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Effects of sodium chloride on membrane fusion and on the formation of aggregates of potassium channel KcsA in Escherichia coli membrane

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Key words: Potassium channel KcsA; Escherichia coli lipid; Sodium chloride; Aggregation; Phosphatidylethanolamine; Phosphatidylglycerol; Phosphatidylcholine; Vesicle fusion

Abstract

In the current study, we found that NaCl induces fast vesicle fusion and aggregation of the bacterial potassium channel KcsA in Escherichia coli (E. coli) membranes. Conventional gel electrophoresis and tryptophan fluorescence experiments were performed to detect NaCl-induced aggregation or supramolecular complexes. Interestingly, in planar lipid bilayer, increasing NaCl concentration dramatically increased the total internal current indicating enhanced vesicle fusion and transfer of high number of channels to the lipid bilayer. Such channels were appeared in clusters of variable size exhibiting higher conductivity, increased open probability and efficient blocking by K⁺-channel blocker tetraethylammonium (TEA). Furthermore, NaCl-induced KcsA aggregation was found to be specific for E. coli membrane as compared to the artificial membrane system. Our preliminary data suggest the role of NaCl in formation of KcsA aggregates as well as in enhancement of membrane fusion capability in E. coli membranes.

1. Introduction

The formation of pathologic ion channels in cellular membranes and other aggregates is preceded by changes in important steps of protein processing. An understanding of the conditions that enhance aggregation is important in many therapeutic strategies [1]. For example, amyloid protein aggregation under physiological conditions is modulated by environmental conditions, such as temperature, pH, and ionic strength. These environmental conditions, e.g., acidic pH values, have been shown to promote the self-assembly of several peptides, elucidating their aggregation behavior under these conditions and the subsequent effects of these aggregates, may be crucial for understanding acute diseases [1].

Many membrane proteins are active as stable oligomers. Physical clustering of ion channels into closely packed assemblies is among the possible consequences of intermolecular interactions, which is often accompanied by coupled channel gating [2-12]. One major strategy for identifying structure-function relationships of ion channels is to reconstitute isolated channel proteins into planar lipid membranes [13, 14]. Vesicles are fused to preformed planar lipid membranes and transfer of channel proteins from vesicles to planar membranes requires the presence of divalent ions like Ca^{2+} , of osmotic gradients across the vesicles and the planar membrane, and of negatively charged lipids [15].

It is also known that di- or tri-valent cations induce fusion of acidic phospholipid vesicles (except for phosphatidylinositol) in either pure or mixed form [16]. Among the neutral phospholipids, phosphatidylcholine (PC) inhibits but phosphatidylethanolamine (PE) sustains or enhances the fusion capacity of acidic phospholipid vesicles [16]. Monovalent cations induce reversible aggregation of negatively charged vesicles, but they inhibit the fusion induced by divalent cations such as Ca^{2+} or Mg^{2+} [16]. Furthermore, NaCl-induced aggregation and membrane fusion was reported in synthetic dioctadecyl-dimethylammonium bromide (DODAB), dioctadecyl dimethylammonium (DODAC), and dihexadecyl phosphate

(DHP) vesicles [17, 18]. Salt-induced vesicle fusion was explained on the basis hydration repulsion in addition to electrostatic repulsion and van der Waals attraction involved in the establishment of short-range interactions.

It is not clear whether monovalent ions induce aggregation and vesicle fusion in natural membranes and if so how does this phenomenon affect the properties of membrane proteins? In the present study, we demonstrate the effect of NaCl in induction of aggregates of bacterial potassium channel KcsA in E. coli membranes. In addition, our data suggest the role of NaCl in enhanced membrane fusion in E. coli membranes.

2. Materials and methods

2.1. Reagents

E. coli total lipid extract, Diphatynoyl phosphatidylcholine (DPhPC), Diphatynoyl phosphatidylethanolamine (DPhPE) and Diphatynoyl phosphatidylglycerol (DPhPG) were purchased from Avanti Polar Lipids Inc. For clarity, the names of these lipids are abbreviated to PC, PE and PG, respectively. n-decyl- β -D-maltoside (DM) and CHAPS were from Fluka. Ni²⁺-NTA agarose beads were obtained from Qiagen. The 100 nm membrane filters were obtained from Avestin Inc. The following chemical reagents were purchased either from Fluka (Switzerland), Merck (Germany) or Aldrich (Germany), available in the highest purity: Tris, 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 pQE60-KcsA in *E. coli* strain BL-21 (DE3). Purification was performed in buffer containing 150 mM KCl, 50 mM Tris (pH 7.5). The solubilized membranes in 40 mM DM were incubated with pre-washed Ni²⁺-NTA agarose beads for 30 min at 4 °C. The bound His-tagged proteins were eluted with 500 mM imidazole pH 7.5 and 10 mM DM. The proteins were purified with a yield of 2-3 mg/l culture. The purity of proteins was assessed by SDS-PAGE. The presence of DM precluded the use of the Bradford assay [19], and therefore the protein concentration was assessed by SDS gel after staining with Coomassie Blue, using a standard of BSA as described previously [20].

2.3. Preparation of liposomes and protein reconstitution

Small unilamellar vesicles (SUVs) were prepared from lipids by extrusion with filters of 100 nm pore diameter [21]. SUV's (10mg/ml) prepared in vesicle buffer (150mM KCl, 10mM KH₂PO₄) at pH 7.0 were solubilised with 35mM CHAPS and mixed with DM

solubilised KcsA proteins at a 1:200 protein: lipid molar ratio. The detergent was removed by dialysis [22]. The reconstituted vesicles were collected by centrifugation (1 h, 40 000 rpm, 4 °C). The proteoliposomes were finally resuspended in vesicle buffer (pH 4.0).

2.4. Analysis of KcsA aggregation by SDS-PAGE

To facilitate detection of NaCl-induced aggregated KcsA in different lipid systems, gel electrophoresis was performed in SDS detergent. Gels were run at 120 V until the blue dye-front reached the edge of the gel. Proteins were detected by staining with Coomassie Brilliant Blue G-250.

2.5. Tryptophan fluorescence and acrylamide quenching

All fluorescence experiments were performed in vesicle buffer (pH 4.0) at room temperature using a Hitachi F-4500 fluorescence spectrometer in a quartz cuvette. For selective excitation of tryptophan fluorescence, the excitation wavelength was fixed at 295 nm (to minimize interference from tyrosine as well as the background signal) and fluorescence emission scans were collected from 300 to 400 nm. The bandwidths for both excitation and emission monochromators were 5 nm and the data were corrected as described previously [20, 23]. All fluorescence experiments were performed at 3 μM protein concentration present in proteoliposomes dissolved in a vesicle buffer. 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 [24]:

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

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. All data were

corrected for inner filter effects due to acrylamide absorbance according to the standard method [24].

2.6. *Analysis of functional reconstitution*

Functional reconstitution of KcsA mutants into a bilayer made from lipids was confirmed by simultaneous measurements of membrane conductance [22]. Briefly, planar lipid bilayer was formed from a solution of lipid (1-2 mg/ml), thus opposing the two monolayers within the aperture in the Teflon septum (150-200 μm diameter) in a homemade Teflon chamber between aqueous bathing solutions of vesicle buffer (pH 4.0). After the bilayers were formed, proteoliposomes were added to one (cis) side of a preformed planar membrane to a final concentration of $\sim 5\mu\text{g/ml}$ in 1-2 mg/ml lipid with gentle mixing. As a control, similar experiments were also performed with planar lipid bilayer without KcsA. All experiments were performed at room temperature.

2.7. *Recording and data analysis*

The cis compartment (voltage command side) was connected to the head stage input, and trans compartment was held at virtual ground via a pair of matched Ag-AgCl reference electrodes which were immersed into the buffer solutions at both sides of the planar bilayers. Under voltage clamp conditions, the membrane current was measured by a patch clamp amplifier (model EPC9; HEKA Electronics). The recording filter was a 4-pole Bessel with 3-dB corner frequency of 0.1 kHz. The acquired raw data were analyzed with the help of the TAC software package (Bruxton Corp., Seattle, WA). A Gaussian filter of 0.3 Hz was applied to reduce noise. The open channel histograms were calculated using TAC and TAC-fit programs (Bruxton Corp.).

3. Results

E. coli is recognized as one of the foremost prokaryotic model organisms; the bacterium has only three main membrane phospholipids which occur frequently in prokaryotic as well as eukaryotic organisms; and the regulation of the lipid composition in cells is brought about by changes in the acyl chain structure, above all in the degree of unsaturation of the acyl chains [25], which is a very common response to changes in the environmental temperature among a variety of organisms [26]. The E. coli inner membrane is composed of ~75% of the zwitterionic phosphatidyl-ethanolamine (PE) and ~20% and 5% of the negatively charged lipids phosphatidylglycerol (PG) and cardiolipin, respectively [27]. A large number of biochemical and physicochemical studies have been carried out on E. coli membranes and membrane lipids [28, 29].

The simplicity of the protein and the ease of expression in *E. coli* make KcsA a good model protein to study channel stability and oligomerization in a particular lipid environment [20]. Herein, we report on salt-induced enhanced fusion of E. coli lipid vesicle containing purified KcsA channel and ultimately KcsA aggregation and compare it to the artificial membranes, i.e., PC:PG (7:3 mol%) or PE:PG (7:3 mol%). We used 30 mol% PG in PC or PE bilayers due to inefficient assembly of KcsA and lack of channel activity in the absence of PG [30, 31]. All experiments were performed in a buffer containing similar but relatively high concentrations of KCl or NaCl which gives the advantage of rendering significant comparison of their effects on KcsA oligomerization and channel activity more straightforward in all experimental systems. We first performed experiments to determine the effect of NaCl on KcsA reconstituted in E. coli membranes and compared to the completely different artificial membrane system PC:PG (7:3 mol%).

3.1. Analysis of NaCl-induced aggregation of KcsA by SDS-PAGE

Because fully assembled KcsA channel is relatively stable in detergent micelles, its oligomeric state is easily assessed using conventional SDS-PAGE. Figure 1 shows the SDS-gel of KcsA reconstituted in E. coli lipid and PC:PG (7:3 mol%). In the presence of 150 mM KCl, the tetramer runs at ~68 kDa for both samples. However, after 100 mM NaCl addition for KcsA reconstituted in E. coli lipid, few bands running at higher molecular weight above the tetrameric fraction can be seen suggesting aggregation (indicated by A) or formation of supramolecular complexes of KcsA. The formation of such complexes has been reported previously [12]. However, such complexes were observed after addition of NaCl which is known to destabilize the tetrameric structure of KcsA [33]. Addition of 100 mM NaCl in PC:PG samples did not induce such complexes as observed for E. coli lipid vesicles. In contrast, a significant amount of monomer running at ~18 kDa was detected indicating that NaCl affected the tetrameric stability of KcsA. These results suggested that NaCl-induced KcsA aggregation might be related to some special properties of E. coli membrane.

3.2. Analysis of channel aggregation by Trp fluorescence

A complicating factor in determining the aggregated state of KcsA from gel electrophoresis experiments is the presence of detergent in the media. As a complementary approach, we therefore also used Trp fluorescence to determine KcsA aggregation in E. coli membranes and compared it to PC:PG (7:3 mol%) lipid bilayers in the presence and absence of 100 mM NaCl in 150 mM KCl containing medium. KcsA contains five tryptophan residues located at the membrane water interfacial regions [34]. The Trp fluorescence emission spectra of KcsA in E. coli lipid vesicles [$\lambda_{\text{ex}} = 295\text{nm}$; $\lambda_{\text{em}} = 328\text{ nm}$ for K^+ , 328 nm after Na^+ addition] and in PC:PG (7:3 mol%) bilayer [$\lambda_{\text{ex}} = 295\text{nm}$; $\lambda_{\text{em}} = 328\text{ nm}$ for 150 mM KCl, 329 nm after 100 mM NaCl addition] are shown in Figure 2A and B, respectively. KcsA exhibits a

typical emission maximum as shown previously [34]. After 100 mM NaCl addition, increase in fluorescence intensity was observed (Fig. 2A) probably due to Trp shielding. In contrast, a slight decrease in fluorescence intensity followed by 1 nm red-shift in maximum was observed for KcsA-PC:PG complex indicating Trp exposure to a slightly hydrophilic environment. The data suggest distinct conformational changes in the protein as a function of different lipid systems.

3.3. Effect of KcsA aggregation on Trp accessibility

The observed changes in fluorescence intensity and shifts in emission maxima suggested several distinct conformational changes in KcsA. 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 [24, 35]. In addition, no charge interaction takes place between this quencher and the negatively charged residues and lipid head groups [24, 35]. The Stern–Volmer quenching plots of KcsA reconstituted in *E. coli* lipid or PC:PG lipid bilayers in which F_0/F is plotted against the acrylamide concentration were linear and are shown in Fig. 2C and D, respectively. The Stern–Volmer constants representing acrylamide accessibility are compiled in Table 1.

In *E. coli* membranes (Fig. 2C), significantly less accessibility to the quencher was observed after 100 mM NaCl addition as compared to K^+ containing sample. This effect again indicated significant protection of Trp residues most probably due to the formation of aggregates or supramolecular complexes as detected by SDS-PAGE. In PC:PG bilayers (Fig. 2D), no significant change in acrylamide accessibility was observed after NaCl addition. The data indicate that NaCl induces aggregation of KcsA in *E. coli* membrane via distinct conformational changes in the protein.

3.4. *Single channel properties and Na⁺ blocking of KcsA in E. coli membranes*

As a next step, we investigated the effect of Na⁺ on KcsA channel activity in *E. coli* membranes. All experiments were performed at pH 4.0 since KcsA opens at pH 4.0 and closes at pH 7.0 [36-38]. In symmetrical 150mM KCl solution KcsA exhibited outward channel rectification as reported previously [36-38]. Fig. 3A (0 mM Na⁺) shows a representative single channel recording of KcsA at +100 mV in which main current level of ~9 pA (equivalent to 90 pS at this voltage) was observed. Fig. 3B represents an open channel histogram exhibiting low open probability of ~0.2. At -100mV (the corresponding reversal potential), the channel of the 5 pA current was appeared. These results are in accordance to KcsA channel properties as shown in previous studies [36, 37].

Blockade by Na⁺ is considered a hallmark of potassium channels [33, 38]. We first checked if low Na⁺ has a blocking effect on KcsA channel activity as shown previously [37]. Upon addition of symmetrical 10 mM Na⁺, single channel current was reduced to 7.5 pA (~75 pS at +100 mV) and open probability (Po) of ~0.1 was calculated (Fig. 3A and B). At -100mV, the channel current was also reduced to 2.5 pA. The data indicate that Na⁺ has a significant blocking effect on KcsA [38] which might differ when KcsA is reconstituted in different conditions [39-41].

3.5. *Formation of NaCl-induced aggregated conductive complexes of KcsA in E. coli membranes*

In principle, decrease in channel current would be expected to be more successive with increasing NaCl concentration. This was not observed. Upon increasing NaCl concentration (50 mM), KcsA exhibited increased channel conductivity and high open probability (HOP). Fig. 4A, upper panel, shows a representative recording from KcsA containing bilayer in the presence of 50 mM NaCl at +100 mV. Most strikingly, after ~80s (patch 1), the total internal

current was increased and multiple channels were recorded. For instance, the total internal current increased to ~55 pA (patch 2) and later several other current levels were recorded (patch 4 and 5). Upon analysis of patch 4, main current level of ~8 pA was observed. Furthermore, an open probability of ~0.5 was calculated as shown in open channel histogram (Fig. 4B, right-panel). Upon applying -100 mV (Fig. 4A, lower panel), the current level increased after ~50 s. Patch 1 exhibited channels of HOP patterns which was followed by the opening of patch 2 and 3 consisting of variable channels. Upon analysis of patch 3, several 'subconductance' current levels were calculated. The ~8 pA current (Fig. 4C, level II) was the most frequently observed event. In addition, larger channels of the ~22 and ~25-pA current (levels III and IV, respectively) were also appeared. The data indicate that Na⁺ induces fast vesicle fusion to the planar lipid bilayer resulting in increase in total internal current of KcsA.

Increasing NaCl concentration to 100 mM resulted in double increase in magnitude of total internal current. Fig. 5A, upper panel, shows in detail a representative long recording taken at +100 mV. Similar patterns were recorded as observed for the effect of 50 mM NaCl. For instance, the current increased to ~110 pA (patch 1) and after few seconds several different current levels were recorded (patch 2, 3 and 4) due to increase in number of channels to the planar lipid bilayer as a result of vesicle fusion. Upon analysis of patch 2, main current level of ~16 pA (equivalent to ~160 pS at this voltage) with open probability of ~0.8 was observed (Fig. 5B, right panel). Two detectable current levels were observed at -100 mV (patch 1). A closer view of current levels (Fig. 5C) reveal that, indeed, ~9 pA current (level I) was the most frequently observed events and, as far as instrumental resolution permitted, another single channel-like openings (~27-pA, level II) also appeared. These patterns exhibited fast gating kinetics and were stable for several hours at room temperature. Increase in total internal current, increased channel conductivity and high open probability clearly indicate that NaCl concentration has a drastic effect on vesicle fusion as well as on channel's

gating characteristics most likely due to the formation of large aggregates of KcsA in the membrane. Disregarding the differences in the open channel probability, this is reminiscent of coupled gating and clustering/aggregation of KcsA as reported previously [12]. However, this is interesting and surprising that such changes were observed after high NaCl addition. Furthermore, experiments performed in buffer containing Na⁺ as the only ion species (in the absence of K⁺) resulted in lack of any channel current (not shown) indicating that active channels (in the presence of K⁺) are required for salt-induced aggregation and vesicle fusion.

3.6. *Effect of TEA on the activity of NaCl-induced aggregated complexes of KcsA in E. coli membranes*

We assumed that NaCl-induced membrane fusion and KcsA aggregation might alter the function of KcsA when it is assembled in large clusters. As a unique tendency KcsA blocking by TEA [36], we next performed TEA blocking experiments. After formation of large conductive complexes/aggregates at 100mM NaCl, 10mM TEA was added on cis-side of the chamber. Fig. 6 shows a long recording taken at +100mV (upper panel) and -100mV (lower panel) in which the magnitude of total internal current decreased and upon analysis of these traces no defined channels were detected. The data clearly indicate that, although, NaCl promotes KcsA aggregation/clusterization, it still does not interfere with TEA binding mode of KcsA.

3.7. *Effect of Na⁺ on KcsA channel activity in PC:PG lipid bilayers*

We next determined whether NaCl-induced vesicle fusion and KcsA aggregation is lipid specific. Therefore, similar experiments were performed for KcsA reconstituted in a mixture of bilayer forming lipids PC:PG (7:3 mol%). Fig. 7 shows series of records of KcsA in the absence and in the presence of increasing concentrations of Na⁺ at +100mV. In symmetrical

150 mM K^+ solution at pH 4.0, KcsA exhibited outward channel rectification as reported previously [36-38]. The main current level of ~ 9.5 pA (equivalent to 95 pS at this voltage) was observed. In addition, small channels of ~ 3 pA were also appeared indicating the insertion of the channel orientated in opposite direction. Upon addition of symmetrical 10 mM Na^+ , single channel current was reduced to only 3.5 pA (~ 35 pS). Addition of 50 and 100 mM Na^+ completely blocked the channels and no significant currents could be recorded. The data indicate that Na^+ blocks the activity of KcsA channel in PC:PG membranes.

3.8. *Effect of Na^+ on KcsA oligomerization and channel activity in PE:PG lipid bilayers*

PE is one of the major constituents of E. coli membranes [42]. The small size of the PE head group facilitates the initial interfacial insertion of the bacterial potassium channel KcsA, similar as was postulated for the interfacial insertion of the catalytic domain of leader peptidase [43]. Remarkably, PE seems to be the most effective lipid in stimulating tetramerization most probably due to the non-bilayer property of PE [30, 44]. To determine whether Na^+ -induced aggregation is related to PE we next performed experiments in PE:PG (7:3 mol%) bilayers.

Figure 8A shows the SDS-gel of KcsA reconstituted in PE:PG (7:3 mol%) in the presence of 150 mM KCl and after 100 mM NaCl addition (indicated as $K^+ + Na^+$). Surprisingly, addition of 100 mM Na^+ did not affect the oligomerization state of KcsA as observed in E. coli membranes. Furthermore, no significant change in acrylamide accessibility was observed after 100 mM Na^+ addition (see Table 1). In symmetrical 150 mM K^+ solution at pH 4.0, again, KcsA exhibited outward channel rectification (Fig. 8B). The main current level of 8 pA (equivalent to 80 pS at 100 mV) was observed. Addition of symmetrical 10 mM Na^+ reduced the single channel current to 5.6 pA (~ 56 pS at this voltage). Increasing concentration of Na^+ had a drastic effect on channel activity (3.3 and 1.8 pA at 50

and 100 mM Na⁺, respectively). Preliminary experiments performed with mixtures of 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) and phosphatidylglycerol (POPG) also showed similar Na⁺-dependent channel blocking (not shown) as reported previously [37, 38]. The data indicate that unlike in E. coli membrane Na⁺ blocks the activity of KcsA both in PC:PG and PE:PG lipid system. Hence, the effects observed for E. coli lipid system are unique.

4. Conclusions

Although, KcsA clusterization and Na⁺ conductance through HOP patterns of KcsA has been reported previously [12], the finding that KcsA, one of the structurally simplest ion channels known to date, shows such a NaCl-induced aggregation behavior 'in vitro' is fascinating. In KcsA, polyphosphates and polyhydroxy-butyrate, which are abundant reservoir materials in prokaryotic cells, have been reported to interact with KcsA to form conductive complexes [45, 46]. Therefore, these or similar compounds, such as prokaryotic alternatives to PDZ-domains or other anchoring proteins observed in the clustering of other channels [2, 8, 47-49], provide an evidence for existence of potential cluster-inducing or cluster-stabilizing molecules. Our data provide for the first time NaCl-induced aggregation of KcsA and fast vesicle fusion of 'aggregated' KcsA containing vesicles in E. coli lipid system which is unique as compared to the artificial membrane system.

Upon combining our data, it seems most likely that Na⁺ plays a special role in inducing vesicle fusion and ultimately aggregation of either channel itself or channel-lipid complex in a natural membrane system like in E. coli membranes. Schematic model of protein aggregation and fast vesicle fusion was derived (Fig. 9) in which the fast vesicle fusion takes place in the presence of high Na⁺ (panel B) as compared to the Na⁺ free solution (panel A). Upon vesicle

fusion, aggregated KcsA channels are incorporated into planar lipid bilayer which function as large conductive complexes of different integrated behavior.

Negatively charged substrate could play an important role in the early stages of protein aggregation via electrostatic interactions [1]. These interactions may disturb the lipid-water interphase of the membrane [1]. KcsA contains several positively charged residues which directly interact with negatively charged lipid head groups via electrostatic interactions [20, 34]. Because monovalent ions are also known to interact with membranes [16, 48] they seem to interfere with electrostatic interactions particularly in E. coli membranes and might also be responsible for KcsA aggregation (see Fig. 9B).

We would like to provide the generalized mechanism of salt-induced fusion in E. coli membranes. We propose that hydration repulsion is the limiting barrier for fast fusion process as described for synthetic membranes [17, 18]. It seems most likely that NaCl affects the surrounding hydration water thus making E. coli membrane less bulky. In other words, the hydration repulsive force due to water structure might be lower for E. coli membrane than artificial lipid vesicles which might then lead to protein aggregation and further fusion of vesicles into large aggregates.

Additional experiments are still required to get more insight into salt-induced membrane fusion and protein aggregation. The data demonstrate that the role of monovalent ions in vesicle fusion and organizing the membrane protein and the role of the native membrane itself as a buffer for ions thus far has been underestimated.

Abbreviations:

CHAPS; 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, SDS-PAGE; sodium dodecylsulphate–polyacrylamide gel electrophoresis, BSA; bovine serum albumin, Trp; tryptophan

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Table 1

Comparison of the effect of K^+ and Na^+ addition on acrylamide accessibility of KcsA in different lipid systems

Ionic condition ^a	Lipid	$K_{SV} (M^{-1})^b$
K^+	<i>E. coli</i> lipid	5 ± 0.8
	PC:PG (7:3)	7.5 ± 1.1
	PE:PG (7:3)	6 ± 0.8
$K^+ + Na^+$	<i>E. coli</i> lipid	2.6 ± 1.6
	PC:PG (7:3)	7.1 ± 0.5
	PE:PG (7:3)	5.5 ± 0.7

^aThe ionic strength was kept as 150 mM for K^+ and 100 mM for Na^+ .

^bThe Stern-Volmer quenching constants were determined from the slopes of the lines of $F_0/F = 1 + K_{SV} [Q]$ as shown in Fig. 2C and D. The quenching plots of PE:PG are not shown. Values are the means \pm S.D. of three experiments.

Figure legends

Fig. 1. SDS-PAGE (11% gel) showing the effect of 150 mM KCl, and 100 mM NaCl addition on KcsA oligomerization in E. coli and PC:PG (7:3 mol%) lipid bilayers. The positions of tetramer (T), monomer (M) and aggregated/supramolecular complexes (A) are indicated.

Fig. 2. Effect of 100 mM NaCl on Trp fluorescence emission spectra of KcsA reconstituted in E. coli (A) and PC:PG (B) membranes. Corrected fluorescence emission spectra of 3 μ M of KcsA in the presence of 150 mM KCl (solid line) and after 100 mM NaCl addition (dash line) are shown. *B*, Stern–Volmer plots of Trp fluorescence quenching by acrylamide in E. coli (C) or PC:PG (D) lipid membranes. The data points show the mean values of three independent experiments. The slopes of the best fit linear regression lines for each data set (K_{SV} values) are shown in Table 1. a.u. = arbitrary units.

Fig. 3. (A) Representative KcsA single channel recording and (B) detailed histogram of the recording showing the open probability of the channel in 150 mM K⁺ (0mM Na⁺) and after addition of 10mM Na⁺ in E. coli lipid bilayer.

Fig. 4. (A) Induction of vesicle fusion in E. coli membranes and aggregation of KcsA after 50mM NaCl addition. Representative whole records after applying +100 and -100mV voltages are shown representing an increase in total internal current after Na⁺ addition. Several patterns are indicated by numbers. (B) Close-view of patch 4 from multiple channel patterns with detailed histogram of the recording showing the open probability of patch 4 at +100mV. (C) Close-view of patch 3 from multiple channel patterns with detailed histogram of the recording showing the open probability of patch 3 at -100mV. Traces were analyzed to determine single channel current (pA) and open probability (P_o). The closed state is indicated by '0'.

Fig. 5. (A) Induction of vesicle fusion E. coli membranes and aggregation of KcsA after 100mM NaCl addition. Representative whole records after applying +100 and -100mV

voltages are shown representing an increase in total internal current after Na^+ addition. Several patterns are indicated by numbers. (B and C) Close-views of patch 2 at +100 mV and patch 1 at -100mV, respectively. Traces were analyzed as described in the figure legend 4.

Fig. 6. Effect of TEA on NaCl-induced vesicle fusion and KcsA aggregation in E. coli membranes. After 100 mM Na^+ addition TEA was added on *cis* side of the membrane. Traces were further analyzed to determine single channel current as shown in upright sub panels.

Fig. 7. (A) Representative single channel recording of KcsA reconstituted in PC:PG bilayers in the presence of 150 mM K^+ (0mM Na^+) and after addition of 10, 50 and 100 mM Na^+ . Single channel currents are described in the section 3.7.

Fig. 8. (A) SDS-PAGE (11% gel) showing KcsA tetramer in the presence of 150mM KCl, and after 100mM NaCl addition in PE:PG (7:3 mol%) lipid bilayers. (B) Representative single channel recording of KcsA reconstituted in PE:PG bilayers in the presence of 150 mM K^+ (0mM Na^+) and after addition of 10, 50 and 100 mM Na^+ . Single channel currents are described in the section 3.8.

Fig. 9. Schematic model of Na^+ -induced KcsA aggregation and transfer of aggregated channels via fast vesicle fusion in planar lipid bilayer formed from E. coli membranes. The red arrow indicates combination or "fusion" of vesicles with protein to a preformed lipid bilayer. For comparison, models of vesicle fusion and channel transfer in the absence of Na^+ (only K^+ species) (A) and KcsA aggregation after Na^+ addition (B) are shown. Each tetramer is represented as a single green subunit in the membrane. Green and red headgroups denote zwitterionic and negatively charged lipids, respectively. The accumulation or aggregation of charged lipids (green headgroups) around KcsA upon Na^+ addition has also been deduced.

Figure 1

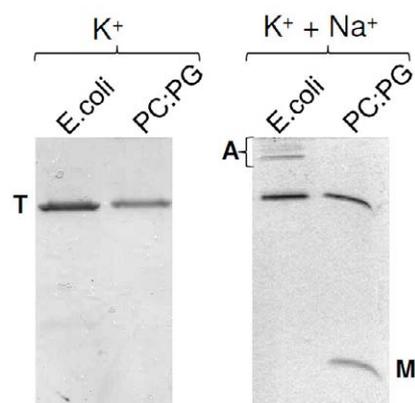


Figure 2

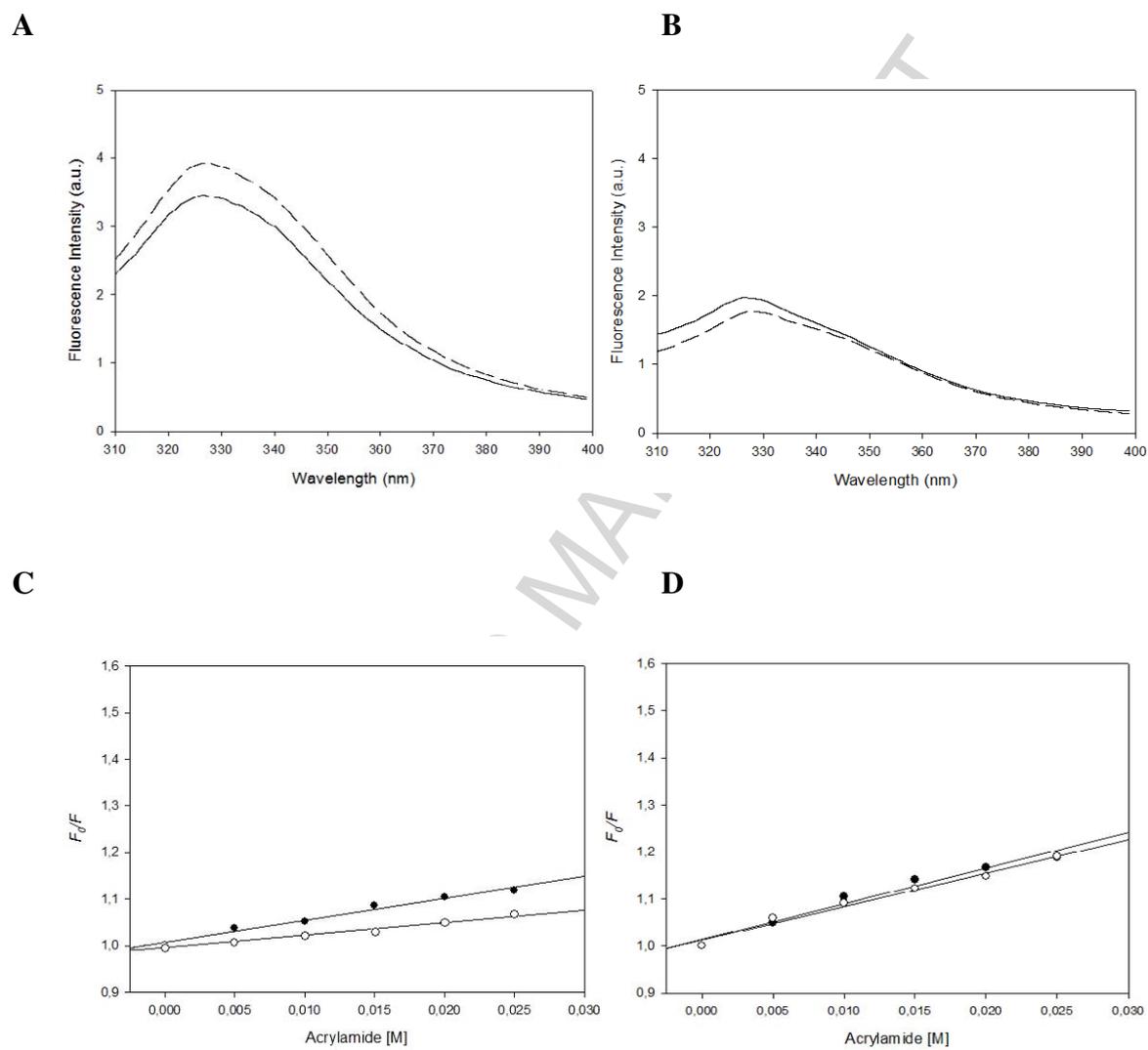


Figure 3

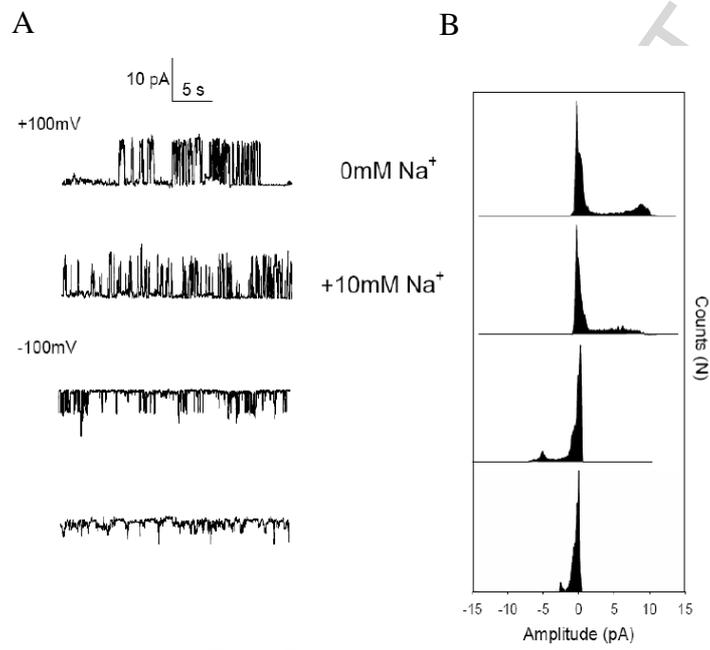
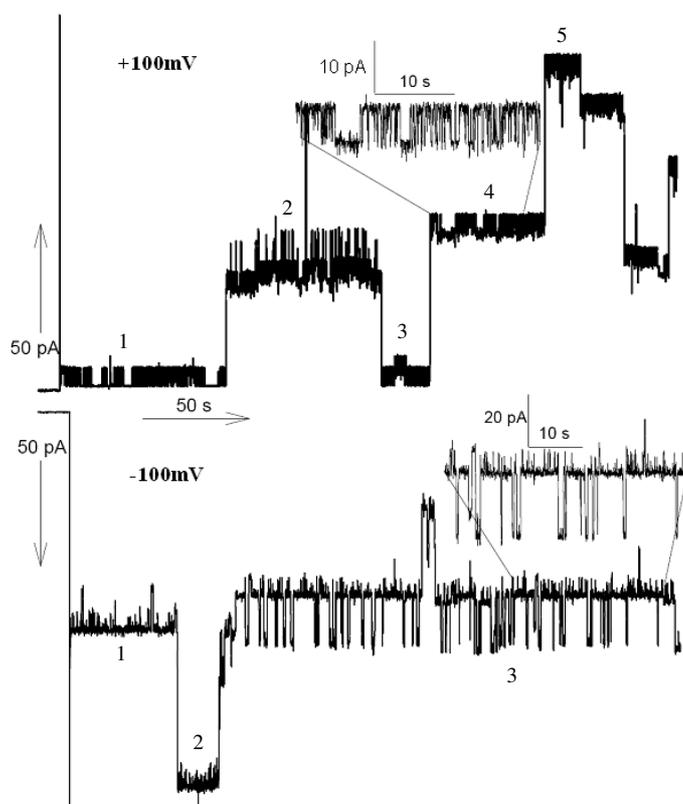
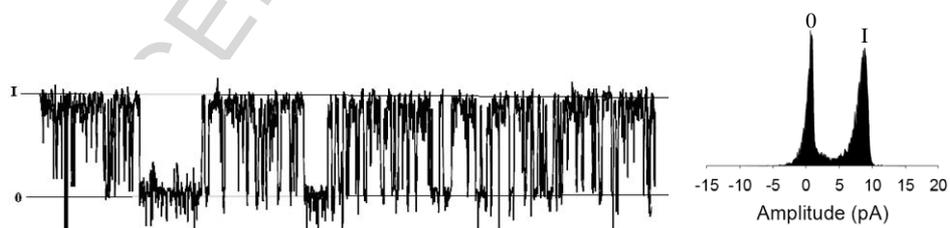


Figure 4

A



B



C

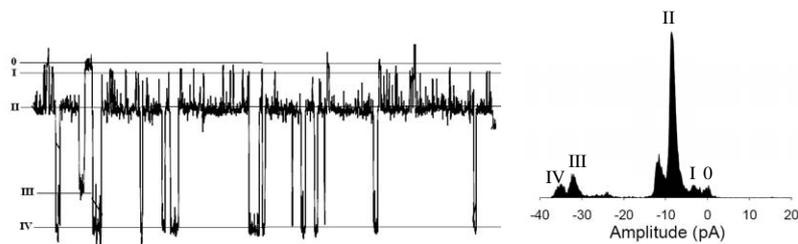
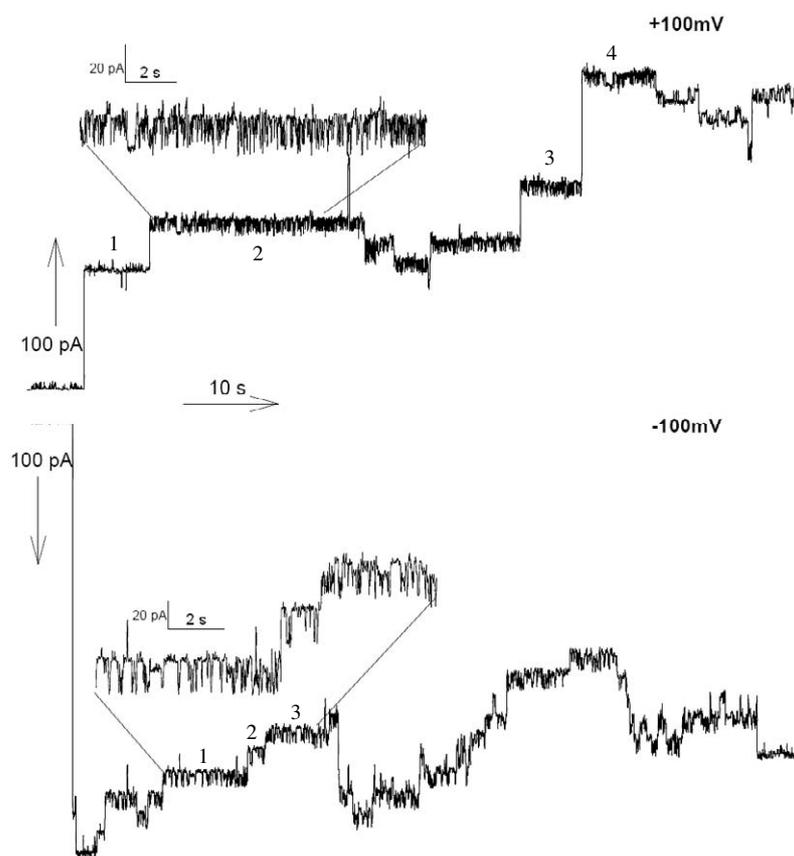
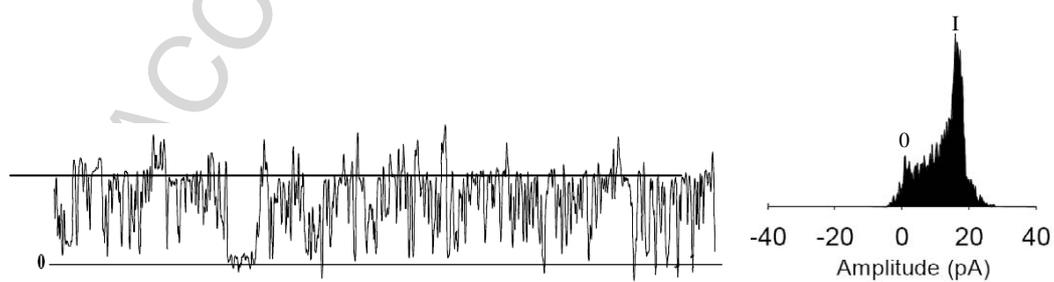


Figure 5

A



B



C

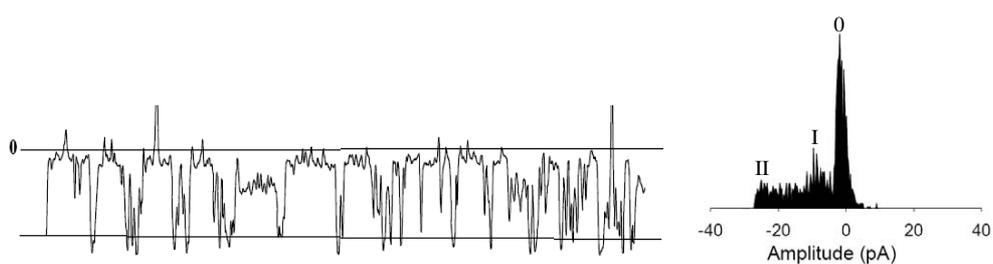
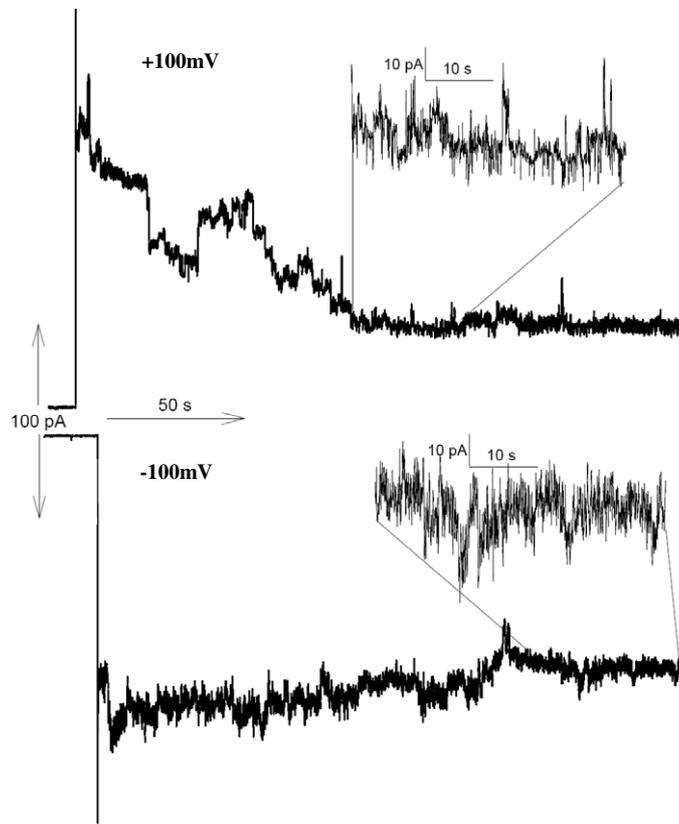


Figure 6



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Figure 7

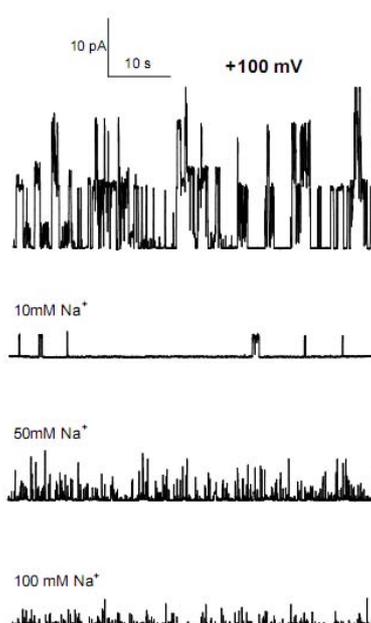


Figure 8

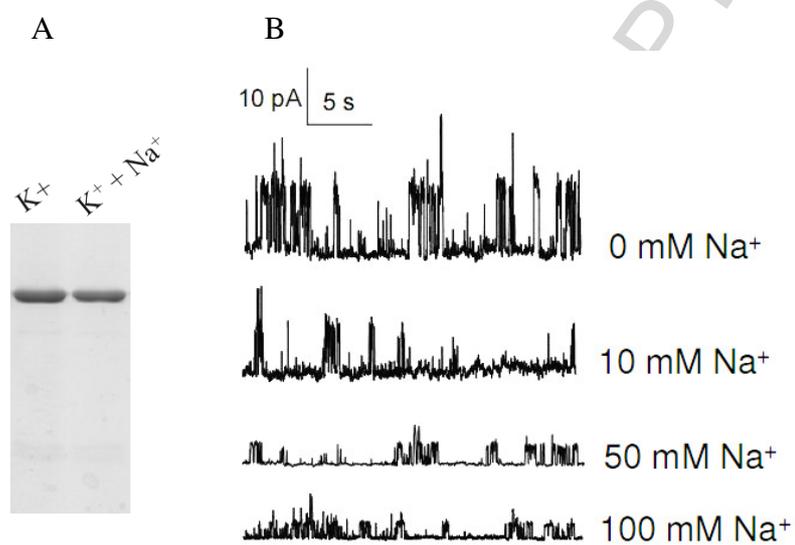


Figure 9

