Published in final edited form as:

Phys Chem Chem Phys. 2017 June 14; 19(23): 15285–15295. doi:10.1039/c7cp00718c.

Cations induce shape remodeling of negatively charged phospholipid membranes

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Abstract

The divalent cation Ca^{2+} is a key component in many cell signaling and membrane trafficking pathways. Ca^{2+} signal transduction commonly occurs through interaction with protein partners. However, in this study we show a novel mechanism by which Ca^{2+} may impact membrane structure. We find an asymmetric concentration of Ca^{2+} across the membrane triggers deformation of membranes containing negatively charged lipids such as Phosphatidylserine (PS) and Phosphatidylinositol-4,5-bisphosphate ($\text{PI}(4,5)\text{P}_2$). Membrane invaginations in vesicles were observed forming away from the leaflet with higher Ca^{2+} concentration, showing that Ca^{2+} induces negative curvature. We hypothesize that the negative curvature is produced by Ca^{2+} -induced clustering of PS and $\text{PI}(4,5)\text{P}_2$. In support of this notion, we find that Ca^{2+} -induced membrane deformation is stronger for membranes containing $\text{PI}(4,5)\text{P}_2$, which is known to more readily cluster in the presence of Ca^{2+} . The observed Ca^{2+} -induced membrane deformation is strongly influenced by Na^+ ions. A high symmetric $[\text{Na}^+]$ across the membrane reduces Ca^{2+} binding by electrostatic shielding, inhibiting Ca^{2+} -induced membrane deformation. An asymmetric $[\text{Na}^+]$ across the membrane, however, can either oppose or support Ca^{2+} -induced deformation, depending on the direction of the gradient in $[\text{Na}^+]$. At a sufficiently high asymmetric Na^+ concentration it can impact membrane structure in the absence of Ca^{2+} . We propose that Ca^{2+} works in concert with curvature generating proteins to modulate membrane curvature and shape transitions. This novel structural impact of Ca^{2+} could be important for Ca^{2+} -dependent cellular processes that involve the creation of membrane curvature, including exocytosis, invadopodia, and cell motility.

TOC image

Introduction

The Ca^{2+} ion is a well-known secondary messenger that regulates diverse signaling processes and cellular phenomena including vesicle trafficking, cell growth, membrane repair, and cell motility^{1–7}. Intracellular Ca^{2+} concentrations within a resting cell are comparatively low (about 100 nM free Ca^{2+}), while extracellular Ca^{2+} concentration is orders of magnitude higher (a few millimolar). Influx of Ca^{2+} from the cell exterior or from Ca^{2+} stores within the endoplasmic reticulum elevates the internal Ca^{2+} concentration to micromolar levels (or possibly much higher near the influx site), triggering cellular signaling cascades. Ca^{2+} influx at the synapse is a signal that triggers exocytosis and neurotransmitter release⁵. Ca^{2+} enacts its signaling role through several mechanisms: the most well-known mechanism is through direct interaction with proteins, such as Calmodulin and Calcineurin. Ca^{2+} -binding leads to rearrangement of calmodulin and allows it to interact with a host of protein partners⁸.

Ca^{2+} may also impact cellular signaling through direct interactions with the negatively charged phospholipids within the membrane. Recent studies have suggested that Ca^{2+} interacts strongly with the signaling lipid phosphatidylinositol-4,5-bisphosphate [$\text{PI}(4,5)\text{P}_2$], and can trigger formation of $\text{PI}(4,5)\text{P}_2$ -rich clusters *in vitro* at micromolar Ca^{2+} concentration^{9–11}. Ca^{2+} has also been suggested to alter lipid packing or induce local clustering of other negatively charged lipids, including phosphatidylserine (PS), albeit to a lesser extent^{12–18}. The interaction of Ca^{2+} with lipids may impact cellular signaling by altering lipid packing and local concentration of signaling lipids. In addition, Ca^{2+} ions have been shown to inhibit some $\text{PI}(4,5)\text{P}_2$ -binding proteins by shielding $\text{PI}(4,5)\text{P}_2$ ¹⁹. Recent work has also suggested that Ca^{2+} may alter the spontaneous curvature of the membrane through its interaction with negatively charged lipids^{20–22}. Ca^{2+} can also promote vesicle fusion^{23–26}. Ca^{2+} promotion of vesicle fusion may be partly induced by increased adhesion between vesicles in the presence of Ca^{2+} , but likely also involves a curvature effect of Ca^{2+} stabilizing intermediate fusion states^{25, 27}. Ohki et al., show that Ca^{2+} increases surface hydrophobicity and surface tension, which may also promote vesicle fusion^{28–30}.

In this study, we show that Ca^{2+} can alter membrane shape by inducing negative spontaneous curvature (defined as local membrane curving towards the bound ion). Specifically, we find that Ca^{2+} ions trigger membrane invaginations in vesicles consisting of bilayers that contain negatively charged lipids in the presence of an excess Ca^{2+} ion concentration on the vesicle exterior relative to the interior, i.e. an asymmetric distribution of ions across the membrane. These results imply that Ca^{2+} can play a direct role in modulating membrane curvature, which may have significance for the role of Ca^{2+} in exocytosis and cell motility.

Experimental

Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and L- α -phosphatidylinositol-(4,5)-bis-phosphate ($\text{PI}(4,5)\text{P}_2$; Porcine Brain, ammonium salt) lipids

were obtained from Avanti Polar Lipids (Alabaster, AL) as lipid stock solutions in chloroform or 2:1 chloroform:methanol. The concentration of lipid stock solutions was periodically verified by phosphate assay. The lipid dye Texas Red-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt) (Texas Red-DHPE) was purchased from Invitrogen/Life Technologies (Grand Island, NY). CaCl_2 , MgCl_2 , NaCl , Casein, Tris, HEPES, DTT and EDTA were obtained from Fisher Scientific (Rochester, NY). All salts were obtained with the Cl^- counterion to prevent any potential impact from varying the counterion. 98% hydrolyzed 146–186 kDa PVA was purchased from Sigma Aldrich (St. Louis, MO). All commercial reagents were used without further purification.

Preparation of giant unilamellar vesicles (GUVs)

GUVs were prepared in sucrose solution by the standard method of electroformation³¹. Briefly, lipid stocks in organic solvent were prepared with the desired lipid composition and spread on indium tin oxide (ITO) coated slides. Typically, 0.3% of the lipid fluorophore Texas Red-DHPE was included in each lipid mixture. The organic solvent was removed by applying vacuum for at least two hours to form a dry lipid film³². The electroformation chamber was formed with two lipid-coated ITO slides using 0.8 mm thick rubber spacers. The lipid film was hydrated with 400–450 μL of 0.3 M sucrose. Electroformation was performed using a 10 Hz and 2–4 V_{pp} electrical field for two hours to produce the final GUV dispersion.

To prepare GUVs in salt solutions a PVA gel hydration based method was used as in Weinberger et al³³. A 5% PVA solution was made by dissolving PVA with a molecular weight of 146–186 kDa in water. To dissolve the PVA, the solution was heated to 90 °C while stirring for several hours. A dry PVA film was formed by spreading 50–100 μL of the 5% PVA solution onto two glass slides and drying at 50 °C (roughly 1 hr). If necessary, glass slides were plasma cleaned before use to prevent dewetting of the PVA film. Lipid stock solution was then spread onto each dried PVA patch and dried via vacuum for at least one hour. The lipid film was then hydrated with 450 μL of buffer solution and left to swell for 30 min to an hour. GUVs formed using different vesicle preparation methods both had similar behavior under the same experimental conditions (data not shown).

GUV dispersions were kept covered throughout the preparation process to limit photo-bleaching and photo-induced oxidation. After a couple days of use, the GUV dispersion was discarded and fresh GUVs were prepared.

Image analysis procedures

The contour of GUVs was fitted with a Gaussian ring using MATLAB (The MathWorks, Natick, MA), following the equation below:

$$I(x, y) = a \cdot \exp \left[- \frac{\left[\sqrt{(x - x_0)^2 + (y - y_0)^2} - r_0 \right]^2}{\sigma_{mem}^2} \right] + b \quad (1)$$

Here, I is the fluorescence intensity of a pixel at coordinate (x, y) of the image, a is the height of the Gaussian ring, (x_0, y_0) is the center position of the GUV, r_0 is the radius of the GUV, σ_{mem} is the width (between 1/e positions) of the Gaussian peak which also provides a measure of the width of the fluorescent trace of the membrane, and b is the background intensity of the image. For pipette-aspirated GUVs, the pipette region is excluded during the fitting.

Single GUV shape instability assay

Two chambers, a GUV chamber and an imaging chamber, were formed between two coverslips (20 mm $\times$ 40 mm, pre-treated with 2 μL of 2.5 mg·ml⁻¹ casein, 20 mM Tris, and 2 mM EDTA) overhanging a glass microscope slide (2 mm thick). The GUV chamber has a total volume of 375 μL and is made by diluting ~ 10 μL of the GUV stock dispersion into a buffer containing glucose, sucrose, NaCl and HEPES. For GUVs made using electroformation (which were used for CaCl₂ measurements), this results in an asymmetry of [Na⁺] across the membrane (with 37.5 mM NaCl on the exterior of the GUV and 0 mM on the interior), as is commonly used in experiments with proteins and GUVs^{20, 34–36}. The osmolarity of the buffer was selected to be 20% higher than the GUV stock dispersion (measured with a micro-osmometer from Advanced Instruments Inc. (Norwood, MA)) to ensure that the vesicles had enough excess area (relative to the area of a sphere with the same volume) for micropipette aspiration. The salt imaging chamber had a total volume of 375 μL . Stock salt solutions were diluted to designated concentrations, using the same buffer as used for diluting GUVs. For both chambers, the pH was maintained at 7.0 with 7 mM HEPES. Sucrose and glucose concentrations were adjusted to balance total osmolarities of the two chambers to prevent any membrane structural changes due to osmotic pressure. In addition, for MgCl₂ and CaCl₂ experiments, total levels of Na⁺ and Cl⁻ were balanced between the two chambers (to within a few mM) to prevent any impact from changing Na⁺ or Cl⁻ concentration on the observed Mg²⁺ or Ca²⁺ induced effects. Micropipettes and transfer capillaries were prepared and casein-treated through incubation with saturated casein solutions followed by rinsing, to prevent GUVs from sticking to the capillaries^{32, 37}. In the rare cases where GUV membranes were observed to stick to pipette walls, data from such vesicles were discarded.

The GUV transfer was a four-step process as shown in Fig. 1b. The moment when the GUV was not protected anymore by the transfer capillary was defined as time zero in the GUV instability analysis. Zero aspiration pressure was checked before and after the GUV transfer process to ensure absence of pressure drifts³⁸. All the transfer and imaging processes were carried out at room temperature (23.7 ± 0.3 °C; Mean $\pm$ SD measured on different days).

Calcium binding measurements

Ca²⁺ binding affinities were determined via titration using a perfectION Ca²⁺ Ion Selective Electrode (ISE) from Mettler Toledo. Lipid vesicles were formed by hydrating dried lipid films with an aqueous buffer composed of 7 mM HEPES, 37.5 mM NaCl, and 250–300 mM Sucrose at pH 7.0 ± 0.1 . The lipid film was suspended by vortexing and sonicated to break apart large multilamellar vesicles. The vesicles were then extruded at least 41 times through a 50 nm membrane to form unilamellar vesicles. Lipid vesicles were diluted using the same

aqueous buffer to 1–3 mM before titration. The Ca^{2+} titrant consisted of a 10 mM CaCl_2 buffer with 7 mM HEPES, 37.5 mM NaCl, and 250–300 mM sucrose at $\text{pH } 7.0 \pm 0.1$. The sucrose concentrations were balanced carefully so that osmolarity did not change during the titration. Titrations were performed at room temperature ($23 \pm 2^\circ\text{C}$) and all measurements were made at a roughly constant stirring rate. The Ca^{2+} ISE was calibrated before and after each titration by titrating the Ca^{2+} buffer into the lipid buffer in the absence of lipid vesicles. In between each titration, the electrode was rinsed with copious amounts of MilliQ water and soaked in MilliQ water for at least 10 min to help eliminate vesicles that may bind to the ISE. The concentration of each lipid solution was verified by phosphate assay after titration to obtain the exact lipid concentration.

Results and Discussion

Calcium binds negatively charged vesicles

The binding of Ca^{2+} to lipid membranes has been the focus of extensive study^{18, 39–45}. Ca^{2+} has been shown to weakly associate with zwitterionic membranes, while it interacts strongly with membranes containing negatively charged lipids⁴⁵. Previous studies have measured association constants with a variety of PS contents and overall saline concentrations. For conditions with 10 mM NaCl, Sinn et al. reported an apparent binding affinity of 650 M^{-1} for Ca^{2+} binding 80:20 mol% DOPC/DOPS membranes, and 250 M^{-1} for Ca^{2+} binding pure DOPC membranes⁴⁵. In contrast, the apparent binding affinity of Ca^{2+} to DOPC membranes in 150 mM NaCl is reported as $\sim 20 \text{ M}^{-1}$ ⁴⁶. Clearly, the overall proportion of negatively charged lipid within the membrane as well as the bulk NaCl concentration can have a significant impact on the fraction of Ca^{2+} that associates with the membrane^{45, 46}. To assess the density of Ca^{2+} ions on the membrane in our experiments, it is necessary to determine the Ca^{2+} association constant for the relevant membrane composition and saline concentration used in our studies. Ca^{2+} ion concentration at the membrane was determined by measuring the concentration of free Ca^{2+} using a Ca^{2+} sensitive electrode and titrating in a known concentration of Ca^{2+} . The apparent binding affinity was then determined by fitting a Langmuir binding isotherm to the titration data:

$$Ca_{bound}^{2+} = \frac{B_{max}}{\left(1 + \frac{1}{K_a [Ca_{added}^{2+}]}\right)} \quad (2)$$

Where Ca_{bound}^{2+} is the bound Ca^{2+} per accessible lipid (taken as half of the total lipid concentration, based on the assumption that half of the LUV lipids constitute the outer leaflet of the LUV), B_{max} is the maximum Ca^{2+} ions per lipid, K_a is the binding affinity (i.e. the association constant), and $[Ca_{added}^{2+}]$ is the bulk Ca^{2+} concentration. The results for pure DOPC vesicles in 7 mM HEPES and 37.5 mM NaCl are shown in Fig. 2. The NaCl concentration was chosen to keep total salt concentrations throughout the titration in the same range as those used for GUV experiments (total salt concentration ranges from 37.5 to 42.5 mM as CaCl_2 is titrated into the LUV solution). As expected, Ca^{2+} binds weakly to DOPC vesicles, with a binding affinity of $410 \pm 141 \text{ M}^{-1}$ and a maximum of 0.4 Ca^{2+} per accessible lipid. The maximum Ca^{2+} per accessible lipid is only reached at high bulk $[\text{Ca}^{2+}]$

(above the highest tested bulk $[Ca^{2+}]$). Ca^{2+} binding to vesicles containing the negatively charged lipid DOPS is much stronger, as shown in Fig. 2 (compare black vs. blue). For 55:45 mol% DOPC/DOPS vesicles the calculated binding affinity is $5150 \pm 142 M^{-1}$, with a maximum of 0.19 Ca^{2+} per accessible lipid. This represents roughly one Ca^{2+} ion per every two PS lipids. We should note that the maximum ion per lipid number here reflects only the first step of high affinity binding to PS and PI(4,5) P_2 lipids. As bulk Ca^{2+} increases beyond the tested range, the numbers of ions per lipid will increase as ions start to bind to PC as well. The calculated binding affinity for these vesicles is significantly higher than the value reported for 80:20 mol% DOPC/DOPS vesicles above due to the higher overall charge of the vesicles. In addition to PC:PS vesicles, vesicles containing the highly negatively charged phospholipid PI(4,5) P_2 were also tested. PI(4,5) P_2 has been shown to bind Ca^{2+} with high affinity^{9, 11, 47}. The binding of Ca^{2+} to vesicles containing 5 mol% PI(4,5) P_2 , 35 mol% DOPC, 30 mol% DOPE, and 30 mol% DOPS is shown in red in Fig. 2. For this lipid composition, the additional charge from PI(4,5) P_2 is compensated by a reduction in the PS content, resulting in an equivalent overall charge compared to the previous lipid composition that did not include PI(4,5) P_2 (assuming PS with a charge of -1 and PI(4,5) P_2 with an effective charge of $-3^9, 47$). The zwitterionic lipid PE is included in this mixture to more closely match the lipid composition of the inner leaflet of the plasma membrane, but is unlikely to significantly influence the binding affinity due to its zwitterionic nature²². The binding affinity resulting from the association curve is $3910 \pm 103 M^{-1}$, with a maximum of 0.24 Ca^{2+} per accessible lipid. The binding affinities for the two negatively charged lipid compositions are similar, as the 15 mol% reduction in total PS is compensated by the higher Ca^{2+} affinity of the added 5 mol% PI(4,5) P_2 . This confirms that overall membrane charge is the primary contributor towards Ca^{2+} binding affinity to lipid vesicles.

Calcium ions induce membrane invaginations

Given the known impact of Ca^{2+} on lipid spontaneous curvature and its role in exocytosis and vesicle trafficking, the question arises whether Ca^{2+} ions may be able to directly impact the structure of the membrane. To assess the effects of Ca^{2+} on membrane shape we employed a single GUV transfer assay that enables us to determine the onset of a membrane curvature transition^{34–36}. By utilizing single GUVs in our experiment, we are able to exclude any effect from Ca^{2+} -induced vesicle aggregation and focus exclusively on the effect of Ca^{2+} on membrane curvature. The GUV is held at a controlled membrane tension by an aspiration pipette and transferred into a Ca^{2+} solution. The onset of a membrane curvature transition is determined by a decrease in apparent area as tubules with a high surface area to volume ratio form. As membrane excess area (relative to a perfect sphere) is consumed by Ca^{2+} -induced invaginations, we observe a decrease in the length of the aspirated region (see Fig. 3a). The formation of tubule invaginations indicates a Ca^{2+} -induced destabilization of the essentially (averaging over thermal fluctuations) planar membrane state (on a molecular scale) of the GUV, leading to a transition to a membrane with highly curved tubules.

As shown in Fig. 3d, the apparent area of the GUV membrane decreased after being transferred into the Ca^{2+} solution. The area decrease was accompanied by a significant increase of fluorescence intensity inside the GUV due to the formation of tubule

invaginations towards the interior of the GUV (Fig. 3a, also see supplemental movie 1). The fluorescence intensity inside the GUV and the area remain constant before transferring the vesicle into the Ca^{2+} solution (Fig. 3c), indicating that changes in these two quantities are a result specifically of exposure to Ca^{2+} solution. The formation of membrane invaginations consumes membrane area from the aspirated region of the vesicle, resulting in a decrease in measured apparent membrane area. The Ca^{2+} -induced membrane shape instability does not influence the overall stability of the membrane in terms of permeability, as we observe no increase in permeability after Ca^{2+} -induced membrane deformation (see supplementary information, Fig. S1).

An alternative explanation for the decrease in membrane area might be that the Ca^{2+} ions are triggering simultaneous condensation of both leaflets without causing bending. However, neither the fluorescence intensity per arc-length of the membrane nor the apparent membrane thickness (measured from the width of the fitted Gaussian ring) changed during the decrease of membrane area (Fig. 3d). This would be expected if bilayer condensation led to increased local lipid fluorophore density. In addition, condensation without bending cannot explain the dynamic tubules and increased fluorescence intensity we observe within the GUV (Fig. 3a and d). Thus, we can see that the Ca^{2+} is not changing overall fluorescence on the membrane, the fluorescence is simply redistributed as tubules form in the interior of the GUV.

Previously, for tubules formed by peripheral proteins, we found that tubule formation was inhibited by high membrane tension^{34–36}. Similarly, we find that Ca^{2+} -induced membrane invaginations are inhibited by increased tension. The high membrane tension opposes the formation of membrane curvature, thus increasing the shape stability of the planar membrane. In this case, we observe neither decreased apparent membrane area nor increased fluorescence intensity within the GUV (Fig. 3b).

As discussed in the previous section, Ca^{2+} ions bind zwitterionic lipids much more weakly than negatively charged lipids (See Fig. 2)^{45, 46}. Based on these results, we would expect to see a decreased impact of Ca^{2+} on DOPC vesicles compared to charged vesicles. To confirm this, we transferred pure DOPC vesicles into a 6 mM Ca^{2+} solution (Fig. 3e). In contrast to the DOPC/DOPS vesicles, there is no apparent membrane area decrease phenomenon for vesicles containing only DOPC. Therefore, we conclude that Ca^{2+} ions only induce membrane instability in GUVs containing negatively charged lipids, at least at physiologically relevant Ca^{2+} concentrations (either extracellular or intracellular).

Shape Instability diagram of calcium-induced membrane deformation

To understand the effect of Ca^{2+} ions on membrane shape stability in more detail, single GUV transfer experiments were carried out for a range of Ca^{2+} concentrations. For each Ca^{2+} concentration, multiple GUVs were measured with the goal of finding the tension value that defines the shape transition point for membranes under the investigated Ca^{2+} concentration. The shape stability diagram for Ca^{2+} ions was constructed with a lipid composition of 55:45 mol% DOPC/DOPS as shown in Fig. 4a. Two states of the membrane are mapped in Fig. 4a: the planar/stable state and the deformed/unstable state. A planar

membrane becomes unstable and is deformed by Ca^{2+} ions either through increasing Ca^{2+} concentration or through decreasing membrane tension.

Based on the data shown in Fig. 4a a stability boundary can be determined between the planar and curved membrane states. Firstly, at a certain Ca^{2+} concentration, the transition tension that separates the two states can be represented by the range of tension values where GUVs showed both stable and unstable behaviors. Therefore, the transition tension can be determined by averaging the overlapped or closest ‘Stable’ and ‘Unstable’ tension values at a given Ca^{2+} concentration in Fig. 4a. Secondly, based on our calculated Ca^{2+} binding affinity and the bulk Ca^{2+} concentration we can calculate the coverage of Ca^{2+} ions on the membrane. The resulting stability boundary describing the relation between the critical tension threshold and the coverage of Ca^{2+} ions on the membrane is shown in Fig. 4b.

Calcium induced membrane shape instability is $\text{PI}(4,5)\text{P}_2$ sensitive

As shown in Fig. 4a, on GUVs containing 55:45 mol% DOPC/DOPS, the Ca^{2+} induced membrane shape instability vanishes at Ca^{2+} concentrations below 0.8 mM. However, most Ca^{2+} -mediated events in mammalian cells involve Ca^{2+} concentrations no higher than 0.5 mM, thus questioning if purely Ca^{2+} -induced membrane instabilities are relevant to mammalian cells.

However, the simple PC/PS mixture that we tested first is not an accurate representation of the inner leaflet plasma membrane composition, which also contains the highly charged lipid $\text{PI}(4,5)\text{P}_2$. Therefore, we compared the Ca^{2+} effect for DOPC/DOPS vesicles with the more biologically relevant lipid composition 35:30:30:5 mol% DOPC/DOPE/DOPS/ $\text{PI}(4,5)\text{P}_2$. As shown in Fig. 5, even though the total amount of charge is similar for both compositions, Ca^{2+} exhibits a significantly stronger ability to induce membrane shape instability and form tubule invaginations on the composition containing $\text{PI}(4,5)\text{P}_2$. More specifically, higher critical tensions are found on the $\text{PI}(4,5)\text{P}_2$ containing composition than on the non- $\text{PI}(4,5)\text{P}_2$ containing composition in both 4 mM and 0.8 mM CaCl_2 solutions. With 4 mM Ca^{2+} , membranes containing $\text{PI}(4,5)\text{P}_2$ are deformed at higher tension values than we have observed for PS vesicles at any Ca^{2+} concentration (see Fig. 5a). With 0.8 mM Ca^{2+} , PS vesicles are only deformed at low tension values (0.018 mN/m), while $\text{PI}(4,5)\text{P}_2$ vesicles are still deformed at higher tensions (0.173 mN/m) (see Fig. 5b). Moreover, membrane curvature instability can be induced on the $\text{PI}(4,5)\text{P}_2$ containing composition at an even lower Ca^{2+} concentration of 0.1 mM (data not shown). We should note that due to the slightly lower affinity of Ca^{2+} to the $\text{PI}(4,5)\text{P}_2$ vesicles (see Fig. 2), the Ca^{2+} coverage on $\text{PI}(4,5)\text{P}_2$ vesicles is actually slightly lower at the same bulk concentration, compared to PS vesicles. This further supports the notion that $\text{PI}(4,5)\text{P}_2$ increases the intrinsic capacity for Ca^{2+} to generate membrane curvature and effect membrane shape instabilities.

Magnesium has a reduced capacity to induce membrane shape instability

Ca^{2+} ions are well documented to induce clustering or area compression of charged lipids, with the effect most prominent on polyphosphoinositides such as $\text{PI}(4,5)\text{P}_2$ ^{9, 11, 12, 14–18, 48–51}. The clustering of PS and/or $\text{PI}(4,5)\text{P}_2$ on the Ca^{2+} -binding leaflet has the potential to induce membrane shape changes by reducing the headgroup area

of the clustered lipids, thereby imposing a negative spontaneous curvature to the bilayer (see Fig. 3f). The membrane will become unstable towards the formation of invaginations when the imposed spontaneous curvature results in an energy gain that is large enough to overcome the elasticity of the bilayer⁵². Since Ca^{2+} ions have a much stronger ability to cluster $\text{PI}(4,5)\text{P}_2$ than PS lipids¹¹, this mechanism also agrees with the observation that Ca^{2+} ions showed a much stronger effect towards $\text{PI}(4,5)\text{P}_2$ containing membranes (Fig. 5). In contrast to Ca^{2+} ions, Mg^{2+} ions do not induce clustering of PS lipids^{15–17}, although they do increase the order of PS lipids⁵³. In addition, Mg^{2+} has a weaker capacity to induce fusion of PS vesicles than Ca^{2+} ^{54, 55}. The reduced fusion capacity is likely due to the reduced impact of Mg^{2+} on membrane hydrophobicity and surface tension^{28–30}. Therefore, we investigated whether Mg^{2+} ions have a similar impact on membrane structure as Ca^{2+} ions. The membrane shape instability tension threshold was compared for 55:45 mol% DOPC/DOPS vesicles transferred into solutions containing either 4 mM Ca^{2+} or Mg^{2+} (Fig. 6). Compared to Ca^{2+} , Mg^{2+} showed a much weaker capacity to induce membrane shape instability, with a transition tension of only 0.048 mN/m with 4 mM Mg^{2+} compared to 0.243 mN/m with 4 mM Ca^{2+} . Since Mg^{2+} has a similar affinity for PS compared to Ca^{2+} ^{44, 56}, the large difference in the capacity of Mg^{2+} and Ca^{2+} to trigger membrane curvature cannot be due to differing coverage of the ions on the membrane. Rather, we suggest that the capacity of the ion to cluster membrane lipids may influence the capacity of the ion to trigger membrane shape instability.

To conclude, we observed Mg^{2+} to have a weaker capacity to induce membrane curvature compared to Ca^{2+} .

Increased saline concentration (symmetric across the bilayer) reduces calcium-induced membrane deformation

In this section, we consider how the presence of saline in addition to Ca^{2+} may impact Ca^{2+} -induced membrane deformation. This is especially important due to the high concentration of salt ions in the cytosol, which may significantly influence the observed Ca^{2+} effect in the cellular environment. Previously we have used an asymmetric $[\text{Na}^+]$, with 37.5 mM outside and 0 mM inside. Here we determined the impact of saline by comparing the membrane shape instability tension threshold for DOPC/DOPE/DOPS/ $\text{PI}(4,5)\text{P}_2$ GUVs in the presence of 0.8 mM Ca^{2+} and either 37.5 mM NaCl on the exterior of the vesicle, as we have previously used, or 150 mM NaCl on both the interior and exterior of the GUV (see Fig. 7). In both conditions, we observe membrane shape instability at moderate tension values. However, the shape instability tension threshold is significantly reduced in the presence of 150 mM NaCl (0.173 vs 0.053 mN/m). Clearly, this shows that higher saline concentration impedes Ca^{2+} -induced membrane deformation. As discussed above, electrostatic screening significantly reduces the binding affinity of Ca^{2+} to negatively charged phospholipids and thus decreases the coverage of Ca^{2+} ions and the resulting shape instability tension threshold. It is important to note that the high saline concentration does not eliminate Ca^{2+} -induced membrane deformation, showing that the observed Ca^{2+} effect may still be relevant even for physiologically high salt concentrations.

Monovalent sodium ions (asymmetrically distributed across the bilayer) induce membrane structure change in opposition to calcium

In the previous section, we observed that NaCl reduces the impact of Ca^{2+} on membrane curvature instability. We suggested that this effect is largely due to NaCl reducing the coverage of Ca^{2+} on the membrane via electrostatic shielding. In the section above, NaCl had the same concentration on both sides of the membrane. In the following section, we are considering the consequences of an iso-osmolar concentration gradient of NaCl across the membrane.

Indeed, recent papers have suggested that monovalent ions may influence membrane curvature^{20, 21}. If monovalent ions do influence the curvature of the membrane, this could lead to an additional impact on Ca^{2+} -induced deformation. Additionally, any monovalent ion-induced membrane deformation could have important implications for cells under saline stress conditions. We therefore examined monovalent Na^+ ion-induced membrane deformation by transferring vesicles containing sucrose solution from low $[\text{Na}^+]$ (50 mM) into high $[\text{Na}^+]$ solutions (150 mM), thus creating an asymmetric ion distribution with 0 mM $[\text{Na}^+]$ inside the vesicles and 150 mM $[\text{Na}^+]$ outside (a few vesicles were also tested with the reverse gradient of 150 mM inside and 0 mM outside). Upon transfer, at sufficiently low tension values, we observe an apparent area decrease similar to what we observe for vesicles in Ca^{2+} . This suggests that the monovalent Na^+ ions may be generating membrane curvature like the Ca^{2+} ions. The observed shape instability tension threshold for a 150 mM asymmetric distribution of NaCl is displayed in Fig. 8a. In the case of Ca^{2+} -induced membrane deformation, we observed an increased effect in the presence of PI(4,5) P_2 . In contrast, for Na^+ , there is a much smaller difference between the transition threshold in the presence of 5% PI(4,5) P_2 , or no PI(4,5) P_2 (with total lipid charge roughly balanced) (see Fig. 8a). Thus, Na^+ -induced membrane deformation is not highly sensitive to the specific lipid species.

To further illuminate the role of Na^+ in modulating membrane structure, we evaluated the impact of an asymmetric $[\text{Na}^+]$ on Ca^{2+} -induced membrane deformation. Holding outside $[\text{Na}^+]$ fixed, we transferred GUV vesicles with varied internal $[\text{Na}^+]$ into 0.1 mM Ca^{2+} solutions. Specifically, we fixed the outside $[\text{Na}^+]$ to 50 mM, and varied inside $[\text{Na}^+]$ from 0 to 150 mM. Following GUV transfer, we observed an apparent area decrease and tubule formation below a tension threshold as shown in Fig. 8b. In each case, Ca^{2+} interacts with the membrane in the presence of an identical $[\text{Na}^+]$ concentration, so the degree of Ca^{2+} binding to the membrane is equivalent in each case. Therefore, our observation of an altered critical tension threshold can only be due to the impact of the asymmetric Na^+ concentration across the membrane. The asymmetric Na^+ concentration opposes the Ca^{2+} effect, with excess $[\text{Na}^+]$ on the exterior inhibiting Ca^{2+} (reducing the tension threshold from 0.281 to 0.136 mN/m), while excess $[\text{Na}^+]$ on the interior of the vesicle enhances the Ca^{2+} effect and thus increases the critical tension threshold (from 0.281 to 0.327 mN/m).

We conclude that the asymmetric $[\text{Na}^+]$ -induced membrane deformation opposes the negative curvature generation of Ca^{2+} , when Na^+ is enriched on the side of the membrane that contains Ca^{2+} . This suggests that Na^+ may be inducing positive curvature. Na^+ -induced positive curvature is consistent with simulations, which suggest that the membrane should

curve away from the Na^+ ions to increase the separation between Na^+ ions on the membrane²¹. This is also consistent with monolayer studies which show that increased $[\text{NaCl}]$ expands membranes containing DOPS or $\text{PI}(4,5)\text{P}_2$ ^{14, 57, 58}. Na^+ ion-induced lipid expansion likely occurs due to binding of ions between lipids in the membrane, thus spreading the leaflet and increasing the average area per lipid^{14, 59–61}. Several studies have suggested that Na^+ may penetrate deeply into the bilayer, and may interact with the carbonyl oxygens of the phospholipid acyl chains^{60, 61}. Additionally, the increased $[\text{Na}^+]$ could disrupt hydrogen bonding between lipids (particularly for $\text{PI}(4,5)\text{P}_2$, which has a high capacity for hydrogen-bonding), leading to additional lipid expansion (as suggested by Levental et al.)⁵⁸. With asymmetric $[\text{Na}^+]$, the leaflet in the presence of higher $[\text{Na}^+]$ will expand further than the leaflet with lower $[\text{Na}^+]$, resulting in positive membrane curvature towards the higher $[\text{Na}^+]$. On the other hand, this conflicts with recent results performed with tether experiments, which suggested that Na^+ creates negative curvature²⁰. However, these experiments utilized tethers with high curvature, as compared to our GUVs, which are effectively flat on a microscale at the onset of the curvature stability. This higher curvature of pulled tethers may alter the nature of the interaction between the Na^+ ions and the lipids.

Discussion and biological relevance

Mammalian cells exhibit oscillatory Ca^{2+} waves that couple with an oscillation of local $\text{PI}(4,5)\text{P}_2$ concentrations to organize signaling events as well as cell migration⁴. Our findings suggest that the increase of local Ca^{2+} concentration (to a few hundred micromolar) during these waves could mediate the formation of protrusions from the plasma membrane as the transient high concentration of Ca^{2+} ions induces local membrane deformation. While these transient $[\text{Ca}^{2+}]$ increases within the cell do not match the high $[\text{Ca}^{2+}]$ outside the cell, due to the relative lack of charged lipids on the exterior of the cell membrane the Ca^{2+} ions within the cell are likely to have a much larger influence on potential Ca^{2+} -induced membrane deformation. During cell migration, ‘flickers’ or short bursts of Ca^{2+} ions are found predominantly at the leading edge of the cell³. These bursts of high $[\text{Ca}^{2+}]$ could play a direct role in helping to push the membrane out and extend the cell in the direction of motion. Ca^{2+} oscillations also play a role in the formation of invadopodia and podosomes². Whereas previously these effects were presumed to be generated by Ca^{2+} primarily interacting with proteins, our results suggest that Ca^{2+} could play a role through direct interaction with the membrane as well. We should note that due to the high Ca^{2+} coverage required to produce membrane deformation, it is unlikely that Ca^{2+} alone will generate membrane curvature *in vivo*. Rather, we suggest that Ca^{2+} ‘flickers’ could act in concert with curvature generating proteins, providing a trigger for initiation of the transition of the membrane from a planar state to a curved state.

In this work, we have found that each of the three biologically relevant cations we have studied can induce membrane deformation. However, the relative strength of the membrane deformation of these ions varies widely between three tested ions, with Ca^{2+} showing the strongest influence (deforming the membrane at sub-millimolar concentration), followed by Mg^{2+} (requiring millimolar concentration) and Na^+ (requiring tens of millimolar levels). Why is there such a large difference between these ions? According to the Hofmeister series, these ions can be arranged as $\text{Na}^+ > \text{Ca}^{2+} > \text{Mg}^{2+}$, from the most chaotropic (and most

weakly hydrated) to the most kosmotropic (and more strongly hydrated)⁶². It has previously been shown by the Cremer group that cations follow the Hofmeister series when partitioning to a negatively charged solid interface, with lower cations in the series such as Mg^{2+} , partitioning to a larger extent than higher ions such as Na^+ ⁶³. This effect is largely based on charge density, and might explain the small impact on membrane deformation by the Na^+ ion. However, charge density differences do not explain the stronger effect on membrane curvature via Ca^{2+} , which has a lower charge density than Mg^{2+} . We also note that Na^+ seems to deform the membrane in the opposite direction from Ca^{2+} and Mg^{2+} (see Fig. 8b). This is likely due to the following differences in the lipid-cation complex formed with Na^+ , as compared to Ca^{2+} and Mg^{2+} : as we mentioned earlier, the formation of Mg^{2+} and Ca^{2+} complexes with lipids tends to compress the membrane^{9–18, 53}, while Na^+ tends to expand the membrane^{14, 57, 58, 64}. In contrast, the difference between Ca^{2+} and Mg^{2+} is likely due to hydration. The Mg^{2+} ion has a higher free energy of hydration and does not dehydrate upon binding to the membrane¹¹, thus decreasing the strength of the Mg^{2+} -lipid complex and the impact on membrane hydrophobicity^{28–30}. This in turn decreases the effect of Mg^{2+} on the spontaneous curvature of the membrane. To summarize, we suggest that the curvature effect of the ion is highly dependent on both the charge density and the hydration free energy of the ion, with higher charge density and decreased free energy of hydration leading to a larger influence on membrane curvature (as in the case of Ca^{2+}).

Our experiments showed that Ca^{2+} ions lead to membrane invaginations on GUVs (Fig. 3), meaning the spontaneous membrane curvature generated by Ca^{2+} should be negative. This does agree with recent measurements regarding the effect of Ca^{2+} ions on lipid spontaneous curvature, where Ca^{2+} ions were found to render the spontaneous curvature of (negatively charged) DOPA, but not (zwitterionic) DOPE, significantly smaller (more negative)²². On the other hand, the notion of negative membrane curvature generated by Ca^{2+} ions is in discrepancy with several recent predictions^{20, 21, 65} that result, however, from consideration of scenarios that likely do not apply to our experimental study, as we discuss in the following.

Firstly, according to a simplifying analytical model, when a membrane is asymmetrically covered by particles much smaller than the thickness of the bilayer (such as sugar or ions), the membrane will prefer to bend away from the side with a higher cover fraction of the particle, in order to maximize the average distance between the bound particles^{21, 66}. This model would imply that the binding of Ca^{2+} ions to only the exterior of the GUV will lead to a positive spontaneous curvature of the bilayer. However, this model entirely neglects the lipid clustering/condensing effect of Ca^{2+} ions discussed above and therefore likely cannot predict the direction of membrane spontaneous curvature induced by Ca^{2+} binding.

Secondly, the spontaneous membrane curvature generated Ca^{2+} binding has recently been evaluated by measuring the effect of Ca^{2+} ions on either the tether pulling force or the radius of a tether pulled from a GUV, again concluding positive spontaneous curvature induced by Ca^{2+} ²⁰. The discrepancy between the C_0 values measured on tethers to the one measured on GUVs is likely due to different behavior of Ca^{2+} ions on membranes of drastically different curvatures. On the highly curved membrane tether, it may be easier (than on low curvature

GUV membranes) for Ca^{2+} ions to bind to the phosphate group of the lipids thereby producing a positive spontaneous curvature^{67, 68}.

Conclusions

In this work, we have demonstrated the capacity of Ca^{2+} to remodel the membrane. We find that Ca^{2+} binding to negatively charged membranes triggers the formation of membrane invaginations in vesicles at millimolar and sub-millimolar bulk concentrations of Ca^{2+} . The invaginations are likely triggered by clustering of lipids due to interaction with Ca^{2+} , leading to negative spontaneous curvature in the membrane leaflet with bound Ca^{2+} ions. Ca^{2+} mediated-membrane remodeling is membrane tension sensitive, and can be blocked by high tension. The presence of $\text{PI}(4,5)\text{P}_2$ within the membrane allows Ca^{2+} -mediated membrane remodeling at lower Ca^{2+} coverage, likely related to the capability of Ca^{2+} to trigger $\text{PI}(4,5)\text{P}_2$ clustering. Consistent with this interpretation, we find that Mg^{2+} , which has been shown to have a poor capacity to trigger lipid clustering, has a much lower capacity to effect membrane remodeling. These results suggest a potential new role for Ca^{2+} as a mediator of curvature formation in the membrane. While Ca^{2+} -induced membrane deformation is weak on its own, local Ca^{2+} signals could act in concert with curvature generating proteins to trigger membrane deformation.

In addition to Ca^{2+} , we also find that Na^+ ions may influence membrane structure in the absence of Ca^{2+} . The observed Na^+ -effect requires a high asymmetric distribution of Na^+ ions across the membrane, and thus may not play a role in modulating the membrane structure of healthy cells. However, Na^+ -induced membrane structural change may be important for cells experiencing saline stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge financial support from NIH grant R01 GM 097552 and NIH grant U54CA193417. Furthermore, we thank R. Jin and S. Wilner for helpful discussions.

References

1. Berridge MJ, Lipp P, Bootman MD. Nat Rev Mol Cell Bio. 2000; 1:11–21. [PubMed: 11413485]
2. Sun J, Lu F, He H, Shen J, Messina J, Mathew R, Wang D, Sarnaik AA, Chang WC, Kim M, Cheng H, Yang S. The Journal of cell biology. 2014; 207:535–548. [PubMed: 25404747]
3. Wei C, Wang X, Chen M, Ouyang K, Song LS, Cheng H. Nature. 2009; 457:901–905. [PubMed: 19118385]
4. Wu M, Wu X, De Camilli P. Proceedings of the National Academy of Sciences. 2013; 110:1339–1344.
5. Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC. Nature. 2001; 410:41–49. [PubMed: 11242035]
6. Cooper ST, McNeil PL. Physiological reviews. 2015; 95:1205–1240. [PubMed: 26336031]
7. Grienberger C, Konnerth A. Neuron. 2012; 73:862–885. [PubMed: 22405199]
8. Klee CB, Crouch TH, Richman PG. Annual review of biochemistry. 1980; 49:489–515.

9. Graber ZT, Wang W, Singh G, Kuzmenko I, Vaknin D, Kooijman EE. RSC Advances. 2015; 5:106536–106542.
10. Sarmiento MJ, Coutinho A, Fedorov A, Prieto M, Fernandes F. Biochimica et biophysica acta. 2013; doi: 10.1016/j.bbame.2013.11.020
11. Wang YH, Collins A, Guo L, Smith-Dupont KB, Gai F, Svitkina T, Janmey PA. Journal of the American Chemical Society. 2012; 134:3387–3395. [PubMed: 22280226]
12. Boettcher JM, Davis-Harrison RL, Clay MC, Nieuwkoop AJ, Ohkubo YZ, Tajkhorshid E, Morrissey JH, Rienstra CM. Biochemistry. 2011; 50:2264–2273. [PubMed: 21294564]
13. Pabst G, Hodzic A, Strancar J, Danner S, Rappolt M, Laggner P. Biophysical journal. 2007; 93:2688–2696. [PubMed: 17586572]
14. Sovago M, Wurfel GW, Smits M, Muller M, Bonn M. Journal of the American Chemical Society. 2007; 129:11079–11084. [PubMed: 17696532]
15. Ohnishi S, Ito T. Biochemistry. 1974; 13:881–887. [PubMed: 4360353]
16. Ohnishi S, Ito T. Biochemical and biophysical research communications. 1973; 51:132–138. [PubMed: 4349320]
17. Haverstick DM, Glaser M. Proceedings of the National Academy of Sciences of the United States of America. 1987; 84:4475–4479. [PubMed: 3474616]
18. Melcova A, Pokorna S, Pullanchery S, Kohagen M, Jurkiewicz P, Hof M, Jungwirth P, Cremer PS, Cwiklik L. Scientific reports. 2016; 6
19. Seo JB, Jung SR, Huang W, Zhang Q, Koh DS. PloS one. 2015; 10:e0144432. [PubMed: 26658739]
20. Simunovic M, Lee KY, Bassereau P. Soft Matter. 2015; 11:5030–5036. [PubMed: 26016587]
21. Rozycki B, Lipowsky R. The Journal of chemical physics. 2015; 142:054101. [PubMed: 25662630]
22. Chen YF, Tsang KY, Chang WF, Fan ZA. Soft Matter. 2015; 11:4041–4053. [PubMed: 25907686]
23. Leventis R, Gagne J, Fuller N, Rand RP, Silvius JR. Biochemistry. 1986; 25:6978–6987. [PubMed: 3801406]
24. Ellens H, Bentz J, Szoka FC. Biochemistry. 1985; 24:3099–3106. [PubMed: 4027232]
25. Papahadjopoulos D, Nir S, Duzgunes N. Journal of bioenergetics and biomembranes. 1990; 22:157–179. [PubMed: 2139437]
26. Duzgunes N, Wilschut J, Fraley R, Papahadjopoulos D. Biochimica et biophysica acta. 1981; 642:182–195. [PubMed: 7225377]
27. Rand RP, Kachar B, Reese TS. Biophysical journal. 1985; 47:483–489. [PubMed: 3986279]
28. Ohki S, Ohshima H. Biochimica et biophysica acta. 1984; 776:177–182.
29. Ohki S, Arnold K. J Membrane Biol. 1990; 114:195–203. [PubMed: 2325117]
30. Ohki S. Abstr Pap Am Chem S. 1990; 199:91-Coll.
31. Angelova MI, Dimitrov DS. Faraday Discuss. 1986; 81:303–+.
32. Tian A, Baumgart T. Biophysical Journal. 2009; 96:2676–2688. [PubMed: 19348750]
33. Weinberger A, Tsai FC, Koenderink GH, Schmidt TF, Itri R, Meier W, Schmatko T, Schroder A, Marques C. Biophysical journal. 2013; 105:154–164. [PubMed: 23823234]
34. Chen Z, Atefi E, Baumgart T. Biophysical journal. 2016; 111:1823–1826. [PubMed: 27806264]
35. Chen Z, Shi Z, Baumgart T. Biophysical journal. 2015; 109:298–307. [PubMed: 26200865]
36. Shi Z, Baumgart T. Nature communications. 2015; 6:5974.
37. Capraro BR, Shi Z, Wu TT, Chen ZM, Dunn JM, Rhoades E, Baumgart T. Journal of Biological Chemistry. 2013; 288:12533–12543. [PubMed: 23482561]
38. Heinrich M, Tian A, Esposito C, Baumgart T. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:7208–7213. [PubMed: 20368457]
39. Zhang T, Cathcart MG, Vidalis AS, Allen HC. Chemistry and physics of lipids. 2016; doi: 10.1016/j.chemphyslip.2016.06.001
40. Altenbach C, Seelig J. Biochemistry. 1984; 23:3913–3920. [PubMed: 6487586]
41. Lau A, McLaughlin A, McLaughlin S. Biochimica et biophysica acta. 1981; 645:279–292. [PubMed: 7272290]

42. Grasdalen H, Eriksson LEG, Westman J, Ehrenberg A. *Biochimica et biophysica acta*. 1977; 469:151–162. [PubMed: 561615]
43. Akutsu H, Seelig J. *Biochemistry*. 1981; 20:7366–7373. [PubMed: 6895698]
44. McLaughlin S, Mulrine N, Gresalfi T, Vaio G, McLaughlin A. *Journal of General Physiology*. 1981; 77:445–473. [PubMed: 7241089]
45. Sinn CG, Antonietti M, Dimova R. *Colloid Surface A*. 2006; 282:410–419.
46. Seelig J. *Cell biology international reports*. 1990; 14:353–360. [PubMed: 2191790]
47. Toner M, Vaio G, McLaughlin A, McLaughlin S. *Biochemistry*. 1988; 27:7435–7443. [PubMed: 2849993]
48. Wang YH, Slochower DR, Janmey PA. *Chemistry and Physics of Lipids*. 2014
49. Kucerka N, Dushanov E, Kholmurodov KT, Katsaras J, Uhríkova D. *Langmuir : the ACS journal of surfaces and colloids*. 2017; doi: 10.1021/acs.langmuir.6b03228
50. Graber ZT, Gericke A, Kooijman EE. *Chemistry and physics of lipids*. 2014; 182:62–72. [PubMed: 24309195]
51. Flach CR, Brauner JW, Mendelsohn R. *Biophysical journal*. 1993; 65:1994–2001. [PubMed: 8298029]
52. Leibler S. *J Phys-Paris*. 1986; 47:507–516.
53. Schultz ZD, Pazos IM, McNeil-Watson FK, Lewis EN, Levin IW. *The journal of physical chemistry B*. 2009; 113:9932–9941. [PubMed: 19603842]
54. Wilschut J, Duzgunes N, Papahadjopoulos D. *Biochemistry*. 1981; 20:3126–3133. [PubMed: 7248275]
55. Duzgunes N, Nir S, Wilschut J, Bentz J, Newton C, Portis A, Papahadjopoulos D. *The Journal of membrane biology*. 1981; 59:115–125. [PubMed: 7241577]
56. Ohki S. *Biochimica et biophysica acta*. 1982; 689:1–11. [PubMed: 7104344]
57. Luna C, Stroka KM, Bermudez H, Aranda-Espinoza H. *Colloids and surfaces B, Biointerfaces*. 2011; 85:293–300. [PubMed: 21440423]
58. Levental I, Cebers A, Janmey PA. *Journal of the American Chemical Society*. 2008; 130:9025–9030. [PubMed: 18572937]
59. Pandit SA, Berkowitz ML. *Biophysical journal*. 2002; 82:1818–1827. [PubMed: 11916841]
60. Mukhopadhyay P, Monticelli L, Tieleman DP. *Biophysical journal*. 2004; 86:1601–1609. [PubMed: 14990486]
61. Bockmann RA, Hac A, Heimburg T, Grubmuller H. *Biophysical journal*. 2003; 85:1647–1655. [PubMed: 12944279]
62. Okur HI, Hladilkova J, Rembert KB, Cho Y, Heyda J, Dzubiella J, Cremer PS, Jungwirth P. *Journal of Physical Chemistry B*. 2017; 121:1997–2014.
63. Flores SC, Kherb J, Konelick N, Chen X, Cremer PS. *J Phys Chem C*. 2012; 116:5730–5734.
64. Leontidis E. *Adv Colloid Interface Sci*. 2017; doi: 10.1016/j.cis.2017.04.001
65. Magarkar A, Jurkiewicz P, Allolio C, Hof M, Jungwirth P. *The journal of physical chemistry letters*. 2017; :518–523.doi: 10.1021/acs.jpclett.6b02818 [PubMed: 28067523]
66. Lipowsky R. *Faraday Discuss*. 2013; 161:305–331. [PubMed: 23805747]
67. Baumgart, T., Capraro, BR., Zhu, C., Das, SL. *Annual Review of Physical Chemistry*, Vol 62. Leone, SR.Cremer, PS.Groves, JT., Johnson, MA., editors. Vol. 62. 2011. p. 483–506.
68. Campelo F, McMahon HT, Kozlov MM. *Biophysical journal*. 2008; 95:2325–2339. [PubMed: 18515373]

Figure 1.

the procedure of a single guv transfer. (a) sketch of a micropipette aspirated guv. Δp is the pressure difference between the inside and outside of the pipette used for guv aspiration. r_p and r_v represent the radius of the pipette and the spherical part of the guv, respectively. L_p represents the aspiration length of the guv. (b) the process of transferring an aspirated guv from the guv dispersion (red) into an imaging chamber with the desired salt solution (grey). the guv transfer is a four-step process: ① a guv is aspirated into a micropipette to adjust the desired membrane tension. ② the transfer capillary is manually positioned to cover the guv. ③ the guv is transferred from the guv chamber into the imaging chamber using a motor-controlled micromanipulator (luigs and neumann, ratingen, germany). ④ the transfer capillary is removed to expose the guv to the salt solution.

Figure 2.

binding of Ca^{2+} ions is determined by membrane charge. titration curves of bound Ca^{2+} versus added Ca^{2+} for Ca^{2+} binding to lipid vesicles composed of (black) 100% dopc, (blue) 55:45 mol% dopc/dops, or (red) 35:30:30:5 mol% dopc/dope/dops/pi(4,5)p₂. all titrations were performed in 37.5 mM nacl and 7 mM hepes at ph 7, to match saline concentrations used in guv experiments. error bars in the y axis are from the propagation of error in the ise measurement, while x axis error is from the propagation of error from the lipid (determined by phosphate assay) and CaCl_2 (determined gravimetrically) stock concentrations. solid lines are fit results using eqn. 2.

Figure 3.

binding of Ca^{2+} ions induces membrane invaginations as revealed by single guv transfer. (a) confocal images showing an aspirated guv (labeled with texas red-dhpe) before and after being transferred into solutions containing 4 mM CaCl_2 (37.5 mM NaCl , 7 mM HEPES , pH 7). membrane tension is 0.17 mN/m. the dashed line separates images before and after transfer. as the projection length decreases the fluorescence intensity within the guv increases as tubules form (the tubules are too dynamic to be resolved clearly) (see supplemental movie 1). (b) confocal images of a stable vesicle that was transferred into 2.4 mM Ca^{2+} solution at high membrane tension (0.35 mN/m). vesicle area remains constant and no tubules are observed forming before or after transferring the vesicle into Ca^{2+} solution (on either side of the dashed line). this shows that the vesicle deformation is dependent on Ca^{2+} concentration and membrane tension. (c) and (d) quantification of the guv apparent membrane area (black), apparent membrane thickness (red), fluorescence intensity per arc-length on the guv contour (green), and fluorescence intensity inside (blue) for a typical guv before (b) and after (c) being transferred into 4 mM CaCl_2 . all values are normalized to the corresponding values at the first frame of the image sequence. lipid composition: 55:45 mol% dopc/dops (labeled with 0.3% texas red-dhpe). (e) Ca^{2+} ions do not induce membrane instability on pure dopc guvs. representative traces of membrane area change after a guv of pure dopc (blue, closed) or 55:45 mol% dopc/dops (blue, open) is transferred into 6 mM CaCl_2 solution. time zero is defined as the time point when the guv is exposed to CaCl_2 solution. the time gap around time zero is due to the time taken to transfer the vesicle into the Ca^{2+} solution. membrane tensions for both guvs are 0.05 ± 0.01 mN/m. (f) diagram of the proposed

mechanism of Ca^{2+} -induced membrane deformation. Ca^{2+} ions bind to negatively charged lipids leading to condensation and/or clustering, resulting in decreased membrane area in the leaflet with bound Ca^{2+} ions and negative spontaneous curvature. at sufficiently low tensions, the membrane then bends to form invaginations to compensate for the negative spontaneous curvature and reduced area in the Ca^{2+} -bound leaflet.

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Figure 4.

membrane deformation is dependent on tension and Ca^{2+} concentration. (a) the black data points represent guvs that became unstable towards a curvature transition (showed area decrease) when transferred to the indicated concentration of Ca^{2+} . the white data points represent guvs that are still stable (showed no area decrease) at least 5 mins after being transferred into the indicated concentration of $CaCl_2$. buffer conditions: (50x/36 + 37.5) mM NaCl, 7 mM HEPES, pH 7 (before transfer, lipid chamber); x mM $CaCl_2$, 37.5 mM NaCl, 7 mM HEPES, pH 7 (after transfer, Ca^{2+} chamber) with x being the concentration of Ca^{2+} ions. the osmolarity in both chambers are balanced with glucose to result in a final osmolarity ~20% higher than the osmolarity of guv stock solutions. (b) shape stability boundary for 55:45 mol % DOPC/DOPS with Ca^{2+} . the data points represent the transition tensions for a certain Ca^{2+} coverage. the Ca^{2+} coverage is determined from the $CaCl_2$ concentration using the previously determined Ca^{2+} binding affinity (see fig. 2). the transition tension for each Ca^{2+} coverage is determined by taking averages from either the overlapped tension values of stable and unstable guvs or the two closest tension values for stable and unstable guvs (values from fig. 4a).

Figure 5.

presence of pi(4,5)p₂ in the membrane significantly amplifies the ca²⁺-induced curvature instability. (a) comparison of the critical tensions for guvs with (5% pi(4,5)p₂) and without (no pi(4,5)p₂) pi(4,5)p₂ in 4 mm cacl₂. lipid composition: no pi(4,5)p₂: 55:45 mol% dopc/dops; 5% pi(4,5)p₂: 35:30:30:5 mol% dopc/dope/dops/pi(4,5)p₂. averages of the overlapped tension values for the two compositions are 0.243 ± 0.005 mn/m (no pi(4, 5)p₂) and 0.391 ± 0.072 mn/m (5% pi(4,5)p₂), both are mean ± sd. student t-test of the overlapped tensions: p = 0.004. (b) comparison of the critical tensions for guvs with (5% pi(4,5)p₂) and without (no pi(4,5)p₂) pi(4,5)p₂ in 0.8 mm cacl₂. averages of the 2ped tension values for the two compositions are 0.018 ± 0.008 mn/m (no pi(4,5)p₂) and 0.194 ± 0.025 mn/m (5% pi(4,5)p₂), both are mean ± sd. student t-test of the overlapped tensions: p = 0.007.

Figure 6. Mg^{2+} has a significantly weaker ability to induce membrane deformation than Ca^{2+} . under the same concentration (4 mM), the transition tension for Ca^{2+} (0.243 ± 0.005 mN/m) induced guv instability is significantly higher than for Mg^{2+} (0.048 ± 0.008 mN/m). both are mean $\pm$ sd. student t-test of the overlapped tensions: $p < 0.001$. lipid composition: 55:45 mol % dopc/dops.

Figure 7.

a high symmetric Na^+ distribution reduces the membrane instability tension threshold for Ca^{2+} -induced deformation. comparison of the critical tension for guvs transferred into 0.8 mM Ca^{2+} with varying Na^+ concentration. the transition tension threshold for Ca^{2+} with 37.5 mM NaCl (0.194 ± 0.025 mN/m) (from fig. 5b) is significantly higher than when 150 mM NaCl is present (0.053 ± 0.018 mN/m). both are mean $\pm$ sd. student t-test of the overlapped tensions: $p < 0.001$. lipid composition: 35:30:30:5 mol% dopc/dope/dops/pi(4,5)p₂.

Figure 8.

asymmetric Na^+ distribution induces membrane deformation of negatively charged vesicles in opposition to Ca^{2+} . guvs containing sucrose solution with no Na^+ ions were transferred into solution containing 150 mM NaCl at controlled tension values. the resulting asymmetric Na^+ concentration across the membrane was 150/0 mM. the reverse gradient was also tested (150 mM inside, 0 mM outside) and had similar results. below a transition tension threshold an apparent area decrease is observed, suggesting membrane deformation. the transition tension threshold for Na^+ with 5% PI(4,5)P₂ (0.181 ± 0.051 mN/m) is similar to the threshold in the absence of PI(4,5)P₂ (0.210 ± 0.022 mN/m). tension threshold is mean $\pm$ sd. student t-test of the overlapped tensions: $p = 0.18$. lipid compositions were 35:30:30:5 mol% dopc/dope/dops/PI(4,5)P₂ (5% PI(4,5)P₂) and 55:45 mol% dopc/dops (no PI(4,5)P₂). (b) Na^+ opposes Ca^{2+} -induced membrane deformation. guvs with varying internal Na^+ concentration were transferred into solutions with 0.1 mM Ca^{2+} and 0 or 50 mM NaCl. the varied asymmetric Na^+ content influences the critical tension threshold, resulting in 0.111 ± 0.020 mN/m for +50 mM [Na⁺], 0.234 ± 0.031 mN/m for 0 mM [Na⁺], and 0.283 ± 0.018 mN/m for -50 mM [Na⁺] (Na⁺ concentration is the exterior [Na⁺] minus interior [Na⁺]). tension threshold is mean $\pm$ sd. student t-test of 50o/0i vs 50o/50i: $p < 0.001$, 50o/50i vs 50o/100i: $p = 0.007$. lipid composition: 35:30:30:5 mol% dopc/dope/dops/PI(4,5)P₂.