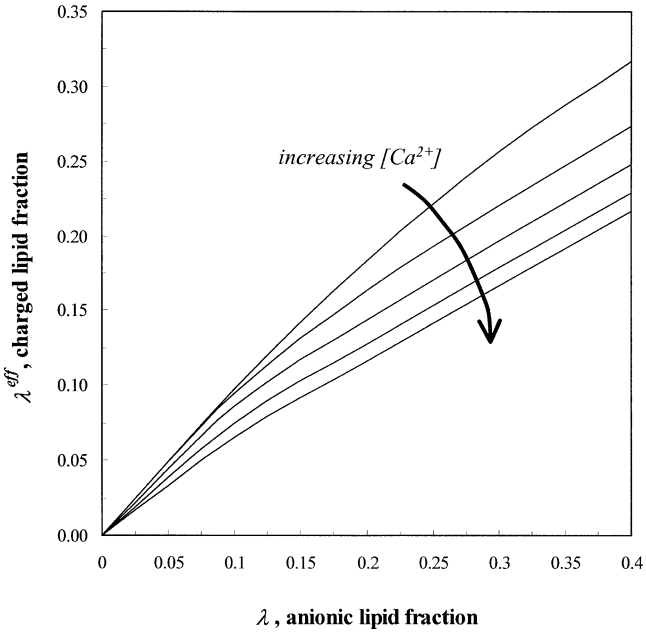

Fig. 1. Predictions of the effectively charged lipid fraction, λ^{eff}, for PG/PC vesicles in 1 mM KCl and various concentrations of $CaCl_2$ (0, 0.1, 1, 10, and 100 μM). Predictions are based on the simultaneous solutions to Eqs. (4), (5), and (7).

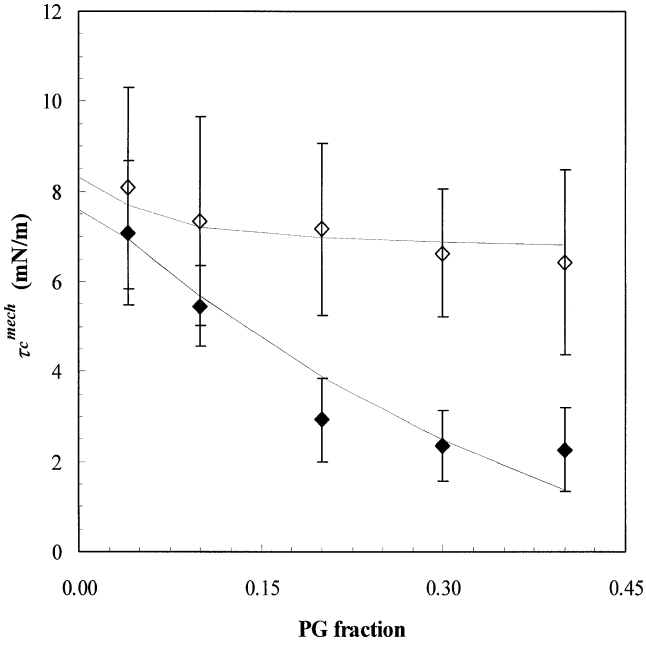

Fig. 2. Critical tension data for PG/PC vesicles in 1 mM KCl, 10 μM $CaCl_2$ (hollow symbols), and EDTA-chelated 1 mM KCl (solid symbols) solutions. Lines are fits using the electrostatic destabilization model with $B^+ = 0.4\ \text{M}^{-1}$ and $B^{2+} = 8.5\ \text{M}^{-1}$. EDTA-chelated data are from [19]. Data points represent the average ± standard deviation.

The critical tensions of PG/PC membranes are shown in Fig. 2. There is a small but clear decrease as the PG fraction is increased from 0.04 to 0.40. Over this range, critical tension drops ∼20%. For comparison, also shown in Fig. 2 are data from PG/PC vesicles in 1 mM KCl solutions that were

chelated with EDTA to remove divalent ion impurities [19]. As seen from the figure, the two data sets have similar shapes but the τ_c^{mech} decrease for PG/PC/Ca^{2+} membranes is substantially less than the 70% seen in the chelated case.

Results from DSC [9] and NMR [6,8] demonstrate that membranes of PG/PC are completely miscible both in the absence and in the presence of calcium. The vesicles under examination here are therefore homogeneous liquid crystalline membranes whose surface charge is reduced due to K^+ and Ca^{2+} binding. The material properties of the resulting partially neutralized membranes should be predictable using the electrostatic destabilization model developed in the previous section.

Before comparing our data and model on a quantitative basis, it is instructive to compare the qualitative trends. The model predicts that τ_c^{mech} should decrease in a nonlinear but monotonic fashion as PG fraction is increased, as the data in Fig. 2 show. Also, as is demonstrated in Fig. 1, calcium binds efficiently to PG membranes. As a result, PG/PC/Ca^{2+} vesicles should be much more neutralized and therefore more stable than PG/PC membranes in chelated solutions. Comparing the two data sets in Fig. 2 shows that this is indeed the case.

The two-electrolyte electrostatic model was used to quantitatively fit the τ_c^{mech} data of PG/PC vesicles in 1 mM KCl and 10 μM $CaCl_2$. There are three free parameters in the model: τ_c^{total}, B^+, and B^{2+}. In previous micropipette experiments performed in EDTA-chelated solutions, we found B^+ for PG/PC vesicles in 1 mM KCl was 0.4 M^{-1}, which was in the range of literature values normally cited [19]. Setting this value for the present experiments allows us to fit the data by modulating only τ_c^{total} and B^{2+}. As shown by the solid line in Fig. 2, we see excellent agreement between our data and the model when τ_c^{total} is set to 8.3 mN/m and B^{2+} equals 8.5 M^{-1}. This value of τ_c^{total}, which is equivalent to the critical tension required to rupture a neutral lipid membrane, is somewhat higher than our previously reported critical tension of POPC vesicles in a nonelectrolyte solution of 6.6 ± 1.4 mN/m [30]. The difference is attributable to subsequent improvements in micropipette experimental design including the use of a syringe pump to apply a smooth, continuous pressure ramp rather than the abrupt and uneven pressure increase that comes from modulating pressure by hand. More importantly, the value found for B^{2+} exactly corresponds to the one found by Lau et al. for Ca^{2+} binding to PG membranes [5]. This suggests that we have successfully captured the electrostatic effects on τ_c^{mech} in the two-electrolyte model.

The micropipette technique was also used to determine the areal stretch elasticity, K, of PG/PC vesicles as a function of λ. Similarly to results for PG/PC vesicles in chelated solutions [19], K values measured for PG/PC/Ca^{2+} membranes were not a function of anionic lipid fraction (data not shown). Averaging across all PG lipid fractions tested gives an elasticity of 154 ± 17 mN/m, similar to the value of 144 ± 15 mN/m found in the absence of calcium [19]. We demonstrated earlier that in the experimentally accessible range, electrostatic interactions are not expected to alter K values [19]. The lack of an effect of λ on elasticity and the numerical agreement between PG/PC vesicles in calcium-rich and calcium-free solutions are thus expected. This, together with the excellent agreement of the model and τ_c^{mech} data seen in Fig. 2, combines with our results on anionic lipid vesicles in monovalent electrolyte solutions [19] to produce a large body of experimental evidence that indicates that (1) electrostatic interactions have important consequences for vesicle mechanical properties and (2) these consequences can be successfully modeled based on the idea of an electrostatically induced tension.

3.3. Mechanical properties of PA/PC vesicles in calcium solutions

The micropipette aspiration technique was used to determine the physical properties of PA/PC vesicles in 1 mM KCl, 10 μM $CaCl_2$ solutions. Figure 3 shows the tension required to rupture the membrane as a function of PA content. Critical tensions were relatively constant with respect to PA fraction (maximum change in $\tau_c^{\text{mech}} = 0.9$ mN/m) and had an average value of 5.2 mN/m. This is substantially lower than the average for PG/PC in the same buffer of 7.1 mN/m (for comparison, the model fit for the PG/PC/Ca^{2+} system is shown in Fig. 3 by the solid line).

In the context of the electrostatic destabilization model, the identity of the lipid (i.e., PG vs PA) is irrelevant except in

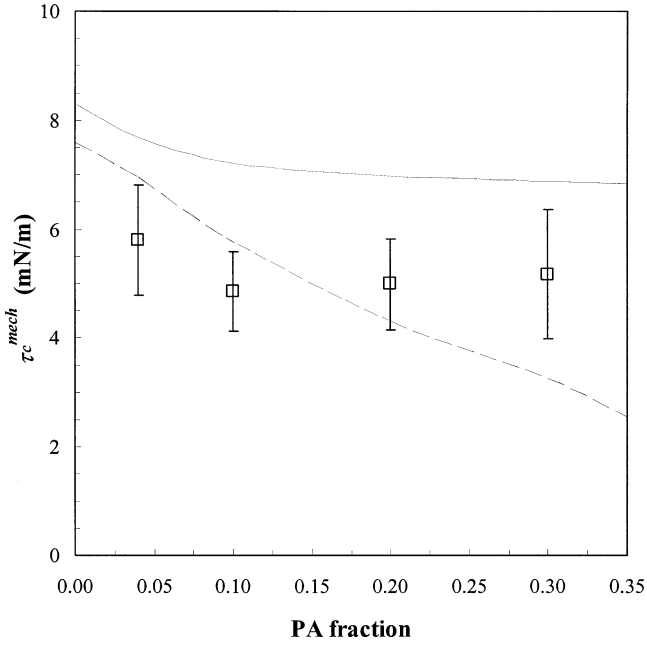

Fig. 3. Critical tension data for PA/PC vesicles in 1 mM KCl, 10 μM $CaCl_2$ (shown by symbols). For comparison, the solid line is the best model fit of PG/PC vesicles in the same buffer (fitting parameters: $B^+ = 0.4$ M^{-1}, $B^{2+} = 8.5$ M^{-1}) and the dashed line is the best model fit of PA/PC vesicles in EDTA-chelated 1 mM KCl solution ($B^+ = 0.8$ M^{-1}, from [19]). Data points represent the average ± standard deviation.

the determination of binding constants. Given that PA generally binds ions with a higher affinity than PG (1), the τ_c^{mech} data for PA/PC should lie slightly above the solid line in Fig. 3 instead of a uniform 25–33% below. The behavior of PA/PC in 10 μM calcium also appears to conflict with results from PA/PC vesicles in EDTA-chelated solutions (indicated by the dashed line in Fig. 3). At low (<0.2) PA fractions, the addition of calcium actually lowers the mechanical stability rather than raise it. These facts clearly contradict the electrostatic destabilization model, indicating that calcium exerts some other effect on the membrane rather than simple charge neutralization, an effect that significantly alters the mechanical properties of PA/PC membranes.

The characteristics of PA/PC membranes and their interaction with calcium solutions have been widely studied, in part because of a possible relevance to the membrane fusion process [3]. Using primarily NMR, DSC, and fluorescence techniques, it has been shown that mixtures of PA and PC lipids with the same tail composition (i.e., POPA/POPC, DMPA/DMPC) are fully miscible in chelated solutions but extensively phase separate in the presence of calcium [11–13,31]. In particular, this phenomenon has been seen in eggPA/eggPC [3], which are natural mixtures dominated by the lipids under examination here, POPA and POPC. We note one key exception found in the work of Garidel and Blume [31]. Instead of a phase separation, these authors reported increasingly nonideal mixing in the fluid phase for 20% DMPA/80% DMPC membranes in 1–10 mM electrolyte even with excess calcium. However, given the number of observations of calcium-induced phase separation of PA/PC membranes in the literature and the fact that this phenomenon uniquely affects PA/PC (and not PG/PC), we conclude that it is highly likely that the unusual mechanical properties in Fig. 3 are due to the phase separated state of PA/PC/Ca^{2+} vesicles.

Under the hypothesis of phase separation, there are two logical reasons for lowered values of τ_c^{mech}, namely breakdown in the interior of one of the phases and breakdown at the domain interface. In the first, electrostatic interactions destabilize either the cochleate or the liquid crystalline phase compared to a purely liquid crystalline membrane. The cochleate phase is an obvious candidate as it is enriched in PA. Although extensively neutralized in the presence of calcium, the high local anionic lipid density could provide relatively large local electrostatic interactions, potentially lowering the mechanical stability of that phase. Because increasing the PA fraction in the membrane serves to increase the amount of cochleate phase present but not its composition, the stability of phase-separated membranes would theoretically be independent of PA fraction, as is seen in Fig. 3. We feel, however, that this scenario is unlikely. Lipids in the cochleate phase are tightly packed in the all trans configuration and are extremely motionally restricted compared to the liquid crystalline phase [10, 13]. This tight packing (and resulting increase in cohesive chain–chain interactions) should confer increased mechanical stability similar to the ∼fivefold increase seen when single-component liquid crystalline membranes are frozen into the tightly packed gel phase [32]. Indeed, unless the increase in cohesive forces compensates for the increase in repulsive electrostatic forces that result from concentrating PA, it is unclear that a cochleate phase would occur at all. This leads to the conclusion that electrostatically driven breakdown in the cochleate phase is unlikely. Additionally, because anionic lipids are locally depleted, the PC-rich liquid crystalline phase should actually have a higher τ_c^{mech} than non-phase-separated membranes. We can therefore dismiss breakdown in either of the phases as the reason or lowered mechanical stability of PA/PC/Ca^{2+} vesicles.

The second possible reason that a Ca^{2+}−induced phase separation could lower the mechanical stability of lipid vesicles is the effect of phase domain boundaries. Lipids located at the interface between gel and liquid crystalline phases experience large density fluctuations [33]. These fluctuations could serve as membrane defect nucleation sites, thereby lowering vesicle mechanical stability. In an earlier work, we showed that the critical tension of two-component neutral lipid membranes (POPC/DPPC mixtures) decreased when vesicles were cooled from a single-phase liquid crystalline into the two-phase liquid crystalline/gel regime [34]. While perhaps a coincidence, it is interesting to note that despite the large differences between the two systems, τ_c^{mech} is reduced by ∼30% on phase separation in both PA/PC/Ca^{2+} and DPPC/POPC membranes. Thus a reduction in mechanical stability may be a general feature of two-phase membranes, implicating a nonspecific effect such as defect nucleation at phase boundaries.

In addition to unusually low τ_c^{mech} values, PA/PC vesicles in calcium have areal stretch elastic moduli that differ from other lipid systems. Although K values for PA/PC/Ca^{2+} again show no dependence on anionic lipid fraction, the average value across all fractions tested was 136 ± 12 mN/m. This is significantly less than the value for PG/PC/Ca^{2+} vesicles of 154 ± 17 mN/m and for PA/PC in chelated solutions of 152 ± 13 mN/m [19]. Because electrostatic interactions have a negligible effect on lipid membrane elasticity in the experimentally accessible range [19], the change in K cannot be due to simple binding/charge neutralization. Instead, this may be the result of the calcium-induced lateral phase separation. Vesicle volume is assumed constant in micropipette aspiration experiments, an assumption that is usually justified [35]. However, Clerc and Thompson showed that vesicles in the liquid crystalline–gel coexistence regime exhibited passive permeation an order of magnitude greater than pure liquid crystalline membranes [33]. Increased solute transport across phase-separated membranes would result in an artificial decrease in the calculated value of K, which might be the cause of the lowered elasticities measured for PA/PC/Ca^{2+} membranes.

Despite the widespread interest in calcium-induced phase separations, little is known about the resulting membrane

properties including the size and configuration of the separated lipid domains. Micromanipulation can offer some insight here, as it is sensitive not just to the presence of two-phase coexistence, but to the structure and properties of the individual domains. For example, two-phase POPC/DPPC vesicles frequently appear nonspherical before aspiration and have nonlinear stress/strain curves. It was concluded that this was because the DPPC-enriched gel phase is present as an extended percolated network, providing a solid "skeleton" in the otherwise fluid membrane [34]. In the case of phase-separated PA/PC/Ca^{2+} membranes, these effects are absent. Prior to aspiration, membranes always appear perfectly spherical and show small thermal fluctuations that are typical for liquid crystalline vesicles. Linear stress/strain curves were obtained at all PA fractions (data not shown). This suggests one of three things about cochleate domains in PA/PC/Ca^{2+} vesicles. First, despite literature evidence suggesting the contrary, perhaps our vesicles are not phase separated. While possible, we find this doubtful, as we could no longer explain the τ_c^{mech} effects seen in Fig. 3. Second, the cochleate phases may be perfectly fluidlike. This would be somewhat surprising, considering the tightly packed and well-ordered nature of the phase. Finally, the cochleate phase may be present in small, disconnected domains. In this configuration, the cochleate phase could have completely solidlike properties and still not give POPC/DPPC-like behavior.

In an attempt to learn more about the configuration of the lipid domains in PA/PC/Ca^{2+} membranes, tagged vesicles were visualized with fluorescence microscopy. This technique has been used to see phase separation in a number of immiscible lipid mixtures [34,36,37]. Vesicles made from PG/PC mixtures appeared homogeneous both in the presence and in the absence of calcium, consistent with single-phase membranes (data not shown). The same is true for PA/PC vesicles in EDTA-chelated solutions (data not shown), again consistent with the phase diagram for calcium-free PA/PC membranes.

Images taken of PA/PC/Ca^{2+} vesicles also appeared completely uniform under fluorescence microscopy (data not shown). This could have three distinct causes. First, it again raises the possibility that the lipid vesicles are not actually phase separated, which again we label as unlikely. The second possible cause is incorrect probe choice; fluorescence imaging of phase coexistence relies on the probe being preferentially enriched in one of the phases. If our probes, NBD-PC or NBD-PA, were not enriched in the liquid crystalline and cochleate phases, respectively, no contrast could be seen. However, Graham et al. used the same probes to monitor phase separation in the closely related DOPA/DOPC/Ca^{2+} membrane [11], indicating that the probes should be an appropriate choice for imaging coexistence. We are left with the final possibility, namely that the cochleate phase is present in very small isolated domains.

4. Summary

We have determined the mechanical properties of PG/PC and PA/PC lipid vesicles in the presence of excess calcium. In a previous work, we developed a model for the effects of membrane electrostatic interactions on the vesicle mechanics [19]. In particular, we predicted a decrease in the mechanical stability of lipid membranes with increasing surface charge. In this paper, we extended that model to treat membranes in two-electrolyte solutions.

For PG/PC vesicles in 1 mM KCl, 10 μM $CaCl_2$ solutions, we found that the tension required for rupture decreases by 20% with increasing PG fraction. As a comparison, PG/PC vesicles in chelated solutions show decreases in τ_c^{mech} of 70% over the same compositional range. In both cases, elastic moduli are independent of membrane composition and show identical values. We showed that the large stabilizing effect of calcium is due to charge neutralization from calcium binding, as critical tension data can be well fit by the two-electrolyte model. These data provide solid further evidence of the destabilizing effect that intramembrane electrostatic interactions have on lipid membranes. Due to the prevalence of anionic lipid in biological membranes, we contend that the effects of membrane electrostatics must be considered in any discussion of membrane mechanics.

For membranes of PA/PC mixtures, very different results were obtained. Calcium had a significant effect on τ_c^{mech}, but comparison to the chelated case shows that mechanical stability is lowered for low PA fractions, rather than increased. The elastic moduli are again independent of PA fraction but are 5–12% lower than elasticities obtained with other anionic vesicles. We describe these effects using the concept of phase separation between a PC-rich liquid crystalline phase and a PA:Ca^{2+}-rich cochleate phase. By combining micromanipulation and fluorescence microscopy, we postulate that the cochleate phase is present in the form of small disconnected domains.

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