Biochimica et Biophysica Acta 1788 (2009) 608–614

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Role of polyphosphate in regulation of the *Streptomyces lividans* KcsA channel

Alexander Negoda, Elena Negoda, Mo Xian, Rosetta N. Reusch *

*Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA*

ARTICLE INFO

*Article history:*
Received 6 August 2008
Received in revised form 1 October 2008
Accepted 29 December 2008
Available online 10 January 2009

*Keywords:*
*Streptomyces lividans*
KcsA
Inorganic polyphosphate
Ion channel
Selectivity

ABSTRACT

We examine the hypotheses that the *Streptomyces lividans* potassium channel KcsA is gated at neutral pH by the electrochemical potential, and that its selectivity and conductance are governed at the cytoplasmic face by interactions between the KcsA polypeptides and a core molecule of inorganic polyphosphate (polyP). The four polypeptides of KcsA are postulated to surround the end unit of the polyP molecule with a collar of eight arginines, thereby modulating the negative charge of the polyP end unit and increasing its preference for binding monovalent cations. Here we show that KcsA channels can be activated in planar lipid bilayers at pH 7.4 by the chemical potential alone. Moreover, one or both of the C-terminal arginines are replaced with residues of progressively lower basicity–lysine, histidine, valine, asparagine–and the effects of these mutations on conductance and selectivity for $K^+$ over $Mg^{2+}$ is tested in planar bilayers as a function of $Mg^{2+}$ concentration and pH. As the basicity of the C-terminal residues decreases, $Mg^{2+}$ block increases, and $Mg^{2+}$ becomes permeant when medium pH is greater than the pI of the C-terminal residues. The results uphold the premise that polyP and the C-terminal arginines are decisive elements in KcsA channel regulation.



## 1. Introduction

The structure of the membrane portion of the *Streptomyces lividans* potassium channel, KcsA has been determined by X-ray crystallography [1,2], and the arrangement of the residues in the full channel (Fig. 1A) has been defined by site-directed spin labeling and electron paramagnetic resonance spectroscopy [3,4], nuclear magnetic resonance spectroscopy [5], neutron and X-ray small-angle solution scattering [6]; however, despite extensive structural and functional studies, the manner by which the channel selects for and transports $K^+$ is still unresolved.

Initial functional studies of KcsA showed significant channel activity in neutral solutions, first in planar lipid bilayers between $K^+$ gradients at pH 7.2 by [7] and then in liposome flux assays at pH 7.4 by [8]. Subsequent planar lipid bilayer studies were conducted with symmetric $K^+$ solutions, and in this case a strong outward proton gradient was required to produce brief and sporadic channel activity [9,10]. Zakharian and Reusch [11] showed that a low intracellular pH is not required for channel activation at neutral pH when the outward $K^+$ gradient is>2:1, and both conductance and open time increase proportionately with increase in $K^+$ gradient size. The role of the $K^+$ gradient in activation of KcsA channels at physiological pH is further examined in this study.

The composition of the channel is also still unresolved. Reusch [12] reported that KcsA polypeptides are modified by short polymers of R-3-hydroxybutyrate (PHB) while the KcsA tetramer also contains inorganic polyphosphate (polyP). The presence of PHB in KcsA monomers and tetramers was revealed by chemical and Western blot assays, and the presence of polyP in KcsA tetramers by reaction to o-toluidine blue stain on SDS-PAGE gels and by an enzymatic assay. A large difference between the high theoretical pI of 10.3 of the monomer and the near neutral experimental pI of the tetramer provided further evidence of the presence of the polyanion polyP. A relationship between KcsA and polyP was also demonstrated by Hegermann et al. [13] in studies in which energy-filtered electron microscopy (EFTEM) and lead sulfide precipitation were used to visualize polyP in *S. lividans* cells. Structured polyP precipitates were observed at the inner face of the cytoplasmic membrane and extending ~50 Å into the cytoplasm of wild-type *S. lividans* cells but were absent in KcsA-minus mutant cells.

PolyP, a flexible chain of tetrahedral phosphate ions, creates a framework for cation transport This polyanion also has cation-selecting properties [14]. The $pK_1$ of polyP is ~2, thus the chain phosphates have a monovalent negative charge, but the $pK_2$ of polyP is ~6.8, thus the end units have a divalent negative charge above $pK_2$ and consequently a preference for divalent cations, but lose this preference when pH is lowered below $pK_2$. Accordingly, channels formed by polyP complexes with poly-(R)-3-hydroxybutyrate in planar lipid bilayers are highly selective for divalent $Ca^{2+}$ over monovalent $Na^+$ at pH 7.4 [15], but display a preference for $Na^+$ over $Ca^{2+}$ at acidic pH [16]. Since cytoplasmic pH of *S. lividans* is consistently>7, a premise of this study is that KcsA polypeptides moderate the charge of the end polyP unit by surrounding it with a collar of arginine residues (Fig. 1B). Zakharian and Reusch [17] provided functional evidence for this hypothesis, and recently Negoda et al [18] showed that mutating the C-terminal

* Corresponding author. 6163 Biomedical Physical Sciences Building, Michigan State University, East Lansing, MI 48823, USA. Tel.: +517 884 5388; fax: +1 517 353 8957.
*E-mail address:* rnreusch@msu.edu (R.N. Reusch).

0005-2736/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.12.017

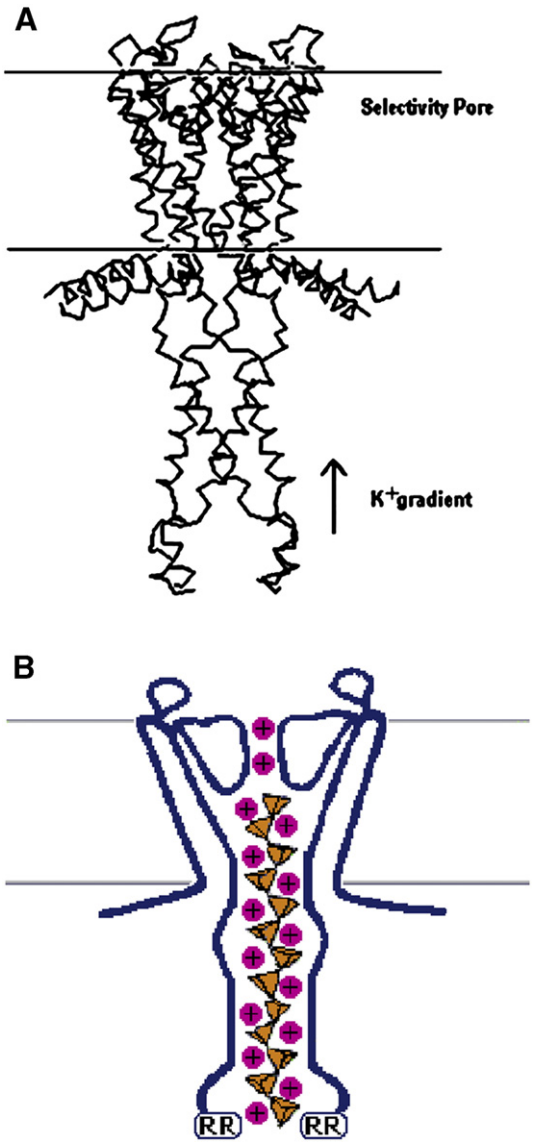


**Fig. 1.** (A) Molecular architecture of full-length KcsA (from Cortes et al. 2001). (B) Schematic diagram of a cross-section of KcsA showing the postulated arrangement of polyP (tan tetrahedra) and $K^+$ (pink circles) within KcsA. RR–C-terminal arginines. PHB is postulated to line the channel vestibule but is not shown for clarity.

arginines to neutral residues altered the selectivity of KcsA from $K^+$ to $Mg^{2+}$. Here we further test the hypothesis by gradually reducing the basicity of the C-terminal residues and observing the effects on KcsA channel selectivity and conductance.

## 2. Materials and methods

### 2.1. Preparation of KcsA mutants

Wild type KcsA, His-tagged at the C-terminal and cloned into pQE60, was a gift from C. Miller. All mutants were made using the Quik-change Site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

### 2.2. Purification of S. lividans KcsA wild-type and mutants

pQE60 plasmids were transformed into *E. coli* BL21 (Novagen), overexpressed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM (Calbiochem) and purified by Ni-affinity chromatography as previously described [11,17]. The proteins when unheated formed single bands at MW ~65 kDa on SDS-PAGE gels, corresponding to the tetrameric form, and they were converted to the monomeric form, ~19 kDa, when heated in 2% SDS. Protein concentrations were determined using the detergent-compatible (DC) assay (Bio-Rad).

### 2.3. Assays for polyP and PHB

PolyP was determined by measuring phosphate with ammonium molybdate, Malachite green reagent before and after hydrolysis in 1N HCl for 15 min in a boiling water bath using $polyP_{15}$ (Sigma) as standard, and by DAPI fluorescence assay [19] using $polyP_{15}$ (Sigma) as standard and turkey egg albumin (Sigma) as protein control. PHB was determined by a chemical assay as previously described [12] in which PHB is converted to crotonic acid by heating in concentrated sulfuric acid. PHB was also converted to its monomer, R-3-hydroxybutyrate (R-3HB), by hydrolysis in 1N NaOH at 60 °C for 30 min. R-3HB was then determined by enzymatic assay (Pointe Scientific) using R-3-HB dehydrogenase in the presence of NAD at 37 °C to form acetoacetate and NADH. The NADH was converted to a colored product using 2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyl Tetrazolium (INT) and diaphorase, and the absorbance at 505 nm was read in a Shimadzu Bio Spec-1601 Spectrophotometer. Standards were PHB (Sigma) and R-3-hydroxybutyrate (Sigma).

### 2.4. Planar lipid bilayer measurements

The KcsA tetramer was reconstituted into liposomes by incubation at 42 °C in a micellar solution composed of a mixture of synthetic 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC), synthetic 1-palmitoyl, 2-oleoyl phosphatidylethanolamine (POPE), and synthetic 1-palmitoyl,2-oleoyl phosphatidylglycerol (POPG) (3:3:1) (Avanti Polar Lipids), for periods of 1–2 h. Lower temperatures required longer incubation times. Planar lipid bilayers were formed from a solution of POPC:POPE:POPG (3:3:1) in n-decane (Aldrich). The lipid solution was used to paint a bilayer on an aperture of ~150 μm diameter in a Delrin cup (Warner Instruments, Hamden CT) between aqueous bathing solutions as described in Results. All salts were ultrapure (>99%) (Aldrich). Bilayer capacitances were in the range of 25–50 pF.

### 2.5. Recording and data analysis

Unitary currents were recorded with an integrating patch-clamp amplifier (Axopatch 200A, Axon Instr.). The *cis* compartment (voltage command side) was connected to the CV 201A head stage input, and *trans* compartment was held at virtual ground via a pair of matched Ag–AgCl electrodes connected to the solutions by an agar bridge containing 3 M KCl. Currents through the voltage-clamped bilayers (background conductance <1–2 pS) were low-pass-filtered at 10 kHz (−3 dB cutoff, Bessel type response) and recorded after digitization through an analog-to-digital converter (Digidata 1322A, Axon Instr.).

Data were filtered through an eight-pole Bessel filter (902LPF, frequency devices) and digitized at 1 kHz using pClamp9 software (Axon Instruments). Singe-channel conductance events were identified automatically and analyzed by using Clampfit9 software (Axon Instruments). The data for each experiment were averaged from>20 independent recordings. The concentration gradient was created and the junction potential offset was compensated before membrane painting. Nernst potentials were calculated using ion activities. Permeability ratios were determined from the Goldman Hodgkin–Katz voltage equation [20].

## 3. Results

### 3.1. Activation of KcsA by $K^+$ gradient at zero voltage

Previous studies showed that KcsA channels in planar lipid bilayers between symmetric solutions of KCl at neutral pH cannot be activated by voltage alone [9–11]. Application of voltages up to 200 mV has no effect; larger potentials cannot be tested since they disrupt the bilayer. However, Zakharian and Reusch showed that the channels are readily activated by applied voltages of ≤100 mV when the $K^+$ gradient is >2:1 [11], indicating the important contribution of the $K^+$ chemical gradient to the electrochemical potential at neutral pH. As the $K^+$ chemical gradient ($[K^+]_{in}/[K^+]_{ex}$) was increased from 200 mM/80 mM to 200 mM/10 mM, conductance at +80 mV increased from 2.5 to 24 pS and open time increased from 0.4 to 0.9. Here we examine the activation of KcsA channels in planar lipid bilayers at pH 7.4 by the $K^+$ chemical potential alone, i.e. at 0 mV.

Purified wild-type KcsA was reconstituted and incorporated into lipid bilayers of synthetic POPC:POPE:POPG (3:3:1) between gradient solutions of KCl, symmetric 2 mM $MgCl_2$, 20 mM Hepes, pH 7.4 at 30 °C. Channel activity was observed at zero voltage when KCl gradients were 5:1 or greater, and there was an increase in current magnitude and open time with increase in gradient size (Fig. 2). The results indicate that a $K^+$ chemical gradient is itself sufficient for the transport of $K^+$ through the KcsA channel at pH 7.4.

### 3.2. Influence of C-terminal residues on KcsA selectivity

In a previous study [18], KcsA mutants in which C-terminal arginines were changed to asparagines were shown to be permeant to $Mg^{2+}$. Here we further examine the role of the C-terminal arginines by observing the selectivity of KcsA mutants, in which one or both arginines (pI 10.8) are replaced with progressively less basic residues—lysines (pI 9.6), histidine (pI 7.5), valine (pI 6.0) or asparagine (pI 5.4). $Na^+$ and $Ca^{2+}$ concentrations are very low in the cytoplasm and both cations have inward gradients. $Mg^{2+}$ is the major divalent cation in the cytoplasm; consequently, the ability of KcsA to discriminate between $K^+$ and $Mg^{2+}$ was considered to be most physiologically relevant. Moreover, Schrempf et al. [7] showed that $Mg^{2+}$ at the intracellular side stabilizes KcsA and is essential for channel activity. The importance of intracellular $Mg^{2+}$ in modulating potassium channel activity has also been demonstrated for diverse eukaryotic potassium channels [21–24].

The selectivity for $K^+$ over $Mg^{2+}$ was examined for each mutant as a function of increasing $Mg^{2+}$ concentration. Selectivity was determined by determining the reversal potential (Erev), i.e. the potential at which the current reversed direction, from current/voltage (I/V) relationships. Under the experimental conditions of these studies, the Nernst theoretical potential [25], indicating perfect selectivity for $K^+$, is −54 mV. A significant increase in the reversal potential would indicate that other cations, in this case $Mg^{2+}$, are permeant.

Wild-type KcsA (RR) and mutants VR, KK, NK, HH, NH and NN were prepared, overexpressed in *E. coli* BL 21 cells, and purified by Ni-agarose chromatography. At least five separate preparations were made of the wild-type and each mutant. The ratio of PHB and polyP to protein was the same within experimental error for wild-type and mutant proteins. PHB concentrations were 39±7 μg/mg protein and polyP concentrations were 18±4 μg/mg protein. Since PHB is covalently attached to each monomer whereas polyP is held only in the tetramer [12], this corresponds to 7–10 PHB units/monomer and 13–20 polyP units/tetramer.

The purified proteins were individually incorporated into liposomes of mixed synthetic phospholipids (POPC:POPE:POPG; 3:3:1) and fully reconstituted by heating at 42 °C for>1 h. [17]. The proteins were then incorporated into planar lipid bilayers of the same lipids between 10:1 gradients of $K^+$ (200 mM *cis*; 20 mM *trans*) and symmetric $Mg^{2+}$ (ranging from 0.1 mM to 5 mM) in 20 mM KHepes at pH 7.4 at 30 °C. The current was measured as a function of applied potential at intervals from −100 mV to +100 mV,

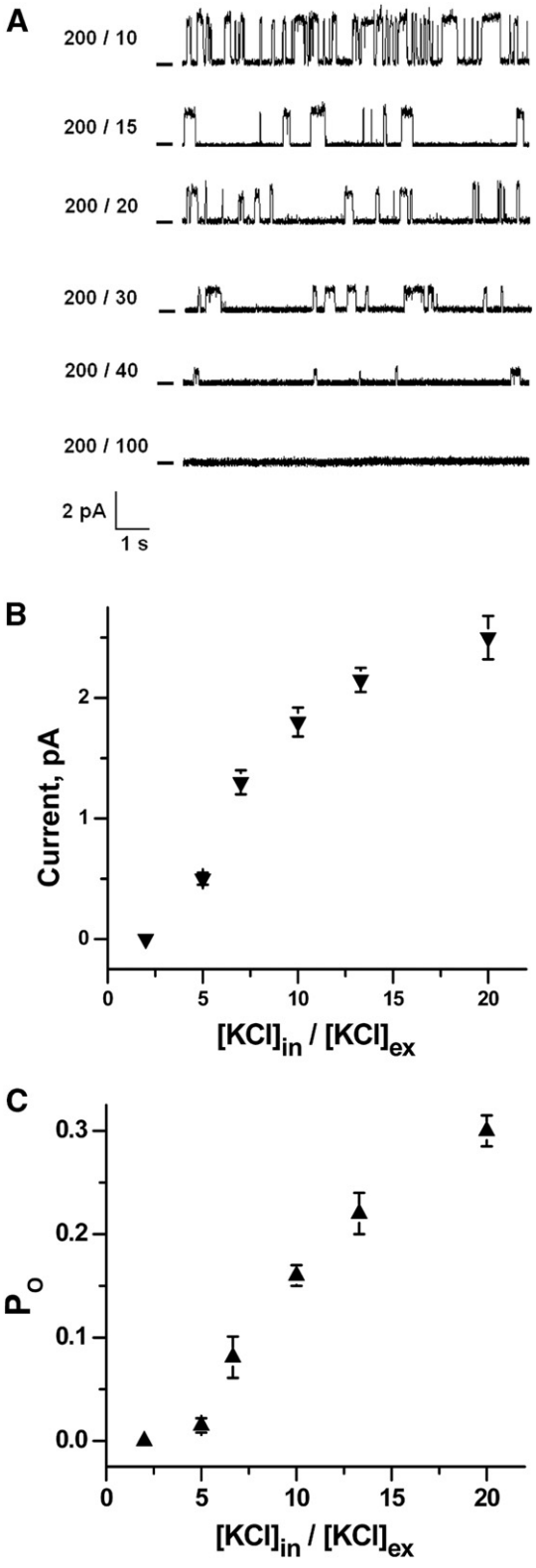


**Fig. 2.** (A) Representative current traces for KcsA at pH 7.4, 30 °C. Single-channel currents at 0 mV for KcsA in planar lipid bilayers of POPC:POPE:POPG (3:3:1) between gradient solutions of KCl, symmetric $MgCl_2$, 20 mM KHepes, pH 7.4. Cis side was maintained at 200 mM; trans side was varied from 10 to 40 mM. (B) Current magnitude as a function of the potassium gradient. (C) Open probability as a function of the potassium gradient.

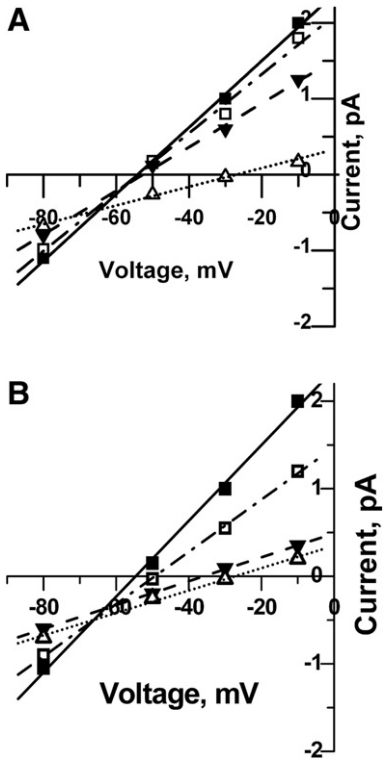


**Fig. 3.** Current/voltage (I/V) relationships for wild-type and mutant KcsAs near the reversal potential (A) at 5 mM $Mg^{2+}$, pH 7.4, (B) at 5 mM $Mg^{2+}$, pH. Wild-type RR: solid line; KK: dash–dot line; HH: dashed line; NN: dotted line.

and Erev was determined from I/V graphs. More than twelve separate observations were made of each preparation over a period of days or weeks, and the variation in Erev was within experimental error (±6%). Fig. 3A shows the region in an I/V graph near the reversal potential for wild-type and mutants RR, KK, HH and NN at 5 mM $Mg^{2+}$.

As seen in Fig. 4A, Erev of the wild-type channel RR and mutants VR, KK, NK, and HH remained within ±3.0 mV of the Nernst potential, indicating strong selectivity for $K^+$ over $Mg^{2+}$ at physiological pH. Mutant NH showed only slight permeance to $Mg^{2+}$ at higher $Mg^{2+}$ concentrations. However, mutant NN, as previously reported [18], was significantly permeant to $Mg^{2+}$ at physiological pH; the Erev with 5 mM $Mg^{2+}$ was only about half the Nernst theoretical potential (−28±3 mV).

The selectivity for $K^+$ over $Mg^{2+}$ was further examined as a function of pH from 5.0 to 10.0 in order to assess the effect of brief local pH changes on channel selectivity. These studies were conducted with 10:1 gradients of KCl (200 mM cis; 20 mM trans) and symmetric 5 mM $Mg^{2+}$ in 20 mM buffer at indicated pH at 30 °C (see Materials and methods). Fig. 3B shows the region of an I/V graph near the reversal potential for wild-type and mutants RR, KK, HH, and NN at pH 9. As the Erev shown in Fig. 4B indicate, at pH<6, wild-type and all mutants were highly selective for $K^+$ over $Mg^{2+}$. However, as the pH of the medium approached the pI of the C-terminal residues, the selectivity of the channels started to diminish. The NN mutant began to show $Mg^{2+}$ permeance>pH 6, NH>pH 7.4, HH and NK>pH 8, KK>pH 9, VR>pH 10. Only the wild-type channel, RR, remained highly selective for $K^+$ over $Mg^{2+}$ at pH 10.0.

### 3.3. Influence of C-terminal residues on KcsA conductance

The effects of $Mg^{2+}$ concentration in the range 0.1 mM to 5 mM on conductance magnitude and open probability (Po) was determined for wild-type and C-terminal mutants of KcsA. As above, at least 5 separate preparations of each protein were examined. Representative current records (from 10 or more separate observations) are shown in Fig. 5 for 5 mM $Mg^{2+}$ at +100 mV.

Wild-type KcsA in planar bilayers between 10:1 KCl gradients (200 mM *cis*; 20 mM *trans*), 0.1 mM $MgCl_2$, 20 mM Hepes, pH 7.4 displayed a chord conductance of 155±5 pS (Fig. 6A). The conductance magnitude remained essentially unchanged when $Mg^{2+}$ concentrations were increased to 3 mM, and then decreased slowly to 147 pS, indicating only slight $Mg^{2+}$ block. The open probability (Po) remained essentially unchanged at 0.91±0.02 (Fig. 6B).

Mutation of the penultimate arginine residue to valine (VR) resulted in channels whose conductance was more sensitivity to increasing $Mg^{2+}$ concentrations. As $Mg^{2+}$ increased from 0.1 mM to 5 mM, pH 7.4, the conductance magnitude decreased gradually from 154 pS to 134 pS while the open time decreased more conspicuously from 0.90 to 0.72 (Fig. 6A). Since the Erev selectivity of the channel was not significantly affected (Fig. 4A), the results signify increasing $Mg^{2+}$ block but not $Mg^{2+}$ permeance.

When both C-terminal arginines were replaced with lysines (KK), the channels displayed slightly lower conductance −152 pS and 123 pS, respectively, at 0.1 mM and 5 mM $Mg^{2+}$, pH 7.4 (Fig. 6A). The Po was also significantly reduced from 0.90 at 0.1 mM $Mg^{2+}$ to 0.50 at 5 mM $Mg^{2+}$ (Fig. 6B). As in the case of VR, the Erev was not significantly affected (Fig. 4A) thus the reduction in conductance magnitude and open time is indicative of increasing block by $Mg^{2+}$.

A KcsA mutant with a single lysine at the C-terminus, NK, displayed still lower conductance −147 pS and 93 pS, respectively, with 0.1 mM and 5 mM $Mg^{2+}$ at pH 7.4 (Fig. 6A). The most striking feature of this mutant was the pronounced decrease in Po, which was only 0.20 at 0.1 mM $Mg^{2+}$ and 0.045 at 5 mM $Mg^{2+}$ (Figs. 4 and 6B). $Mg^{2+}$ was still not permeant (Erev −53.0 mV with 5 mM $Mg^{2+}$) (Fig. 4A), but it essentially blocked the channel.

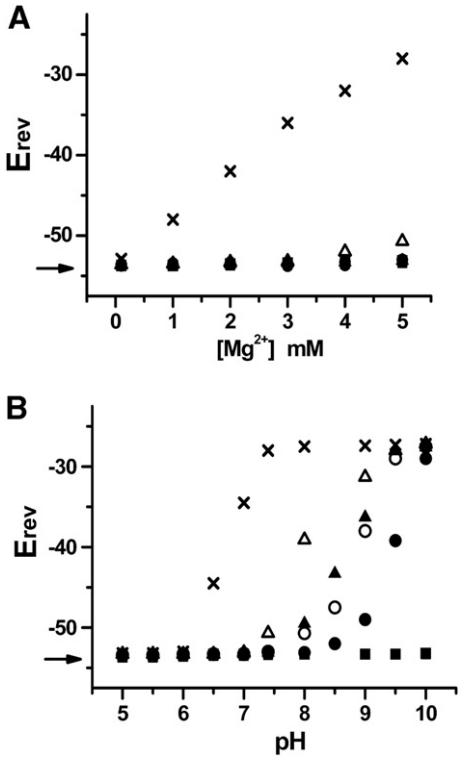


**Fig. 4.** Reversal potentials (Erev) of KcsA in planar lipid bilayers of POPC:POPE:POPG (3:3:1) between aqueous gradient solutions of 200 mM KCl *cis*, 20 mM KCl *trans* at 30 °C. (A) as a function of $Mg^{2+}$ concentration at pH 7.4, (B) as a function of pH at 5 mM $Mg^{2+}$. Error in the measurements is±6%. Arrow indicates the Nernst theoretical potential. C-terminal residues are ■ RR; □ VR; ● KK; ○ NK; ▲ HH; △ NH; X NN.

When the C-terminal residues were two histidines (HH) or a single histidine (NH), the channels displayed lower and nearly identical conductance magnitudes −143 pS and 140 pS, respectively, at 0.1 mM $Mg^{2+}$ and 61 pS and 56 pS, respectively, at 5 mM $Mg^{2+}$ (Fig. 6A). However, the open times were markedly greater for channels with two C-terminal histidines than for those with a single C-terminal histidine, 0.60 and 0.20 for HH compared to 0.09 and 0.003 for NH, at 0.1 mM $MgCl_2$ and 5 mM $Mg^{2+}$, respectively (Fig. 6B). Most of the decrease in conductance may be attributed to increased $Mg^{2+}$ block, but the Erev for NH at 5 mM $Mg^{2+}$ is −51 mV (Fig. 4A), suggesting very slight $Mg^{2+}$ permeance.

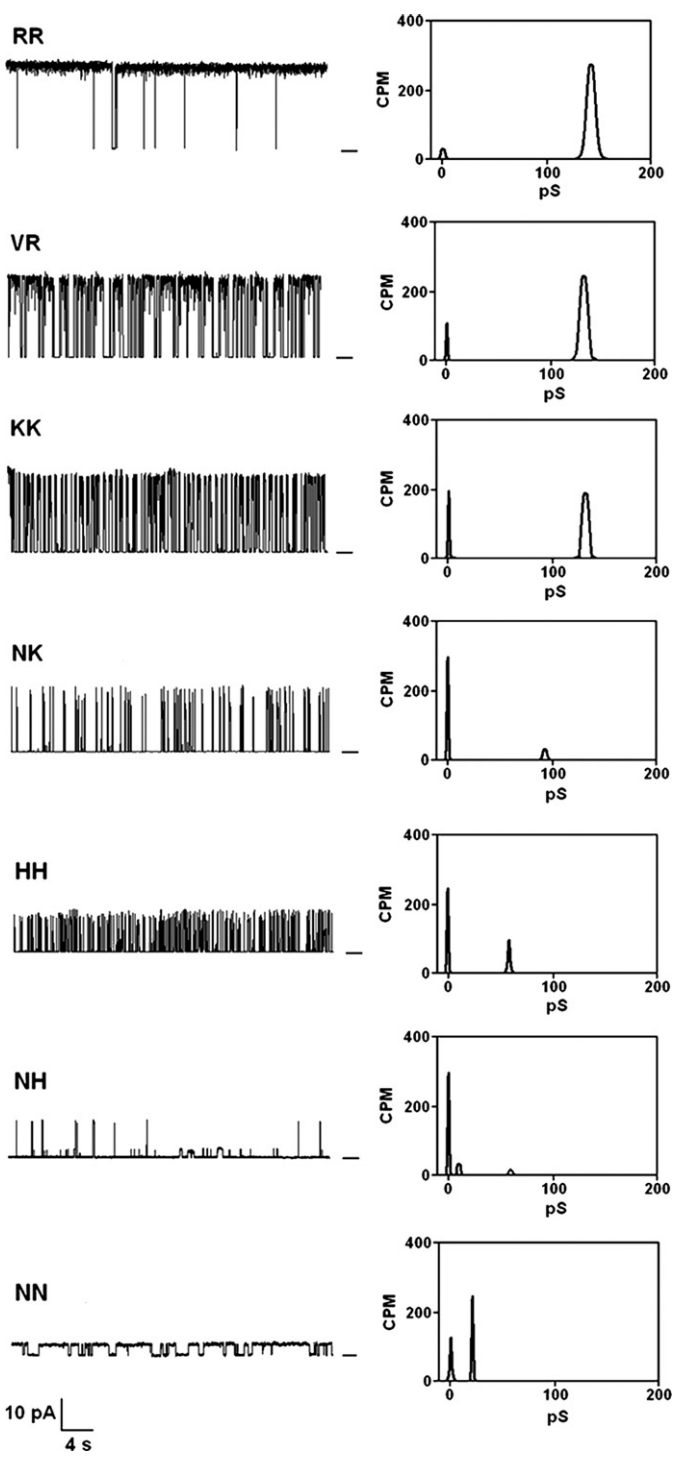


**Fig. 5.** Representative current traces at 100 mV for KcsA wild-type (RR) and C-terminal mutants (VR, KK, NK, HH, NH, NN) in planar lipid bilayers of POPC:POPE:POPG (3:3:1) between aqueous gradient solutions of 200 mM KCl *cis*, 20 mM KCl *trans*, symmetric 5 mM $Mg^{2+}$ at pH 7.4, 30 °C. Conductance histograms are shown to the right of each trace.

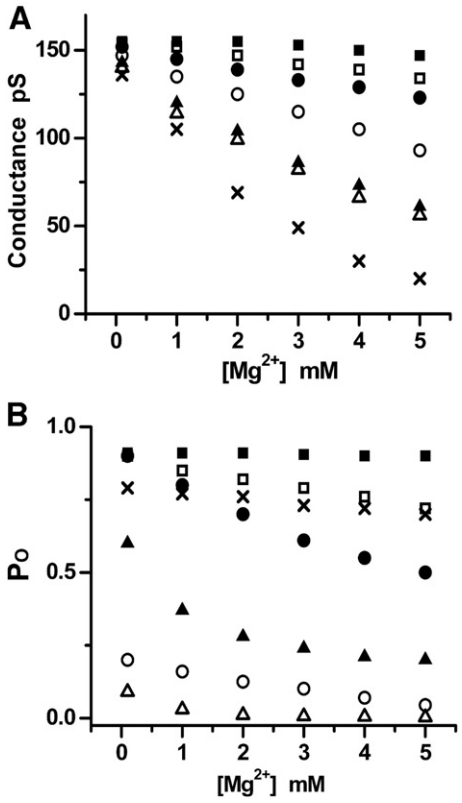


**Fig. 6.** Conductance of KcsA in planar lipid bilayers of POPC:POPE:POPG (3:3:1) between aqueous gradient solutions of 200 mM KCl *cis*, 20 mM KCl *trans*, pH 7.4, 30 °C as a function of $Mg^{2+}$ concentration. A. Conductance magnitude (pS); B. Open probability (Po). Error in the measurements is ± 10%. C-terminal residues are ■ RR; □ VR; ● KK; ○ NK; ▲ HH; △ NH; X NN.

Replacement of the arginines by asparagines (NN), as evidenced by the Erev of −28 mV (Fig. 4A) and reported previously [18], made the channels substantially permeant to $Mg^{2+}$. The permeance of $Mg^{2+}$ and its presence in symmetric and low concentrations, results in a high open time but low conductance.

## 4. Discussion

Our studies support the postulate that a guest polyP molecule plays a critical role in KcsA channel function. They further demonstrate the importance of the $K^+$ chemical gradient in activating the KcsA channel at neutral pH, and they illustrate the decisive role of the C-terminal residues, which are located in the cytoplasm more than 70 Å from the narrow pore at the external end of the channel, on channel selectivity and conductance.

KcsA has a relatively high sequence similarity to other $K^+$ channels, especially in the region lining the narrow pore at the external end [26,27]. In the currently accepted model of KcsA function, this narrow pore is the site of $K^+$ selectivity. The channel is said to be activated by a shift in intracellular pH from 7.4 to<5.5 [9,10], which stimulates movements of the helices that open a 'gate' through which ions enter a water-filled vestibule [28,29]. $K^+$ ions move out of the vestibule through the narrow pore in single-file as water of hydration is replaced by carbonyl oxygens of the pore residues [30]. Other ions are rejected as a result of their inappropriate size, coordination geometry and/or hydration energy.

This model is inconsistent with the data presented here and it also raises a number of questions that have not yet been addressed.

Intracellular pH in *S. lividans* is essentially invariant and no physiological activator that could substitute for a low pH has been identified. Experimental observations show that even with a pH of 4 at the intracellular side, the channel has a low open probability, the duration of the openings are very brief, and the channel rapidly inactivates [9,10,31–33]. Furthermore, it is not clear how, in the face of the strong $K^+$ gradient pressure, impermeant ions are removed from the vestibule, and there is no apparent function for the helical bundles formed by the ~40 C-terminal residues of the polypeptides (Fig. 1A).

Our results indicate that KcsA is activated and regulated at neutral pH by the $K^+$ electrochemical potential, and they demonstrate the importance of the chemical potential in planar bilayer studies. Levels of available $K^+$ are very low in soils; accordingly, KcsA is designed to function in cells with strong outward $K^+$ gradients. *In vivo*, the large positive chemical potential is balanced in the closed state by strong ionic bonds between $K^+$ and polyP and the negative membrane potential. $K^+$ flow outward when there is an increase in the $K^+$ gradient or decrease in the membrane potential. In planar bilayers, KcsA cannot be activated by the electrical potential alone simply because the fragile bilayer cannot withstand the magnitude of electrical potential required to overcome the binding energy of $K^+$ to polyP without assistance from the $K^+$ chemical potential. Zakharian and Reusch [11] showed that the failure of KcsA channels to be activated by voltage in planar lipid bilayers at neutral pH is readily corrected by applying a small outward $K^+$ gradient (>2:1). This conclusion is supported here by the activation of KcsA channels in the planar bilayer at pH 7.4 by a $K^+$ chemical gradient alone (Fig. 2). Moreover, the fact that a voltage pulse is not needed to activate the channel signifies that channel opening does not involve a physical gate. A 5:1 gradient is strong enough to provide a small current at zero potential and both the current and the open time increase directly with the size of the gradient. The currents become increasingly irregular as the gradient size increases, suggesting that a potential difference has a stabilizing effect on the conformation of the channel in the bilayer.

After many years in which potassium channels have been viewed as principally proteinaceous structures, many may find it difficult to accept the presence of the polyanion polyP in the core of the KcsA channel and acknowledge its definitive role in ion selection and transport. However, once the supramolecular structure of the channel is recognized, its performance under physiological conditions is readily understood in terms of simple chemical principles. In the proposed model, four identical protein subunits associate to form a symmetric complex around a central ion-conducting core of polyP (Fig. 1B shows a cross-section of the model). At the intracellular side, polyP attracts cations. Cations that bind are stripped of much of their water of hydration; remaining water of hydration is replaced by carbonyl oxygens of amino acid residues and/or PHB. Accordingly, unlike extracellular cations which are fully hydrated, intracellular cations are dehydrated when they arrive at the narrow pore at the external end of KcsA. The narrow pore may be viewed as a one-way valve that permits the exit of dehydrated $K^+$ (149 pm radius [34]) while denying entry to hydrated cations with inward gradients, namely $Na^+$ (358 pm) and $Ca^{2+}$ (412 pm). The major competitor to $K^+$ at the intracellular side is $Mg^{2+}$, which is impermeant when hydrated (428 pm) but permeant if dehydrated (72 pm). As a divalent cation, $Mg^{2+}$ is strongly attracted to polyP, but its binding to polyP is minimized by the collar of eight C-terminal arginines of the polypeptides which repel divalent cations more strongly. The arginines also attenuate the divalent negative charge of the polyP end unit, thus decreasing its preference for divalent cations. Intracellular $Na^+$ and $Ca^{2+}$ are not competitive due to their very low cytoplasmic concentrations and inward gradients. Accordingly, the polyP within KcsA is made to preferentially bind $K^+$ over $Mg^{2+}$ at physiological pH. The dehydrated $K^+$ ions are held by strong ionic bonds along the polyP backbone which extends from the cytoplasmic face to the narrow pore. $K^+$ ions move out through the narrow pore when the binding energy of $K^+$ to polyP is overcome by one or both components of the electrochemical potential. The channel is 'open' when the electrochemical potential is sufficient and 'closed' when it is not.

The importance of the C-terminal arginines in attenuating the preference of polyP for divalent cations at physiological pH is illustrated in Fig. 5 which shows visually the remarkable effects of the two C-terminal residues on conductance magnitude and open time at pH 7.4. The data in Figs. 4 and 6 further confirm that two arginines at the end of each polypeptide, as in the wild-type channel (RR ■), are most effective at preventing $Mg^{2+}$ blocking. At pH 7.4, the high selectivity for $K^+$ (Fig. 4A) and large open time indicate that $Mg^{2+}$ block is minimal (Fig. 6B). Changes in local pH (Fig. 4B) have no significant effect on selectivity. The replacement of one C-terminal arginine by valine (VR □) does not result in $Mg^{2+}$ permeance (Fig. 4A, B), but the slightly lower conductance and significantly lower open time indicate increased $Mg^{2+}$ block putatively due a lower positive charge (Fig. 6A, B).

Erev in Fig. 4A indicates that lysines (KK●, NK○) are also effective at preventing $Mg^{2+}$ permeance at pH 7.4, but the decreases in conductance and open time (Fig. 6A, B) signify greater $Mg^{2+}$ block. Again, two basic residues are more effective than one at repelling $Mg^{2+}$. As expected, lysines are also more sensitive than arginines to increases in local pH. Wild-type (RR) and mutant VR remain selective to pH 10, whereas mutant KK retains selectivity up to pH~9 whereas NK begins to show permeance to $Mg^{2+}$ at pH ~8 (Fig. 4B).

When histidines are substituted for arginines (HH▲, NH Δ), there is a substantial decrease in both conductance magnitude and open time (Fig. 6A, B). At pH 7.4, the two mutants show nearly identical decreases in conductance magnitude but NH has a much lower open time and its Erev is significantly higher than the Nernst potential (Fig. 4A) indicating not only stronger $Mg^{2+}$ block but also some $Mg^{2+}$ permeance. When both arginines are substituted by asparagines (NN X), the Erev (Fig. 4A) indicates that $Mg^{2+}$ is highly permeant at pH 7.4. $Mg^{2+}$ permeance results in a large increase in open time, but also a sharp decrease in conductance magnitude due to the substantially higher binding energy of divalent $Mg^{2+}$ to polyP, and the low symmetric $Mg^{2+}$ concentrations.

The model for the KcsA channel offered here is consistent with all previous structural studies. PolyP has not been observed in X-ray structures; however, there is at present no X-ray structure of whole KcsA, much less functionally active KcsA. The association between macromolecules in supramolecular complexes is non-covalent. The narrow polyP chain (~4 Å across) is likely to be physically held within the channel by the C-terminal helical bundles (Fig. 1B). The only bonds between polyP and KcsA would be relatively weak coordinate bonds *via* $K^+$ to carbonyl oxygens of amino acid residues and/or PHB. Since KpolyP is water soluble, it may be lost during protein purification or in the planar bilayer, especially when its presence is not suspected and measures are not taken to preserve it. KpolyP is lost when the tetramer is dissociated into monomers [12] and also may be lost when sections of the protein are excised. In this regard, X-rays structures that show putative binding sites for $Ba^{2+}$ [35], or tetraethylammonium [36] and tetrabutylammonium [37] are partial structures, sometimes with bound antibodies. They do not necessarily represent the *in vivo* binding sites of these cations.

Our functional studies are consistent with those of Schrempf et al. [7] and Heginbotham et al. [8], but not with later studies conducted under nonphysiological conditions, i.e with symmetric $K^+$ solutions and intracellular pH~4 for reasons discussed above. The sporadic, brief, rapidly inactivated channel openings produced under those conditions do not resemble the channels with long open times observed with $K^+$ gradients at neutral pH (Fig. 5).

In summary, our studies support a supramolecular structure for KcsA in which a guest polyP molecule plays a cooperative role in ion selection and decisive role in ion transport. The important contribution

of polyP to selective ion transport is further indicated by its presence in *E. coli* $Ca^{2+}$-selective channels [15], the CaATPase pump in human erythrocytes [38], porin Omp P5 of *Haemophilus influenzae* [39], and the voltage-dependent channel of rat liver mitochondria [40–42].

## Acknowledgements

We thank Christopher Miller, Brandeis Univ. for a clone of KcsA and NSF grant MCB 0445067 for support of this project.

## References

[1] D. Doyle, A.J. Morais Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, R. MacKinnon, The structure of the potassium channel: molecular basis of $K^+$ conduction and selectivity, Science 280 (1998) 69–77.
[2] Y. Zhou, J.H. Morais-Cabral, A. Kaufman, R. MacKinnon. Chemistry of ion coordination and hydration revealed by a $K^+$ channel-Fab complex at 2.0 A resolution, Nature 414 (2001) 43–48.
[3] E. Perozo, D.M. Cortes, L.G. Cuello, Three-dimensional architecture and gating mechanism of a $K^+$ channel studied by EPR spectroscopy, Nat. Struct. Biol. 5 (1998) 459–469.
[4] D.M. Cortes, L.G. Cuello, E. Perozo, Molecular architecture of full-length KcsA: role of cytoplasmic domains in ion permeation and activation gating, J. Gen. Physiol. 117 (2001) 165–180.
[5] J.H. Chill, J.M. Louis, C. Miller, A. Bax, NMR study of the tetrameric KcsA potassium channel in detergent micelles, Protein Sci. 15 (2006) 684–698.
[6] J. Zimmer, D.A. Doyle, J.G. Grossmann, Structural characterization and pH-induced conformational transition of full-length KcsA, Biophys. J. 90 (2006) 1752–1766.
[7] H. Schrempf, O. Schmidt, R. Kummerlen, S. Hinnah, D. Muller, M. Betzler, T. Steinkamp, R. Wagner, A prokaryotic potassium ion channel with two predicted transmembrane segments from *Streptomyces lividans*, EMBO J. 14 (1995) 5170–5178.
[8] L. Heginbotham, L. Kolmakova-Partensky, C. Miller, Functional reconstitution of a prokaryotic $K^+$ channel, J. Gen. Physiol. 111 (1998) 741–749.
[9] L.G. Cuello, J.G. Romero, D.M. Cortes, E. Perozo, pH-dependent gating in the *Streptomyces lividans* $K^+$ channel, Biochem. 37 (1998) 3229–3236.
[10] L. Heginbotham, M. LeMasurier, L. Kolmakova-Partensky, C. Miller, Single *Streptomyces lividans* $K^+$ Channels: functional asymmetries and sidedness of proton activation, J. Gen. Physiol. 114 (1999) 551–560.
[11] E. Zakharian, R.N. Reusch, *Streptomyces lividans* KcsA channels are regulated by a potassium electrochemical gradient, Biochem. Biophys. Res. Commun. 316 (2004) 429–436.
[12] R.N. Reusch, *Streptomyces lividans* potassium channel contains poly-(R)-3-hydroxybutyrate and inorganic polyphosphate, Biochem 38 (1999) 15666–15672.
[13] J. Hegermann, H. Lünsdorf, J. Overbeck, H. Schrempf, Polyphosphate at the *Streptomyces lividans* cytoplasmic membrane is enhanced in the presence of the potassium channel KcsA, J. Microsc. 229 (2007) 174–182.
[14] D.E.C. Corbridge, Phosphorus: an outline of its chemistry, biochemistry and technology, 5th ed., Elsevier Amsterdam, 1995, pp. 226–242.
[15] R.N. Reusch, R. Huang, L.L. Bramble, Poly-3-hydroxybutyrate/polyphosphate complexes from the plasma membranes of *Escherichia coli* form voltage-gated $Ca^{2+}$ channels in planar lipid bilayers, Biophys. J. 69 (1995) 754–766.
[16] S. Das, R.N. Reusch, pH regulates cation selectivity of poly-(R)-3-hydroxybutyrate/polyphosphate channels from *E coli* in planar lipid bilayers, Biochemistry 40 (2001) 2075–2079.
[17] E. Zakharian, R.N. Reusch, Functional evidence for a supramolecular structure for the *Streptomyces lividans* potassium channel KcsA, Biochem. Biophys. Res. Commun. 322 (2004) 1059–1065.
[18] A. Negoda, M. Xian, R.N. Reusch, Insight into the selectivity and gating functions of *Streptomyces lividans* KcsA, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 4342–4346.
[19] R. Aschar-Sobbi, A.Y. Abramov, C. Diao, M.E. Kargacin, G.J. Kargacin, R.J. French, E. Pavlov, High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach, J. Fluoresc. 18 (2008) 859–866.
[20] B. Hille, in Ionic Channels of Excitable Membranes 2nd edit. Sinauer Assoc. Inc. Sunderland MA, pp. 341–348
[21] C.J. Lingle, $Mg^{2+}$-dependent regulation of BK channels: importance of electrostatics, J. Gen. Physiol. 131 (2008) 5–11.
[22] B.C. Suh, B. Hille, Electrostatic interaction of internal $Mg^{2+}$ with membrane PIP2 seen with KCNQ K+ channels, J. Gen. Physiol. 130 (2007) 241–256.
[23] T.W. Claydon, D.C. Kwan, D. Fedida, S.J. Kehl, Block by internal $Mg^{2+}$ causes voltage- dependent inactivation of Kv1.5, Eur. Biophys. J. 36 (2006) 23–34.
[24] P. Bednarczyk, K. Dołowy, A. Szewczyk, Matrix $Mg^{2+}$ regulates mitochondrial ATP- dependent potassium channel from heart, FEBS Lett. 579 (2005) 1625–1632.
[25] G. Eisenman, R. Horn, Ionic selectivity revisited: the role of kinetic and equilibrium processes in ion permeation through channels, J. Membr. Biol. 76 (1983) 197–225.
[26] R. MacKinnon, S.L. Cohen, A. Kuo, A. Lee, B.T. Chait, Structural conservation in prokaryotic and eukaryotic potassium channels, Science 280 (1998) 106–109.
[27] Z. Lu, A.M. Klem, Y. Ramu, Ion conduction pore is conserved among potassium channels, Nature 413 (2001) 809–813.
[28] Y.S. Liu, P. Sompornpisut, E. Perozo, Structure of the KcsA channel intracellular gate in the open state, Nat. Struct. Biol. 8 (2001) 883–887.
[29] Y. Jiang, A. Lee, J. Chen, M. Cadene, B.T. Chait, R. MacKinnon, The open pore conformation of potassium channels, Nature 417 (2002) 523–526.
[30] Y. Zhou, R. MacKinnon, The occupancy of ions in the $K^+$ selectivity filter: charge balance and coupling of ion binding to a protein conformational change underlie high conduction rates, J. Mol. Biol. 333 (2003) 965–975.
[31] J.F. Cordero-Morales, L.G. Cuello, Y. Zhao, V. Jogini, D.M. Cortes, B. Roux, E. Perozo, Molecular determinants of gating at the potassium-channel selectivity filter, Nat. Struct. Mol. Biol. 13 (2006) 311–318.
[32] J.F. Cordero-Morales, V. Jogini, A. Lewis, V. Vásquez, D.M. Cortes, B. Roux, E. Perozo, Molecular driving forces determining potassium channel slow inactivation, Nat. Struct. Mol. Biol. 14 (2007) 1062–1069.
[33] L. Gao, X. Mi, V. Paajanen, K. Wang, Z. Fan, Activation-coupled inactivation in the bacterial potassium channel KcsA, Proc. Natl Acad. Sci USA 102 (2005) 17630–17635.
[34] A.G. Volkov, S. Paula, D.W. Deamer, Two mechanisms of permeation of small neutral molecules and hydrated ions across phospholipid bilayers, Bioelectrochem. Bioenergetics 42 (1997) 153–160.
[35] Y. Jiang, R. MacKinnon, The barium site in a potassium channel by x-ray crystallography, J. Gen. Physiol. 115 (2000) 69–272.
[36] M.J. Lenaeus, M. Vamvouka, P.J. Focia, A. Gross, Structural basis of TEA blockade in a model potassium channel, Nat. Struct. Mol. Biol. 12 (2005) 454–459.
[37] S. Yohannan, Y. Hu, Y. Zhou, Crystallographic study of the tetrabutylammonium block to the KcsA K(+) channel, J. Mol. Biol. 366 (2007) 806–814.
[38] R.N. Reusch, R. Huang, D. Kosk-Kosicka, Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump, FEBS Lett. 412 (1997) 592–596.
[39] E. Zakharian, R.N. Reusch, *Haemophilus influenzae* outer membrane protein P5 is associated with inorganic polyphosphate and polyhydroxybutyrate, Biophys. J. 92 (2006) 588–593.
[40] R.N. Reusch, Poly-beta-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes, Proc. Soc. Exp. Biol. Med. 191 (1989) 377–381.
[41] E. Pavlov, E. Zakharian, R.N. Reusch, R. French, A large, voltage-dependent channel, isolated from mitochondria by water-free chloroform extraction, Biophys. J. 88 (2005) 2614–2625.
[42] E. Pavlov, C. Grimbly, C.T. Diao, R.J. French, A high-conductance mode of a poly-3-hydroxybutyrate/calcium/polyphosphate channel isolated from competent *Escherichia coli* cells, FEBS Lett. 579 (2005) 5187–5192.