Importance of Direct Interactions with Lipids for the Function of the Mechanosensitive Channel MscL[†]

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ABSTRACT: We have studied the effects of lipid structure on the function of the mechanosensitive channel of large conductance (MscL) from *Escherichia coli* to determine whether effects follow from direct interaction between the lipids and protein or whether they follow indirectly from changes in the curvature stress in the membrane. The G22C mutant of MscL was reconstituted into sealed vesicles containing the fluorescent molecule calcein, and the release of calcein from the vesicles was measured following opening of the channel by reaction with [2-(triethylammonium)ethyl] methanethiosulfonate (MTSET), which introduces five positive charges into the region of the pore constriction. The presence of anionic lipids in the vesicle membrane changed the rates and amplitudes of calcein release, the effects not correlating with calculated changes in lipid spontaneous curvature. Mutation of charged residues in the Arg-104, Lys-105, Lys-106 cluster removed high-affinity binding of anionic lipids to MscL, and the presence of anionic lipid no longer affected calcein flux through MscL. Changing the zwitterionic lipid from phosphatidylcholine to phosphatidylethanolamine resulted in a large decrease in the rate of calcein release, the change in rate varying linearly with lipid composition, as expected if spontaneous curvature affected the rate of release. However, rates of release of calcein measured in the presence of phosphatidylethanolamine-*N*-methyl and phosphatidylethanolamine-*N*,*N*-dimethyl did not fit the correlation between rate and curvature established for the phosphatidylcholine/phosphatidylethanolamine mixtures. Rather, the effects of zwitterionic lipid headgroup on calcein flux suggested that what was important was the presence of a proton in the headgroup, able to take part in hydrogen bonding to MscL. We conclude that the function of MscL is likely to be modulated by direct interaction with the surrounding, annular phospholipids that contact the protein in the membrane.

The activities of many integral membrane proteins have been observed to change with changes in the composition of the surrounding lipid bilayer (*1, 2*). These changes in protein activity could follow from changes in the bulk physical properties of the bilayer; alternatively, they could follow directly from the changes in lipid chemical structure if interactions at the molecular level between lipids and proteins were important for function.

Several of the bulk physical properties of a lipid bilayer have been suggested to be of possible importance for membrane protein function. Recently, interest has focused on distortions of the lipid bilayer caused by the presence of a membrane protein and on how these distortions could depend on elastic properties of the bulk lipid bilayer such as its spontaneous curvature (*3*−*7*). The spontaneous curvature of a lipid, C_0, is the reciprocal of the spontaneous radius of curvature, R_0, and is the curvature that one monolayer of a bilayer would adopt if it was allowed to bend freely, without the constraint imposed from being packed back to back with another monolayer in a bilayer (*8*). Lipids such as phosphatidylcholine (PC) with an overall cylindrical shape favor a flat monolayer, for which the spontaneous radius of curvature is infinite, so that C_0 will be close to zero. Other lipids, such as phosphatidylethanolamine (PE), with a small headgroup and a conical shape, form curved inverted hexagonal phases (HII) at physiological temperatures, for which C_0 will have a large negative value, where the negative sign, by convention, indicates a bend toward the aqueous phase (*9*). A lipid with a large negative spontaneous curvature forced to be in a bilayer will be in a state of curvature stress, and the elastic energy in such a bilayer (*10*) will be available to do work such as changing the conformational state of a membrane protein. The concept of spontaneous curvature and curvature stress affecting membrane protein function has been shown to be equivalent to an effect of lateral pressure profile (*7, 11*).

A correlation between lipid spontaneous curvature and channel formation has been observed for alamethicin (*8, 12*) and gramicidin (*13*), and effects of lipid structure and bilayer additives on the conformational equilibrium of rhodopsin (*5*) and on sodium channel function have also been correlated with changes in lipid spontaneous curvature (*6*). However, a study of the function of the multidrug transporter LmrP suggested that direct interactions between the lipid headgroup and LmrP were more important than changes in spontaneous curvature (*14*).

A protein whose function is likely to be particularly dependent on the nature of the surrounding phospholipid bilayer is the bacterial mechanosensitive channel of large

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conductance MscL;[1] the channel opens in response to an increase in membrane tension when reconstituted into simple lipid bilayers in the absence of any other proteins, showing that the only interactions important for channel opening are those between the channel and the surrounding lipid molecules (*15, 16*). Activation of the channel leads to opening of a large pore across the membrane, the large changes in helix packing responsible for channel opening (*17, 18*) making MscL potentially very sensitive to the bulk physical properties of the membrane. MscL therefore provides an excellent system with which to test the relative importance of lipid spontaneous curvature and lipid chemical structure for membrane protein function.

Effects of lipid structure on MscL function in reconstituted membranes have been studied at the single-molecule level by patch clamping techniques; these studies have shown that the tension required to open the channel is higher in bilayers of PE than in bilayers of PC but that the opening tension is not affected by the presence of the anionic lipids phosphatidylserine (PS) or phosphatidylglycerol (PG) (*19*). An alternative technique, allowing the study of bulk fluxes mediated by MscL in reconstituted lipid vesicles, was introduced by Kocer et al. (*20, 21*). This involved trapping high concentrations of the fluorescent dye calcein within the lumen of the vesicles, so that the intensity of calcein fluorescence was reduced by concentration quenching. Opening of the MscL channel led to the release of calcein into the external medium with a large increase in fluorescence intensity. The channel used in these experiments was a mutant of *Escherichia coli* MscL with a Cys residue at position 22 (G22C) within the region of the pore constriction. Reaction with [2-(triethylammonium)ethyl] methanethiosulfonate bromide (MTSET) introduced five positively charged choline groups within the pore and forced the channel into an open state. Using this approach, we showed that the magnitude of release of calcein was low in vesicles containing PC as the only phospholipid, but that the magnitude and rate of release increased with increasing anionic lipid content, up to 50 mol %, beyond which release decreased in magnitude (*22*). Effects of lipid on the amplitude of release were attributed to a conformation change of the open channel to an inactivated state unable to release calcein, the rate of the conformation change decreasing with increasing anionic lipid content (*22*).

Here we show that the flux of calcein through the MscL channel in mixtures of zwitterionic and anionic lipid is affected strongly by the structure of the zwitterionic lipid, and that these effects do not correlate with the calculated spontaneous curvatures of the lipid mixtures. Effects of anionic lipid on flux through the MscL channel also do not correlate with calculated spontaneous curvatures. An alternative possibility is that the observed effects of lipid structure on flux through the MscL channel depend on molecular interactions between MscL and the lipids in contact with it in the membrane. The data suggest that effects of zwitterionic lipids on MscL function follow from interaction with a relatively large number of sites around the protein, whereas effects of anionic lipids could follow from binding to a specific charged cluster on the cytoplasmic side of the membrane.

[1] Abbreviations: MscL, mechanosensitive channel of large conductance; MTSET, [2-(triethylammonium)ethyl]methanethiosulfonate bromide; OG, octyl β-D-glucoside; $C_{12}E_8$, octa(ethylene glycol)-*n*-dodecyl ether; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; Me-DOPE, dioleoylphosphatidylethanolamine-*N*-methyl; Me_2-DOPE, dioleoylphosphatidylethanolamine-*N*,*N*-dimethyl, DOPA, dioleoylphosphatidic acid; DOPG, dioleoylphosphatidylglycerol; BrPC, di(9,10-dibromostearoyl)phosphatidylcholine; BrPE, di(9,10-dibromostearoyl)phosphatidylethanolamine; BrPA, di(9,10-dibromostearoyl)phosphatidic acid; BrPG, di(9,10-dibromostearoyl)phosphatidylglycerol; PS, phosphatidylserine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

MATERIALS AND METHODS

Calcein and [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) were obtained from Sigma and Toronto Research Chemicals, respectively. The detergents octyl β-D-glucoside (OG) and octa(ethylene glycol)-*n*-dodecyl ether ($C_{12}E_8$) were obtained from Anatrace and Calbiochem, respectively. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE),dioleoylphosphatidylethanolamine-*N*-methyl (Me-DOPE), dioleoylphosphatidylethanolamine-*N*,*N*-dimethyl (Me_2-DOPE), dioleoylphosphatidic acid (DOPA), and dioleoylphosphatidylglycerol (DOPG), were obtained from Avanti Polar Lipids. DOPC, DOPE, DOPA, and DOPG were brominated to give di(9,10-dibromostearoyl)phosphatidylcholine (BrPC), di(9,10-dibromostearoyl)phosphatidylethanolamine (BrPE), di(9,10-dibromostearoyl)phosphatidic acid (BrPA), and di(9,10-dibromostearoyl)phosphatidylglycerol (BrPG), as described in East and Lee (*23*).

E. coli strain BL21(λDE3)pLysS and plasmid pET-28a were obtained from Novagen. The *E. coli mscL* gene, *EcmscL*, with a poly-His epitope at the N-terminus was the generous gift of Professor B. Martinac. The *E. coli mscL* gene with a poly-His epitope at the C-terminus was generated by cloning the gene into plasmid pET-28a, by use of restriction sites *Hin*dIII and *Nco*I.

Mutation and Expression. Site-directed mutagenesis was performed according to the Quik-change protocol from Stratagene. *E. coli* BL21(λDE3)pLysS transformants carrying the pET-28a plasmid (Novagen) with the G22C-*EcmscL*-6×His gene were generally grown in 6 L of Luria broth to midlog phase ($OD_{600} = 0.6$) and then induced for 3 h in the presence of isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM). MscL was purified as described by Powl et al. (*24*) and stored at −80 °C until use.

Reconstitution into Sealed Vesicles. MscL was reconstituted into sealed lipid vesicles containing calcein as described (*22*). Briefly, lipid in buffer (20 mM Hepes, 100 mM KCl, 40 mM OG, and 50 mM calcein, pH 7.2) was sonicated to optical clarity in a bath sonicator. MscL was then added to the sonicated suspension to give a 500:1 molar ratio of lipid:MscL pentamer, and detergent was removed by the addition of SM2 Bio-Beads. Unencapsulated dye was removed by passage through two G-50 Sephadex columns (100−300 μm); the eluted sample was kept on ice until use.

Calcein Efflux Assay. Full experimental details of the assay are given in Powl et al. (*22*). A 50 μL aliquot of the reconstituted vesicles was diluted into 2.95 mL of buffer (20 mM Hepes, 100 mM KCl, and 0.5 M sucrose, at pH 7.2) in a stirred fluorescence cuvette at 25 °C, unless otherwise stated. Conditions were chosen to minimize any osmotic imbalance across the vesicle membrane (*22*). A baseline was

recorded for 50 s, followed by the addition of 1 mM MTSET from a freshly prepared stock solution of 300 mM MTSET in buffer (20 mM Hepes and 100 mM KCl, pH 7.2), after which the fluorescence was monitored for up to 30 min. Vesicles were finally burst by the addition of 200 μM $C_{12}E_8$ to determine the fluorescence intensity when all the trapped calcein had been released. Fluorescence intensity was recorded at 520 nm on an SLM 8100C fluorometer (Urbana, IL) with excitation at 490 nm. Fluorescence signal changes following addition of MTSET were expressed as a fraction of the total fluorescence change seen following the addition of both MTSET and $C_{12}E_8$ (*22*) and were fitted to single exponentials with the nonlinear least-squares routine in the SigmaPlot package (SPSS, Chicago, IL). Quoted rates and amplitudes of release correspond to the results of at least three separate reconstitutions.

Fluorescence Quenching Studies with Brominated Lipids. Purified MscL was reconstituted into lipid bilayers by mixing lipid and MscL in cholate at a 100:1 molar ratio of lipid to MscL monomer, followed by dilution into buffer to decrease the concentration of cholate below its critical micelle concentration, as described (*24*). Trp fluorescence intensities were measured at 325 nm with excitation at 280 nm, for 0.98 μM MscL in buffer (20 mM Hepes, 200 mM KCl, and 1 mM EGTA, pH 7.2) at 25 °C, on an SLM 8100 fluorometer. Intensities were corrected for light scatter by subtraction of a blank consisting of lipid alone in buffer.

Quenching of Trp fluorescence by brominated phospholipid in a mixture of a brominated phospholipid with the equivalent nonbrominated phospholipid fits to the equation (*25, 26*)

$$F = F_{min} + (F_0 - F_{min})(1 - x_{Br})^n \quad (1)$$

where F_0 and F_{min} are the fluorescence intensities for MscL in nonbrominated and brominated lipid, respectively; F is the fluorescence intensity in the phospholipid mixture when the mole fraction of brominated lipid is x_{Br}; and n is the number of lipid binding sites on MscL from which the fluorescence of the Trp residue can be quenched. In a mixture of a nonbrominated lipid A and a brominated lipid B, an equilibrium will be established at each lattice site on MscL:

$$PA + B \rightleftarrows PB + A$$

where PA and PB are MscL bound to lipids A and B, respectively, and the binding constant for B relative to A is given by

$$K = ([PB][A])/([PA][B]) \quad (2)$$

Fluorescence quenching in the mixture is described by the equation

$$F = F_{min} + (F_0 - F_{min})(1 - f_{Br})^n \quad (3)$$

where f_{Br}, the fraction of sites on MscL occupied by brominated lipid, is given by

$$f_{Br} = Kx_{Br}/(Kx_{Br} + [1 - x_{Br}]) \quad (4)$$

The value for n for *E. coli* MscL is ca. 1.5 (see below), corresponding to three fatty acyl chains from two phospholipid molecules binding close to a lipid-exposed Trp residue. It is possible that the binding sites for these two phospholipid molecules have different affinities for some lipids (*27*). The simplest case is to assume that there are two sets of sites with different affinities, but from which the level of quenching is equal. In this case quenching can be expressed as

$$F = F_{min} + (F_0 - F_{min})(1 - f^1_{Br})^{n/2}(1 - f^2_{Br})^{n/2} \quad (5)$$

where

$$f^1_{Br} = K_1x_{Br}/(K_1x_{Br} + [1 - x_{Br}]) \quad (6)$$

and

$$f^2_{Br} = K_2x_{Br}/(K_2x_{Br} + [1 - x_{Br}]) \quad (7)$$

where K_1 and K_2 are the relative binding constants for the brominated lipid at the two sites (*27*). Equations 1 and 3 were fitted to the experimental data by the nonlinear least-squares routine in the SigmaPlot package. An iterative procedure was used to fit the data for a set of experiments (A with brominated B and B with brominated A) to eq 5.

RESULTS AND DISCUSSION

Effects of Lipid on MscL Function. MscL is opened by an increase in tension in the surrounding lipid bilayer, so that understanding the interactions between MscL and the lipid bilayer is critical to understanding how the protein works. It is well established that changing the structures of the lipids in the bilayer leads to changes in function of MscL. The tension required to open MscL is larger in a 1:1 mixture of DOPE and DOPC than in a bilayer of DOPC, although the presence of DOPS or DOPG did not affect the opening tension (*19*), and in bilayers of PC, increasing the fatty acyl chain length also led to an increase in the tension required to open the channel (*28*). In studies of the effects of lipid structure on the rate of bulk flux of calcein through MscL, a richer repertoire of effects was seen, with the presence of anionic lipids up to 50 mol % leading to an increase in the rate and amplitude of release of calcein, attributed to effects on the rates of transition from the closed to the open state of the channel, and to effects on the rate of the transition from the open state to a desensitized or subconductance state of the channel, unable to pass calcein (*22*).

We ask here whether the observed effects of lipid on MscL function depend on particular physical properties of the membrane or whether they depend on direct interactions between MscL and the lipid molecules that make contact with it in the membrane, that is, the boundary or annular lipids. One physical property of the membrane that has been suggested to be important is the shape of the lipid molecules (*3*). Lipid molecules can have different effective shapes in a membrane: lipids with small headgroups, such as PE or phosphatidic acid (PA), have a conical shape, whereas lipids with larger headgroups, such as PC or PG, have a cylindrical shape. Lipids with a conical shape prefer to adopt a curved rather than a planar structure, shown by a large spontaneous curvature (*3*). Such lipids, when forced to adopt a bilayer structure, will exist in a state of curvature frustration and it has been argued that the curvature energy stored in the bilayer can be used to drive conformational changes in a protein. For example, if there is a mismatch between the hydrophobic thickness of a protein and the surrounding lipid bilayer, the lipid bilayer may be compressed or stretched around the protein to reduce the mismatch, and the energy

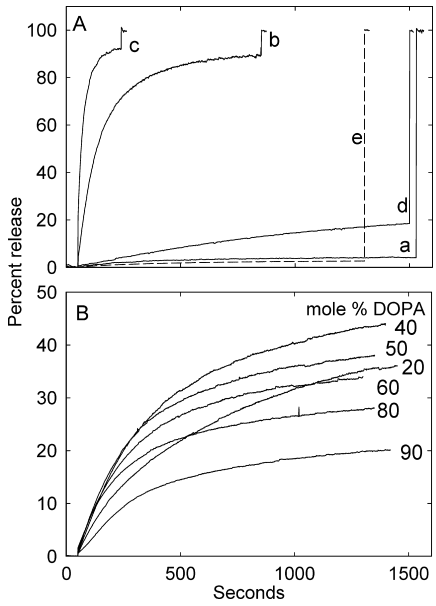


FIGURE 1: Effect of lipid mixtures on calcein flux through MscL. (A) The G22C mutant of *E. coli* MscL was reconstituted into lipid vesicles containing the following lipid mixtures: (a) DOPC, (b) DOPC/DOPA 1:1, (c) DOPC/DOPG 1:1, (d) DOPA, and (e) DOPC/DOPE 85:15. Vesicles were diluted into buffer (20 mM Hepes, 100 mM KCl, and 0.5 M sucrose, at pH 7.2), and after 50 s, 1 mM MTSET was added, followed finally by 200 μM $C_{12}E_8$ to burst the vesicles and define the 100% release level. (B) G22C was reconstituted into lipid vesicles containing mixtures of DOPE and DOPA with the given mole % DOPA. After 50 s, 1 mM MTSET was added; at the end of the experiment, 200 μM $C_{12}E_8$ was added to burst the vesicles and define the 100% release level.

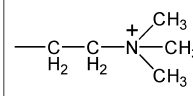
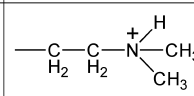
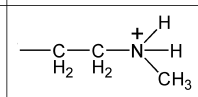
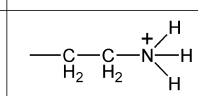
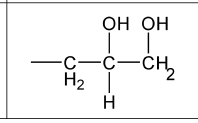
required to distort the lipid bilayer in this way could depend on the spontaneous curvature of the bilayer (*29*). The conventional iris model for opening of MscL suggests a significant change in helix tilt, and so in hydrophobic thickness, on opening (*28*), making MscL a likely candidate for a membrane protein sensitive to lipid spontaneous curvature (*29*).

Do Effects of Anionic Lipids on MscL Function Correlate with Spontaneous Curvature? The G22C mutant of the *E. coli* MscL channel was reconstituted into sealed vesicles and reacted with MTSET to open the channel. The vesicles contained a high concentration of calcein, whose fluorescence intensity was low as a result of concentration quenching of fluorescence; release of the calcein through the open MscL channel resulted in recovery of calcein fluorescence. The molar ratio of lipid:MscL pentamer in the vesicles was 500:1. The relative dimensions of the lipid and MscL molecules suggest that about 30 lipid molecules are required to form a complete annular shell of lipid around the MscL pentamer (*30*), so that at a molar ratio of lipid:MscL pentamer of 500:1 there would be enough lipid to form an average of about seven lipid shells around each MscL pentamer.

As shown in Figure 1A, and as reported previously (*22*), addition of MTSET to G22C in vesicles of DOPC leads to very little release of calcein (curve a), whereas when the vesicles contain a 1:1 mixture of DOPC/DOPA or DOPC/DOPG, almost total release of calcein is observed (curves b and c); in vesicles of DOPA alone, release of calcein is slow and of lower magnitude (curve d).

One possible explanation for the observed effects of anionic phospholipid is that the presence of these lipids

Table 1: Lipid Spontaneous Curvatures C_0 (Å^{-1})

Lipid	Headgroup structure[a]	C_0 (Å^{-1})	Reference
DOPC		-0.011	31
Me_2-DOPE		-0.023	31
Me-DOPE		-0.035	31
DOPE		-0.048	31
DOPA[b]		-0.022	32
DOPG[c]		-0.001	33,34

[a] Structure of the alcohol group attached to the phosphate in the lipid headgroup. [b] pH 7.2, 150 mM NaCl or 150 mM NaCl plus 25 mM Ca^{2+}. [c] pH 7.4, 150 mM NaCl.

changes the curvature stress of the lipid bilayer. Values for spontaneous curvature are listed in Table 1; the spontaneous curvatures of a variety of lipid mixtures have been shown to vary linearly with lipid composition (*9, 12, 31*−*34*). The observation that DOPA and DOPG have similar effects on calcein release (Figure 1A) but have very different spontaneous curvatures would argue against an important role for curvature stress in the release process. The argument can be taken further. The spontaneous curvature of a 1:1 mixture of DOPC/DOPA is equal to that of a mixture of 15 mol % DOPE and 85 mol % DOPC, but as shown in Figure 1A (curve e), release of calcein from vesicles with this lipid composition is very like that observed from vesicles of just DOPC (curve a) and very unlike that seen in 1:1 mixtures of DOPC/DOPA (curve b). These results argue that the observed effects of anionic lipid on calcein release do not follow from changes in curvature stress in the lipid bilayer.

Do Effects of Zwitterionic Lipids on MscL Function Correlate with Spontaneous Curvature? To address the possibility that charge interactions could dominate the effects of anionic lipids on calcein release but that curvature stress could be important in explaining any effects of the zwitterionic lipid component of the membrane, we also studied the effects of the zwitterionic lipid headgroup structure on the function of MscL. Figure 1B shows calcein release from vesicles containing mixtures of DOPE and DOPA. Sealed vesicles capable of retaining calcein could not be prepared from DOPE and MscL alone. Vesicles containing MscL with 80 mol % DOPE and 20 mol % DOPA retained calcein and showed a burst of release following reaction with MTSET, but this was followed by a slow, continual release of calcein; this slow phase of release was largely absent from vesicles containing a lower mole fraction of DOPE (Figure 1B). Levels of release from vesicles containing DOPE and DOPA were considerably lower than from vesicles containing DOPC and DOPA at the same levels of DOPA (*22*). The maximum level of release was observed for a 3:2 ratio of DOPE:DOPA,

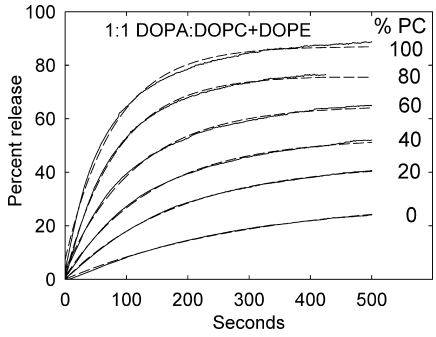


FIGURE 2: Effect of DOPC:DOPE ratio in mixtures with DOPA on calcein flux through MscL. The G22C mutant of *E. coli* MscL was reconstituted into lipid vesicles containing a fixed 1:1 molar ratio of DOPA to DOPC + DOPE, with the given mole % DOPC in the DOPC + DOPE mixture. MTSET was added at time zero to start release. Solid lines show experimental data, and dotted lines show best fits to single exponentials, giving the amplitudes and rates plotted in Figure 3.

whereas with DOPC maximum release was observed at a 1:1 molar ratio of DOPC:DOPA. Thus DOPC and DOPE support very different levels of flux of calcein through the MscL channel. In previous studies we have shown that labeling of G22C with MTSET is complete under these conditions (*22*) and the rate of reaction of G22C with MTSET in the presence of DOPE is not rate-limiting since varying the concentration of MTSET between 0.5 and 2 mM had no effect on the observed rate or amplitude of release observed in the presence of DOPE (data not shown).

Figure 2 shows calcein release from vesicles containing a fixed 1:1 molar ratio of DOPA to DOPC + DOPE as a function of the relative contents of DOPC and DOPE; both the rates and the amplitudes of release decrease with decreasing DOPC content and increasing DOPE content. The data fit well to single exponentials (Figure 2). The dependence of the amplitude of release on DOPC content is complex (Figure 3A), and indeed, there is no reason to expect a simple relationship between the magnitude of release of calcein measured at long time and the rates of the various conformational changes on MscL involved in calcein release. We have therefore chosen to concentrate on the relationship between DOPC content and the rate of calcein release, which is linear (Figure 3B). The linear dependence of rate of calcein release on DOPC content might suggest an important role for curvature stress in explaining the effects of zwitterionic lipids, since curvature stress varies linearly with composition in mixtures of DOPC and DOPE (*12, 31*). However, a linear dependence of rate on the chemical composition of the membrane could also arise in a number of other ways, particularly if DOPC and DOPE interacted relatively nonspecifically with MscL at a large number of sites. We therefore also studied the effects on calcein release of gradually changing curvature stress by changing the level of methylation of the PE headgroup. Figure 4 shows calcein release in 1:1 mixtures of DOPA with the lipids Me-DOPE or Me_2-DOPE, whose structures are shown in Table 1; data again fit well to a single-exponential process. The spontaneous curvature for Me-DOPE is the same as that for a 1:2 mixture of DOPC and DOPE (*12*), and that for Me_2-DOPE is predicted to be the same as for a 2:1 mixture of DOPC and DOPE (*31*). When the rates and amplitudes of calcein release in the presence of Me-DOPE and Me_2-DOPE are plotted as a function of the number of methyl groups, they

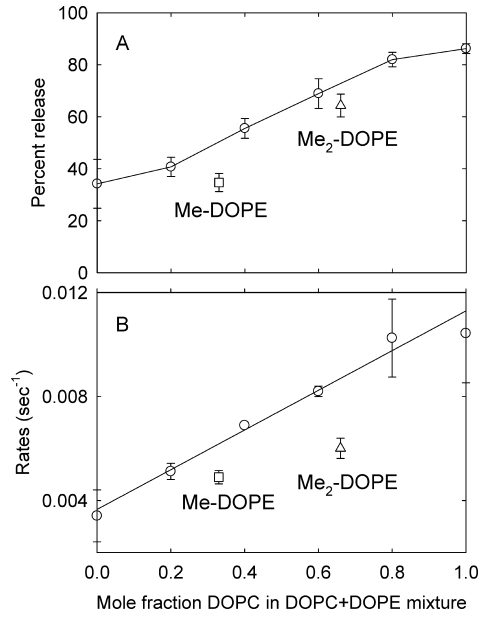


FIGURE 3: (A) Amplitudes and (B) rates of release of calcein from vesicles containing a fixed 1:1 molar ratio of DOPA to DOPC + DOPE, plotted against the mole fraction of DOPC in the DOPC + DOPE mixture (○). In panel B, the line shows a best fit of the rates to a straight line. Also shown are data for calcein release in vesicles containing 1:1 molar ratios of DOPA to Me-DOPE (□) and to Me_2-DOPE (Δ). The plotted rates and amplitudes of release are the averages of at least three separate reconstitutions.

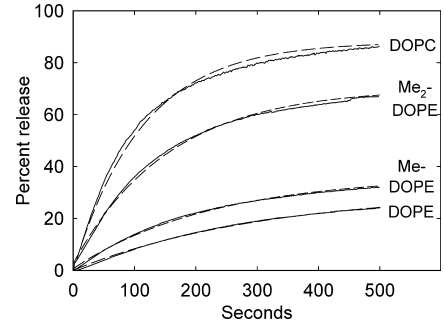


FIGURE 4: Effects of Me-DOPE and Me_2-DOPE on release of calcein. The G22C mutant of *E. coli* MscL was reconstituted into lipid vesicles containing a 1:1 molar ratio of DOPA to DOPC, Me_2-DOPE, Me-DOPE, or DOPE. MTSET was added at time zero to start release. Solid lines show experimental data and dotted lines show best fits to single exponentials, giving the amplitudes and rates for the mixtures of DOPA with Me_2-DOPE and Me-DOPE plotted in Figure 3.

do not fit on the curves established by the DOPC/DOPE data (Figure 3). In particular, the rates of release in the presence of Me-DOPE and Me_2-DOPE are much lower than expected from the DOPC/DOPE data. The observation that the rate of release of calcein varies rather little with the number of methyl groups in the lipid headgroup from 0 to 2, but then changes markedly between 2 and 3, suggests that what is important is the presence in the headgroup of a proton able to take part in hydrogen bonding.

In mixtures of DOPA with Me-DOPE or Me_2-DOPE, the largest amplitude of calcein release is seen at a 1:1 molar ratio of DOPA to zwitterionic lipid, as in mixtures with DOPC (data not shown). Similar effects are seen in mixtures of these lipids with DOPG, with larger amplitudes and rates of release observed in a 1:1 mixture of DOPG with Me_2-

Table 2: Effect of Lipid Headgroup Structure on Calcein Release following Osmotic Shock[a]

lipid composition	% release of calcein
DOPA/DOPC 1:1	24 ± 2
DOPA/Me_2-DOPE 1:1	18 ± 2
DOPA/Me-DOPE 1:1	11 ± 2
DOPA/DOPE 1:1	7 ± 1
DOPC	1 ± 1

[a] Vesicles containing G22C were diluted into buffer (20 mM Hepes and 100 mM KCl, at pH 7.2) in a stirred fluorescence cuvette and left to equilibrate for 3 min, and the calcein fluorescence intensity was determined. $C_{12}E_8$ was then added to burst the vesicles, to determine the fluorescence intensity when all the trapped calcein had been released. Data are corrected for release of calcein from vesicles lacking MscL.

DOPE than in a 1:1 mixture of DOPG with Me-DOPE (data not shown).

To show that the effects of Me-DOPE and Me_2-DOPE reported here do not only apply when the MscL channel is opened by reaction with MTSET, we have also studied the effects of opening the channel by osmotic shock. The 50 mM calcein trapped within the vesicles in these experiments exerts a large osmotic effect because calcein contains five ionizable carboxyl groups. In the experiments described above, this has been balanced by the presence of 0.5 M sucrose in the external buffer. When vesicles are diluted 60-fold into buffer in the absence of sucrose, the initial osmotic pressure difference is high enough to open the MscL channel, leading to release of calcein; eventually sufficient calcein is released to drop the osmotic pressure difference to below that required to maintain the MscL channel open, and release stops and the level of trapped calcein remains stable for at least 5 min (*22*). The rate of release of calcein is too fast to measure in these hand-mixing experiments; we therefore just determined the final amplitude of release. The level of release was very low if the vesicles contained DOPC as the only lipid but increased in the presence of DOPA (Table 2). Levels of release in mixtures of DOPA with Me_2-DOPE or Me-DOPE were lower than for mixtures of DOPA with DOPC (Table 2), paralleling the effects on the amplitude of release seen when the channel was opened by reaction with MTSET (Figure 3A); with Me_2-DOPE and Me-DOPE, levels of release were 75% and 46%, respectively, compared with that seen for DOPC in the osmotic shock experiments (Table 2) and 75% and 40%, respectively, compared with the experiments where the channel was opened with MTSET (Figure 3A).

Are There Specific Binding Sites for Anionic Lipid on E. coli MscL? If the effects of anionic lipid on calcein flux through MscL described above do not follow from effects on membrane spontaneous curvature, then it is possible that they follow from binding to a specific set of sites on the MscL channel. We have shown that binding of lipid to MscL can be studied by fluorescence quenching methods, observing the effects of bromine-containing phospholipids on the intensity of Trp fluorescence of a Trp residue introduced into MscL (*24, 27, 30, 35*). Bromine-containing phospholipids are made by addition of bromine across the double bonds of phospholipids containing two oleoyl chains. These lipids behave much like conventional phospholipids with unsaturated fatty acyl chains because the bulky bromine atoms have effects on lipid packing similar to those of a cis double bond (*23*). Because bromine is a short-range quencher of Trp fluorescence (*24*), only a bromine-containing phospholipid bound close to a Trp residue will quench its fluorescence. Thus the level of fluorescence quenching observed for a Trp-containing mutant of MscL reconstituted into a mixture of brominated and nonbrominated phospholipids will depend on the fraction of phospholipid molecules next to the Trp residue that are brominated and thus on the strength of binding of the brominated phospholipid at sites close to the Trp residue. Because native MscL from *E. coli* contains no Trp residues, we are able to introduce single Trp residues into specific regions of interest.

In previous studies with MscL from *Mycobacterium tuberculosis*, we found that quenching of the fluorescence of a Trp residue at position 87 on the cytoplasmic side of the membrane in mixtures of DOPC and BrPA was different from that in mixtures of BrPC and DOPA, suggesting the presence of two sites close to the introduced Trp residue, one with higher affinity for PA than for PC and the other with similar affinities for these two phospholipids (*27*). A similar result was obtained for the anionic lipid PG, although the affinity for PG was less than for PA (*27*), suggesting that the binding site could be rich in Lys and Arg residues, since these have been shown to interact particularly favorably with the PA headgroup (*36*). The crystal structure for the closed state of MscL from *M. tuberculosis* (*37*) shows a cluster of three residues, Arg-98, Lys-99, and Lys-100, as the only charged residues in a position to interact with an anionic phospholipid headgroup, and we showed that mutation of any of these residues in *M. tuberculosis* MscL led to a decrease in affinity for anionic lipids (*27*). The equivalent cluster of charged residues in *E. coli* MscL is Arg-104, Lys-105, and Lys-106.

We have studied anionic lipid binding on the cytoplasmic side of *E. coli* MscL using the F93W mutant, where the Trp residue is located at the position equivalent to that used in our previous studies with MscL from *M. tuberculosis* (*30, 35*). As shown in Figure 5, fluorescence quenching results for *E. coli* MscL are very similar to those reported for the *M. tuberculosis* channel (*27*). In mixtures of PC with either PA or PG, the data fit to eq 3, but the relative binding constants obtained from experiments with brominated anionic lipids are greater than those obtained from experiments with BrPC (Table 3), consistent with the presence of at least two classes of binding site on MscL from which the fluorescence of Trp-93 can be quenched, one with a higher relative binding constant for anionic lipid than the other. The value of n, the number of lipid binding sites from which the fluorescence of Trp-93 can be quenched is ca. 1.5 (Table 4), corresponding to approximately three bromine-containing fatty acyl chains from two phospholipid molecules binding close enough to the Trp residue to result in efficient quenching. In this case data can be fitted to eq 5 to give estimates for the affinities of these two phospholipid binding sites (*27*). As shown in Table 5, one site binds anionic lipid with an affinity about half that of PC, whereas the second site binds anionic lipid with much higher affinity than PC, the effect being particularly marked for PA.

Fluorescence quenching in mixtures of PE and anionic lipid show a pattern very similar to that observed for mixtures with PC (Figure 5), showing that the site of high affinity for anionic lipid is present in the presence of either PC or PE (Table 5).

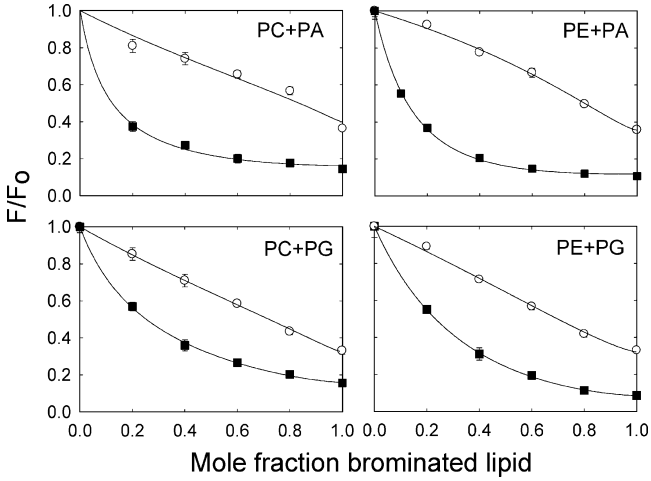


FIGURE 5: Quenching of Trp fluorescence of F93W in mixtures containing anionic lipid. F93W was reconstituted into mixtures of PC and PE with PA and PG, as marked: (○) mixtures with BrPC or BrPE; (■) mixtures with BrPA or BrPG. Fluorescence intensities are expressed as a fraction of fluorescence for MscL reconstituted in the nonbrominated lipid and are plotted against the mole fraction of brominated lipid in the mixture. Solid lines show best fits to the one-set-of-sites model (eq 3), giving the relative binding constants given in Table 3. The data were also fitted to the two-sets-of-sites model (eq 5), giving lines identical to the solid lines shown and giving the relative binding constants listed in Table 5.

Table 3: Relative Lipid Binding Constants for MscL When One Set of Sites Is Assumed[a]

anionic phospholipid	relative binding constant: measured with BrPC or BrPE	relative binding constant: measured with BrPA or BrPG
Phosphatidylcholine		
PA	1.39 ± 0.4	4.1 ± 0.5
PG	1.3 ± 0.1	2.7 ± 0.1
Phosphatidylethanolamine		
PA	2.0 ± 0.1	3.9 ± 0.1
PG	1.4 ± 0.1	2.5 ± 0.1

[a] Binding constants relative to DOPC or DOPE were obtained by fitting the quenching data in Figure 5 to eq 3, with the values for n given in Table 4.

Table 4: Fluorescence Quenching of MscL in Brominated Phospholipids as a Function of Lipid Headgroup[a]

phospholipid	mutant F93W[b] n	mutant F93W[b] F/F_0	mutant G22C:F93W:QQQ n	mutant G22C:F93W:QQQ F/F_0
BrPC	1.35 ± 0.02	0.34 ± 0.01	2.12 ± 0.18	0.68 ± 0.01
BrPA	1.89 ± 0.09	0.23 ± 0.02	2.36 ± 0.21	0.54 ± 0.01
BrPG	1.42 ± 0.02	0.24 ± 0.01	2.20 ± 0.02	0.53 ± 0.02
BrPE	1.36 ± 0.02	0.30 ± 0.02		

[a] F_0 and F are fluorescence intensities for MscL reconstituted in nonbrominated and brominated phospholipid, respectively, measured at pH 7.2. The value of n is obtained by fitting fluorescence quenching data for MscL, reconstituted into mixtures of brominated lipid with the corresponding nonbrominated phospholipid, to eq 1. [b] Data from ref *24*.

Does the High-Affinity Binding Site for Anionic Lipid on MscL Correspond to the Charge Cluster? A potential binding site for anionic lipids on MscL is provided by the Arg-104, Lys-105, and Lys-106 cluster on the cytoplasmic side of the membrane. Kloda et al. (*38*) showed that mutation of these three positive charges to Gln in an N-His-tagged MscL resulted in a channel that was functional but required a higher tension than normal to open. However, N-His-tagged MscL is known to have abnormal gating behavior (*39*). We were unable to prepare the corresponding C-His-tagged G22C/QQQ mutant, suggesting that this construct could have been a severe gain of function mutant. However, we were able to overexpress the corresponding mutant containing a Trp residue at position 93, G22C/F93W/QQQ. This could be related to the observation by Chiang et al. (*40*) that the F93W mutant of MscL required a markedly higher tension than the wild-type protein to open, so that the F93W mutation might act as a compensatory mutation to the mutation of the three charged residues; this possibility is discussed further below.

Table 5: Relative Lipid Binding Constants for MscL When Two Sets of Sites Are Assumed[a]

anionic phospholipid	relative binding constants: site 1	relative binding constants: site 2
Phosphatidylcholine		
PA	0.6 ± 0.2	10.1 ± 1.1
PG	0.6 ± 0.1	5.7 ± 0.3
Phosphatidylethanolamine		
PA	1.0 ± 0.1	7.8 ± 0.1
PG	0.7 ± 0.1	4.9 ± 0.3

[a] Binding constants relative to DOPC or DOPE were obtained by fitting the quenching data in Figure 5 to eq 5 as described under Materials and Methods.

Table 6: Fluorescence Properties of the Triple Charge Mutant of MscL[a]

lipid	mutant F93W λ^{max} (nm)	mutant F93W ω (nm)	mutant G22C/F93W/QQQ λ^{max} (nm)	mutant G22C/F93W/QQQ ω (nm)
DOPC	326.5 ± 0.1	51.4 ± 0.1	334.6 ± 0.1	56.6 ± 0.2
DOPA	326.7 ± 0.1	50.7 ± 0.2	334.4 ± 0.1	57.0 ± 0.1
DOPG	325.5 ± 0.1	51.9 ± 0.2	334.0 ± 0.1	57.7 ± 0.2
DOPE	327.2 ± 0.1	51.9 ± 0.1	334.2 ± 0.1	56.2 ± 0.1

[a] Mutants were reconstituted into bilayers of the given phospholipid. Values of λ^{max}, the wavelength of maximum emission, and ω, the peak width at half-height, were determined by fitting the fluorescence emission spectra to the equation for a skewed Gaussian (*43*).

G22C/F93W/QQQ could be purified in yields comparable to those for the wild-type protein, showing that the mutant was not a severe gain of function mutant, for which yields are very low (*27*). Nevertheless, the fluorescence emission for G22C/F93W/QQQ, when reconstituted into lipid bilayers, was at significantly longer wavelengths than for F93W (Table 6), suggesting that the conformational state of the mutant channel was different from that of the wild-type channel. Comparison of the emission maximum of ca. 326 nm for F93W with the emission maxima for Trp residues in MscL from *M. tuberculosis* (*35*) suggests a location for the Trp residue in F93W facing the lipid bilayer, about midway between the bilayer center and the cytoplasmic face of the membrane. The ca. 8 nm shift to longer wavelength observed for G22C/F93W/QQQ suggests a shift of the Trp residue to a more polar environment in G22C/F93W/QQQ. The wavelength of maximum emission for G22C/F93W/QQQ varied only very slightly with changes in lipid structure (Table 6). Fluorescence quenching properties for G22C/F93W/QQQ in bilayers containing a mixture of a brominated lipid with the corresponding nonbrominated lipid are also different from those for F93W, with less efficient quenching and large values for n (Table 4). These quenching results would also be consistent with movement of the Trp residue away from the lipid bilayer in G22C/F93W/QQQ.

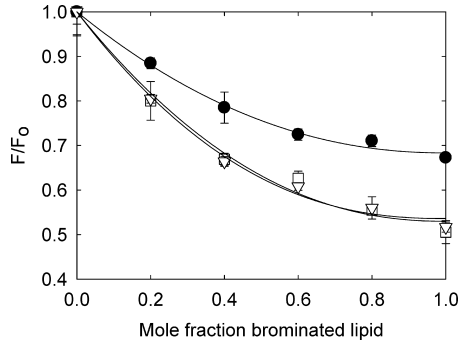


FIGURE 6: Quenching of Trp fluorescence of G22C/F93W/QQQ. G22C/F93W/QQQ was reconstituted into mixtures of DOPC and BrPC (●), DOPG and BrPG (□), and DOPA and BrPA (▽). F/F_0 is plotted against the mole fraction of the brominated lipid in the mixture. The solid lines show best fits to eq 1, giving the values for n listed in Table 4.

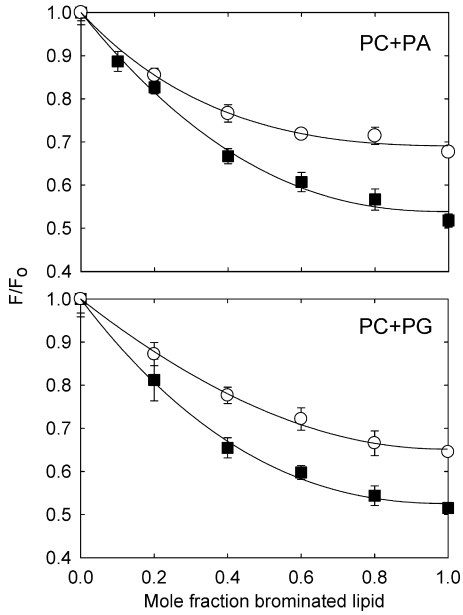


FIGURE 7: Quenching of Trp fluorescence of G22C/F93W/QQQ in mixtures of PC and anionic lipid. G22C/F93W/QQQ was reconstituted into mixtures of PC with PA or with PG: (○) mixtures with BrPC; (■) mixtures with BrPA or with BrPG. Sold lines show best fits to the one-set-of-sites model (eq 3) using the values for n given in Table 4, giving the relative binding constants given in Table 7.

Table 7: Relative Lipid Binding Constants for the Charge Mutant G22C/F93W/QQQ When One Set of Sites Is Assumed[a]

	relative binding constants	
phospholipid	measured with BrPC	measured with BrPA or BrPG
PA	0.8 ± 0.1	1.0 ± 0.1
PG	1.1 ± 0.1	1.1 ± 0.1

[a] Binding constants relative to DOPC were obtained by fitting the quenching data in Figure 7 to eq 3, with the values for n given in Table 4.

Analysis of fluorescence quenching plots for G22C/F93W/QQQ in mixtures of PC with PA or with PG give very similar binding constants whether the bromine is on the PC or on the anionic lipid (Figures 6 and 7; Table 7). This is consistent with simple competitive binding of lipids at one set of sites, showing that mutation of Arg-104, Lys-105, and Lys-106 has led to loss of the high-affinity binding of anionic lipid observed in F93W. This, in turn, would be consistent with the proposal that Arg-104, Lys-105, and Lys-106 are part of the high-affinity binding sites for anionic lipids.

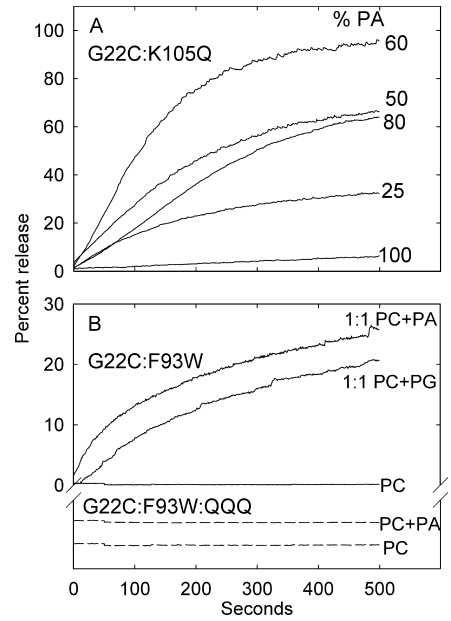


FIGURE 8: Effects of charge mutations on release of calcein. (A) The G22C/K105Q mutant of *E. coli* MscL was reconstituted into lipid vesicles containing mixtures of DOPC and DOPA with the given mole % DOPA. (B, upper panel) G22C/F93W mutant was reconstituted into bilayers of DOPC/DOPA 1:1 or DOPC/DOPG 1:1 or DOPC. (B, lower panel) G22C/F93W/QQQ mutant was reconstituted into bilayers of DOPC/DOPA 1:1 or DOPC. The traces have been offset vertically for clarity; traces recorded at molar ratios of DOPC:DOPA up to 1:9 are identical to those shown. In all cases MTSET was added at time zero to start release.

Do Effects of Anionic Lipid on Calcein Release Follow from Binding to the Charge Cluster? To test for the possibility that binding of anionic lipid to the charged cluster of Arg-104, Lys-105, and Lys-106 was responsible for the effects of anionic lipid on calcein release, we studied the effects on calcein release of mutating residues in the charged cluster. The G22C/K105Q mutant, in which one of the charges in the positively charged cluster has been removed, still releases calcein on reaction with MTSET in an anionic lipid-dependent manner (Figure 8A). However, the amplitude of release observed for G22C/K105Q in mixtures of DOPC and DOPA containing 50 mol % DOPA is markedly reduced compared to that seen with G22C (compare with Figure 1A), and for G22C/K105Q the highest level of release is seen at 60 mol % DOPA. Thus mutation of Lys-105 appears to result in a reduced affinity for DOPA, consistent with Lys-105 being part of an anionic lipid binding site important in the effects of anionic lipid on flux of calcein through the MscL channel.

As described above, we have been unable to obtain the mutant G22C/QQQ in which all three residues in the charge cluster were mutated to Gln, but we were able to produce and overexpress the mutant G22C/F93W/QQQ. In part, this could be because, unexpectedly, we found that introduction of a Trp residue at position 93 resulted in a marked reduction in flux through the channel. Thus, as shown in Figure 8B, the mutant G22C/F93W shows release of only ca. 30% of the trapped calcein in vesicles of a 1:1 mixture of DOPC/DOPA compared to ca. 85% for G22C (Figure 1A), with a rate of release of $4.0 \times 10^{-3} \pm 4.3 \times 10^{-5}$ s^{-1} compared to

$0.010 \pm 0.002\ s^{-1}$ for G22C. However, as for G22C, release of calcein by G22C/F93W was still strongly dependent on the presence of anionic lipid: no release was observed in vesicles of DOPC alone, and the effect of DOPG was comparable to that of DOPA (Figure 8B).

For G22C/F93W/QQQ, no release of calcein was observed in vesicles where DOPC was the only lipid (Figure 8B), as for G22C or G22C/F93W, but we also observed no release with G22C/F93W/QQQ in vesicles containing mixtures of DOPC and DOPA with up to 90 mol % DOPA (Figure 8B). These results are consistent with, but do not prove, the proposal that anionic lipids bind to the Arg-104, Lys-105, and Lys-106 cluster to modify calcein flux through the channel.

CONCLUSIONS

We asked here whether the observed effects of lipid on calcein flux through the MscL channel depended on the particular physical properties of the membrane or on direct interactions between MscL and the surrounding annular lipid molecules. One particular physical property of a membrane that has been suggested to be important for membrane protein function is the spontaneous curvature of the lipid molecules in the membrane. Since, for example, PE favors curved structures, it will, when forced to adopt a bilayer structure, be in a state of curvature frustration and the curvature energy consequently stored in the bilayer could result in conformational changes for a protein in the membrane.

A number of experiments reported here suggest that, in fact, the function of MscL is not sensitive to spontaneous curvature. Thus, for example, the amplitude and rates of calcein release through MscL increase on addition of up to 50 mol % DOPA or DOPG to bilayers of DOPC (Figure 1A). Since the spontaneous curvatures of DOPA and DOPG are very different (Table 1), the observed effects of DOPA and DOPG on MscL function cannot follow from effects of spontaneous curvature. This is confirmed by the observation that calcein efflux in a 1:1 mixture of DOPC and DOPA and in a mixture of DOPC and DOPE with the same spontaneous curvature, are very different (Figure 1A). Although, therefore, effects of anionic lipid cannot be explained in terms of curvature stress, it remains possible that curvature stress could explain the effects of zwitterionic lipids on MscL function. To test this possibility, we studied the effects on calcein flux through MscL of mixtures of DOPE/DOPC/DOPA in which the DOPA content was fixed (Figure 2). We found that the rate of release of calcein varied linearly with the proportion of the DOPE/DOPC mixture that was DOPC (Figure 3B). Since the spontaneous curvature of mixtures of DOPC and DOPE also varies linearly with composition (*12, 31*), this might suggest that spontaneous curvature was exerting a significant effect on MscL function when the anionic lipid content was kept constant. However, the correlation between spontaneous curvature and function failed when spontaneous curvature was varied by varying the methylation level of the lipid headgroup. The spontaneous curvature of Me-DOPE is the same as that of a 1:2 mixture of DOPC and DOPE, and the spontaneous curvature of Me_2-DOPE has been predicted to be the same as that of a 2:1 mixture of DOPC and DOPE (Table 1). However, the rates of release of calcein in mixtures of DOPA with Me-DOPE or Me_2-DOPE are similar to that in mixtures of DOPA with DOPE and do not fit on the straight-line relationship established for release in DOPC/DOPE mixtures (Figure 3B). Thus we can conclude that spontaneous curvature is also not an important factor in explaining the effects of the zwitterionic lipid component in mixtures with anionic lipid.

In the experiments where the methylation level of the headgroup was changed, the largest change in the rate of calcein release occurred in going from Me_2-DOPE to DOPC (Figure 3B), a change corresponding to replacement of the final proton in the lipid headgroup by a methyl group. It is therefore likely that hydrogen bonding between the headgroup and MscL is important in keeping the protein in a conformation showing slow, low amplitude release of calcein. A possible explanation for the observed linear dependence of the rate of calcein release on DOPE/DOPC content (Figure 3B) could be that the zwitterionic lipid headgroup interaction with MscL is of low specificity, involving a large number of lipid binding sites on the transmembrane surface of MscL. A molecular dynamics simulation of MscL in bilayers of PE showed a large number of hydrogen bonds forming between the lipid molecules and MscL, about half of which involved the NH_3^+ group of the lipid headgroup (*41*). A simulation of MscL in bilayers of PC suggested that the loss of hydrogen bonding observed in bilayers of PC is compensated for by a conformational change in the C-terminal region of MscL, bringing the C-terminal region closer to the membrane and leading to stronger interactions with the membrane (*42*). This compensating conformation change could be the reason why the binding constants of MscL for DOPE and DOPC are very similar (*24, 27*) as well as the reason for the different effects of DOPE and DOPC on function reported here.

Effects of anionic lipid on MscL function are likely to follow from more specific interaction with MscL. Fluorescence quenching studies show the presence of a binding site with high affinity for anionic lipids on the cytoplasmic side of the membrane for MscL from *E. coli* (Table 5), as previously observed for MscL from *M. tuberculosis* (*27*). Mutational studies suggest that this binding site consists of the charged cluster Arg-104, Lys-105, and Lys-106, corresponding to Arg-98, Lys-99, and Lys-100 in the *M. tuberculosis* protein. A mutant channel lacking the three charged residues shows a very low rate of release of calcein in either the presence or absence of anionic lipid (Figure 8), consistent with the suggestion that the charged cluster is the functionally important binding site for anionic lipid.

Overall, the results presented above suggest that effects of phospholipids on the function of MscL are best understood in terms of direct lipid−protein interactions. Effects of anionic lipid on function are likely to follow from binding of anionic lipid to a charged cluster on the cytoplasmic side of the membrane. Effects of zwitterionic lipid on the function of MscL appear to depend on the ability of the lipid headgroup to take part in hydrogen bonding to MscL. Effects of zwitterionic lipids on the function of the multidrug transporter LmrP were also found to depend on the hydrogen-bonding capability of the headgroup rather than on curvature stress (*14*).

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