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Phosphatidic acid plays a special role in stabilizing and folding of the tetrameric potassium channel KcsA

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Abstract In this study, we investigated how the presence of anionic lipids influenced the stability and folding properties of the potassium channel KcsA. By using a combination of gel electrophoresis, tryptophan fluorescence and acrylamide quenching experiments, we found that the presence of the anionic lipid phosphatidylglycerol (PG) in a phosphatidylcholine (PC) bilayer slightly stabilized the tetramer and protected it from trifluoroethanol-induced dissociation. Surprisingly, the presence of phosphatidic acid (PA) had a much larger effect on the stability of KcsA and this lipid, in addition, significantly influenced the folding properties of the protein. The data indicate that PA creates some specificity over PG, and that it most likely stabilizes the tetramer via both electrostatic and hydrogen bond interactions.

Keywords: Potassium channel; Phosphatidic acid; Oligomerization; Stability; Anionic lipid; Tryptophan fluorescence; Electrostatic interaction; Hydrogen bonding

1. Introduction

Many membrane proteins are active as stable oligomers. The stability of these complexes is crucial for their function as they, e.g., contribute to the control of ion flow, cell volume, release of hormones and neurotransmitters [1–4]. However, how these complexes are stabilized is not clear. Besides protein–protein interactions, also protein–lipid interactions play an important role in oligomerization and determining the stability and function of such complexes [2]. These protein–lipid interactions may be either specific [5] or non-specific, in which case they depend on more general properties of lipids, such as the extent of hydrophobic mismatch [6], the presence of non-bilayer lipids [7,8] or the charge of the lipid headgroup [9].

Negatively charged lipids constitute 20–30% of biomembrane lipids [10]. It is well established that negatively charged membranes act as a site of attraction for positively charged (basic) protein domains [11,12]. Such electrostatic interactions may guide the membrane insertion and orientation of membrane proteins [13]. There is also strong evidence for the existence of specific binding of small numbers of lipid molecules to some membrane proteins. Some of these lipid molecules bind between transmembrane α-helices, often at protein–protein interfaces in oligomeric membrane proteins, and most of the lipid molecules identified in X-ray crystal structures of membrane proteins correspond to lipid molecules of this type [14]. Nevertheless, X-ray crystal structures of some membrane proteins, including bacteriorhodopsin [15], succinate dehydrogenase from *Escherichia coli* [16], and the ADP/ATP carrier from mitochondria [17], show resolved lipid molecules bound to the surface of the protein, not buried within deep clefts [18]. Although bacteriorhodopsin may represent a special case, being located in quasi-crystalline membrane patches in the membranes of *Halobacterium salinarum*, the other examples suggest that the lipid-bound surface of a membrane protein could be heterogeneous, with some lipid molecules binding more tightly than others. Evidence is now emerging for the existence of 'hotspots' on the surface of such proteins showing marked selectivity for anionic phospholipids. However, how different lipids and the amount of charge on their headgroup affect membrane proteins and the folding and stability of oligomeric complexes is far from clear.

The potassium channel KcsA from S*treptomyces lividans*, is a convenient model protein to study the role of lipids in membrane protein oligomerization. The tetrameric structure of KcsA is highly stable in a wide range of detergents, even in SDS [19]. Within the tetramer, the four individual subunits are arranged symmetrically around the central pore. Each subunit consists of a positively charged N-terminal helix lying at the membrane interface, two transmembrane helices separated by a pore region, and a large C-terminal cytoplasmic domain [20,21]. There are several indications that KcsA has a special interaction with anionic lipids. For example, it has been shown that KcsA has a functional requirement for anionic phospholipids [22,23], that anionic lipids are important for efficient biosynthetic insertion and assembly of the channel [24] and that anionic lipids bind more strongly to KcsA than the zwitterionic lipid phosphatidylcholine (PC) [25]. Furthermore, the anionic lipid phosphatidylglycerol (PG) has been copurified with KcsA and was suggested to be present in the crystal structure [23], while by mass spectrometry it was found that KcsA can form strong complexes with both PG and the anionic lipid phosphatidic acid (PA) [26,27]. Finally, recent molecular dynamics (MD) simulations on the KcsA tetramer suggested that PG and PA strongly interact with positively charged residues on KcsA, but that in

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Abbreviations: SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; CF, carboxyfluorescein; Trp, tryptophan

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particular the interaction with PA is stabilized by extensive hydrogen bonding [28].

In the present study, we investigated the importance of anionic lipids for the folding and stability of KcsA. For this purpose, wild type KcsA was purified and reconstituted in bilayers of PC in the absence and presence of the anionic lipids PG and PA. PG is a typical and abundant bacterial membrane lipid, whereas PA occurs in small amounts in many membranes, being a key intermediate in phospholipid biosynthesis and an important signalling molecule [29,30]. Tetramer stability was assayed by analyzing the concentration of the membrane-active alcohol trifluoroethanol (TFE) required to dissociate the tetramer on SDS gel [31,32]. The effects of TFE on the folding properties of KcsA were determined by tryptophan fluorescence quenching experiments using acrylamide. The most striking result was that PG and PA appear to affect the stability of KcsA very differently, with the tetramer being much more stable in the presence of PA. The fluorescence quenching experiments indicated that folding and unfolding of the tetramer by TFE are related to its stability. Taken together, the data suggest that PA binds stronger than PG presumably via the establishment of electrostatic and hydrogen bond interactions with the positively charged residues located at the cytoplasmic side of KcsA.

2. Materials and methods

2.1. Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids Inc. For clarity, the names of these lipids are abbreviated to PC, PG and PA, respectively. *n*-Dodecyl-β-D-maltoside (DDM) was from Anatrace Inc. Ni^{2+}-NTA agarose was obtained from Qiagen and Bio-Beads SM-2 Adsorbent from Bio-Rad Laboratories. TFE was obtained from Merck. The 200 nm membrane filters were obtained from Anotop 10, Whatman, UK. The following chemical reagents were purchased either from Fluka (Switzerland), Merck (Germany) or Aldrich (Germany), available in the highest purity: α-chymotrypsin from bovine pancreas, HEPES, KCl, NaCl, imidazole and isopropyl-β-D-thiogalactopyranoside (IPTG).

2.2. Protein expression and purification

KcsA was expressed with an N-terminal His-tag from pT7-KcsA [33] in *Escherichia coli* strain BL21(DE3). Purification was performed in buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM KCl. The solubilized membranes in 3 mM DDM were incubated with pre-washed Ni^{2+}-NTA agarose beads overnight at 8 °C. The bound His-tagged proteins were eluted with 300 mM imidazole pH 7.5 and 1 mM DDM. KcsA was purified with a yield of ~1 mg/l culture as previously reported [32]. The purity of proteins was assessed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The presence of DDM precluded the use of the Bradford assay [34], and therefore the protein concentration was assessed by SDS gel after staining with Coomassie Blue, using a standard of bovine serum albumin (BSA).

2.3. Vesicle preparation and reconstitution

Large unilamellar vesicles (LUVs) were prepared by extrusion through 200 nm filters [46]. The proteins were reconstituted in different lipid mixtures to obtain a final protein concentration of 0.1 mg/ml. Briefly, LUV's (10 mM phospholipids) were prepared in vesicle buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM KCl), solubilized with 1% Triton X-100 (w/v) and mixed with DDM solubilized KcsA proteins at a 1:200 protein:lipid molar ratio. The detergent was removed using pre-washed Bio-Beads. The reconstituted vesicles were collected by centrifugation (1 h, TLA 100.1 rotor, 100 000 rpm, 4 °C). The proteoliposomes were finally resuspended in vesicle buffer.

2.4. Dissociation of KcsA tetramer by TFE

Small aliquots (10 μl) of proteoliposomes resuspended in vesicle buffer were incubated with variable amounts of pure TFE (vol%) for 1 h at room temperature. The TFE containing samples were mixed with 5 μl of an electrophoresis sample buffer (50 mM Tris–HCl, pH 6.8, 50% glycerol, 0.01% bromophenol blue and 10% SDS) and directly run on 15% acrylamide gel in the presence of 0.1% SDS at room temperature. After staining by silver nitrate [35] the gels were scanned by a densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The tetramer band intensity as a total amount of tetramer present at 0 vol% TFE was set to 100%. The amount of tetramer (%) was plotted against TFE (vol%) for the stability assay.

2.5. Carboxyfluorescein leakage experiments

Large unilamellar vesicles (10 mM phospholipids) were prepared as described above in buffer containing 50 mM CF, 10 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM KCl. Subsequently, the carboxyfluorescein (CF) outside the vesicles was removed by a Sephadex G-25 spin column, prepared in vesicle buffer. For the leakage experiments, a continuously stirred 10 × 4 mm cuvette was filled with 1 mL of vesicle buffer and placed in a QuantaMaster QM-1/2005 spectrofluorometer (Photon Technology International, NJ). The excitation wavelength was set at 491 nm and the emission wavelength was at 517 nm, both with a bandwidth of 2.5 nm. A variable amount of TFE was added to 20 μL of the CF vesicles in an eppendorf tube. After vortexing, 25 vol% of this sample (5 μL for a sample without TFE) was added to the cuvette, and the fluorescence was measured immediately. The percentage of CF leakage was calculated after subtracting the fluorescence without TFE, relative to a sample in which full leakage was induced by the addition of 1% Triton X-100 (w/v).

2.6. Tryptophan fluorescence and acrylamide quenching

All fluorescence experiments were performed in vesicle buffer at room temperature using a QuantaMaster QM-1/2005 spectrofluorometer (Photon Technology International, NJ) in a quartz cuvette. The samples were excited at 280 nm and emission spectra were collected between 300 and 400 nm. The bandwidths for both excitation and emission monochromators were 5 nm. Acrylamide quenching of tryptophan fluorescence was performed to check the accessibility of tryptophans in lipid bilayers as a function of TFE. Proteoliposomes were prepared as described above and diluted in vesicle buffer to achieve a final protein concentration of 0.6 μM. The samples were treated with 30 vol% TFE for 1 h at room temperature. Acrylamide was added in aliquots from a 5 M stock solution to each sample up to a concentration of about 25 mM. The Stern–Volmer equation was used to analyze the quenching data [47]:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 is the tryptophan fluorescence in the absence of quencher and F is the observed fluorescence at the concentration $[Q]$ of the quencher. K_{SV} is the collisional quenching constant, which was determined from the slope of Stern–Volmer plots. As a control, similar experiments were performed for a L-Trp solution (3 μM). All data were corrected for inner filter effects due to acrylamide absorbance according to the standard method [47]. The inner filter effects of 30 vol% TFE were found to be weak, as determined for a L-Trp solution (3 μM); hence, these effects of TFE are not considered further in our measurements.

3. Results

3.1. Stability of KcsA and effect of negatively charged lipids

We first tested the stability of KcsA in lipid bilayers of PC as compared to the stability in bilayers containing 30 mol% of the anionic lipids PG and PA. The silver-stained gels of tetramer dissociation as a function of TFE in different lipid bilayers are shown in Fig. 2A. The tetramer runs at a molecular weight

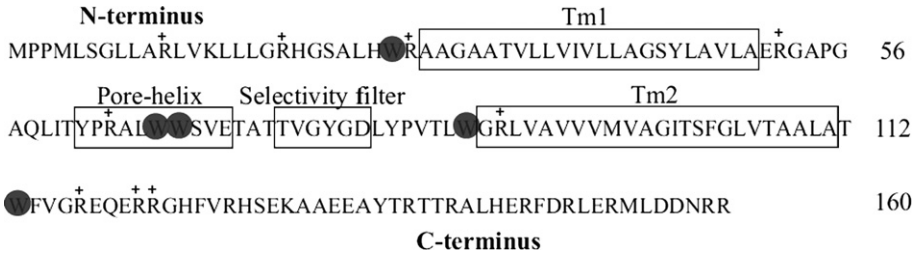

Fig. 1. Amino acid sequence of the KcsA potassium channel from *S. lividans* (SWISS-PROT accession number Q54397). The open boxes indicate the transmembrane segments Tm1 and Tm2 and the characteristic pore-helix and selectivity filter. Arginine residues at the positions 11, 19, 27, 52, 64, 89, 117, 121 and 122 are indicated as positive charges (+). Five tryptophans (W) at the positions 26, 67, 68, 87 and 113 are also highlighted in dark grey circles.

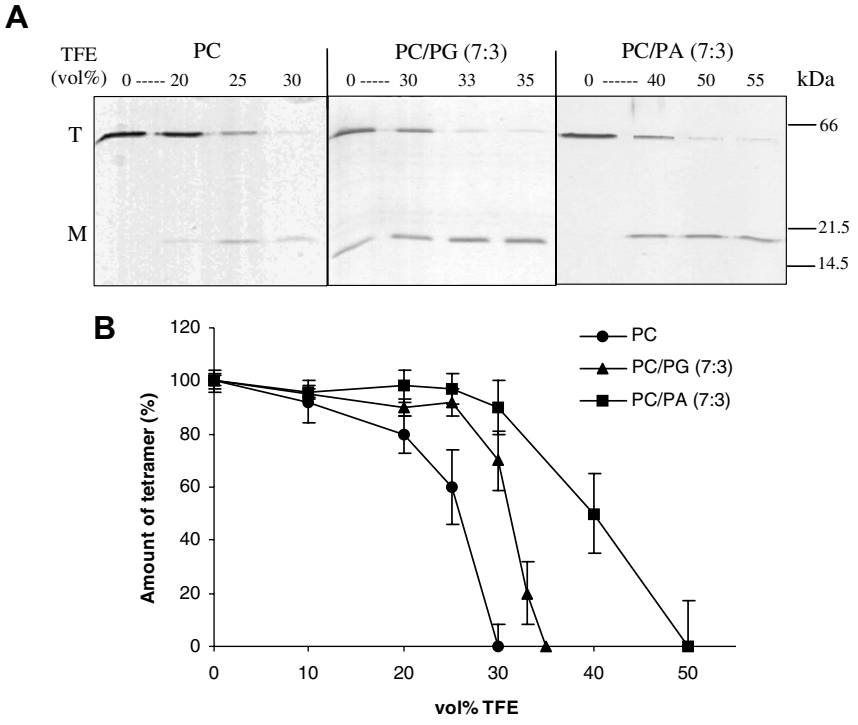

Fig. 2. (A) TFE-induced tetramer dissociation. TFE treated samples were analyzed by SDS–PAGE and stained with silver nitrate. Monomeric (M), tetrameric (T) KcsA are indicated and a protein marker (in kDa) is shown on the right. (B) Quantification of gels by densitometry. The intensity of tetramer bands were assigned as a relative value of 100% tetramer observed for TFE untreated (0 vol% TFE) sample. Data points correspond to the average and ±S.D. of two or three experiments.

around 66 kDa and the monomeric band runs at about 18 kDa. In some preparations of proteoliposomes, we found a monomeric band also in the absence of TFE, as for example shown here in the case of PC/PG bilayers. The reason for this variability is not known, but it has been reported before [32]. Upon incubation with increasing concentration of TFE in pure PC, the tetramer band intensity decreases and finally disappears at ~30 vol% TFE. As observed previously [31], a monomer band also appears, but its intensity is relatively low, possibly because the monomer is not stable and forms larger insoluble aggregates. In the presence of PG and PA, the tetramer band intensity also decreases as a function of TFE, but higher alcohol concentrations are needed of more than 30 vol% of TFE in case of PG and of more than 40 vol% in case of PA to fully dissociate the tetramer. In the samples containing anionic lipids, the monomer band intensity is increased as compared to the situation in PC, which may suggest proper folding of the monomer in the presence of charged lipids [23].

The effect of TFE on tetramer stability in the different lipid systems is quantified in Fig. 2B, where the amount of tetramer (%) is shown as a function of TFE (vol%). It is concluded that KcsA is slightly more stable in the presence of PG than in pure PC, but that PA has a strong stabilizing effect.

3.2. Influence of charge density on KcsA stability

To test to what extent each charged lipid contributes to tetramer stability, titration experiments were performed by increasing the amount of PG or PA in PC bilayers. Quantification of the results shows that the presence of 30–40 mol% PG and 20 mol% PA is sufficient to obtain maximal stability (Fig. 3).

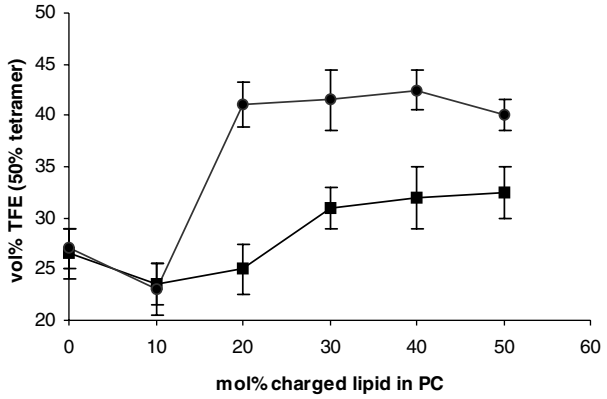

Fig. 3. Influence of negatively charged lipids on KcsA tetramer stability. KcsA was reconstituted in LUV's containing varying amounts of PG (■) or PA (●) in PC (mol%). Samples were analyzed as described in the legend of Fig. 2 and vol% TFE concentration required to dissociate the 50% tetramer was calculated. Data points correspond to the average and ±S.D. of two or three experiments.

3.3. Effect of TFE on bilayer integrity

The remarkable difference in amount of TFE required to destabilize the tetramer in PA containing bilayers as compared to PC or PC/PG bilayers, could in principle result from an increased amount of 'space' available for partitioning of TFE at the lipid/water interface, due to the small headgroup of PA. If this were the case, one would expect that also for direct effects of TFE on the bilayer, such as its ability to compromise membrane barrier properties, a higher concentration of the alcohol would be required in the presence of PA than in the presence of PG. To test this possibility, carboxyfluorescein leakage experiments were performed on bilayers of PC with and without PG or PA. Fig. 4 shows that these bilayers all become leaky around 15 vol% TFE. Thus, there is no lipid dependency of TFE-induced vesicle leakage, suggesting that the increased KcsA tetramer stability observed in the presence of PA is not due to increased partitioning of TFE, but that it is related to some special properties of PA (see Section 4).

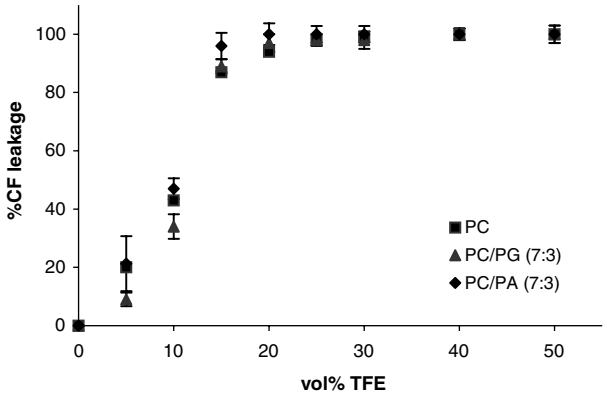

Fig. 4. Carboxyfluorescein leakage of membrane vesicles induced by TFE. TFE was added to carboxyfluorescein-loaded vesicles (10 mM phospholipids, without KcsA). The leakage was determined immediately after dilution with buffer in the cuvette (see Section 2). The standard deviation is based on three experiments.

3.4. Effect of lipids and TFE on Trp fluorescence

A complicating factor in interpreting the effects of lipids on the stability of KcsA from gel-electrophoresis experiments is the presence of SDS in the media. As a complementary approach, we therefore also used Trp fluorescence to test the lipid dependence of folding and of TFE-induced unfolding of KcsA. The positions of five tryptophan (W) residues located at the membrane water interfacial regions are indicated in Fig. 1. The Trp fluorescence emission spectra [λ_{ex} = 280 nm; λ_{em} = 324 nm for PC, 318 nm for PC:PG (7:3) and 320 nm for PC:PA (7:3)] in the presence and absence of 30 vol% TFE are shown in Fig. 5. As quantified in Table 1, for all systems the fluorescence intensity decreases upon addition of TFE. In principle, this loss of intensity in the absence of acrylamide may be due to TFE quenching, but if it were solely due to quenching, then the loss would be expected to be progressive and to rapidly

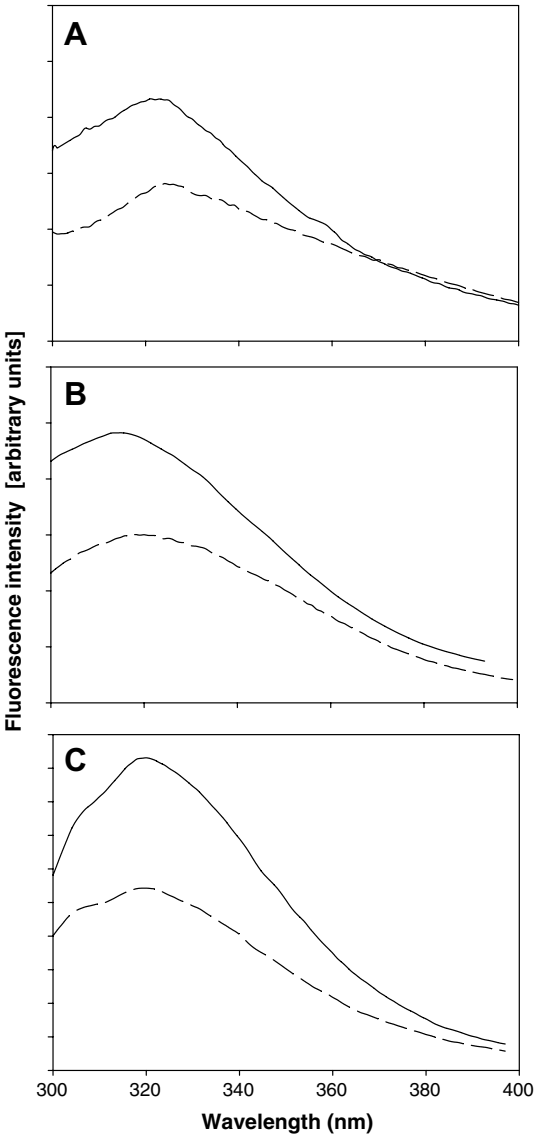

Fig. 5. The effect of lipids and TFE on fluorescence emission spectra of KcsA in pure PC (A), PC/PG (7:3 mol%) (B) and PC/PA (7:3 mol%) (C). Samples were investigated with (dashed line) or without (solid line) 30 vol% TFE. The corrected spectra and the effect of TFE (30 vol% TFE) on the fluorescence intensities are shown.

Table 1
Effect of lipids and TFE on Trp fluorescence and acrylamide accessibility

TFE (vol%)	Fluorescence quenching (%)[a]			Red-shift in maximum (nm)[b]			$K_{SV}(M^{-1})$[c]		
	PC	PC/PG	PC/PA	PC	PC/PG	PC/PA	PC	PC/PG	PC/PA
0	–	–	–	–	–	–	7 ± 1.1	6 ± 0.8	3 ± 0.6
5	~5	nd	~20	0	nd	0	10 ± 2	5.5 ± 2	2.6 ± 0.8
10	~22	~52	~35	2	2	0	8 ± 2	9 ± 2	2.5 ± 1.2
20	~52	~30	~38	2	4	0.5	7 ± 2	11 ± 2	2.7 ± 0.8
30	~55	~40	~40	4	4	0.5	18 ± 3	16 ± 2.5	8 ± 1.8
40	~75	~33	~48	4	5	1	15± 2	11 ± 2.5	10 ± 1.2
50	nd	nd	~35	nd	nd	1	nd	nd	3 ± 0.8

nd, not determined.
[a,b]The parameters were derived from Trp fluorescence spectra (not shown). The data for the effect of 30 vol% TFE are shown in Fig. 5.
[c]The Stern–Volmer quenching constants were determined from the slopes of the lines of $F_0/F = 1 + K_{SV}$ [Q]. The data derived for 30 vol% TFE is shown in Fig. 6. Values are the means ± S.D. of two or three experiments.

increase with the TFE concentration [48]. This was not observed. Therefore, these results indicate a conformational change of the protein upon TFE addition. In addition, a strong red-shift in fluorescence maximum was noticed for PC and PC/PG, but not for PC/PA, suggesting a different conformational behavior of KcsA in the presence of PA (see below).

3.5. Effect of TFE on Trp accessibility by collisional quenching

The observed shifts in fluorescence emission maxima suggested several distinct lipid dependent conformational changes in the protein. This was assessed in a more direct manner by using the collisional quencher acrylamide to detect changes in the 'availability' of Trp to the aqueous environment. This quencher has the advantage that it has a very low permeability to lipid membranes [46]. In addition, no charge interaction takes place between this quencher and the negatively charged lipid head groups [24]. The Stern–Volmer quenching plots of KcsA reconstituted in PC, PC/PG (7:3) and PC/PA (7:3) in which F_0/F is plotted against the acrylamide concentration in the presence and absence of 30 vol% TFE were linear and are shown in Fig. 6. The Stern–Volmer constants representing acrylamide accessibility at increasing concentrations of TFE (vol%) are compiled in Table 1.

In the absence of TFE more accessibility of KcsA to the quencher was observed for PC and PC/PG as compared to PC/PA, again indicating conformational differences in the presence of PA. At 30 vol% TFE, the quenching constants increased by ~2–3-fold in all lipid systems. These data suggest that TFE addition results in exposure of Trp residues to a more hydrophilic environment where PA induces a more compact and stable conformation of KcsA than PC and PC/PG. At 40 vol% TFE decreased accessibility to acrylamide was observed for PC and PC/PG and at 50 vol% TFE for PC/PA. This behavior might be related to KcsA dissociation into monomers and/or formation of larger aggregates at higher TFE concentrations.

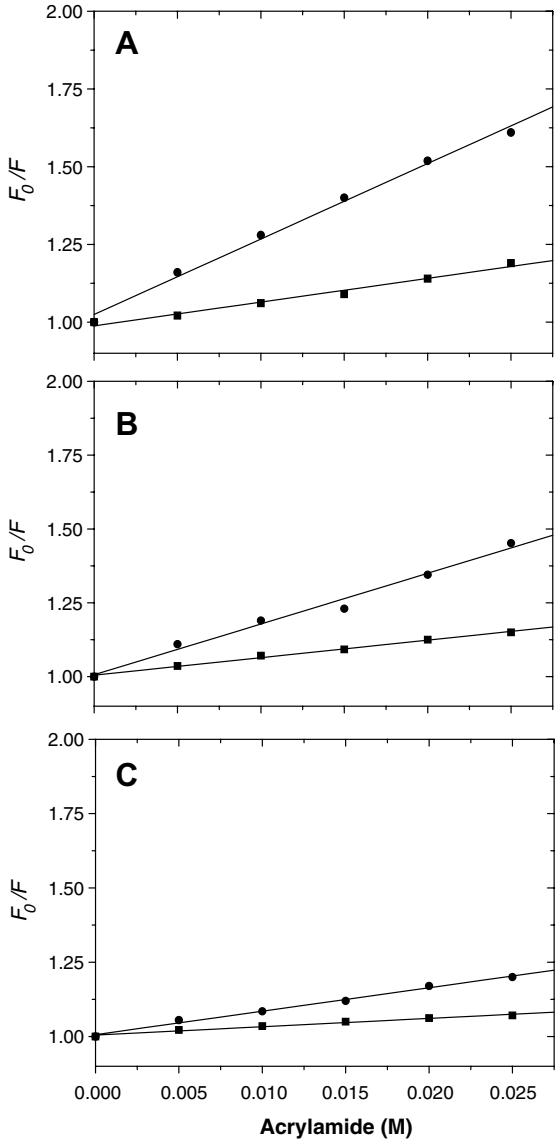

Fig. 6. Stern–Volmer plots of Trp fluorescence quenching by acrylamide. Quenching experiments were conducted as described under Section 2. KcsA was reconstituted in lipid bilayers of different compositions. The samples include pure PC (A), PC/PG (7:3) (B) and PC/PA (7:3) (C) and were investigated with (●) or without (■) 30 vol% TFE. The slopes of the best fit linear regression lines for each data set (K_{SV} values) are shown in Table 1.

4. Discussion

Elucidation of the role of protein–lipid interactions in determining the stability of oligomeric membrane proteins has been hindered by the lack of proper experimental approaches and difficulties in finding good solubilization conditions. Here, we optimized an assay to study the stability of the tetrameric

potassium channel KcsA, as an example of an oligomeric membrane protein, by combining the techniques of gel electrophoresis and tryptophan fluorescence. We chose the zwitterionic lipid PC as a stable bilayer forming lipid to compare the stability of KcsA upon incorporation of the anionic lipids PG and PA.

Our gel electrophoresis results showed a mild stabilizing effect when PG was included in the PC bilayer and a much stronger effect for PA. Since KcsA has many positively charged residues (Fig. 1) it is likely that anionic lipids in general stabilize the tetramer due to electrostatic interactions. This is supported by the observation that deletion of the positively charged N-terminus does not affect the stability in PC, but abolishes the slight stabilizing effect of PG (Raja et al., unpublished results).

The stability of the KcsA tetramer is known to be sensitive to ion composition [50]. For example, Na^+ has been reported as a destabilizer of the KcsA tetramer [51]. We performed stability assays in a buffer containing a relatively high Na^+ and low K^+ concentration. Under these conditions, the tetramer is slightly less stable, which gives the advantage of rendering comparison of lipid specificity more straightforward because smaller amounts of TFE are required.

Rather surprisingly, in the presence of PA about twice as much TFE was required to destabilize the tetramer suggesting a special role for PA in stabilizing the tetramer, which is dominating over the possible contribution of electrostatic interactions as suggested for PG with the positively charged residues.

As a complementary approach to test the effects of lipids on properties of the KcsA tetramer, Trp fluorescence and acrylamide quenching experiments were performed (Table 1). The quenching constants for different lipid systems in the absence of TFE agreed well with the determined stability of KcsA. The most intriguing observation of this study was that the presence of PA results in significantly less tryptophan fluorescence quenching as compared to PG and PC, suggesting a more condensed state of the molecule.

Our fluorescence quenching experiments also in 'simpler' systems, like in the absence of TFE, gave strong indications for differences in conformational behavior in the presence of PA. Addition of increasing concentrations of TFE to KcsA resulted in conformational changes of the protein in all lipid systems tested. This can be concluded from the loss of fluorescence intensity, the red shift in fluorescence maximum and the increased accessibility of the tryptophans to the aqueous quencher (Table 1), which all are indicative of an increased exposure of the tryptophan residues to the aqueous environment. However, in the presence of PA the red shift and accessibility to the quencher were significantly less than observed in the presence of PG or in pure PC. Thus, the results of the fluorescence experiments are in line with the strong stabilizing effect of PA observed by gel electrophoresis experiments.

The above results suggest that tetramer dissociation and protein unfolding are linked processes. Such a conclusion seems reminiscent of that from a somewhat simpler system, the T1 or prime tetramerization domain of Shaker B, a eukaryotic potassium channel, solubilized in DDM, in which folding and tetramerization were found to be coupled [36]. Also, a similar conclusion was recently drawn from Fourier transform infrared measurements on KcsA that was reconstituted into native-like membranes [37].

How do membrane-active alcohols like TFE influence proteins? On one hand, TFE might in fact stabilize helices by enhancing intramolecular hydrogen-bonding and electrostatic interactions [38]. On the other hand, and probably more relevant for its effect on KcsA, TFE could interfere with 'hydrophobic' protein–protein interactions by associating preferentially with hydrophobic sites on the interface between these proteins.

There are also several ways in which TFE may act indirectly on KcsA via the membrane. First, it is possible that the tetramer is destabilized via a change in the membrane lateral pressure profile upon TFE interaction in the membrane interface, as suggested previously [32]. In line with this, recent studies on the effects of TFE on the small bacterial mechanosensitive channel MscS showed that TFE addition to the membrane affects channel properties, possibly by distortion of the bilayer lateral pressure profile [38]. However, a second mechanism that needs to be considered is related to the observation from recent studies on model transmembrane peptides that TFE may cause a loss of the anchoring ability of interfacially localized tryptophans [49]. Such a loss in anchoring function of tryptophans in KcsA also might be a cause for destabilization of the tetramer.

Since TFE can promote ionic interactions [25] it may leave the interaction with the anionic lipids PG and PA intact [27], while weakening the hydrophobic intermolecular interactions between the KcsA subunits. We propose that such interactions play an important role in the folding of KcsA subunits and in the stability of the tetramer against TFE-induced dissociation. But what then makes the interaction of KcsA with PA so special?

Our titration experiments indicate that the special role of PA may be related to a possible requirement for a certain charge density for maximal tetramer stability. PG contains only one net charge, while PA has a net charge of $\sim$1.5 at pH 8.0 [39]. However, it should also be noted that the phosphomonoester headgroup of PA has remarkable H-bonding properties [40–42]. Indeed, two recent studies point to a special role of H-bonding in the interaction of KcsA with PA. First, molecular dynamics (MD) simulations [29] on a KcsA-PA system showed that the phosphate headgroups of all four lipid molecules at the interfaces of the KcsA monomers are able to form electrostatic interactions with Arg64 and Arg89, and that H-bonds, once these are formed, are quite stable and remain intact till the end of the simulation. On the other hand, a simulation of a PcsA-PG system revealed that H-bonding in this case involves the (headgroup) glycerol moiety of only two of the four PG lipid headgroups with these same two arginines. The second recent study in which the H-bonding properties of PA were addressed, involved solid state nuclear magnetic resonance (NMR) experiments on PA containing lipid bilayers in interaction with positively charged model peptides. The results suggested that initially the basic side chains of Arg and Lys on these peptides bind electrostatically to the negatively charged lipids. Upon binding, the side chains can form a H-bond with the phosphomonoester of the PA headgroup. This leads to further deprotonation of the headgroup, which in turn enhances the electrostatic interaction, thereby stabilizing the protein/lipid interaction. It was proposed that this electrostatic/hydrogen bond switch is a key element of specific recognition of PA by basic amino acids in membrane interacting proteins [43]. Because TFE strengthens

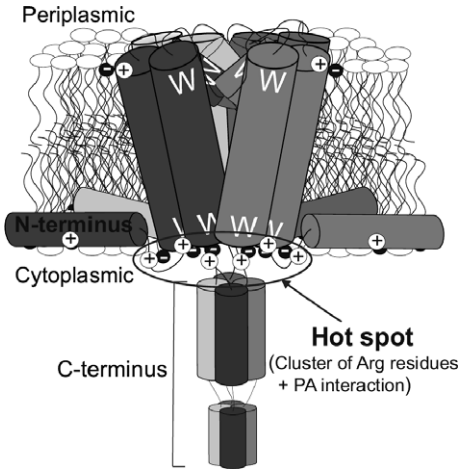

Fig. 7. Schematic two-dimensional model of the interaction of KcsA with PA in a lipid bilayer. For simplicity each monomer is shown in four distinct colours. The positions of five Trp residues (W) are indicated in two monomers. White headgroups represent PC and black headgroups correspond to PA. The interaction of PA (−) with the positively charged arginine (+) has been shown.

hydrogen bond and electrostatic interactions in soluble proteins rather than weakening them, the presence of TFE may make this electrostatic/hydrogen bond switch even more powerful, resulting in an extra-stabilizing interaction between the basic residues and PA at the membraneous parts of proteins.

This information can now be combined with our experimental data. We propose that the key residues Arg64 and Arg89 as well as the highly positively charged region (Arg 27, Arg117, Arg 121 and/or Arg 122) are responsible for establishing a strong stabilizing effect of PA. The reason is that such residues lie very close the membrane surface and therefore might interact specifically with the phosphate headgroup of PA. This holds in particular for the highly charged cluster at the cytoplasmic site of KcsA (see model in Fig. 7) thus creating a similar situation as observed for the mechanosensitive channel of large conductance MscL [44], where it was observed that PA also has a special interaction with basic residues on the so-called hot-spots. The importance of such residues could also explain the observation that deletion of the cytoplasmic C-terminal domain of KcsA (120–124 amino acids) containing Arg 121 and Arg 122 impaired tetrameric assembly of channel subunits in a heterologous *E. coli* expression system [52]. We would like to emphasize that this proposed specific interaction of Arg with PA is basically different from the stabilizing interaction of Arg with phosphodiester groups of phospholipids that has been found to be of functional importance for voltage-sensing in related K^+-channels [45].

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