Biochemical and Biophysical Research Communications 450 (2014) 1537–1540

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A single amino acid gates the KcsA channel

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ARTICLE INFO

Article history:
Received 1 July 2014
Available online 11 July 2014

Keywords:
Ion channel
KcsA channel
pH-dependent gating
Single-channel recording
Rearrangement

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 states 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.

1. 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 pH 7. The protonated-mimicking mutant, in which eight negatively charged amino acids were substituted with neutral ones, showed channel activity even at pH 7 and pH 9 [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.

2. Materials and methods

2.1. 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 *Escherichia coli* and purified as previously described [8].

2.2. Fluorescence labeling and fluorescence measurements

A132C mutant channels were fluorescently labeled by mixing 1 μM protein with 10 μM tetramethylrhodamine (TMR)-maleimide, followed by incubation for 12–16 h 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-phosphoethanol-

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http://dx.doi.org/10.1016/j.bbrc.2014.07.032
0006-291X/

amine (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 μl of a liposome suspension containing TMR-labeled channels was diluted to 200 μl using 200 mM KCl with 10 mM MES at pH 4 or 10 mM Tris-Hepes at pH 7 and centrifuged at 100,000×*g* for 20 min at 4 °C. The precipitation was suspended with the same solution (200 μl) 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 pH 4 was normalized to 100%.

2.3. 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 s using Clampfit software (n = 4–14).

3. Results

3.1. 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).

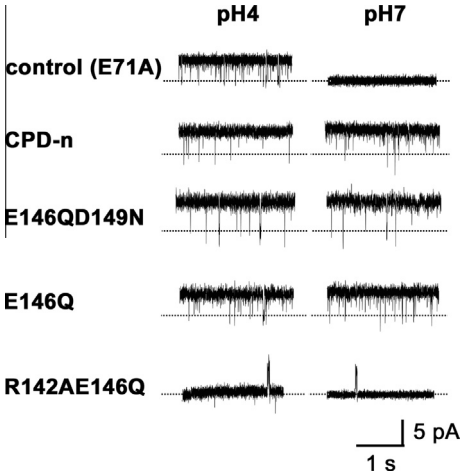

Fig. 1. Effects of CPD charged states on KcsA channel activity. Activities of control (E71A), three protonated-mimicking mutants (CPD-n, E146QD149N, and E146Q) and R142AE146Q were measured. Representative current records at pH 4 and pH 7 at 40 mV are shown.

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 ± SD, n = 4–14).

	pH 4	pH 7
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

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 pH 4 and pH 7 (Fig. 1, Table 1), with open probabilities similar to control (E71A) at pH 4. In contrast, D149N showed varying open probabilities even at pH 4, 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 pH 7. These results suggest that the charged state of E146 plays 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 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 pH 4 and pH 7 (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.

3.2. 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 florescence 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.

Fig. 2 shows results of the fluorescence intensity measurements at pH 4 and pH 7 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 pH 4, 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

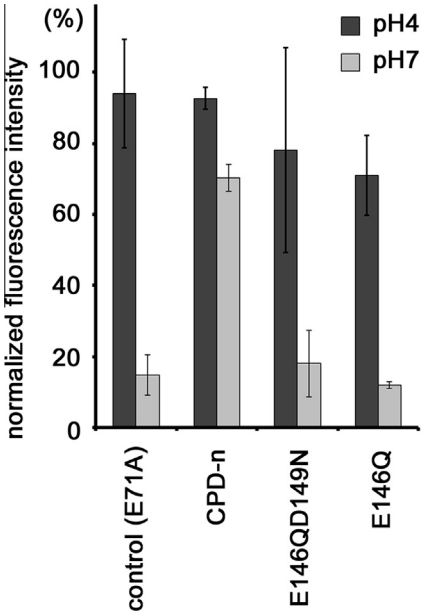

Fig. 2. Conformation of the CPD. Fluorescence intensities of TMR-labeled at the A132 site of control (E71A), CPD-n, E146QD149N and E146Q were measured at 570 nm and pH 4 or pH 7 (mean ± S.D., n = 4–7).

[8]. On the other hand, only the CPD-n mutant showed high fluorescence at pH 7, while the fluorescence intensities of E146QD149N and E146Q more resembled that of the control (E71A). For those mutants with low fluorescence at pH 7, the A132 site is in a hydrophilic environment far from the membrane. Therefore, because E146QD149N and E146Q both showed high open probabilities at pH 7 (Fig. 1) but low fluorescence intensities, A132 may not need to be near the membrane for the opening of the KcsA.

4. 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 pH 4 and pH 7, 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 pH 4. The inner entrance regions of the pores in these mutants do not have repulsive forces at pH 7, 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.

Acknowledgments

We thank Dr. Peter Karagiannis for carefully revising the manuscript. This work was supported by JSPS KAKENHI Grand Number 22370059, 23770190, 24657110 and 25840055, MEXT KAKENHI Grand Number 23113519, Grants-in-aid for Scientific Research of Foundation Advanced Technology Institute (ATI), The Science Research Promotion Fund of The Promotion and Mutual Aid Corporation for Private Schools of Japan, The Uehara Memorial Foundation, Grant for Basic Science Research Projects from The Sumitomo Foundation, Kowa Life Science Foundation, and the Center of Innovation Program from Japan Science and Technology Agency, JST.

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