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Interaction of the K^+ channel KcsA with membrane phospholipids as studied by ESI mass spectrometry

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Abstract In this study we have used electrospray ionization mass spectrometry (ESI-MS) to investigate interactions between the bacterial K^+ channel KcsA and membrane phospholipids. KcsA was reconstituted into lipid vesicles of variable lipid composition. These vesicles were directly analyzed by ESI-MS or mixed with trifluoroethanol (TFE) before analysis. In the resulting mass spectra, non-covalent complexes of KcsA and phospholipids were observed with an interesting lipid specificity. The anionic phosphatidylglycerol (PG), and, to a lesser extent, the zwitterionic phosphatidylethanolamine (PE), which both are abundant bacterial lipids, were found to preferentially associate with KcsA as compared to the zwitterionic phosphatidylcholine (PC). These preferred interactions may reflect the differences in affinity of these phospholipids for KcsA in the membrane.

Key words: Electrospray ionization mass spectrometry; Protein–lipid interaction; Lipid bilayer

1. Introduction

An important characteristic of integral membrane proteins is the presence of hydrophobic membrane spanning segments, which are compatible for interactions with membrane lipids. In order to understand functioning of membrane proteins at the molecular level, it is necessary to gain knowledge about the interactions between these proteins and the lipids which surround them. The enormous diversity of membrane lipids, comprising zwitterionic phospholipids (e.g. phosphatidylcholine (PC) and phosphatidylethanolamine (PE)), anionic phospholipids (e.g. phosphatidylglycerol (PG), phosphatidic acid (PA) and phosphatidylinositol), sphingolipids and sterols suggests a specific involvement of such lipids in particular cellular processes. Indeed, for several membrane lipid classes, roles in functioning and assembly of membrane proteins have been established. Whether such effects are caused by direct interactions between lipids and proteins, or whether they are indirect effects on the proteins involved, is unclear.

A large array of experimental biophysical techniques is available to study interactions between integral membrane proteins and lipids. However, electrospray ionization mass spectrometry (ESI-MS) has to our knowledge never been used for this purpose, even though it has been proven to be a suitable technique to study non-covalent interactions between proteins [1–3] and between soluble proteins and phospholipids [4]. We have shown before that it is possible to perform direct ESI-MS experiments on phospholipid membrane vesicles containing transmembrane peptides [5,6]. This method may also be suitable for investigation of interactions between integral membrane proteins and phospholipids. Here we explore the possible use of ESI-MS to study protein–lipid interactions using KcsA as a model protein.

KcsA is a homotetrameric K^+ channel from *Streptomyces lividans* [7]. The crystal structure is known, as well as many aspects of its channel function [8–13]. It is a predominantly α-helical integral membrane protein with each monomer containing two transmembrane segments. The protein can be expressed with an N-terminal His-tag in *Escherichia coli*, which after purification can be functionally reconstituted into lipid bilayers [7]. It has been found recently that specific lipids can significantly promote assembly and oligomerization of newly synthesized KcsA and that they also can increase the stability of KcsA reconstituted in model membranes [14]. Moreover, it was shown that the anionic lipid PG can be copurified with KcsA, indicating preferential binding of this lipid [15].

Using KcsA reconstituted in lipid vesicles of varying composition we show here that non-covalent interactions between KcsA and phospholipids can be detected by ESI-MS. It is shown that anionic lipids as well as PE have an increased affinity for KcsA as compared to PC. This specificity is very similar to that observed in membrane assembly and tetramer stability, suggesting that direct and specific lipid–KcsA interactions are involved in those processes [14].

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Abbreviations: DDM, *n*-dodecyl-β-D-maltoside; DEiPC, 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleyl-*sn*-glycero-3-phosphate; DOPC, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleyl-*sn*-glycero-3-phosphoglycerol; DPoPC, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine; ESI-MS, electrospray ionization mass spectrometry; LUVETs, large unilamellar vesicles prepared by extrusion; *m/z*, mass-to-charge ratio; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TFE, 2,2,2-trifluoroethanol

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2. Materials and methods

2.1. Materials

The phospholipids 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (DPoPC), 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (DEiPC) were supplied by Avanti Polar Lipids Inc. (Birmingham, AL, USA). *n*-Dodecyl-β-D-maltoside (DDM) was from Anatrece Inc. BioBeads SM-2 Adsorbent from Bio-Rad Laboratories. KcsA was expressed and purified with an N-terminal His-tag as described [14].

2.2. Preparation of LUVETs

Dry films of mixtures of phospholipids were prepared by mixing the appropriate amounts of lipids dissolved in chloroform and evaporation of the solvent under a stream of nitrogen. These films were dried overnight under vacuum to remove all solvent. They were then rehydrated at room temperature in 50 mM Tris–Cl (pH 7.4) to a final concentration of 10 mM phospholipid. The resulting multilamellar vesicles were vortexed and freeze-thawed (10 cycles). Finally, the vesicle suspensions were extruded 10 times through 0.2 μm polycarbonate filters, resulting in the formation of unilamellar vesicles (LUVETs).

2.3. Reconstitution of KcsA

Reconstitution of KcsA in lipid bilayer vesicles was performed as described [14]. Briefly, LUVETs were solubilized with Triton X-100 and mixed with KcsA in 1 mM DDM in 1:150 protein:lipid molar ratio. The detergent was removed using BioBeads, according to the manufacturer's procedure, which resulted in the formation of proteoliposomes. The proteoliposomes were collected by centrifugation for 1 h at 100 krpm at 4°C (TLA 120.2 rotor) and subsequently resuspended in the desired volume of 50 mM Tris–Cl buffer (pH 7.4).

2.4. MS

MS measurements were performed on an electrospray ionization time-of-flight (ESI-ToF) instrument (LC-T; Micromass Ltd., Manchester, UK), operating in positive ion mode and equipped with a Z-spray nano-flow ESI source. Nano-flow ESI capillaries with a relatively large tip opening were prepared from borosilicate glass capillaries (Kwik-Fil™, World Precision Instruments Inc., Sarasota, FL, USA) on a P-97 puller (Sutter Instrument Co., Novato, CA, USA). The capillaries were coated with a thin gold layer (approximately 500 Å) using an Edwards Scancoat sputter-coater 501 (at 40 mV, 1 kV, for 200 s). Basically, three different MS experiments were performed. For measuring the molecular mass of the KcsA monomer, the protein was first extracted from detergent solution with organic solvents, primarily according to [16]. The KcsA solution in 90% formic acid was sprayed using a capillary voltage of 1500 V and a cone and extraction voltage of 50 and 0 V, respectively. When proteoliposome dispersions with reconstituted KcsA at neutral pH were directly introduced into the mass spectrometer (according to [5]), voltages were set to relatively high values to allow detection of protein signals. Typically, the capillary voltage was set to 2800 V, and the cone and extraction cones to 200 and 50 V, respectively. Finally, by mixing these proteoliposome suspensions with the organic solvent 2,2,2-trifluoroethanol (TFE) in a 1:1 or 1:5 (v/v) ratio, protein ions could be observed using more gentle experimental parameters (i.e. capillary voltage 1800 V, cone voltage 80 V; the extraction cone voltage was varied between 0 and 50 V).

3. Results

For characterization of KcsA, first the exact mass of the protein was measured. Fig. 1 shows the mass spectrum of purified KcsA including the His-tag in 90% formic acid. The numbers indicate the charge states. The deconvoluted spectrum (inset) reveals a major peak with a total mass of 19274.33 ± 0.1 Da, which is in agreement with the theoretical value for KcsA of 19274.43 Da and with earlier measurements

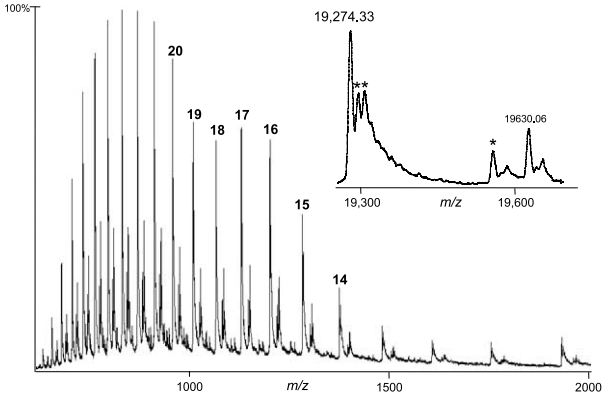

Fig. 1. ESI-ToF mass spectrum of KcsA in 90% formic acid. The deconvoluted spectrum (insert) gives a total mass for KcsA of 19274.33 ± 0.1 Da. The extra peaks indicated by asterisks were only observed when the protein was sprayed from formic acid and may represent formylation products of the protein. The second series of peaks in the spectrum with an average intensity of about one fourth of the KcsA peaks represents a compound with a mass of 19630.06 ± 0.9 Da (see text for details).

[16], indicating that the majority of the protein is not post-translationally modified when overexpressed in *E. coli*. Besides the peak series representing KcsA, there is a series representing a minor compound with a mass of 19630.06 ± 0.9 Da. This compound most likely represents a covalently modified KcsA protein. The identity of this modification (≈355 Da) will be subject of a forthcoming study. Here we focus only on the full-length KcsA protein.

In order to study possible interactions of KcsA with specific phospholipid classes, KcsA was first reconstituted in proteoliposomes of DOPC and DOPG, in which the KcsA tetramer is highly stable. Fig. 2 shows the spectrum of DOPC/DOPG (1:1 molar ratio) vesicles with reconstituted KcsA directly introduced into the mass spectrometer by ESI from an aqueous buffer solution. The major peaks represent phospholipid multimers. In addition, several low-intensity peaks around mass-to-charge ratio (*m*/*z*) 1800 representing charge states of KcsA are clearly visible. Only monomeric KcsA was detected in the mass spectrum, although under the used conditions KcsA is present as a tetramer in the bilayer [17]. A likely explanation is that the settings were too harsh to allow non-covalent interactions to stay intact (see Section 4). Also, no protein–phospholipid complexes could be detected under these conditions.

Because the proteoliposome spraying technique required harsh conditions and because it was difficult to obtain reproducible protein signals of sufficient intensity with this technique, dispersions were mixed with TFE, which is often used as a membrane-mimicking solvent. Spectra of vesicle dispersions with TFE, using more gentle experimental conditions, showed much higher signal-to-noise ratios than obtained for the direct proteoliposome approach (Fig. 3). Nevertheless, again only monomeric KcsA could be detected. However, under these conditions, satellite peaks of KcsA signals were observed, which were assigned to non-covalent complexes of the protein and phospholipid molecules.

Fig. 3 shows a mass spectrum of KcsA, reconstituted in vesicles of DPoPC/DOPG (1:1), with the insert showing the satellite peaks. Comparison of inserts A and B reveals that increasing the extraction cone voltage results in a decrease in

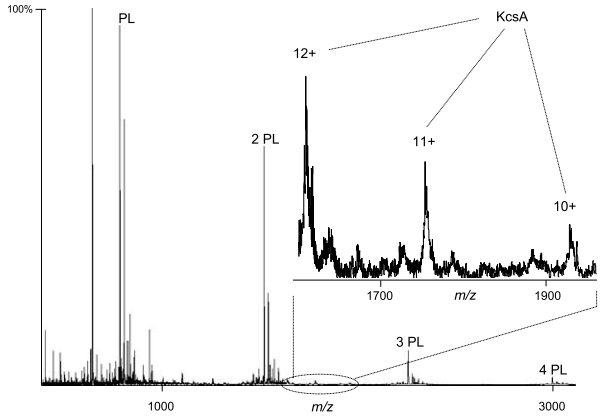

Fig. 2. ESI-ToF mass spectrum of KcsA reconstituted in lipid vesicles (DOPC/DOPG, 1:1) sprayed directly from an aqueous 50 mM Tris–Cl buffer solution (pH 7.4). The most intense phospholipid peaks are from DOPC ions and singly or multiply charged oligomers of DOPC and/or DOPG ions. PC ions are generally better observed than PG ions, since PC is more easily ionized in ESI-MS in the positive mode. KcsA is visible by its 10+, 11+ and 12+ charge states (insert). The intensity of the KcsA peaks is only about 1–2% of that of the base peak in the spectrum, but still the signal-to-noise ratio is substantial.

the relative intensities of these satellite peaks as compared to the KcsA peaks, demonstrating that these satellite peaks indeed represent non-covalent complexes.

The presence of non-covalent protein/lipid complexes in principle allows analysis of preferential binding of specific phospholipid species by KcsA. To discriminate between binding of PC and PG, the combination of DPoPC (molecular mass: 730.1 Da) and DOPG (molecular mass: 774.0 Da) was used to ensure a sufficiently large difference in mass in order to characterize the observed complex and to avoid overlapping peaks in the mass spectrum. A closer look at the KcsA–phospholipid complexes that are formed in these mixtures (Fig. 4) shows that the fine structure of the peaks due to

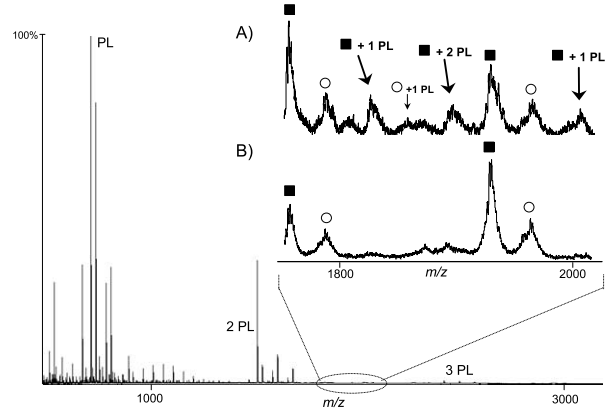

Fig. 3. ESI-ToF mass spectra of KcsA reconstituted vesicles of DPoPC/DOPG sprayed from an aqueous buffer/TFE mixture: effect of the extraction cone voltage on detection of non-covalently bound KcsA–phospholipid complexes. At low extraction cone voltage (A) the charge states 10+ and 11+ of KcsA (black squares), as well as KcsA–phospholipid complexes (arrows) are visible. Increasing the extraction cone voltage results in disappearance of these non-covalently bound complexes (B). The peaks indicated by the open circles represent the modified protein (see Fig. 1).

Na^+ and K^+ adducts is similar to that of the protein peak. Strikingly, only the KcsA–PG complex at *m/z* 1823.6 and not the KcsA–PC complex, which would be at *m/z* 1819.6, is observed, although the protein was reconstituted in vesicles of PC/PG in a 1:1 ratio. Only when KcsA is reconstituted in vesicles enriched in PC (DPoPC/DOPG ratio = 5:1), KcsA–PC complexes were detected, as can be observed in Fig. 4B. Nevertheless, the KcsA–PG complex is also in this case the predominant complex, indicating preferential binding of PG. These results are quantified in Fig. 5.

The lipid specificity of binding to KcsA was also tested in a variety of other lipid mixtures (see Fig. 5). First, to test whether preferential binding was specific for PG only or general for anionic phospholipids, KcsA was reconstituted in vesicles of the anionic lipids DOPA (molecular mass: 698.9 Da) and DPoPC (1:1 ratio). The relative intensities of the KcsA–PA complex were similar to those observed in PG binding experiments and again no PC binding to the protein was observed, suggesting that the preferential binding indeed is general for anionic lipids. Next, in order to test whether the abundant zwitterionic bacterial lipid PE shows a similar low affinity for KcsA as the eukaryotic zwitterionic lipid PC, the protein was reconstituted in vesicles of DOPE (molecular mass: 744.0 Da) and DOPG (1:1 ratio) and analyzed by ESI-MS. In this case, about 90% of the lipid associated to KcsA represented PG. Surprisingly, when KcsA in vesicles containing both DOPA and DOPE was analyzed, it showed a similar affinity towards both of the phospholipids. Since no PC association at all was observed in PC/PG and PC/PA vesicles, these results suggest that the affinity of KcsA for PE is significantly higher than for PC. This could be confirmed by analysis of KcsA reconstituted in mixtures of DEiPC (molecular mass: 842.2 Da) and DOPE (1:1 ratio), which showed that indeed binding of PE is favored over PC. However, some PC binding was also observed. Together, the results suggest that the affinity of KcsA for PE is higher than for PC, but that the affinity for PG is higher than for PE.

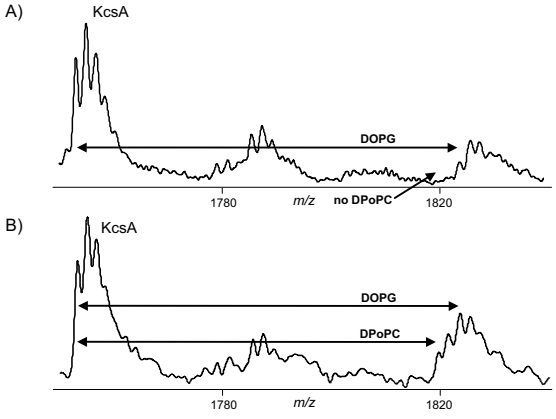

Fig. 4. A: ESI-ToF mass spectra of KcsA reconstituted in equimolar DPoPC/DOPG vesicles sprayed from a buffer/TFE mixture. Only KcsA–DOPG complexes are detected, whereas KcsA–DPoPC complexes are completely absent. A similar peak shape is visible in the KcsA–phospholipid complexes, indicating that only one phospholipid molecule is present in the complex. The extra peaks at the KcsA charge states are due to Na^+ and K^+ adducts. B: When KcsA is reconstituted in vesicles enriched in PC (DPoPC/DOPG, 5:1), some KcsA–DPoPC adducts are visible, although the major adduct complex is represented by KcsA–DOPG.

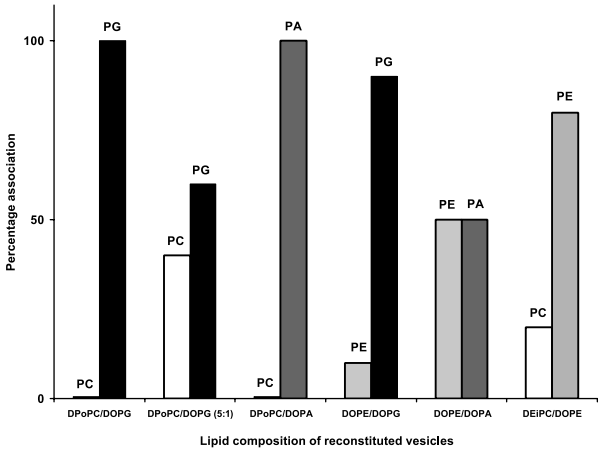

Fig. 5. Quantification of phospholipid binding to KcsA in vesicles composed of the indicated phospholipid mixtures (1:1 ratio, except for the second entry, where the DPoPC/DOPG ratio is 5:1), as derived from KcsA–phospholipid complex peak intensities in the mass spectra. The phospholipid composition of the vesicles is given on the *x*-axis. The sum of the binding percentages for two classes of phospholipids is in every case 100%. The error in the measurements was estimated to be about 10%.

4. Discussion

We show in this paper that ESI-MS can be used to detect non-covalent complexes of KcsA with phospholipid molecules. These complexes were not observed when vesicles with reconstituted KcsA protein were sprayed directly, but could be observed after mixing the vesicle dispersion with TFE. Addition of TFE allowed the analysis of reconstituted vesicles under more gentle mass spectrometric conditions compared to analysis in pure aqueous buffer solutions. Possibly, these bound lipids are those that directly interact with the protein when incorporated in a lipid bilayer. Control experiments with the water-soluble model protein cytochrome *c* showed that protein–lipid complexes cannot be observed when mixtures of lipid vesicles and this protein are analyzed by direct ESI-MS (data not shown). Interestingly, ESI-MS of reconstituted vesicles from pure aqueous buffer solution resulted in the detection of only the monomeric species of KcsA in the mass spectrum, although in lipid bilayer vesicles the protein is solely present as a tetramer, as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis [14]. An explanation for this could be that the high voltages applied during analysis do not allow non-covalent protein–protein interactions to stay intact. On the other hand, SDS–PAGE analysis revealed that in 50% TFE, KcsA runs as a monomer (not shown). The effect of the presence of only TFE, as under ESI-MS conditions, instead of both SDS and TFE, as under SDS–PAGE conditions, is unclear. Nevertheless, it is likely that in 50% TFE also under MS conditions the protein is present as a monomer. However, since TFE, which is often used as a membrane-mimicking solvent, is known to promote α-helix formation [18], also in the monomeric configuration secondary structure and intact lipid binding regions may be retained.

Our data show that the order of preference of KcsA to associate with a phospholipid species was PG$\approx>>$PC. We hypothesize that the preferential binding of KcsA to the phospholipids PG, PA and, to a lesser extent, PE, reflects the order of affinity of KcsA to these lipids in the phospholipid bilayer. The recent observation that PG copurifies with KcsA when isolated from *E. coli* membranes [15], is in agreement with the preferential binding of this lipid. However, we cannot exclude the possibility that this order represents the affinities of association of the lipids to the protein in water/TFE systems. We do not believe that there is a large difference in interaction energies between KcsA and the various phospholipids, as measured by ESI-MS. In that case, significant variations in the cone voltage threshold needed for dissociation of the protein–lipid complex due to variable interaction energies should have been observed for the different lipids, which was clearly not the case in our experiments. However, the affinities as measured by ESI-MS in the gas phase may not be directly related to the affinities in the water phase or in the lipid bilayer. Interestingly, complexes of KcsA and more than one phospholipid molecule were also observed, even though the voltages that had to be applied to achieve sufficient sensitivity were rather high, which normally results in a lower amount of non-covalently bound complexes detected.

For practical reasons, we have used combinations of phospholipids with variable acyl chain lengths. Since the variation in chain length was only small, we assumed that this did not affect the affinity of KcsA for the different classes of lipids. However, in some cases it was possible to use a combination of lipid species with equal chain length, e.g. DOPE and DOPG. The large difference in affinity of KcsA for these lipids suggests that it is indeed the nature of the phospholipid headgroup that governs the interaction of the phospholipid with KcsA. This is supported by preliminary results of an SDS–PAGE assay, which indicate that there is no significant difference in KcsA tetramer stability in bilayers of PC species with acyl chains ranging from $C_{14:1}$ to $C_{20:1}$ (S. Hegger and A. van Dalen, unpublished results), although changes in tilt angle of the α-helices may occur [19].

Our results indicate that KcsA has the highest affinity for anionic lipids. Anionic lipids have been found to be important for integration and of membrane proteins into the membrane in general [20,21] and PG has been suggested to be needed for correct folding of the KcsA monomer [14]. This is in apparent contrast with recent studies using an in vitro folding assay [15]. These studies showed that lipids are required for folding of KcsA from TFE acidified with trifluoroacetic acid (TFA), but there appeared to be no specific requirement for anionic lipids under these conditions. On the other hand, anionic lipids have been shown to be important for several biological functions, including KcsA activity [22], tetramer assembly and stability [14]. Moreover, anionic lipids were copurified with KcsA [15]. Our observation of a preferential interaction of KcsA monomers with anionic lipids correlates well with these latter findings. A possibility to explain the preferred affinity of KcsA for anionic lipids would be that these lipids interact with positively charged residues in the interfacial region. For instance, the N-terminal amphipathic α-helix, which is situated in the membrane–water interface according to a model by Cortes et al. [23], contains several positively charged amino acid residues. Limited proteolysis with trypsin on KcsA reconstituted in lipid vesicles showed that the tryptic peptide containing the N-terminal α-helix associates with the membrane fraction after the vesicles were spun down (data not shown). This demonstrates that the N-terminal part of the protein indeed has a strong interaction with lipids. Alterna-

tively or additionally, the positively charged residues in the KcsA loop region may interact with negatively charged phospholipids [15]. Since we observe lipids bound to the protein when it is presumably in the monomeric conformation, we suggest that these lipids are the lipids bound at the bulk annular sites. The lipids associated with the protein that we observe in our mass spectra are likely to be different from the PG molecule observed in the crystal structure [15], since in that case the lipid seems to be mainly situated in the interfacial region between the protein subunits. Moreover, the mass spectra show complexes of more than one lipid per protein molecule, whereas this was not observed in the crystal structure of the protein–lipid complex.

The preferred interaction of KcsA to PE, compared to PC, may be explained by the capacity of this phospholipid for hydrogen bonding to the protein, which is not possible for PC. Alternatively, PE might preferentially surround KcsA because its relatively small headgroup may result in different and perhaps more favorable packing properties at the protein–lipid interface, as compared to PC. Interestingly, both PG and PE have been found to increase the efficiency of tetramer formation of newly synthesized KcsA in a lipid bilayer as compared to PC alone [14]. Based on the results in this paper, we hypothesize that the roles of PG and PE in KcsA assembly and stability are a consequence of direct, preferential interactions of KcsA with these lipids.

In conclusion, this study shows that the binding affinities of certain classes of phospholipids to an integral membrane protein can be investigated by ESI-MS. The possibility to visualize such interactions could open perspectives for a better understanding of the association of proteins and lipids, which plays an important role in biological membranes. Future challenges will be to improve experimental measurement conditions sufficiently to enable the detection of intact integral membrane protein assemblies (e.g. the tetrameric form of KcsA) and complexes with phospholipids at physiological concentration levels, as well as to avoid the necessity of using organic modifiers in the ESI-MS experiments.

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