Kalata B1 and Kalata B2 Have a Surfactant-Like Activity in Phosphatidylethanolomine Containing Lipid Membranes

Charles G. Cranfield[1*], Sónia Troeira Henriques[2], Boris Martinac[3,4], Paul Duckworth[5], David J. Craik[2] and Bruce Cornell[1,5]

AUTHOR AFFILIATIONS

[1]School of Life Sciences, University of Technology Sydney, Ultimo, NSW 2007, Australia. [2]University of Queensland, Institute for Molecular Bioscience, Brisbane, Queensland 4072, Australia. [3]Victor Chang Cardiac Research Institute, Darlinghurst, NSW 2010, Australia. [4]St Vincent's Clinical School, The University of New South Wales, Darlinghurst, NSW 2010. [5]SDx Tethered Membranes Pty Ltd, Unit 6 30-32 Barcoo St, Roseville NSW 2069, Australia.

CORRESPONDING AUTHOR

[*]Charles Cranfield, School of Life Sciences, University of Technology Sydney, Ultimo, NSW 2007, Australia. Ph +61 9514 4034. E-mail: charles.cranfield@uts.edu.au

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ABSTRACT

Cyclotides are cyclic disulfide-rich peptides that are chemically and thermally stable and possess pharmaceutical and insecticidal properties. The activities reported for cyclotides correlate with their ability to target phosphatidylethanolamine (PE)-phospholipids and disrupt cell membranes. However, the mechanism by which this disruption occurs remains unclear. In the current study we examine the effect of the prototypic cyclotides, kalata B1 (kB1) and kalata B2 (kB2) on tethered lipid bilayer membranes (tBLMs) using swept frequency electrical impedance spectroscopy. We confirmed that kB1 and kB2 bind to bilayers only if they contain PE-phospholipids. We hypothesize that the increase in membrane conduction and capacitance observed upon addition of kB1 or kB2 is unlikely to result from ion channel like pores, but is consistent with the formation of lipidic toroidal pores. This hypothesis is supported by the concentration dependence of effects of kB1 and kB2 being suggestive of a critical micelle concentration event rather than a progressive increase in conduction arising from increased channel insertion. Additionally, conduction behaviour is readily reversible when the peptide is rinsed from the bilayer. Our results support a mechanism by which kB1 and kB2 bind to and disrupt PE-containing membranes by decreasing the overall membrane *critical packing parameter*, as would a surfactant, which then opens or increases the size of existing membrane defects. The cyclotides need not participate directly in the conductive pore, but might exert their effect indirectly through altering membrane packing constraints and inducing purely lipidic conductive pores.

INTRODUCTION

Cyclotides are a large family of plant-derived peptides that possess pharmaceutical (e.g. uterotonic, anti-HIV and anti-cancer) and agricultural (e.g. insecticidal, nematocidal) properties and have attracted much attention as leads in drug design.[1-2] They feature a cyclized backbone and three disulfide bonds arranged in a knot, which confer to them remarkable stability (Figure 1A).[1] Kalata B1 (kB1) and kalata B2 (kB2), Figure 1B, are two of the most studied cyclotides and were originally isolated from the African herb *Oldenlandia affinis*.[3] Both peptides belong to the Möbius subfamily[4] of cyclotides and mode-of-action studies with these and other native cyclotides belonging to this subfamily suggest they act by interacting with the cell membranes of the target organism. In particular, cyclotides bind to and disrupt membranes that contain phosphatidylethanolamine (PE) phospholipids.[5-6] A strong correlation between biological activity and affinity for PE-containing membranes has been reported.[7-8]

An Ala-scan of kB1 in which insecticidal activity was measured for all of the Ala point mutants showed that a patch of residues formed at the surface of kB1 is important for activity (Figure 1C); accordingly this patch is referred to as the bioactive face.[9] This finding was later confirmed with a Lys-scan,[10] which found that, in addition to the bioactive face, a hydrophobic patch also at the surface of the molecule is essential for activity. Later it was found that both the bioactive

and the hydrophobic patches are required for kB1 to bind to lipid membranes.[8] Specifically, the bioactive patch binds to phospholipids containing PE head-groups whereas the hydrophobic patch is important for the peptide to insert into the lipid bilayer. Single mutations in either of these two patches rendered kB1 unable to bind to membranes and it became inactive in all of the biological assays tested so far.[8]

Patch-clamp electrophysiology measurements showed that binding of kB1 to model membranes containing PE-phospholipids induces ion channel-like activity.[11] The ability to disturb lipid bilayers was also supported by a dye leakage study showing that kB1 induces membrane leakage in liposomes but only when they have PE-phospholipids in their composition.[7, 11] Based on these findings it was postulated that kB1 might form pores in membranes through the formation of multimers of tetramers. The proposed oligomerization behaviour is supported by analytical ultracentrifugation studies showing that kB2 can oligomerize to form tetramers and octomers.[12]

An extension to the pore-forming model was proposed by Wang et al in 2012.[13] Based on quartz crystal microbalance and neutron reflectometry data, the extended model predicts that membrane pores are formed when sufficient quantities of peptide monomers or oligomers have bound to the membrane via PE lipids, and increasing concentrations of kB1, or kB2, can permanently disrupt the membrane.

More recently, Nawae et al reported coarse-grained molecular dynamics simulations of kB1 and its association with lipid membranes[14] and suggested that kB1 forms oligomers in aqueous solutions that themselves can conjugate to form either "tower-like" or "wall like" structures. Each of these elongated structures was hollow, with apertures of ~13nm in diameter. According to that study, kB1 is arranged within the outer leaflet of lipid bilayers, and instead of forming ion channels within the membrane, induces a positive membrane curvature. However, that study was done using membranes lacking PE lipids, which are known to be essential for the ability of kB1 and kB2 to bind to model membranes, so does not provide realistic insights into cyclotide membrane binding.[7-8]

Given the contrasting membrane disruption mechanisms proposed for cyclotides, here we conducted studies using tethered bilayer lipid membranes (tBLMs) in conjunction with swept frequency electrical impedance spectrometry (EIS) to gain insights into cyclotide mode-of-action. We consider this experimental approach unique as, unlike other structural biological measures, such as neutron and X-ray scattering or nuclear magnetic resonance (NMR), which require excessively high concentrations of perturbants, EIS permits the direct observation of the peptide induced changes in the membrane conductance and capacitance at peptide concentrations in the range 0.1-10 μM. The data collected by EIS questions the model that kB1 and kB2 might form ion channel like pores. Instead, we suggest that these cyclotides have a surfactant-like behaviour and induce the formation of toroidal pores in membranes. Furthermore, we demonstrate that this surfactant-like behaviour is reversible upon rinsing tBLMs with cyclotide-free electrolyte solution. The putative mechanism of cyclotide-membrane interaction is described here in terms of lowering the membrane *critical packing*

parameter (CPP)[15-19] leading to an increase in the size of membrane defects existent in the bilayer according to a model discussed previously.[20]

MATERIALS AND METHODS

Peptide extraction and synthesis

The cyclotides kB1 and kB2 were extracted from *Oldenlandia affinis* and purified as described previously.[4] kB1 mutant, [W23A]kB1, was synthesised and purified as before.[9] The concentration of kB1 and kB2 in various experiments was quantified by absorbance at 280 nm assuming an extinction coefficient of 5875 M^{-1} cm^{-1}, as determined from the contribution of aromatic residues and disulfide bonds. As the analogue [W23A]kB1 does not contain aromatic residues, the concentration of [W23A]kB1 samples was quantified by weight of lyophilised peptide.

Solutions

The peptides were tested at 1 µM and 10 µM, concentrations at which they were previously shown to have negligible and considerable effects on lipid membranes, respectively.[8] All experiments were conducted using phosphate buffered saline (PBS) at pH 7.2; except for those experiments that necessitated a pH change, which were performed in 100 mM NaCl solutions whose pH was adjusted to pH 5 and pH 7 by the addition of concentrated HCl or NaOH. These unbuffered solutions were used immediately upon creation so as to prevent subsequent alterations in pH by the absorption of atmospheric CO_2.

Tethered bilayer lipid membranes

tBLMs with 10% tethering lipids and 90% spacer lipids (T10 tBLM) were formed using the solvent exchange technique as described previously. Briefly, 2.1 mm^2 electrodes were pre-prepared with tethered benzyl-disulfide (tetra-ethyleneglycol)$_{n=2}$ C_{20}-phytanyl *tethers* (DLP) and benzyl-disulfide-tetra-ethyleneglycol-OH *spacers* (TEGOH) in the ratio of 1:9. The electrodes are 2 mm^2 patterned, 100 nm thick, fresh 5N5 gold surfaces sputter coated onto a polycarbonate slide (*SDx Tethered Membranes Pty Ltd, Australia*).[21] To the tethered monolayer chemistries, 8 µL of a 3 mM solution of a mobile lipid phase dissolved in ethanol were added, and, after a 2 minute incubation, were washed three times with 2 × 200 µL of 100 mM PBS. The mobile lipid phases investigated were: 20% (mol/mol) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) with 80% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Avanti Lipids, USA); and POPC alone.

AC electrical impedance spectroscopy (EIS)

Impedance and phase measurements were performed using an SDx tethaPod™ operated with SDx tethaQuick™ software (SDx Tethered Membranes Pty Ltd). Swept frequency EIS was employed using a 50 mV peak-to-peak AC excitation at frequencies between 0.02 and 10,000 Hz, with 4 steps per decade, at zero bias potential. The data were fitted using an equivalent circuit comprising a *Constant Phase Element* (CPE) in series with a *Resistor/CPE* network using a proprietary adaptation of a Levenberg–Marquardt fitting routine (Figure 4*A*). In this equivalent circuit, Qs is the imperfect series capacitance at the gold electrode interface which is modified by an exponent, α_s, as in the equation:

$$\frac{1}{Z_{CPE}} = Q_{s\,(i\omega)^{\alpha_s}}$$

Where Z_{CPE} is the impedance magnitude of the constant phase element, *i* the imaginary unit and ω the frequency in radians per second. When $\alpha_s = 1$, Q_s is a pure capacitor and when $\alpha_s = 0$, Q_s is a pure resistor. It has been determined in previous studies that the presence of tethering molecules in the tBLM architecture can act as an impediment to ion flow towards the gold tethering electrode. The delay charging the sulphur coated gold surface distorts the capacitive properties of the gold-sulphur-electrolyte interface justifying the use of the CPE[22] as an approximation of the distributed RC network at the interface. In Figure 4*A*, Q_m and α_m represent the imperfect capacitance and exponent of the tethered lipid bilayer and G_m the membrane conduction. Recent work from Valincius and co-workers examined equivalent circuit models for a heterogeneous distribution of membrane conductive elements. Their conclusions further support the use of a CPE[23] in the equivalent electrical circuit model. G_e represents the conduction of the PBS or NaCl electrolyte solutions. In the present work, the fitting of the equivalent circuit model was optimised against the combined, frequency weighted impedance magnitude and phase data.

RESULTS AND DISCUSSION

EIS is particularly suited to measurements of conduction changes that depend on specific lipid-peptide components within lipid bilayers. In particular, the effect of a lipid–peptide complex on the membrane can be identified from the Bode plot of Log_{10} [$Z_{mag.}$(Ohms)] and separately the Phase (degrees), both plotted against Log_{10}[Frequency(Hz)]. Phase versus frequency profiles are represented in Figure 2. Briefly, when the lipid-protein or lipid-peptide form an ion channel in the membrane, the *frequency at the minimum phase* will be shifted to higher frequencies, as shown in Figure 2*A*. This is a typical response seen with many membrane ion channels, such as those formed by *gramicidin-A* or *α-haemolysin*. Small alterations in the membrane thickness and/or water content of the membrane occur when such channels enter the membrane. With a channel, the changes in the imperfect membrane capacitance, or Q_m and α_m of the CPE, remain essentially unaltered. Thinner membranes and/or membranes with greater water content will exhibit higher capacitances or Q_m values. Figure 2*B* shows the effect of a change in the membrane capacitance without a substantial change in G_m (the membrane conductance). An example of a peptide that induces this behaviour is GSMTx-4 toxin[24] from tarantula venom (as seen in Figure 2*B*).

Lipid-peptide or lipid-protein complexes that have surfactant-like properties can, at high concentrations, cause a change in the membrane morphology producing a large increase in the membrane conduction and result in a phase profile similar to that depicted in Figure 2*C*. Here the *phase at phase minima* is increased, resulting from the exposure of the sulphur coated gold surface and an increase in Q_m, as well as exhibiting a shift to higher frequencies.

The addition of either kB1 or kB2 to tBLMs containing POPC/POPE (80:20 molar ratio) phospholipids induced a shift in the EIS phase profile (Figure 3 for kB1; Figure S1 for kB2 in

Supplementary Material) that more resembles surfactant-like properties similar to that depicted in the Figure 2*C* example. When pure POPC membranes were used, neither kB1 nor kB2 had any noticeable effect on the EIS phase profile, consistent with previous reports showing that the presence of PE-containing phospholipids is required for these peptides to bind to membranes.[7-8] Interestingly, following a subsequent PBS wash step, any effect of the cyclotides on the membrane impedance spectra disappeared and the membrane appeared to return to its former state. To gain further evidence of the specificity of kB1 for PE containing membranes, [W23A]kB1, a kB1 analogue with a mutation in the hydrophobic face (see Figure 1C) and previously shown to be inactive in the activities tested[9] was here included as a control. As expected, [W23A]kB1 exhibited no response in either POPE containing membranes or POPC-only membranes (Supplementary Material Figures S3 & S4). This is in agreement with previous studies with kB1 analogues with a mutation in this residue showing that Trp23 is important for kB1 membrane binding properties and activity.[8-10, 25]

The way in which membrane conduction (G_m) and imperfect capacitance (Q_m) are altered as a result of adding kB1 is depicted in Figure 3*B*&*C* (Supplementary Material Fig S2 *B*&*C* for kB2). Membrane conduction increases in response to addition of 10 μM kB1, then returns to baseline levels following a washing step. These responses are repeatable upon subsequent exposure to, and washouts of the cyclotides. Likewise, the imperfect capacitance of the membrane is drastically altered with exposure to kB1 and kB2, and is also reversible. To record such a drastic capacitance change, not only would the membrane have to become substantially thinner as a result of adding the cyclotide, but the dielectric of the membrane would also need to substantially increase, indicating increased water content. However, such a dramatic change would indicate that the membrane has undergone a structural alteration to a non-sealing isotropic phase that exposes the underlying gold electrode.

The effects of kB1 and kB2 were only detected in membranes containing a proportion of POPE in their composition, and not in POPC-only membranes. This observation suggests a *specific* interaction between the PE headgroup and the cyclotide which increases both membrane conduction and permittivity. In addition, the CPE exponent, α_m, decreased from 0.95 to 0.8 apparently indicating an impediment to the charging of the membrane capacitance (Figure 3*D*). In the example where the membrane has undergone a change of morphology such that highly conductive pathways are now bypassing the permeability barrier previously caused by the presence of the lipid bilayer, the two capacitor model collapses to a single capacitor model describing the underlying gold electrode. The fall in α_m is now reflecting α_s (Figure 4*F*). Had the primary effect of kalata been the assembly of multimeric peptide ion channels, the conduction would have increased but the capacitance would be expected to remain substantially unaltered. Furthermore, the Qs minimally changed upon addition of kB1 or kB2, suggesting that the effects induced by these cyclotides are localised within the lipid bilayer (Figure 3*E*).

The effects of cyclotides on a PE containing membrane were observed over a very narrow concentration range. This is in contrast to the progressive increase in conduction experienced with ion channels such as gramicidin-A.[26] No significant effects were detected upon addition of 1 μM kB1, even after 10 hours of incubation (Figure 5*A*&*B*); whereas at 10 μM substantial

changes in Q_m and G_m occur immediately. Such changes over the 1 - 10 μM kalata concentrations are similar to a critical micelle concentration (CMC) event in which the self-assembly of surfactant molecules can incorporate membrane lipids inducing a morphological phase change and a disruption of the membrane. One model, therefore, of the effect of the cyclotides on the tBLMs studied here is that, rather than behaving like an ion channel, kB1 and kB2 interact with PE containing lipid bilayers as a surfactant, inducing major leakage pathways in the membrane and causing an increase in the membrane dielectric constant through the facilitated increase in the water content in the previously strongly hydrophobic region of the membrane.

Another important observation is that the effects of kB1 and kB2 are reversible. Following a PBS rinse, the membrane's conduction and capacitance return to the values observed before the addition of the cyclotides (Figure 4*B-F*). Had the cyclotides acted as a *lytic* surfactant, the system would not show reversibility. The fact that the tBLM intrinsically possesses stabilising tethers that anchor the membrane lipids to the electrode must result in stabilising the conductive state induced by the addition of kB1 and kB2. However, the outer leaflet of the tBLM is not tethered and so would be disrupted, forming an irreversible micellar phase (as would occur when a conventional surfactant, such as *Triton-X 100,* is added). It is noteworthy that the re-addition of kB1 and kB2, after rinsing, repeated the earlier response, indicating there can have been no loss of the PE lipid from the tBLM. These observations raise the possibility of other amphipathic peptides or organic compounds acting as surfactants on lipid bilayers which can induce toroidal pores.

Our analysis focuses exclusively on the steric constraints that determine the membrane's morphology. This is effectively described by a weighted CPP parameter (CPP_w). The CPP_w relates the population weighted metrics of the individual molecular dimensions, such as area (a_o), length (l) and volume (v), to the morphology of the resultant assembled phase formed by the molecules such that $CPP_w = \langle v/(a_o l) \rangle$.[15-16, 20] When the $CPP_w = 1/3$, spherical micelles are predicted, and when the $CPP_w = 1$, the anticipated structure is a planar bilayer. In a model presented here, the interaction that titrates in the range 1 – 10 μM[8] forming cyclotide-PE complexes, reduces significantly the CPP_w of the membrane and results in the generation of regions of high local curvature which, in turn, induce toroidal pores causing the substantial increase in G_m and Q_m.[20]

The CPP_w model is further supported by the data in Figure 6*A*&*B* which shows a typical response of tBLMs to kB1 at pH 5 compared to pH 7 in 100 mM NaCl. In this pH range the conformational change in the structure of kB1 is minimal,[27] and earlier work has indicated that pH has little effect on the mass loading of kB1 to PE within supported membranes in the range of pH 5.5 – 7.4.[28] However, a recent study has shown the membrane organisation is strongly dependent on pH over the same range.[20] At lower pH the membrane is more ordered with a significantly reduced Q_m suggesting an increased inter-molecular attraction of the lipids at lower pH is modifying the interaction of kB1 with the PE population of the lipid bilayer. A consequence of this dependence would be that the effect of kB1 on the membrane conduction will be closely correlated with the pH dependent partition coefficient, consistent with the CPP_w model.

There is no necessity in this model for there to be an interaction between cyclotide molecules. The impact of the cyclotides on the membrane structure is primarily through the alteration of the steric CPP_w which, in the presence of kB1 and kB2 is proposed to fall, inducing a need for regions of high curvature within the membrane structure which, in turn, induces the formation of conductive toroidal pores. The dramatic changes that occur in the membrane capacitance and conduction are difficult to explain by other than a major surfactant effect. It is unlikely that pores arising within the membrane are due to peptide self-assembly will possess sufficiently large central ion conductive pathways to increase the membrane capacitance to the extent observed. The magnitude of the effect of kB1 and kB2 on the membrane strongly suggests these peptides induce a morphological change in the membrane and not simply self-assembly into ion channel-like pores.

CONCLUSIONS

Using tethered bilayer lipid membranes in conjunction with swept frequency electrical impedance spectroscopy we have shown that the prototypical cyclotides, kB1 and kB2, have surfactant-like properties and are unlikely to exert their effects on lipid membranes via an ion channel like pore mechanism. It was further confirmed that the presence of PE lipids in the bilayer is required for the effects of these cyclotides. The formation of cyclotide-PE complexes supports the creation of toroidal pores described by a CPP_w model whereby an alteration of the size of the head groups in proportion to the lipid tails generates regions of curvature in the membrane inducing the formation toroidal pores.

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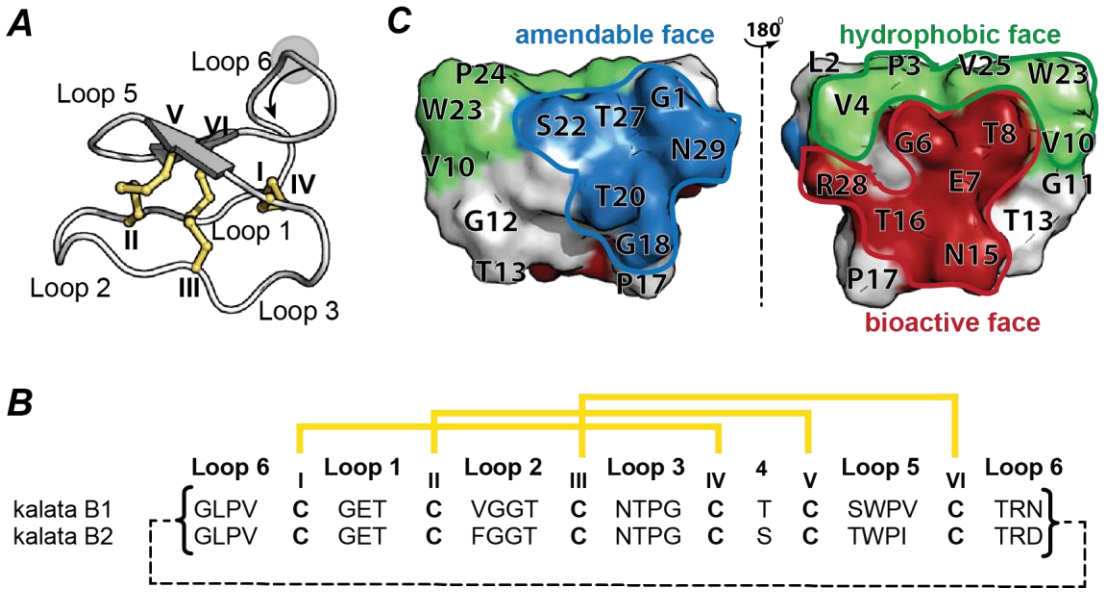

Figure 1. Structure and sequence of cyclotides. ***A,*** Three-dimensional structure of kalata B1 (PDB: 1nb1) showing the cyclic backbone and the three disulfide bonds (yellow) arranged in a knot. The grey circle shows the position of Gly-1, I-VI indicate the location of Cys residues and the black arrow indicates the direction of the peptide chain. The segments between the Cys residues are termed loops and are labelled 1-6. ***B***, Sequences of kalata B1 and kalata B2. Backbone cyclization between Gly-1 and Asn/Asp-29 is indicated with a black dashed line, with the disulfide connectivity shown in yellow; Cys residues are labelled I-VI and loops are labelled 1-6. ***C***, Surface representation of kalata B1 in two views showing the location of the amendable face (blue), hydrophobic face (green) and bioactive face (red).[9-10] The residues that have been shown to be important for the reported activities of kalata B1 are located in the hydrophobic and bioactive faces. Point mutations in these regions might render the peptide inactive and also unable to bind to membranes. Mutations in the amendable face can improve the potency of kalata B1.[10]

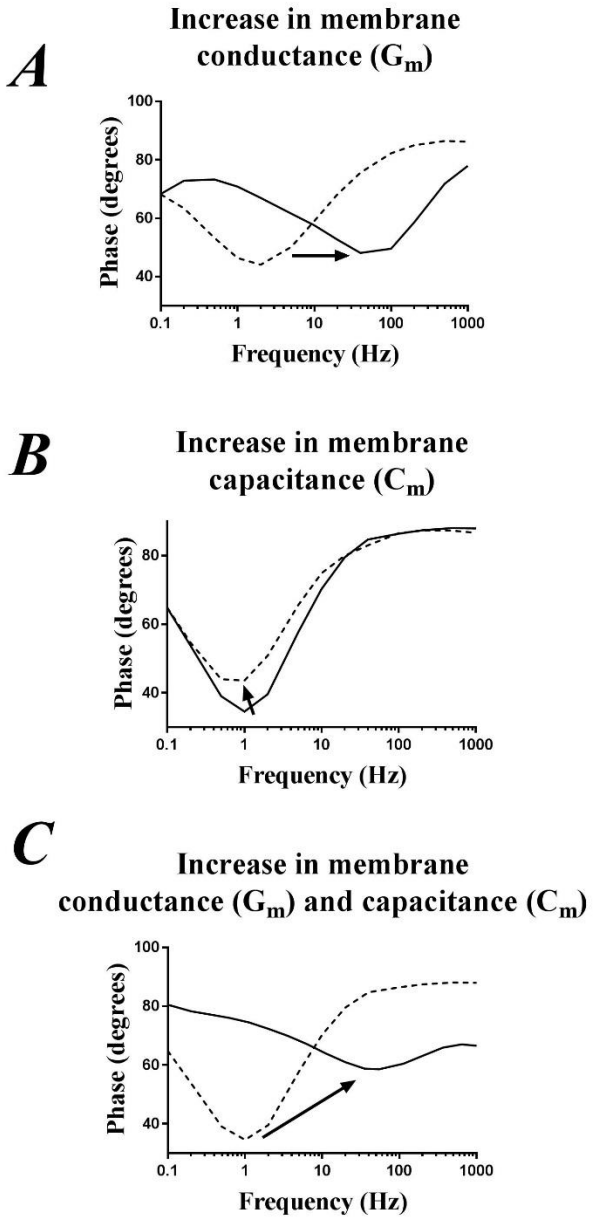

Figure 2 ***A,*** the higher membrane conduction resulting from the incorporation of an ion channel into a tBLM will shift the phase profile to higher frequencies. ***B,*** incorporation of a protein or peptide that induces a thinning of the membrane will cause an *increase* in membrane capacitance resulting in an increase in the phase minima. ***C,*** the typical response seen here, resulting from the addition of kB1 or kB2 to a POPC/POPE membrane was both a shift to higher frequencies of the phase profile and an increase in the phase minima.

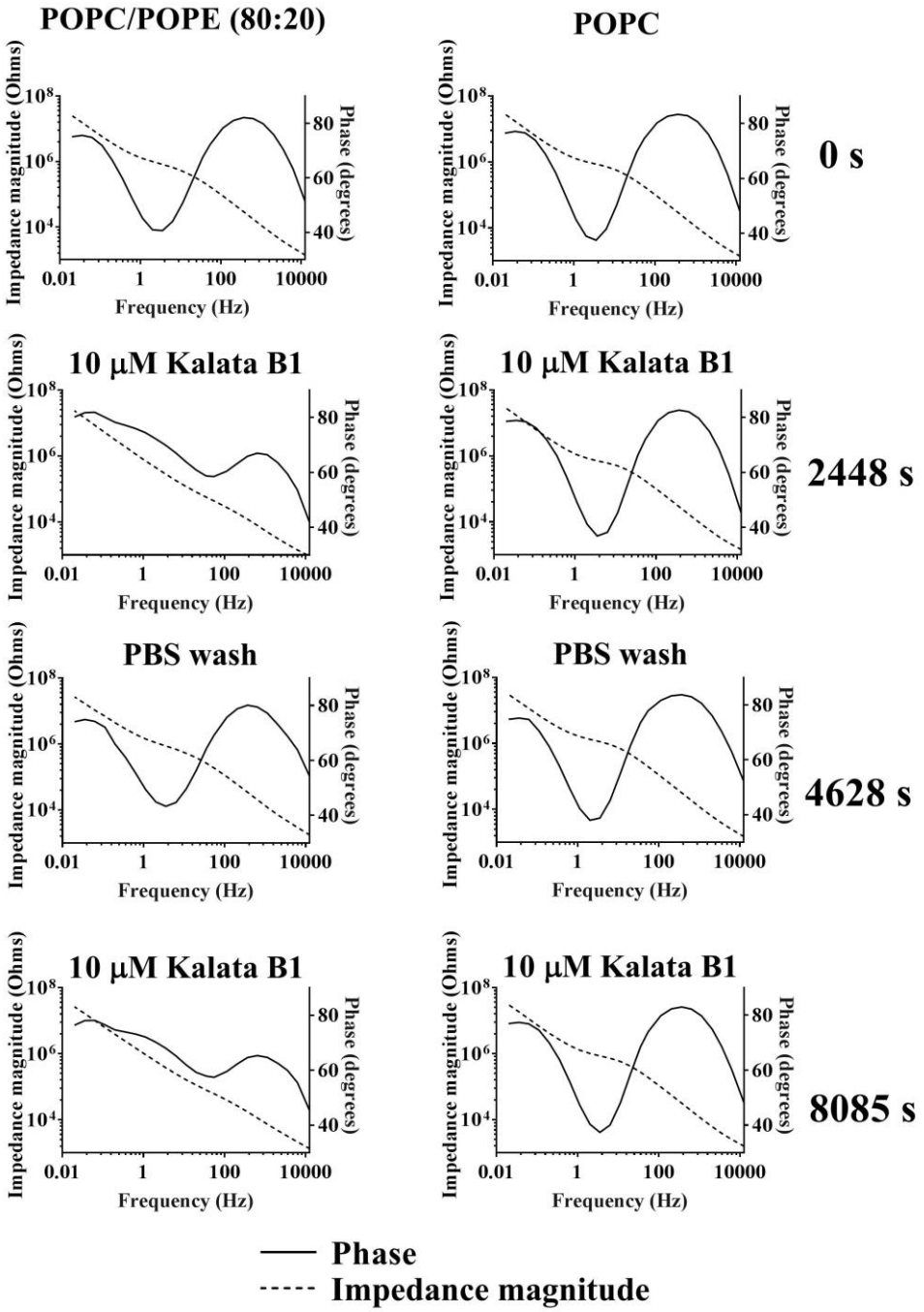

Figure 3. Bode plots highlighting the change in the impedance spectra in response to 10 µM kB1. On the left hand side are Bode plots of tBLMs containing a mixture of POPC/POPE (80:20) lipids before adding kB1, after addition of 10 µM kB1, after a PBS wash and then a subsequent re-addition of 10 µM kB1. The right hand side are Bode plots of corresponding POPC tBLMs. These data confirm that the presence of ethanolamine lipid head groups are required for kB1 activity and that the effects of kB1 on lipid membranes are recoverable following a PBS wash step.

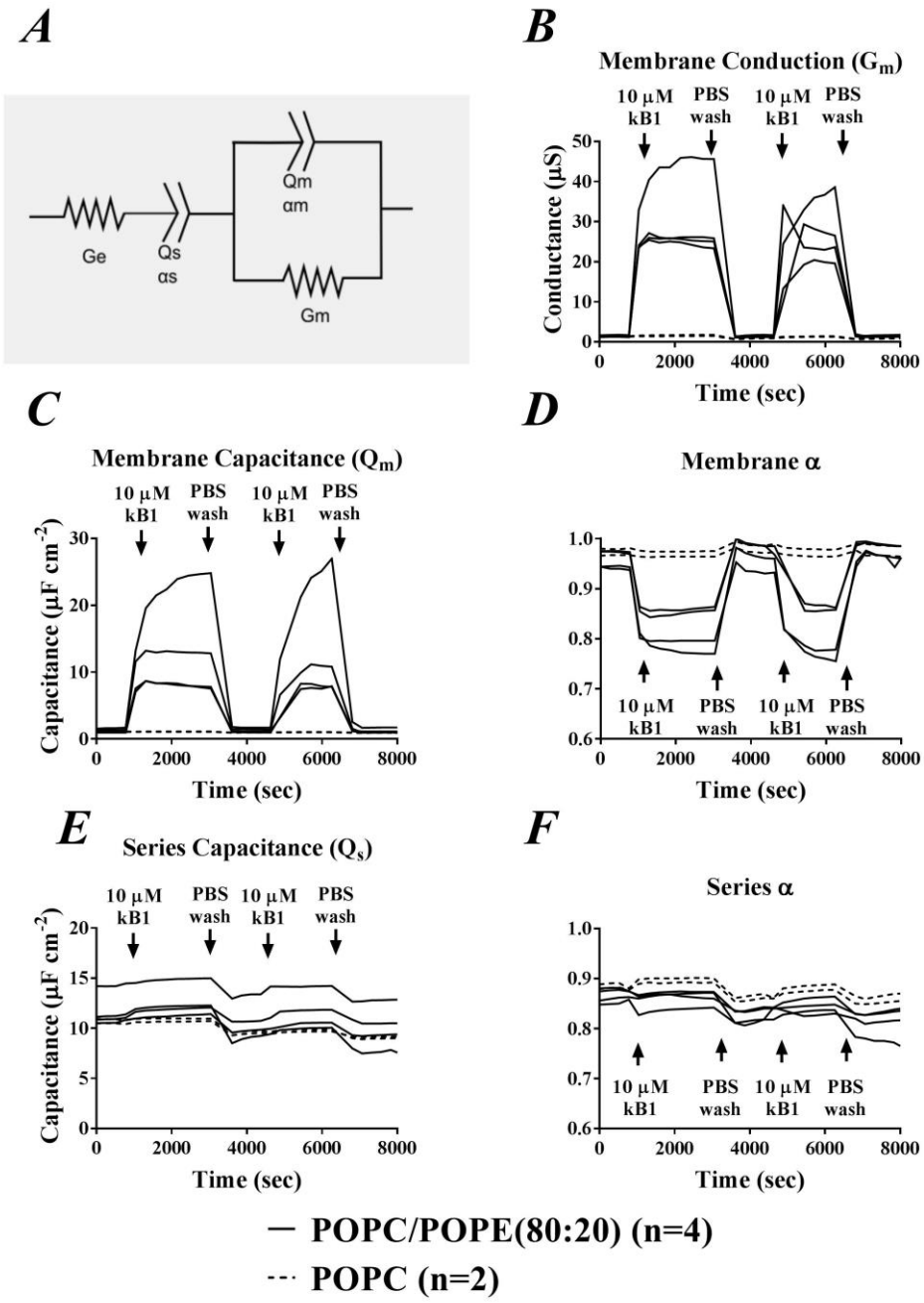

Figure 4 ***A*** The equivalent circuit used to fit the phase and impedance magnitude EIS data. Qs and Qm are the CPE values (imperfect capacitances) at the gold electrode and the lipid membrane, respectively, and α_s and α_m are their respective exponents. G_m is the membrane conductance and G_e the conductance of the PBS bathing solution. ***B*** is a plot of the change in membrane conduction in response to 10 μM KB1 and subsequent wash steps with PBS. ***C*** are the associated changes in membrane capacitances (Q_m) and ***D***, the changes in the exponent values α_m in response to 10 μM kB1 and subsequent wash steps with PBS. ***E*** and ***F*** highlight how the capacitances and exponent values are minimally altered by the presence of 10 μM kB1 and subsequent wash steps.

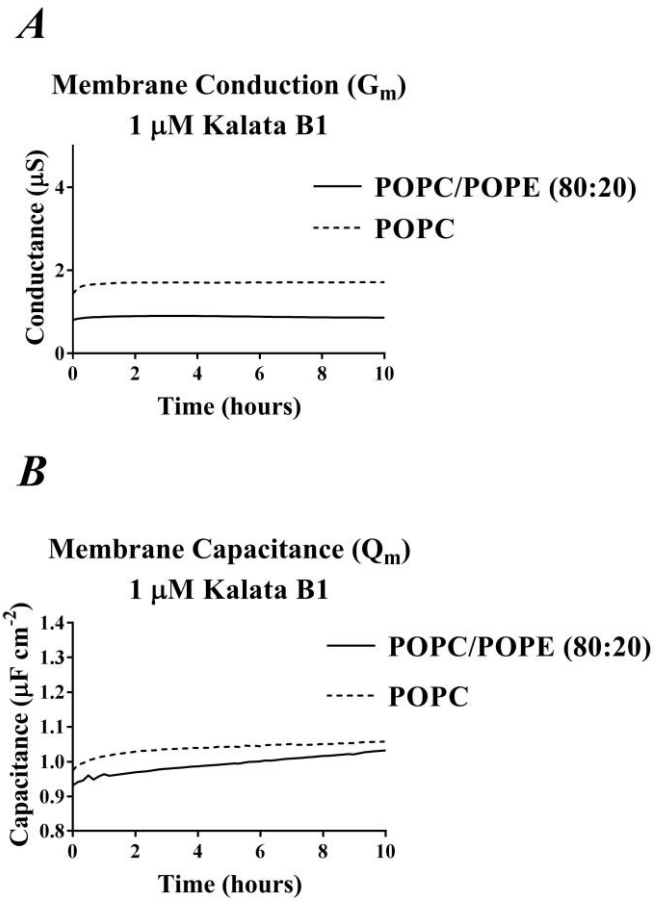

Figure 5 ***A*** Conduction changes (G_m) over a period of 10 hours to a 1 μM dose of kB1. ***B,*** the capacitances (Q_m) over the same period.

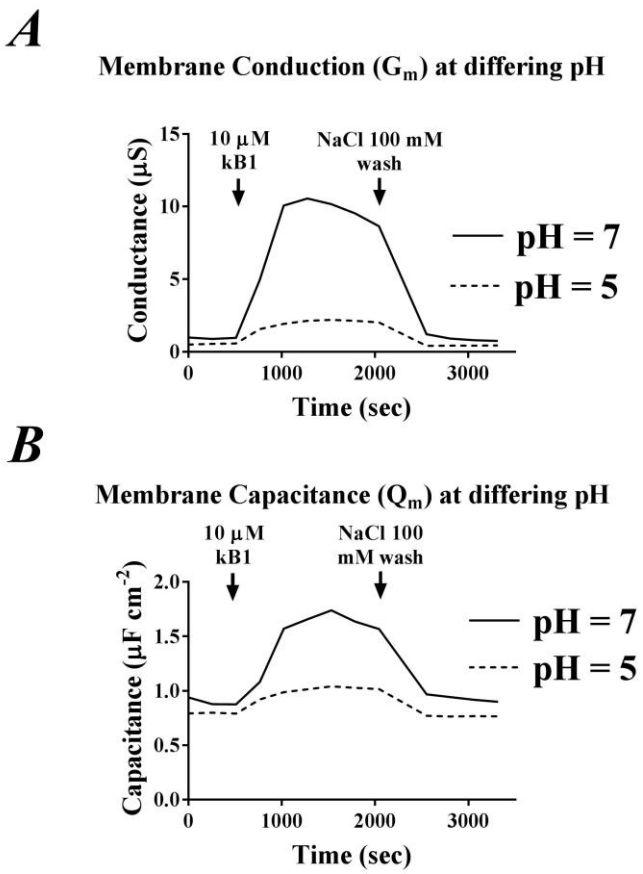

Figure 6. ***A***, the impact of kB1 on tBLM conduction and ***B,*** capacitance, at pH 5 compared to pH 7. There is a clear dampening of the response at pH 5 supporting the model that kB1 lowers the overall CPP of the bilayer inducing a more curved-like membrane. The tBLM contains POPC/POPE (80:20) lipids.

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