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Separation of heteromeric potassium channel Kcv towards probing subunit composition-regulated ion permeation and gating

Qiulin Tan, Ji Wook Shim, Li-Qun Gu *

Department of Biological Engineering, Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211, USA

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ABSTRACT

The chlorella virus-encoded Kcv can form a homo-tetrameric potassium channel in lipid membranes. This miniature peptide can be synthesized in vitro, and the tetramer purified from the SDS–polyacrylamide gel retains the K^+ channel functionality. Combining this capability with the mass-tagging method, we propose a simple, straightforward approach that can generically manipulate individual subunits in the tetramer, thereby enabling the detection of contribution from individual subunits to the channel functions. Using this approach, we showed that the structural change in the selectivity filter from only one subunit is sufficient to cause permanent channel inactivation ("all-or-none" mechanism), whereas the mutation near the extracellular entrance additively modifies the ion permeation with the number of mutant subunits in the tetramer ("additive" mechanism).

1. Introduction

The chlorella virus-encoded membrane protein Kcv can form a K^+ channel in both cell membranes [2–6] and artificial lipid bilayers [1,7]. This 94-amino acid miniature peptide is one of the shortest K^+ channel proteins ever found [8], and possesses the most primitive structure of all K^+ channels. In particular the pore domain of Kcv contains a highly conserved sequence TVGFG for the K^+ selectivity filter [4] (Fig. 1). Because of the small size and primitive structure, Kcv is a unique model for the structure-function research of K^+ channels.

The Kcv channel is a homo-tetramer [1,7]. The tetrameric structure is commonly adopted by most K^+ channels in which the four identical subunits are symmetrically associated and work concertedly in conferring unique conductance, selectivity and gating properties [9–12].We are motivated to explore the role of individual subunits in the Kcv functions. By genetically manipulating individual subunits, we would like to characterize how the channel activity varies with the subunit composition [13–17]. The study of functional stoichiometry would be beneficial for understanding structure-determined channel functions, and for engineering ion channels with programmable activity by manipulating subunit composition. Such a study also has significant medical implications. In many channelopathies such as Andersen's syndrome, the heterogeneous phenotypes are contributed by hetero-multimerization of the pathogenic channel protein (mutants) with other members in a channel family [18–20].

Here we propose a simple, straightforward approach to studying the subunit contribution to the channel functions. This approach takes advantage of the unique Kcv property we uncovered recently: the in vitro synthetic wild-type Kcv can form a stable tetramer in the detergent SDS, and the tetramer purified from the SDS electrophoresis gel retains functions as a K^+ channel [1]. Based on this capacity, we designed a tagged-Kcv (Kcv attached with a polypeptide tag) that can hybridize with an untagged-Kcv (e.g. mutant Kcv) to form hetero-tetramers. As these hetero-tetramers migrate in different mobility during electrophoresis, they can be well separated. The stability of Kcv tetramers in SDS allows us to purify each type of electrophoretically separated tetramers directly from the gel, and examine its single channel functionality. The tagged-Kcv is also required to function as a wild-type channel, thereby providing a "wild-type background" when forming hetero-tetramers with untagged mutant subunits. By detecting the channel activity of each hetero-tetramer, the correlation between the channel function and subunit combination can be established.

Using this approach, we were able to distinguish the "all-or-none" regulatory mechanism by the selectivity filter of Kcv, and the "additive" regulation mechanism by amino acids near the extracellular entrance. The structural change in the selectivity filter

* Corresponding author. Fax: +1 573 884 4232.
E-mail address: gul@missouri.edu (L.-Q. Gu).

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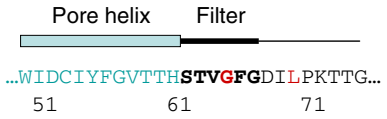

Fig. 1. Part of the Kcv peptide sequence. The demonstrated domain includes the pore helix, the selectivity filter, and the following free chain up to the inner helix domain. The mutation sites Gly65 and Leu70 were marked in red.

(Gly65) from one subunit is sufficient to cause permanent channel inactivation, whereas the mutation near the extracellular entrance (Leu70) additively modifies the ion permeation with increasing the number of mutant subunits in the tetramer.

2. Results and discussion

2.1. Construction and properties of the tagged-Kcv

We designed a series of tagged-Kcv that are distinct from each other in the tag's charge and/or molecular weight (Table S1). Their proteins were synthesized from coupled in vitro transcription and translation (Methods in Supplementary data), and the products were revealed using electrophoresis (Fig. S1). We finally put focus on N8, which carries eight asparagines on the N-terminus. This is because N8 tetramerizes as efficient as the wild-type Kcv (WT), and its tetramer migrates much slower during electrophoresis with a distinguishable gap from the WT tetramer (Fig. S1b). When N8 was co-synthesized with WT at various plasmid ratios, their products split into five tetramer bands on a 12.5% SDS gel running for 16 h (Fig. 2). The single bands in Lane A and Lane E are the WT and N8 homo-tetramers, WT_4 and $N8_4$. Three distinguishable intermediate bands appear in Lane B, C and D, with their protein amount distribution shifting with the DNA ratio. Given the tetrameric stoichiometry studied earlier [1], the three intermediate bands are associated with WT/N8 hetero-tetramers in three subunit combinations: from the fast- to slow-migrating, WT_3N8_1, WT_2N8_2 and WT_1N8_3. The subscripts denote the number of each subunit in the tetramer.

We used the planar lipid bilayer system to examine single channel properties of each WT/N8 tetramer directly purified from the gel (Methods, Supplementary data). Fig. 3a shows their single channel currents recorded at ±40 mV in 150 mM KCl symmetrical recording solutions, and Fig. 3b the current–voltage relations (I–V curves) measured from single channel data. Clearly, all the four tetramers containing N8 subunits form channels with similar conductance to the WT tetramer at various voltages between ±120 mV. For instance, the conductance of the five tetramers at +40 mV are, WT_4, 213 ± 5 pS; WT_3N8_1, 203 ± 36 pS; WT_2N8_2, 216 ± 5 pS; WT_1N8_3, 216 ± 16 pS; and $N8_4$, 194 ± 19 pS (Table S2). The confidence interval is g (1 ± 0.19) at a confidence level of 95%. We further measured the ion selectivity of the five channels under bi-ionic condition. Fig. 3c illustrates their single channel currents at −20 mV and −40 mV with 150 mM NaCl in *cis* chamber vs. 150 mM KCl in *trans* chamber. The corresponding I–V curves in Fig. 3d indicates that all the five WT/N8 hybrid channels produce a positive current at negative voltages with similar reverse potentials (V_r), WT_4, −60.2 ± 5.9 mV, WT_3N8_1, −62.9 ± 7.7 mV; WT_2N8_2, −62.0 ± 5.2 mV; WT_1N8_3, −56.1 ± 2.7 mV; and $N8_4$, −61.5 ± 6.5 mV (Table S2). The confidence interval is V_r (1 ± 0.17) at a confidence level of 95%. Similar reverse potentials suggest the channels formed by the five WT/N8 tetramers are all highly K^+ selective.

Based on findings from both electrophoresis and single channel recordings, the eight asparagines in N8 not only separates hetero-Kcv tetramers using electrophoresis, but also gives the hetero-channels similar conductance and selectivity to the wild-type Kcv. Therefore we selected N8 as a qualified tagged-Kcv to explore subunit contribution to the channel functionality.

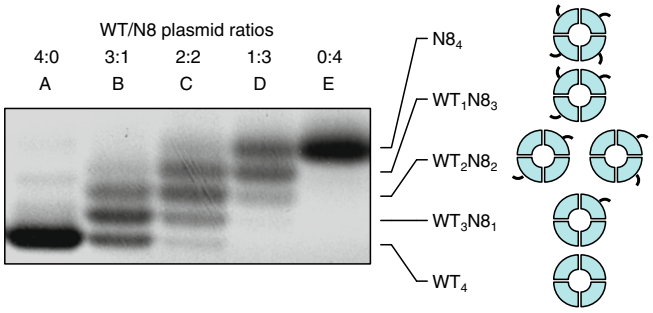

Fig. 2. Electrophoretic separation of WT/N8 tetramers. The synthesized S^{35}-labeled proteins run on a 12.5% SDS–polyacrylamide gel for 16 h. Lane A through E were tetramers formed at WT:N8 plasmid ratios of 4:0, 3:1, 2:2, 1:3 and 0:4. The five bands identified represent all possible subunit combinations, $WT_{4-n}N8_n$ (n = 0, 1, 2, 3 and 4 is the number of N8 in the tetramer).

2.2. "All-or-none" channel inactivation by Gly65 in the selectivity filter

The crystallographic structures have revealed that the selectivity filter of a K^+ channel comprises an symmetric ion pathway that is assembled by the backbone carbonyls contributed from four identical signature sequences [12]. Many studies have confirmed the important role of the selectivity filter in maintaining the rapid and selective conduction of K^+ ions. For instance, the Shaker K^+ channel with an altered selectivity filter has been turned into a non-selective pore for monovalent cations [21]; the KcsA channel with a substitution on the first glycine in the selectivity filter TVGYG (G77A) completely loses the permeability, and the mutant on the second glycine (G79A) cannot even assemble into a tetramer [22]. Similar to KcsA, the Kcv mutant on the first glycine in its filter TVGFG (G65C) is permanently inactivated (non-conducting state) under either symmetrical or bi-ionic condition (described below), and the mutant G67Q on the second glycine can not tetramerize either (Fig. S2).

To detect the functional regulation by individual subunits, we co-synthesized G65C with N8 at various plasmid ratios. Their products form five bands on the electrophoresis gel (Fig. 4a). Similar to the WT/N8 co-expression products, the fastest-migrating (Lane A) and slowest-migrating band (Lane E) are the homo-tetramers $G65C_4$ and $N8_4$. The three intermediate bands appeared in Lane B, C and D are hetero-tetramers, from fast- to slow-migrating, $G65C_3N8_1$, $G65C_2N8_2$ and $G65C_1N8_3$. The proteins of five tetramers were extracted from the gel, and their single channel properties were studied under the bi-ionic condition. The single channel currents in Fig. 4b indicate that, except $N8_4$ that functions as the wild-type Kcv, all other four tetramers containing different number of G65C subunits stay in a permanent inactivation state without open current. Actually, under similar stock protein concentrations (Fig. S3), the $N8_4$ channel can be easily formed a few minutes after addition of 1 μl stock protein, whereas other tetramer channels can never be formed even when recorded for 1 h and with addition of proteins 10 times the amount of $N8_4$. Each inactive tetramer was confirmed by more than ten tests. Overall, the finding from single channel currents (Fig. 4b) confirms an "all-or-none" functional stoichiometry that all the four Gly65 in the selectivity filter are required to retain the K^+ ion conduction. Substitution of one Gly65 among four is sufficient to inactivate the channel permanently.

This "all-or-none" mechanism could be correlated with the selectivity filter's function in gating regulation. There has been increasing evidence supporting that the selectivity filter can regulate the gating of K^+ channels [23–28]. The molecular dynamics