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Special Interaction of Anionic Phosphatidic Acid Promotes High Secondary Structure in Tetrameric Potassium Channel

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Abstract Anionic phosphatidic acid (PA) has been shown to stabilize and bind stronger than phosphatidylglycerol via electrostatic and hydrogen bond interaction with the positively charged residues of potassium channel KcsA. However, the effects of these lipids on KcsA folding or secondary structure are not clear. In this study, the secondary structure analyses of KcsA potassium channel was carried out using circular dichroism spectroscopy. It was found that PA interaction leads to increases in α-helical and β-sheet content of KcsA protein. In PA, KcsA α-helical structure was further stabilized by classical membrane-active cosolvent trifluoroethanol followed by reduction in the β-sheet content indicating cooperative transformation from the β-sheet to an α-helical structure. The data further uncover the role of anionic PA in KcsA folding and provide mechanism by which strong hydrogen bonds/electrostatic interaction among PA headgroup and basic residues on lipid binding domains may induce high helical structure thereby altering the protein folding and increasing the stability of tetrameric assembly.

Keywords Potassium channel · Electrostatic/H-bonding · Anionic phosphatidic acid · Secondary structure · Alpha helix

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Introduction

Negatively charged lipids act as major attraction sites for positively charged protein domains or protein segments (Dowhan 1997; Raetz 1978; Ridder et al. 2001; Heijne 1985). Strong evidences have been accumulated for the existence of 'hotspots' on the surface of several membrane proteins showing distinct selectivity for anionic phospholipids that stabilize oligomeric membrane proteins, including the well-known tetrameric potassium channel KcsA (Yankovskaya et al. 2003; Pebay-Peyroula et al. 2003; Raja et al. 2007). Anionic phosphatidylglycerol (PG) and phosphatidic acid (PA) are known to stabilize KcsA tetramer via H-bonds/electrostatic interactions between the negatively charged lipid headgroups and the positively charged residues or 'hot spots' (arginine, lysine) located at the surface of KcsA. The reduced or enhanced stability has been shown to be dependent on various properties of anionic lipids in terms of their net charge, headgroup size and deprotonation capabilities upon binding of side chains of basic residues (Raja et al. 2007; van den Brink-van der Laan et al. 2004a, b; Raja 2010a, b). Such differences in lipids have been shown to alter the protein structure and function (Yankovskaya et al. 2003; Pebay-Peyroula et al. 2003; Raja et al. 2007). The interfacial interactions between lipid headgroups and the aromatic (tryptophan/phenylalanine) and charged residues (arginine/lysine) are known to act as an organizing element in membrane proteins (Dowhan 1997). Whether or not preferential interactions of anionic phospholipids alter the secondary structure of oligomeric proteins is not yet clear.

Circular dichroism (CD) spectroscopy is a powerful method in structural biology for examining the folding characteristics and secondary structures of proteins in different environments (Blundell and Mizuguchi 2000).

This technique has been limited for investigation of the secondary structure and folding of membrane proteins that are embedded in hydrophobic lipid environments instead of the aqueous milieu of soluble proteins (Wallace et al. 2003). However, by using the crystal structures of membrane proteins as model proteins—that consist of predominantly α-helical and β-strand structures—it is possible to examine the characteristics and accurate analyses of the secondary structures of membrane proteins from their CD spectra (Wallace et al. 2003). Using CD spectroscopy in this study, KcsA secondary structures were estimated in membranes containing either anionic PG or PA. Furthermore, the destabilizing effect of small alcohol, trifluoroethanol (TFE), was used as a tool to study which anionic lipid protects KcsA secondary structure during tetramer unfolding/dissociation. The data further uncover the role of 'special' interaction between PA headgroup and lipid/PA binding domains on KcsA via significant increase in an α-helical and β-sheet contents thereby providing a plausible mechanism by which PA extremely stabilizes the tetrameric structure in membranes.

Materials and Methods

Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) were purchased from Avanti Polar Lipids Inc (Birmingham, AL). For clarity, the names of these lipids are abbreviated to PC and PA, respectively. *n*-Dodecyl-β-D-maltoside (DDM) was from Anatrace (Maumee, OH). Ni^{2+}-NTA agarose was obtained from Qiagen (Chatsworth, CA) and Bio-Beads SM-2 Adsorbent was from Bio-Rad (Richmond, CA). TFE was obtained from Merck (Darmstadt, Germany). The 200 nm membrane filters were obtained from Anotop 10, Whatman, UK.

Protein Expression and Purification

Full-length wild type (WT-KcsA) protein was expressed and purified as described previously (Raja et al. 2007; Raja 2010a). Briefly, KcsA was purified 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. The purity of KcsA protein fractions was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Raja et al. 2007).

Vesicle Preparation and Reconstitution

Large unilamellar vesicles (LUVs) were prepared by extrusion (Hope et al. 1985). KcsA tetramer was reconstituted in different lipid mixtures with a protein concentration of 0.1 mg/ml, and proteoliposomes were obtained according to the method described previously (Raja et al. 2007). LUVs (10 mM phospholipids) were prepared in vesicle buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, and 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) and the resultant pellet was resuspended in a buffer containing 50 mM Tris–HCl, pH 7.0, and 15 mM KCl. The samples were recentrifuged (15 min, 14,000 rpm, 4 °C) to remove insoluble aggregates. The fractions of clear supernatant containing proteoliposomes were directly used for secondary structure analysis.

Circular Dichroism Spectroscopy

The far-UV CD spectra were recorded between 200 and 250 nm (350 µl sample volume) on a Jasco J-810 spectropolarimeter equipped with a temperature-controlled incubator at 20 °C using 1-mm optical path length quartz cell. The step size was 0.5 nm with a 1.0-nm bandwidth at a scan speed of 10 nm/min. An average of 10 scans was obtained for blank and protein spectra, and the data were corrected for buffer, lipid, and volume increase by TFE contributions and then smoothed. The optical chamber was deoxygenated with dry purified nitrogen before use and kept in the nitrogen atmosphere during experiments. Samples were incubated at room temperature for 1 h with or without variable concentrations of TFE. A quantitative estimation of the secondary structure contents was made by applying the K2D2 modeling program (Perez-Iratxeta and Andrade-Navarro 2008; Trujillo et al. 2013). The comparison of CD and K2D2 estimations plots provided visual assessments of the accuracy of the predictions with maximum error (<0.3). The directly recorded raw CD spectra were transformed into mean residue ellipticity (θ) in units of degrees/cm^2/dmol. The mean residue molar ellipticity (θ) MRW $= \theta/(10 \times c_r \times l)$, where c_r is the mean residue molar concentration of the sample (mol/l) and l is the pathlength in cm.

Results

Effects of PG and PA on KcsA Secondary Structure

Figure 1a represents the amino acid sequence of WT-KcsA. KcsA was reconstituted in either PC:PG (7:3 mol%)

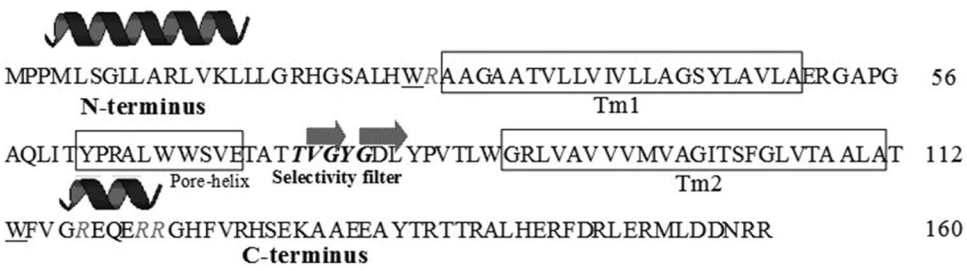

Fig. 1 Amino acid sequence of the KcsA potassium channel (SWISS-PROT Accession Number Q54397). *Open boxes* indicate the transmembrane segments Tm1 and Tm2 and the pore helix and selectivity filter

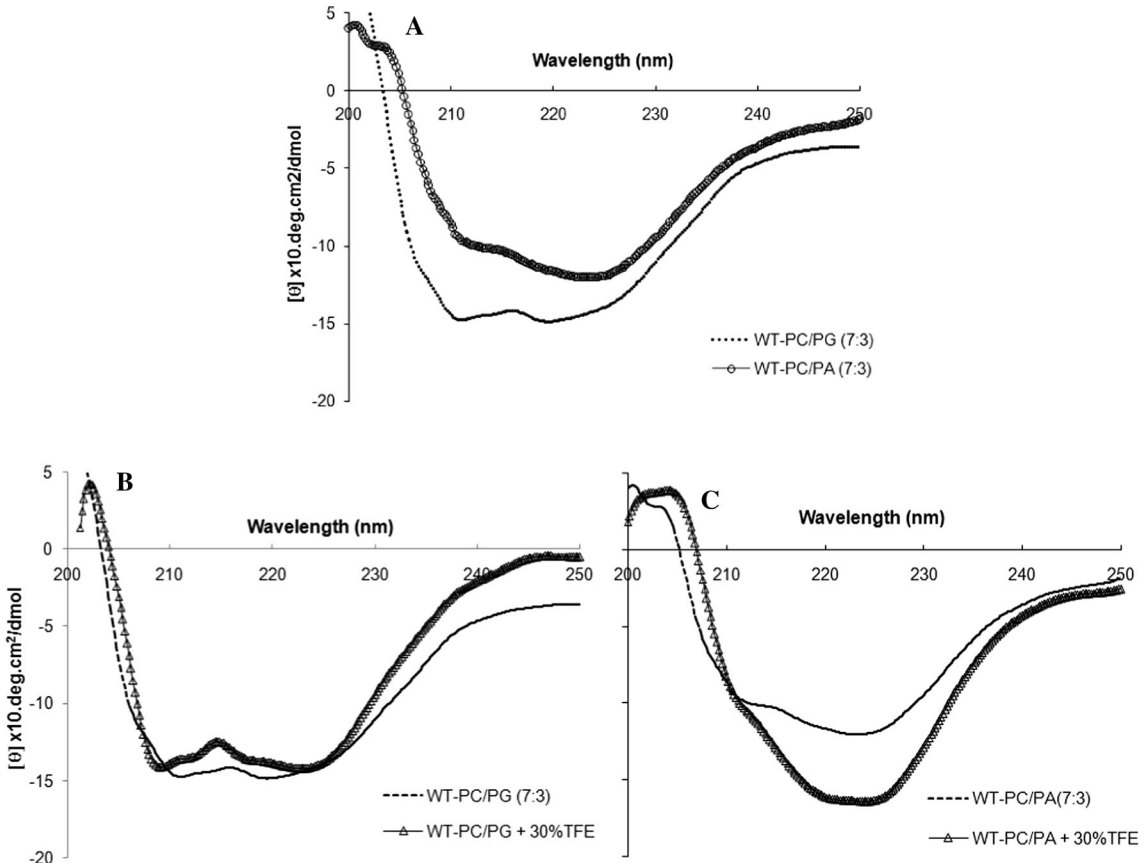

Fig. 2 **a** Far-UV CD spectra of WT-KcsA in PC:PG or PC:PA lipid bilayers. Far-UV CD spectra of WT-KcsA in the absence or presence of 30 vol% TFE in PC:PG (**b**) or PC:PA (**c**) bilayers. The calculated estimated secondary structure contents are compiled in Table 1

or PC:PA (7:3 mol%) lipid bilayers. The characteristics of CD spectra were sufficient to permit accurate analyses of the secondary structure of KcsA in both lipid systems. CD spectra of WT-KcsA in PC/PG and PC/PA lipid systems are shown in Fig. 2a and the calculated % structures are compiled in Table 1. For KcsA-PG, the CD spectrum showed characteristic negative bands at ~222 and 210 nm, which represents predominantly helical structures from which the secondary structure was estimated to consist of ~60 % α-helical and ~5 % β-structures. The CD spectrum of KcsA-PA exhibited negative prominent band at ~225 and less noticeable minimum at ~210 nm indicating that α-helical contents are significantly increased. Considering shifts in maxima, it has been well documented

Table 1 Percent determination of WT-KcsA secondary structure in different lipid bilayers

Lipid bilayer	TFE (vol%)	WT-KcsA	
		α-helical	β-strand
PC:PG	0	58 ± 4*	5 ± 0.2*
	30 %	57 ± 3	4 ± 0.3
PC:PA	0	75 ± 3*	8 ± 1.4*
	30 %	87 ± 4*	4 ± 1.5*

Parameters of secondary structure were derived from data shown in Fig. 2. Structural analyses were performed for the average CD spectra corrected. Values are given as mean ± SEM

Significance was calculated using Student's *t* test. $^*P < 0.05$

that CD spectra of membrane proteins differ from those in solution (Wallace et al. 2003).

Effects of TFE on the Secondary Structure of KcsA-PG or PA Complex

It has been shown that relatively lower concentrations of TFE are required to dissociate detergent-solubilized (DDM) KcsA tetramer into its monomeric subunits which is followed by reduction in the α-helical content and increase in the β-sheet structures (van den Brink-van der Laan et al. 2004b). Because, DDM-solubilized KcsA is directly exposed to TFE the amount of TFE (10–15 vol%) required to dissociate the tetramer, via alteration in secondary structure contents, is considerably less than the amount to dissociate membrane incorporated KcsA tetramer (van den Brink-van der Laan et al. 2004b). We, therefore, monitored the effects of TFE on the secondary structure contents in lipid bilayers containing anionic PG or PA. Figure 2b represents the CD spectra of TFE treated and untreated WT-KcsA in PC/PG membranes. It is important to mention that below 30 vol% TFE concentration (10 or 20 vol%) no significant changes in the protein secondary structures could be found (data not shown). In the presence of 30 vol% TFE, a shift in maximum from 210 to 208 nm wavelength was observed which is most likely related to the solvent polarisability and change in the energies of the transitions that affect the positions of the spectrum peaks (Chen and Wallace 1997a, b). Interestingly, no significant change in secondary structure was noticed (Table 1) indicating that membrane bilayer protects KcsA against direct effects of TFE thereby stabilizing its structure. Incubation of 30 vol% TFE with KcsA in PC/PA bilayers slightly, but significantly increased α-helical contents and significantly decreased the β-sheet structures (Fig. 2c; Table 1) suggesting cooperative transformation from the β-sheet to an α-helical structure. These results strongly suggest that PA interaction with KcsA induces helical structure thereby increasing the intrinsic stability of KcsA tetramer.

Discussion

The purpose of the present study was to examine the 'special' effects of PA on KcsA structure and whether or not PA-dependent changes in the secondary structure of KcsA are related to increasing the stability of tetrameric assembly. CD has been proven to be a valuable technique in examining the folding and particularly secondary structures of soluble proteins. However, investigation of the structure and folding of membrane proteins in hydrophobic lipid environment can be challenging due to the spectral characteristics that are dependent on the dielectric constant of the surrounding medium as examined by studying model peptides and proteins (Chen and Wallace 1997a, b). The analyses of CD spectra also represented distinct spectral properties indicating dissimilar folding patterns of KcsA in different lipid bilayers.

DDM-solubilized KcsA exhibited 50–60 % helical contents as determined by CD spectroscopy in previous studies (van den Brink-van der Laan et al. 2004b; Chill et al. 2006). Similar trend was noted for KcsA in PC/PG bilayers indicating that interaction of anionic PG did not change the α-helical contents of KcsA. However, significant increase in α-helical and β-sheet contents was observed upon interaction of PA with KcsA. In line with these observations, KcsA exhibits rather compact/squeezed folding pattern in the presence of PA as revealed by tryptophan fluorescence and acrylamide quenching experiments indicative of structural changes in the protein (Raja et al. 2007). These results can be further supported by previously reported effects of anionic PA on induction of β-sheets followed by stable α-helical structures in membrane-associated cytochrome c (Sui et al. 1994). In another study, binding of anionic PA to acetylcholine receptor has been shown to increase β-sheet structure via establishment of salt bridges between phosphorylated amino acids and positively charged Arg or Lys residues thereby stabilizing β-structures and altering the protein structure and function (Bhushan and McNamee 1993). The effects of anionic PA on the protein secondary structures are most likely related to an interaction between lipid headgroup and accessibility of basic residues/hot spots that are either exposed at the surface or buried deep inside membrane proteins (Belrhali et al. 1999; Yankovskaya et al. 2003; Lee 2004). Such conformational changes might, therefore, induce either α-helical, β-sheet, or both.

According to KcsA structure (Roux and MacKinnon 1999), there are a few amino acids in the pore region (Gly-Asp-Leu) that account for about 2 % of the β-sheet structure. The molecular dynamics simulation for KcsA in PC bilayers (Bernèche and Roux 2000) indicates that the backbone in the filter/pore region can adopt a configuration which corresponds to a β-sheet for Val76 and Gly77 (as represented by gray arrows in Fig. 1). In the current study, slightly higher contents of β-sheet were observed for WT-KcsA in both lipid bilayers suggesting that addition of anionic lipids might induce β-sheet structure as discussed above. However, which part(s) of KcsA adopt β-sheets in a PA-dependent manner cannot be delineated with data at hand. It is interesting to note that in additional experiments deletion of membrane-associated N-terminus resulted in significant decrease and increase in α-helical and β-sheet

contents, respectively, suggesting some distinct yet unknown conformation of the protein (data not shown).

It has been shown in previous studies that KcsA tetramer is more stable in the presence of PA than PG [7, 10], because the amount of TFE required to completely dissociate the tetramer was considerably higher in PA lipid system than PG. So, TFE has been used as a tool to investigating the intrinsic stability of KcsA tetramer in the presence of different lipids. The reason for using TFE in CD experiments was to investigate what happens to KcsA structure during TFE-induced tetramer dissociation and whether or not anionic lipids, PG or PA, retain KcsA secondary structure in TFE-induced denaturing conditions.

It has been suggested that helices can be stabilized by direct interaction of TFE (Jasanoff and Fersht 1994). Another mechanism is related to the effect in which the H-bonds in the helices are stabilized by weakening the H-bonding among water and peptide carboxyl and amino groups (Cammers-Goodwin et al. 1996). TFE is not only known to stimulate native-like α-helical structures in proteins, but it also induces non-native helical structures in the unordered loops or β-sheet regions of some proteins (Fan et al. 1993; Buch et al. 1995). In particular, TFE has been shown to cause induction of non-native β-sheets and reduction in α-helical structures in detergent-solubilized KcsA (van den Brink-van der Laan et al. 2004b).

In the current experimental conditions, the exposure of KcsA to TFE is limited due to the presence of lipid bilayers. So, the classical effects of TFE on protein structures, as mentioned above, cannot be taken into account for explaining the current data. It has been shown that in lipid bilayers KcsA tetramer is stabilized most likely via changes in membrane lateral pressure profile—an indirect effect which is induced by insertion of TFE molecules in between the lipid headgroups (Raja et al. 2007; van den Brink-van der Laan et al. 2004a, b). CD analyses demonstrate that upon addition of TFE the secondary structure is not altered in PG containing bilayers, but in the presence of PA. So, what is the possible reason for this effect?

It is known that TFE partitions into lipid membranes and the extent of partitioning has been shown to depend on the nature of lipids present in a particular membrane system as studied previously for non-bilayer phosphatidylethanolamine (PE) and bilayer PC lipids (van den Brink-van der Laan et al. 2004a). Compared to PC/PG bilayers, an increased partitioning of TFE molecules into PA containing bilayers is expected due to its smaller size as PE lipid headgroup (Raja 2011) causing significant changes in protein secondary structure. Tryptophans are known to act as anchoring residues for transmembrane domains (Özdirekcan et al. 2008) and stability of KcsA structure (Raja et al. 2007). Each KcsA monomer contains five tryptophan flanking residues (shown as 'W' in Fig. 1). W26 is located in the beginning of transmembrane domain (TM1) close to N-terminus at the intracellular surface. W67 and W68 are present in the pore helix close to the outer surface, whereas W87 and W113 are localized in the interfacial regions of TM2. However, TFE can cause a loss of the anchoring ability of interfacially localized tryptophans which seems to destabilize the tetramer while weakening the hydrophobic interactions between the KcsA monomers (Raja et al. 2007).

A schematic model was derived in which the N-terminus, which is apparently less ordered, is loosely associated with the lipid bilayer. However, strong negative charge and small size of PA headgroup may allow deeper insertion of this domain into lipid bilayer (Kooijman et al. 2007) thereby inducing α-helical structures and increasing the stability of tetrameric assembly (Fig. 3). These studies might also support an important role of the N-terminus in structural stability and proper folding of KcsA tetramer in membranes as shown in previous studies mentioned above. In addition, strong interaction between PA headgroup and arginine cluster (R27, R117, R121/122, highlighted in Fig. 1) at the cytoplasmic surface (Raja et al. 2007) would be expected to cause an increase in the α-helical content. Further studies are required to delineate the role of the structural importance of the PA-binding domains, including N-terminus and other 'hot spots' in altering secondary structures and ultimate changes in activity of oligomeric membrane proteins, like KcsA.

It is emphasized that unlike KcsA-PA complex and ultimate changes in the secondary structure the effects

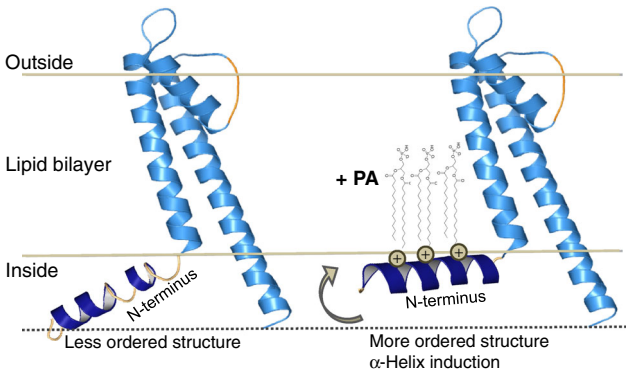

Fig. 3 A schematic model of KcsA monomer depicting the interaction of N-terminus with a lipid bilayer either in the absence or presence of anionic PA. The N-terminus is depicted to be less ordered structure. However, upon interaction of positively charged residues (R11, K14, R19), as indicated by *positive circles*, in the N-terminus with PA headgroup deeper insertion of this domain is favored, as evident from the tryptophan fluorescence quenching data (Raja et al. 2007; Raja 2010a, b), which could increase an α-helical content. The *arrow* indicates positioning of N-terminus very close to lipid bilayer carrying PA. Strong interaction between PA and arginine cluster (R27, R117, R121 or R122) (Raja et al. 2007) at the cytoplasmic side (not highlighted) is also expected to increase in helical structure

observed for other anionic lipids, like phosphatidylserine (PS) or cardiolipin (CL), are not similar. These lipids exhibit dissimilar physical properties and do not seem to alter the secondary structure of other proteins, including nicotinic acetylcholine receptor (nAChR) and calcium binding protein (Zolese et al. 1988; daCosta et al. 2004).

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Conflict of interest None.

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