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

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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

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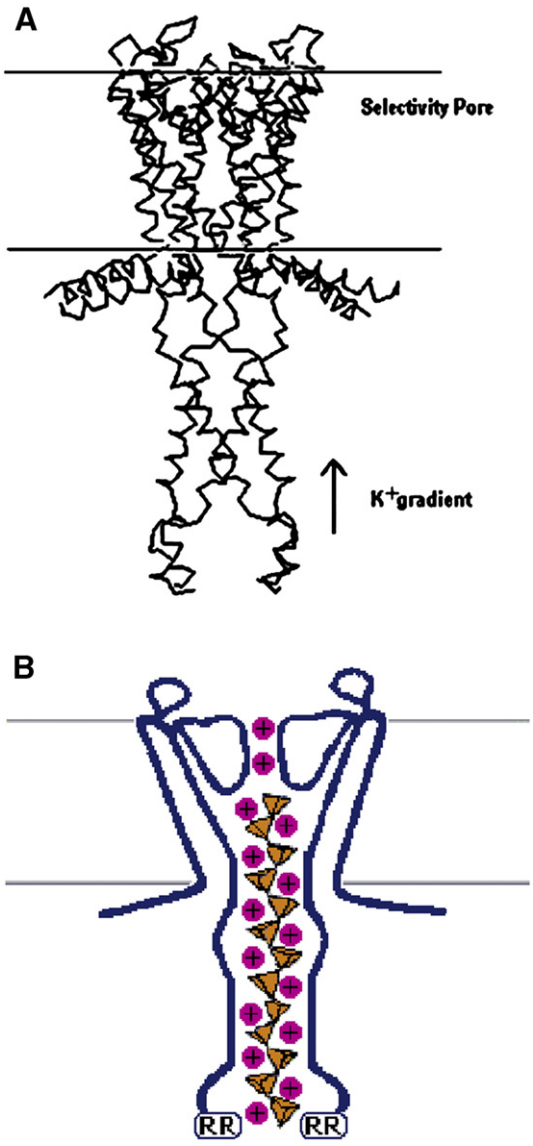


**Fig. 1.** (A) Molecular architecture of full-length KcsA (from Cortes et al. 2001). (B) Schematic diagram of a cross-section of KcsA showing the postulated arrangement of polyP (tan tetrahedra) and $K^+$ (pink circles) within KcsA. RR–C-terminal arginines. PHB is postulated to line the channel vestibule 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].