

Images of oligomeric Kv β 2, a modulatory subunit of potassium channels

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Abstract The Shaker type voltage-gated potassium (K⁺) channel consists of four pore-forming Kv α subunits. The channel expression and kinetic properties can be modulated by auxiliary hydrophilic Kv β subunits via formation of heteromultimeric Kv α -Kv β complexes. Because each (Kv α)₄ could recruit more than one Kv β subunit and different Kv β subunits could potentially interact, the stoichiometry of α - β and β - β complexes is therefore critical for understanding the functional regulation of Shaker type potassium channels. We expressed and purified Kv β 2 subunit in Sf9 insect cells. The purified Kv β 2, examined by atomic force and electron microscopy techniques, is found predominately as a square-shaped tetrameric complex with side dimensions of 100 \times 100 Å² and height of 51 Å. Thus, Kv β 2 is capable of forming a tetramer in the absence of pore-forming α subunits. The center of the Kv β 2 complex was observed to be the most heavily stained region, suggesting that this region could be part of an extended tubular structure connecting the inner mouth of the ion permeation pathway to the cytoplasmic environment.

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1. Introduction

Ion channels are oligomeric proteins that are usually composed of several classes of proteins. The pore-forming subunits define the ion permeation pathway and control much of the channel gating properties. Structural studies of these proteins, including the prokaryotic KcsA potassium channel [1] and the acetylcholine (ACh) receptor [2], have provided important insights into the structure and function. As regulatory modules, auxiliary subunits for a variety of ion channels have also been found. Although their functional roles in subunit assembly and modulation have been increasingly recognized, their structure and stoichiometry have not been studied by biophysical techniques (see reviews by [3–5]).

Voltage-gated Shaker type K⁺ channels are composed of at least two types of subunits, pore-forming transmembrane Kv α subunits and hydrophilic cytoplasmic Kv β subunits. Kv α subunits consist of six putative transmembrane segments with both amino (N)- and carboxy (C)-termini positioned on the cytoplasmic side [6,7]. When expressed individually, most Kv α subunits form functional K⁺ channels. The expression level and inactivation kinetics can be modulated by coexpression of Kv β subunits in heterologous systems [8,9]. Genetic mutation

of the *hyperkinetics* (Hk) protein, a Kv β subunit in *Drosophila*, causes significant muscle dysfunction [10]. Homologous Kv β -like proteins have been found in a variety of species including plants, squid, *Drosophila*, and mammals. At least nine genes encoding various Kv β subunits have been isolated. They share a highly conserved core region at the C-terminal portion, but the N-terminal portions of the β subunits vary significantly in both length and sequence (see a recent review [5]).

Shaker type potassium channels are assembled as Kv α tetramers as determined by toxin binding and kinetic analyses [11] and by electron microscopy studies of purified Shaker protein [12]. The formation of the (Kv α)₄ complex is mediated by associations between the cytoplasmic amino-terminal T1 (or NAB) domains [13–15]. The interaction with Kv β subunits occurs through associations between the T1 domain of α subunits and the conserved core regions of Kv β subunits [16,17]. Crystallographic studies have shown that the T1 domain forms a four-fold symmetric tetrameric complex [18]. Thus, the completely assembled four-fold symmetric Kv α tetramer could contain as many as four sites for interaction with Kv β proteins. Among the cloned Kv β subunits, mammalian Kv β 1 and Kv β 2 are the ones that have been studied most extensively: Kv β 1 induces or accelerates fast inactivation, whereas Kv β 2 does not exhibit significant effects on channel kinetics [8]. Yeast two-hybrid genetic studies have shown that Kv β 2 has the ability to interact homomultimerically or heteromultimerically with Kv β 1 [19]. In transfected cells, coexpression of Kv β 1 and Kv β 2 subunits abolishes the Kv β 1-mediated fast inactivation, which presumably results from the formation of Kv β heteromultimers and/or competition for the binding sites of Kv α subunits [19,20]. However, there is as yet no direct evidence that Kv β subunits self-associate physically to form an oligomeric complex. Because the composition and stoichiometry of the α - β complex could ultimately determine the kinetics and gating properties of the K⁺ channel, imaging a purified Kv β subunit may provide important insights into the mechanism by which these subunits affect channel function. In this study, we expressed and purified the Kv β 2 subunit in the absence of Kv α subunits and visualized the purified protein using atomic force and electron microscopy. The images of the oligomeric Kv β 2 complexes have provided important information on the cytoplasmic dimensions of Shaker type potassium channels.

2. Materials and methods

2.1. Construction of Kv β 2 baculoviruses

Full-length rat Kv β 2 [16] was amplified by PCR and cloned into the *Eco*RI and *Xho*I sites of pBlueBacHis2B (Invitrogen, CA) to generate the plasmid pBlueBacHis2B-Kv β 2. When expressed, the recombinant Kv β 2 contains an extra 37 amino acids at its N-terminus (Fig. 1A), including a hexahistidine tag, an antibody epitope, and an enteroki-

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nase protease cleavage site. Sf9 cells were cotransfected with pBlue-BacHis2B-Kv β 2 and linearized AcMNPV genomic DNA using cationic liposomes (Invitrogen, CA); recombinant plaques were identified using 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside staining. Viral stocks were prepared and amplified using standard methods provided by the manufacturer.

2.2. Expression and purification of Kv β 2

Kv β 2 was expressed by infecting Sf9 cells with recombinant baculoviruses. In a typical purification, $4\text{--}8 \times 10^8$ Sf9 insect cells, in 400 ml of Grace's medium that contained 3.3 mg/ml lactalbumin hydrolysate, 3.3 mg/ml yeastolate, 20 μ g/ml gentamicin, 0.1% Pluronic F-68, and 10% fetal calf serum, were infected with the His-Kv β 2 viruses at an MOI (multiplicity of infection) of 5–10. After shaking at 100 rpm in a 2500 ml low foam culture flask for 48 h at 27°C, cells were harvested by centrifugation at $2000 \times g$ and pelleted cells were stored at -80°C prior to use. For purification, cells were thawed, resuspended in a lysis buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole and 5 mM 2-mercaptoethanol] and lysed by sonication for 2 min on a 30% duty cycle, microprobe setting 6 (model 250/450 Sonifier, Branson Ultrasonics Corporation, NJ). All subsequent steps were carried out at 4°C. The total cell lysates were collected after centrifugation at $12000 \times g$ for 15 min to remove cell debris. His-tagged proteins were purified by incubation with the crude cell lysate with 1 ml Pro-bond nickel-chelated resin (Invitrogen, CA). After washing the column with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole and 5 mM 2-mercaptoethanol, Kv β 2 was eluted with the buffer supplemented with 250 mM imidazole.

2.3. Gel filtration chromatography

Protein in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole, and 5 mM 2-mercaptoethanol was separated by FPLC on Superdex-200 in a running buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 5 mM 2-mercaptoethanol. Generally, 50 μ g His-Kv β 2 in 200 μ l of the running buffer was loaded. The column was developed at a flow rate of 0.5 ml/fraction/min.

2.4. Microscopy

Typically, for atomic force microscopy (AFM), the experimental procedures were essentially identical to a published protocol [21]. Briefly, a solution of purified His-Kv β 2 subunits (at 10 μ g/ml) in 10 mM Tris-HCl (pH 7.2), 150 mM NaCl was incubated on freshly cleaved mica for 30 min at room temperature. Following extensive washing, the sample was imaged, in the same buffer, using a Nanoscope II atomic force microscope (Digital Instruments, Santa Barbara, CA). All images were acquired in the contact mode using oxide-sharpened 'twin tip' Si_3N_4 cantilevers with a spring constant of 0.06 N/m at a typical scan rate of 9 Hz and applied force of ~ 0.1 nN. The piezoscanner was calibrated with mica and the cholera toxin β subunit [22]. Lateral dimensions of the His-Kv β 2 subunits were determined from full width at half height using Nanoscope III software.

For electron microscopy (EM), a solution of His-Kv β 2 subunits (at ~ 15 μ g/ml) in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA and 5 mM 2-mercaptoethanol, was applied to glow discharged carbon-coated grids. After a brief period of incubation, the sample was stained with 2% uranyl acetate and then imaged with a Philips CM12 electron microscope. To determine the lateral dimensions, EM images were converted into Nanoscope III files, and measurements were made of the full width at half intensity with Nanoscope III software.

3. Results and discussion

The Kv β 2 subunit is the most abundant isoform found in native tissues. However, its absolute amount and potential heterogeneity via oligomerization with other proteins render large-scale purification difficult. To facilitate structural studies, we chose to express rat Kv β 2 cDNA in Sf9 cells using a baculovirus system containing a His tag (Fig. 1A). The tagged fusion protein was soluble and affinity-purified on a nickel column. The purified material yielded a single band on Coomassie-stained SDS-polyacrylamide gel (Fig. 1B, lane 3) with

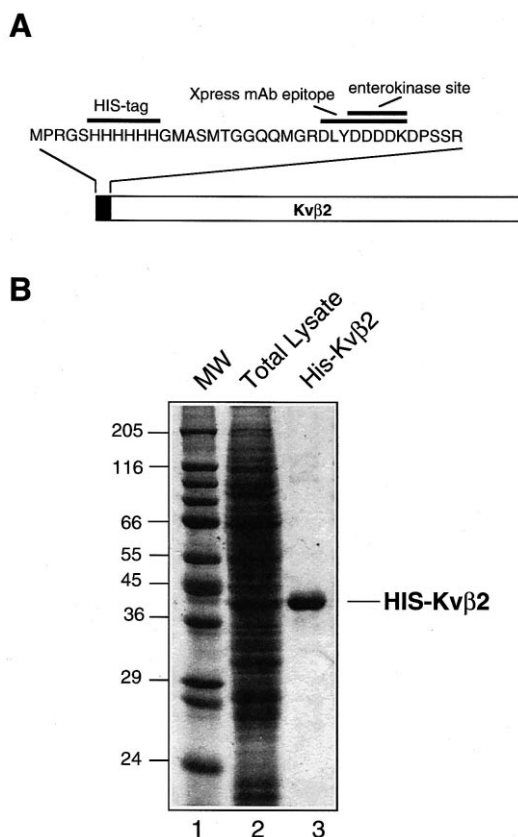


Fig. 1. Expression and purification of Kv β 2. A: A diagram of His-tagged Kv β 2 fusion protein. B: The His-tagged Kv β 2 (His-Kv β 2) protein produced in Sf9 insect cells was affinity purified from crude cell lysates. The cell lysate (lane 2) and the purified Kv β 2 (lane 3) were separated by polyacrylamide gel (12%) and visualized by Coomassie stain.

an apparent molecular weight of 37 kDa, consistent with the molecular weight of the native Kv β 2 [23].

To investigate the hydrodynamic properties of Kv β 2, the purified His-Kv β 2 was separated on a Superdex-200 gel filtration column by FPLC. The A_{280} chromatogram of the Superdex-200 revealed a single peak, corresponding to Kv β 2 as determined by SDS-PAGE (Fig. 2B). Using molecular weight standards, the Stoke's radius of His-Kv β 2 was determined to be 65.4 Å (Fig. 2A), which is considerably greater than a 37 kDa monomer. Therefore, in solution, His-Kv β 2 is oligomeric.

To directly determine the stoichiometry of the His-Kv β 2 complex, the purified protein was examined by EM and AFM. Electron micrographs of negatively stained His-Kv β 2 reproducibly showed square-shaped complexes, approximately 100×100 Å², oriented with the four-fold axis normal to the supporting substrate (Fig. 3A,B). Each of the four individual globular 'domains' of the complex is 37 ± 7 Å ($n=211$) in diameter. Between adjacent domains there is a 23 ± 7 Å ($n=109$) densely stained gap. The most heavily stained region was frequently found to be the center of the complex with a cross dimension of 36 ± 8 Å ($n=48$), suggesting that there may be either a central vestibule or, more likely, a central aqueous pore formed by the assembled His-Kv β 2 subunits.

If each His-Kv β 2 subunit is an ellipsoid with a mass density of 1.23 Å³/Da (a specific volume calculated for the acetylcho-

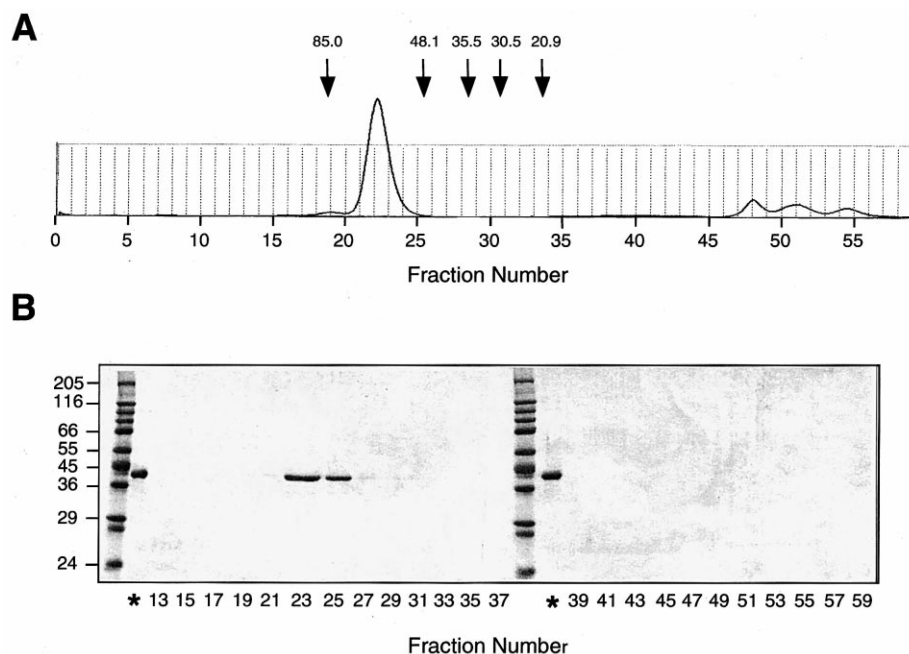


Fig. 2. Purified Kvβ2 proteins are oligomers. A: Gel filtration fractionation of His-Kvβ2. Superdex-200 was used to separate the purified protein and A_{280} chromatogram profiles are shown. Protein Stokes' radius standards (in Å) are thyroglobin (85.0), aldolase (48.1), bovine serum albumin (35.5), chicken ovalbumin (30.5), and chymotrypsinogen (20.9). Their migration positions on Superdex-200 are marked by arrows. B: SDS-PAGE analysis of proteins in fractions after Superdex-200 (FPLC) separation. Lanes marked with an asterisk are the starting material. Numbers under each lane indicate the corresponding fractions in Superdex-200 (B). Molecular weight (in kDa) standards are shown on the left.

line receptor [24]), the dimensions determined from the EM images would predict that such a tetrameric assembly of Kvβ2 subunits would have a total height of ~ 46 Å. To directly determine the height of the complex, purified His-Kvβ2 was adsorbed onto a mica substrate and examined by AFM, a technique that has been used to determine the height of macromolecules [22,25]. Consistent with the EM observations, square-shaped complexes with a side length of ~ 100 Å were also routinely observed in the somewhat densely packed

sample (Fig. 3D). Each of the four globular domains in the complex is 41 ± 10 Å ($n = 266$) in diameter with a 19 ± 4 Å ($n = 136$) groove separating adjacent domains. The total height of the complex, measured from the top of the protein to the mica substrate, in these topographs is 51 ± 6 Å ($n = 292$), remarkably close to the value predicted above. Taken together, the size and symmetry of the complex determined from both EM and AFM studies clearly indicate that purified His-Kvβ2 predominantly forms tetramers.

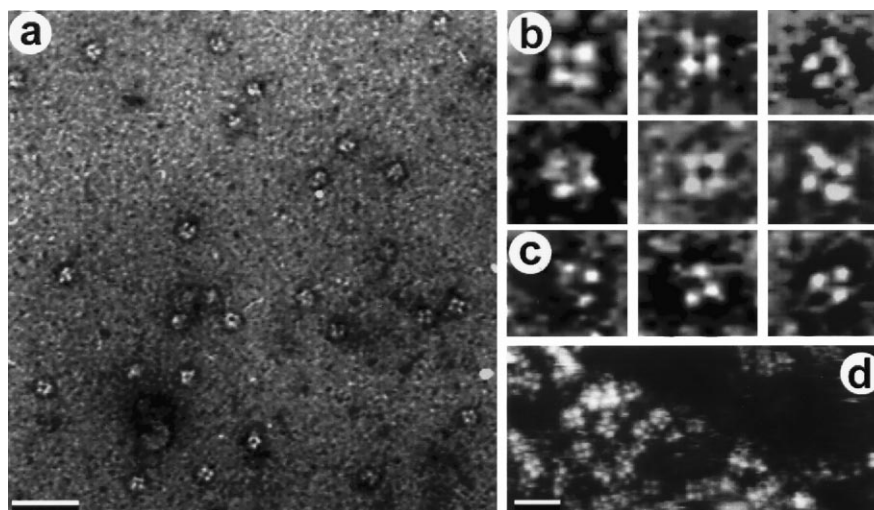


Fig. 3. Images from electron and atomic force microscopy show that purified His-Kvβ2 subunits form tetramers. a: Negative-stain electron microscopy shows that the His-Kvβ2 complex is oriented predominantly with the four-fold symmetry axis perpendicular to the support film, resulting in a square-shaped appearance. (Bar = 500 Å.) b: A panel of six individual His-Kvβ2 complexes was collected from EM. Each image is 240×280 Å². Each complex contains four globular domains, corresponding to each subunit. c: A panel of three individual His-Kvβ2 oligomers containing only three subunits. d: Purified His-Kvβ2 visualized by AFM which also reveal square-shaped complexes. The total height of the complex determined from these topographs is ~ 50 Å. (Bar = 250 Å.)

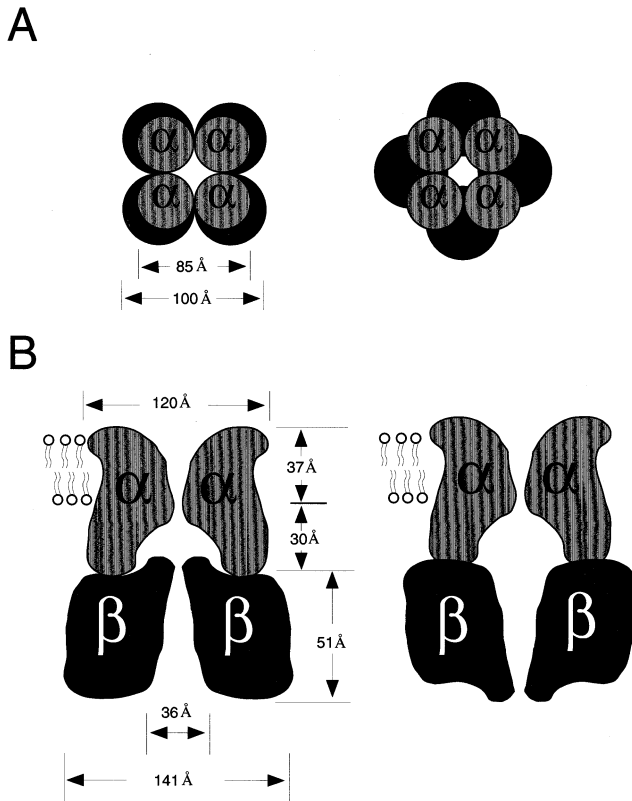


Fig. 4. Dimensions of the $\alpha_4\beta_4$ potassium channel complex. A: Direct (left) and alternative (right) models of $\alpha_4\beta_4$ complexes. The side dimension of α_4 is taken from the EM investigation [12]. The β_4 side dimension of 100 Å is from the present study. B: A side view, along the diagonal of the $\alpha_4\beta_4$ complex. Total height of α_4 is 67 Å that is derived from the height of the KcsA transmembrane core region (37 Å [1]) and the height of T1 domain (30 Å [18]). The height of β_4 is 51 Å (from the present study). The inner and outer cross dimensions of α_4 and β_4 are calculated from the side dimensions. It should be noted that the dimensions obtained from different domains or subunits may not be necessarily additive. The native $\alpha_4\beta_4$ could have deviated dimension due to, for example, interaction-induced conformation changes. The narrow pore of β_4 is based on the crystal structure of $(Kv\beta)_4$ [33].

It should be noted, however, that complexes with only three domains were also observed by both EM and AFM ($\sim 20\%$, Fig. 3C). The domains in these trimeric complexes were of the same size as those in the tetramers, and moreover, were arranged in the form of a right-angle triangle, suggesting that these three-domain complexes represent intermediates in the process of assembly or disassembly of the His-Kv β 2 tetramer. The observed trimeric complex is consistent with the notion that the four-fold symmetric complex is formed by sequential assembly of four individual subunits as suggested by the earlier studies [20].

Kv β 2 is the most abundant subunit in rat brain and early hydrodynamic studies suggested that the native Kv α 1.2-Kv β 2 complex in bovine brain is consistent with an α/β ratio of 1:1 which led to the prediction of $\alpha_4\beta_4$ stoichiometry [23]. The most pronounced activity of Kv β subunits is the induction of fast inactivation by Kv β 1 subunits [8]. Functional tests in heterologous systems suggested that Kv β 1 could incrementally regulate channel activity. The gradual increase of the inactivation rate induced by increasing the level of Kv β 1 expression suggests a functional stoichiometry of $\alpha_4\beta_n$, where n was

thought to equal 0–4 depending upon the Kv β 1 expression level [20]. In contrast, Kv β 2 itself does not exert obvious effects on Kv α subunit inactivation. However, when Kv β 1 and Kv β 2 are coexpressed in tissue culture cells, Kv β 2 could completely inhibit the fast inactivation induced by Kv β 1 [19]. Although the binding affinity between individual Kv α subunits and Kv β subunits has not been determined, the formation of tetrameric Kv β 2 complex creates multivalent interaction that significantly tightens the $\alpha_4\beta_4$ complex. Such multivalent interaction may improve the on-rate of α - β association during the early stage of channel assembly and/or reduce the off-rate for the assembled α - β complex on the cell membrane.

The shape and symmetry of the Kv β 2 oligomer suggests that the four-fold axis of the Kv β 2 tetramer is aligned with the central pore of the K⁺ channel during α - β interaction. The lateral size of the Kv β 2 tetramer is slightly larger than the dimensions of the α subunits of the Shaker α_4 tetramer determined by EM (side length of 80–85 Å [12]) and the tetramer of the T1 domain determined by X-ray crystallography (side length 65–70 Å [18]). During preparation of this work, an independent study reported crystal structure of Kv β 2 with approximate dimensions of $90 \times 90 \times 40$ Å [33]. Except slightly smaller in overall size, the relative dimensions are in general agreement with AFM and EM measurements reported here. Although the angular orientation of the β subunits relative to the α subunits in the $\alpha_4\beta_4$ complex is not known, the lateral dimensions measured here indicate that the outer central cavity of the Kv β 2 tetramer has a dimension of ~ 36 Å. Thus, the 100×100 Å lateral surface of Kv β 2 tetramer should effectively cover the outer surface of the T1 tetramer. Surprisingly, the crystal structure of the Kv β 2 tetramer has a narrow pore (~ 5 Å). It is not known whether the Kv α -Kv β interaction induces widening of the narrow pore. In addition, it would be interesting to determine the orientation by which $(Kv\beta)_4$ is docked to the $(Kv\alpha)_4$ complex (Fig. 4). Thus, it is likely that binding of the Kv β 2 tetramer to the cytoplasmic surface of the T1 tetramer excludes other possible interactions between the N-terminal surface of Kv α tetramer and other soluble proteins.

A surprising characteristic of the Kv β 2 tetramer is its rather large total height (~ 51 Å). If the Kv β 2 complex was only mainly interacting with the α subunits, it might be expected that the Kv β 2 tetramer would be only as high as one or two layers of secondary structures (~ 10 – 20 Å). Instead, there is apparently a large portion of the Kv β 2 subunit far removed from the immediate site of contact with the α subunit. Together with the T1 domains, the $\alpha_4\beta_4$ complex would be expected to protrude as much as ~ 80 Å into the cytoplasm (Fig. 4B). Hence, it may be that the Kv β 2 tetramer acts as a molecular 'extension' of the α subunits into the cytoplasm, providing specific docking sites for additional regulatory factors to act on potassium channel.

Recent evidence suggests the existence of signaling complexes containing both ion channels and closely associated protein kinases and phosphatases [26–31]. Such ion channel-kinase/phosphatase complexes are thought to provide the necessary macromolecular organization for signaling specificity and regulation (for a recent review, see [32]). Purification of Kv β 2 subunit and imaging its oligomeric structure could provide a starting point to learn more about the stoichiometry and geometry of fully assembled potassium channels, which appear to be large, multicomponent complexes containing not

only α and β subunits, but also other associated proteins such as those typically involved in signal transduction pathways.

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