Title: **A single amino acid gates the KcsA channel**

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Abstract

The KcsA channel is a proton-activated potassium channel. We have previously shown that the cytoplasmic domain (CPD) acts as a pH-sensor, and the charged state of certain negatively charged amino acids in the CPD play an important role in regulating the pH-dependent gating. Here, we demonstrate the KcsA channel is constitutively open independent of pH upon mutating E146 to a neutrally charged amino acid. In addition, we found that rearrangement of the CPD following this mutation was not large. Our results indicate that minimal rearrangement of the CPD, particularly around E146, is sufficient for opening of the KcsA channel.

Keywords

ion channel, KcsA channel, pH-dependent gating, single-channel recording, rearrangement

Introduction

The KcsA channel is a proton-activated potassium channel that consists of four subunits [1, 2]. Each subunit includes two transmembrane regions, TM1 and TM2, and a cytoplasmic domain (CPD) at the C-terminal [3, 4]. It is believed that the primary pH-sensing site is located at the intracellular entrance of the pore where the four TM2 helices form a bundle and their charged amino acids regulate the channel gating [5, 6]. However, other reports have indicated that the CPD is also important for pH sensing. We have shown that a chimera mutant channel consisting of the CPD of the KcsA and the transmembrane domain of another potassium channel showed similar pH sensitivity to the WT KcsA [7]. We also detected conformational changes of the CPD in response to changes in pH [8] and reported that the open probability increased when the CPD was pushed toward the membrane using atomic force microscopy [9]. Other groups have found that tetramerization of the CPD is pH-dependent [10, 11].

We have previously demonstrated that pH sensing by the CPD is dependent on its charged amino acids [7]. About half the amino acids in the CPD are charged, with the number of positively and negatively charged amino acids being approximately the same at pH7. The protonated-mimicking mutant, in which eight negatively

charged amino acids were substituted with neutral ones, showed channel activity even at pH7 and pH9 [7]. Moreover, the mutant E146QD149N, in which two of the eight negatively charged amino acids are neutralized, made the KcsA open independent of pH, suggesting the electrostatic charges of these two amino acids are especially important for the pH-dependent regulation of the KcsA.

In the present paper, we further explored these two amino acids, identifying E146 as most essential for pH sensing. Neutralization of only E146 made the KcsA constitutively open independent of pH. In addition, this neutralization resulted in only minimal rearrangement of the CPD, suggesting only slight rearrangements are sufficient for channel opening.

Materials and Methods

Mutagenesis and protein purification.

All KcsA mutants were made with the *Quick-Change*[™] site-directed mutagenesis kit (Stratagene) and included an E71A mutation to prevent gate inactivation [12]. The KcsA and its mutants were expressed in *E. coli* and purified as previously described [8].

Fluorescence labeling and fluorescence measurements.

A132C mutant channels were fluorescently labeled by mixing 1 μ M protein with 10 μ M tetramethylrhodamine (TMR)-maleimide, followed by incubation for 12-16 hours on ice. Excess dye was removed by a Co^{2+} affinity gel column. All TMR-labeled channels (1.0 nmol) were reconstituted into liposomes (1 mg) as previously described (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE): 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG)=3:1) [8].

Fluorescence measurements of the TMR-labeled mutants in liposomes were performed as follows. 20 μ l of a liposome suspension containing TMR-labeled channels was diluted to 200 μ l using 200 mM KCl with 10 mM MES at pH4 or 10 mM Tris-Hepes at pH7 and centrifuged at 100,000 x g for 20 minutes at 4 °C. The precipitation was suspended with the same solution (200 μ l) and sonicated to equilibrate internal and external solution. Fluorophores were excited at 532 nm, and fluorescence intensities at 570 nm were obtained with a fluorescence spectrometer (F-4500, HITACHI). To compare fluorescence intensities between different mutants, data were normalized with concentrations of TMR-labeled channels determined by the fluorescence densities of TMR-labeled KcsA channels in SDS-PAGE gels using fluorography (ImageQuant LAS4000). The average fluorescence of TMR-labeled A132C channels at pH4 was normalized to 100%.

Channel current recordings.

Purified channels were reconstituted into liposomes and their currents were measured by the planar bilayer method as previously described [8, 13]. The bath solution was held at virtual ground such that voltage at the upper solution defined the membrane potential. Liposomes with channels were added to the upper solution. Currents under a KcsA-activated condition were recorded in upper solution (200 mM KCl and 10 mM Tris-Hepes (pH 7.0)) and bath solution (200 mM KCl and 10 mM MES (pH 4.0)). Currents under a KcsA-inactivated condition were recorded in a symmetrical solution containing 200 mM KCl and 10 mM Tris-Hepes (pH 7.0). Open probabilities were calculated from current traces for 20 seconds using Clampfit software (n=4-14).

Results

E146 is critical for pH-dependent gating

Our previous study revealed that the protonated-mimicking mutation, E130QE134QE135QE146QD149NE152QD156ND157N (CPD-n), in which eight negatively charged amino acids in the CPD were substituted for neutral ones, made the KcsA with the non-inactivating mutation E71A constitutively open even under

high pH [7], a property we confirmed here (Fig. 1, Table 1). This result suggests the electrostatic charge of the CPD is important for the pH-dependent gating of the KcsA. In addition, we identified that E146 and D149 have a significant role in pH sensing, since neutralization of only these two amino acids (E146QD149N) opened the channel independent of pH [7] (Fig. 1, Table 1).

To identify which of these two amino acids is most important in the regulation, the activity of E146Q and D149N mutants was measured. E146Q showed current at both pH4 and pH7 (Fig. 1, Table 1), with open probabilities similar to control (E71A) at pH4. In contrast, D149N showed varying open probabilities even at pH4, as five measurements resulted in open probabilities of 1.00, 1.00, 0.78, 0.24 and 0.17. A similar phenomenon was observed at pH7. These results suggest that the charged state of E146 play an important role in regulating the gating of the KcsA, whereas the charged state of D149 contributes to the gating but is insufficient for stable regulation of the activity.

To better understand the gating mechanism and importance of E146, we investigated whether a repulsive force among positively charged amino acids arose when E146 was protonated. In accordance with simulation analysis by Li, Y. et. al. [14], which predicted the E146 and R142 from the same strand interact, a double

mutant, R142AE146Q, was made to examine whether positively charged R142 amino acids opened a channel that had E146 protonated. R142AE146Q showed low open probabilities both at pH4 and pH7 (Fig. 1, Table 1), suggesting that neutralization of R142 in the E146Q mutant changed the channel from the open to closed state. This result suggests a repulsive force between the R142 of each subunit may exist when E146 is protonated.

CPD rearrangement during KcsA channel opening

To clarify how changes occurred in the open state of protonated-mimicking mutants, conformations of the CPD were investigated using the fluorophore tetramethylrhodamine (TMR). The fluorescence intensity of TMR is higher in a hydrophobic environment than in a hydrophilic one [15]. We have previously shown that conformational changes of the CPD can be detected by measuring fluorescence intensity changes when a part of the CPD including A132 is labeled with TMR [8]. Briefly, KcsA mutants that were labeled with TMR at the C-terminal of the transmembrane region and at the adjacent part of the CPD including A132 showed high fluorescence intensities at acidic pH but low intensities at neutral pH. The high intensities at acidic pH decreased in the presence of dipicrylamine, a membrane-localized quencher, suggesting that the above two regions were located

in or near the membrane in the activated condition but in the solution in the inactivated condition.

Figure 2 shows results of the fluorescence intensity measurements at pH4 and pH7 of the control (E71A) and three protonated-mimicking mutants, CPD-n, E146QD149N and E146Q, when A132 in the CPD was labeled with TMR. All mutants showed high fluorescence intensities at pH4, although less than that of the control (E71A). High fluorescence intensity indicates that the A132 site was in a hydrophobic environment, most likely in or near the membrane [8]. On the other hand, only the CPD-n mutant showed high fluorescence at pH7, while the fluorescence intensities of E146QD149N and E146Q more resembled that of the control (E71A). For those mutants with low fluorescence at pH7, the A132 site is in a hydrophilic environment far from the membrane. Therefore, because E146QD149N and E146Q both showed high open probabilities at pH7 (Fig. 1) but low fluorescence intensities, A132 may not need to be near the membrane for the opening of the KcsA.

Discussion

The KcsA is a proton-activated potassium channel which consists of four subunits

that each has two transmembrane regions and a cytoplasmic domain. It is generally accepted that the pH-sensing region is located at the intracellular entrance of the pore [5, 6]. A cluster of charged amino acids there plays an important role in pH-sensing, and it has been proposed that electrostatic repulsions between them induce opening of the channel at acidic pH. However, we previously reported that the CPD also acts as a pH sensor, as charged amino acids there play an important role in the pH-dependent regulation of the gating [7]. Other groups have found the tetramerization of the CPD is pH-sensitive [10, 11] and the pH-sensitivity depends on the charged state of H145 [11], suggesting that the CPD region distal from the transmembrane region plays an important role in the pH-dependent gating of the KcsA. Consistent with this conclusion, we found previously that the charged state of E146 and D149, which are also located at the region distal from the transmembrane region, significantly influences channel activity [7].

Here, we provide further evidence for the importance of E146 in KcsA gating. We showed that rearrangements of the CPD may be caused by electrostatic repulsion between R142s from different strands when E146 is protonated (Fig. 1, Table 1). This rearrangement in turn may lead to the opening of the KcsA, which agrees with a simulation study that found E146 is more important than D149 for the gating [14].

Additionally, a structural report has suggested that D149 interacts with R147 from a neighboring strand to form a bundle at the CPD in the closed state that does not dissociate during the gating [16, 17], suggesting that formation and disruption of the CPD bundle, i.e. association and dissociation between D149 and R147, is not essential for regulating the gating of the KcsA.

Interestingly, although E146QD149N and E146Q mutants showed activity at pH4 and pH7, fluorescence measurements show the conformation of the CPD differed with pH. Such a structural difference is likely to come from the different charged states between the CPD. At acidic pH, both the CPD and the inner entrance of the pore in E146QD149N and E146Q are positively charged, inducing repulsive forces which open the channel. At neutral pH, because in the CPDs of E146QD149N and E146Q only one or two amino acids per subunit were neutralized, the repulsive force between the four CPDs should be smaller than that in these mutants at pH4. The inner entrance regions of the pores in these mutants do not have repulsive forces at pH7, suggesting that only motions of the CPD induced by the protonation of E146 and D149, or even E146 only are sufficient to open the KcsA and that rearrangement of the CPD toward the membrane is not necessary for channel opening. It has been reported that in other potassium channels such as the Kir

channel and calcium-gated potassium channels, twisting motions of the CPD are needed for the open state [18-20]. Such twisting has also been detected in the KcsA channel [21]. It is possible this twisting represents the rearrangement of the CPD by the protonation of E146 and is necessary for KcsA channel opening.

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Figure Legends

Figure 1. Effects of CPD charged states on KcsA channel activity. Activities of control (E71A), three protonated-mimicking mutants (CPD-n, E146QD149N, and E146Q) and R142AE146Q were measured. Representative current records at pH4 and pH7 at 40 mV are shown.

Figure 2. Conformation of the CPD. Fluorescence intensities of TMR-labeled at the A132 site of control (E71A), CPD-n, E146QD149N and E146Q were measured at 570 nm and pH4 or pH7 (mean $\pm$ S.D., n = 4-7).

Table Legends

Table 1. Open probabilities of CPD-activated mutants. Open probabilities of KcsA control (E71A), CPD-n, E146QD149N, E146Q and R142AE146Q were measured at 40 mV in a symmetrical solution containing 200 mM KCl (mean $\pm$ SD, n = 4-14).

Table

	pH4	pH7
control (E71A)	0.84 ± 0.15	0.14 ± 0.22
CPD-n	0.95 ± 0.07	0.96 ± 0.07
E146QD149N	0.98 ± 0.05	0.87 ± 0.20
E146Q	0.88 ± 0.21	0.91 ± 0.18
R142AE146Q	0.04 ± 0.03	0.13 ± 0.06

Figure 1

Figure 2