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Surface-Enhanced IR Absorption Spectroscopy of the KcsA Potassium Channel upon Application of an Electric Field

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Abstract
Surface-enhanced IR absorption spectroscopy (SEIRAS) is a powerful tool for studying the structure of molecules adsorbed on an electrode surface (ATR-SEIRA). Coupled with an electrochemical system, structural changes induced by changes in the electric field can be detected. All the membrane proteins are subjected to the effect of membrane electric field, but conformational changes at different membrane potentials and their functional relevance have not been studied extensively except for channel proteins. In this contribution, background information of potential-dependent functional and structural changes of a prototypical channel, the KcsA channel, is summarized, and SEIRAS applied to the KcsA channel under the application of the potential is shown. The potassium channels allow K^+ to permeate selectively through the structural part named the selectivity filter, in which dehydrated K+ ions interact with backbone carbonyls. In the absence of K^+, the selectivity filter undergoes conformational changes to the non-conductive collapsed conformation. To apply the electric field, the KcsA channels were fixed on the gold surface in either upside or reverse orientation. The SEIRA spectrum in K^+ or Na^+ solution revealed both backbone structural changes and local changes in the OCO-carboxylate groups. Upon application of the negative electric field, the spectrum of OCO was enhanced only in the K^+ solution. These results indicate that the negative electric field accumulates local K+ concentration, which turned the collapsed filter to the conductive conformation. ATR-SEIRA serves as an unprecedented experimental system for examining membrane proteins under an electric field.

Key words: potassium channel, membrane potential, inactivation, selectivity filter, gating.

1. Introduction

The potassium channel is a ubiquitous membrane protein in living organisms from bacteria to humans, and mediates the selective K^+ flux across cell membranes[1]. K^+ permeation is turned on and off (gating) through the conformational changes of the transmembrane pore domain. The physiological role of the potassium channel varies substantially in the expressed cell types, but one of its most fundamental function in all cell types is housekeeping, generating the resting membrane potential across the cell membrane. Accordingly, all the membrane proteins are under the influence of the membrane potential.

Signal transduction function of channel proteins is intimately related to the membrane potential. In fact, the membrane potential plays an integral role in the channel performance: the membrane potential is a driving force for passive ion permeation through the channel; the channel undergoes conformational changes upon changes in the membrane potential, which is called voltage-dependent gating; and the ionic current through channels generates changes in the membrane potential, such as the action potential. Thus, studying channel proteins under electric fields is crucially important to understand the *in situ* channel function[2].

In this review, we focus on the KcsA potassium channel to understand effects of the membrane

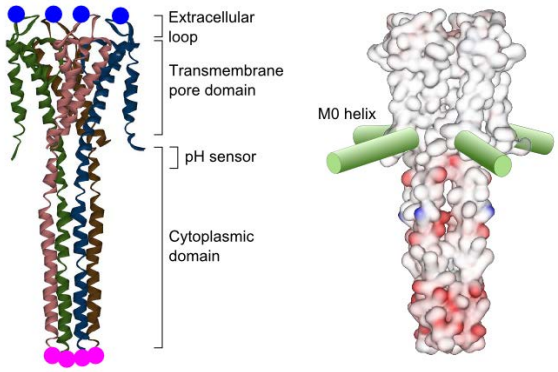

Figure 1. Crystal structure of the KcsA potassium channel in the closed conformation at neutral pH. The left panel shows the full length structure (PDBID: 3EFF). The molecule consists of the transmembrane pore (TM) domain and the cytoplasmic (CP) domain. The positions of R52 and 161 are presented as blue and pink spheres, respectively. The right panel shows a surface representation of the full-length KcsA channel with the M0 helices. The N-terminal M0 helix is an amphipathic helix and should be located at the membrane interface, but it has not been crystallographically resolved. Red represents negatively charged residues, and blue represents positively charged residues.

potential on the structure and function of channel proteins. With its shortest length of amino acid sequence and stability of the channel protein, KcsA, from bacterial origin, has been used frequently. The KcsA potassium channel was the first to be crystallised[3], revealing its fundamental structural features (Fig. 1)[4]. There are two domains in the KcsA channel; the pore domain and the cytoplasmic domain. The transmembrane pore domain is the most important for the ion permeation and gating. Structures of several types of potassium channels have been elucidated, demonstrating that the pore domains from different types of potassium channels share a common architecture. The potassium channels show strict K^+ selectivity, and the mechanism underlying K^+ selective permeation has been studied. Moreover, K^+ concentration-dependent and ion species-dependent changes of the KcsA function have been studied thoroughly.

Although several tens of atomic coordinates of the KcsA channel in various ionic conditions for various types of mutants have been registered in the protein data bank, the crystallography does not provide structural information under electric field because of its technical irrelevance. Similarly, spectroscopic methods generally use detergent-solubilized channel proteins and membrane potential could not be applied. Thus, surface-enhanced IR absorption spectroscopy (SEIRA) in an attenuated total reflection (ATR) mode (ATR-SEIRA)[5] under application of the electric field is a method of choice for studying potential dependent structural changes of channel proteins. SEIRA effects drastically increase the absorption intensity of molecules on the surface and prevent strong interference from the bulk solution. Coupled with an electrochemical system, SEIRA enables studies of the potential-dependent and concomitant ion concentration-induced structural changes of molecules adsorbed on an electrode surface: changes in the electrode potential also induce changes in the concentration of ions at the interface[6].

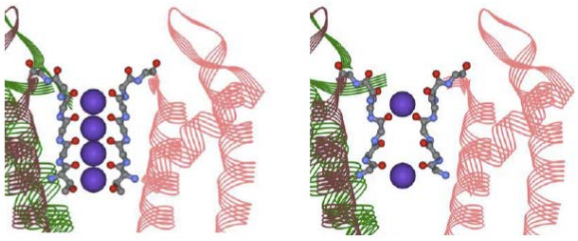

Figure 2. Structure around the selectivity filter at high (left) and low (right) K^+ concentrations. The carbonyl oxygens of the peptide backbone solvate K^+ ions. Only diagonal subunits are shown. The crystal structure represents superimposed images of the ion distribution in the selectivity filter, and under physiological conditions, two K^+ ions occupy the selectivity filter. PDB code: 1K4C (high K^+), 1K4D (low K^+).

In earlier studies, ATR-SEIRA under the application of the electric field has been applied to membrane proteins, such as cytochrome *c*[7], rhodopsin[8], hydrogenase[9], voltage-dependent anion channel[10]. Changes in the electrode potentials induce various changes in protein molecules, such as redox state changes, elongation and shortening of the protein, and orientation changes relative to the electrode surface. In the sensory rhodopsin, a proton released from the retinal by photoactivation is transferred under the applied electric field[8]. In the experiment of the voltage-dependent anion channel, the channels were embedded in the membrane, which is attached to the electrode. Thus electrical properties of the system were examined with the impedance spectroscopy[10]. These elegant experiments open the application of ATR-SEIRA for studying functional membrane proteins. However, effects of the membrane electric field are most closely related to the physiologically relevant channel function[11]. Thus, we demonstrate advantages of ATR-SEIRA under the electric field by applying to the KcsA channel. The background information of channel function under the membrane potential and recent results of studies on the structural changes of the KcsA channel are presented[12].

2. Functional structure of the KcsA channel

The KcsA channel comprises 160 amino acid residues and forms a tetramer to produce the active channel. The mechanism underlying the selective permeation and the gating has been studied extensively[13-16]. Owing to the compact size of each subunit and stability of the functional tetramer, KcsA has undergone various types of experimental studies, including circular dichroism, electron paramagnetic resonance, nuclear magnetic resonance, Fourier transform infrared spectroscopy and diffracted X-ray tracking method [17-23]. The crystal structures of KcsA under various experimental conditions have been revealed [14, 24, 25]. However, they have been obtained in the absence of the membrane, and hence some structural parts remained elusive. For example, the N-terminal M0 helix, which should be located at the membrane interface, has not been resolved (Fig. 1)[16]. Recently, atomic force microscopy revealed the membrane-embedded structure of the KcsA channel[26, 27], and the dynamic behaviour of the channel in the membrane is under investigation.

The KcsA channel is activated by the acidification of the cytoplasmic solution. There are two gates in series along the pore: the activation gate and the inactivation gate. The channel becomes ion conductive only if both gates are open. The pore domain is formed by the bundle of transmembrane helices, and the twisting and untwisting of the bundle provide the conductive and non-conductive conformations of the activation gate[19]. This global twisting underlies the pH-dependent activation.

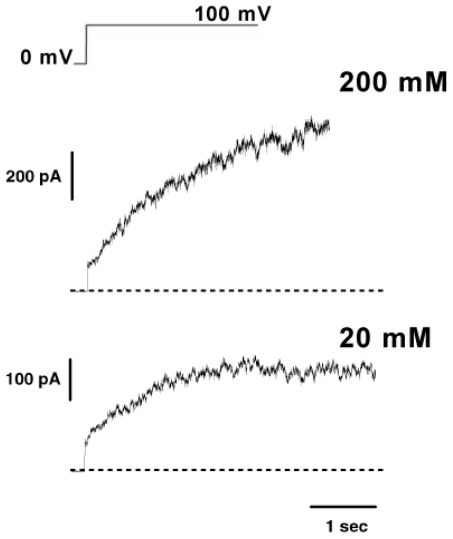

Figure 3. Voltage-dependent activation of the KcsA channel upon application of a positive potential. The macroscopic current of the KcsA channel measured from the KcsA-incorporated liposome (liposome-patch method). The time course of the activation differs significantly at different K^+ concentrations, suggesting the inactivation gate is involved. Reprinted from J. Neuroscience, 31, 120180-12188, 2011, Iwamoto, M. and Oiki, S., Counting Ion and Water Molecules in A Streaming File through the Open-Filter Structure of A K Channel.

Along the outer half of the pore, the diameter narrows to 3 Å for 12 Å in length until reaching the extracellular bulk space. This region is called the selectivity filter, where permeating ions must shed hydrating water and a bare K^+ ion is solvated by eight carbonyl oxygens of the peptide backbone (Fig. 2, left) [24,28,29] . The strict selectivity for K^+ over Na^+ is the most fundamental functional property of the potassium channels, for which the selectivity filter is responsible.

Physiologically, it has been known that the potassium channel becomes non-conductive at low K^+ concentrations[30]. Crystallographic analysis has revealed that the selectivity filter undergoes a structural change from the open conductive conformation in high K^+ solution to the collapsed non-conductive conformation in low K^+ or pure Na^+ solutions (Fig. 2, right)[24]. The conductive filter structure is maintained by the hydrogen bond network in the back of the filter structure, but is subject to change under various conditions, leading to the collapsed, non-conductive conformation. This structural change is called the inactivation gating.

The KcsA channel is not a so-called voltage-gated channel, but its gating shows weak voltage-dependency[31]. At acidic pH, macroscopic current recordings shows low conductance at negative potentials. As the membrane potential becomes positive, the current amplitude increases gradually (Fig. 3). At this pH, the activation gate opens nearly 100%. Thus, the mechanism underlying this increased conductance at positive potentials is attributed to the inactivation gate, rather than the activation gate: The inactivation gate exhibits voltage-dependent opening at positive potentials. In the back of the selectivity filter, there is a glutamate residue (Glu71), and it has been reported that the carboxylic moiety of Glu71 changes its orientation upon changes in the membrane potential[31]. Responses to the changes in the membrane potential are fundamental to operating the channel function, and structural study under different membrane potentials is crucially important.

3. SEIRA study of the structural change of KcsA fixed on an electrode surface

3.1 Oriented adsorption of KcsA molecules on the Au electrode

We first describe the method for applying SEIRA to the KcsA channel protein. The detergent (*n*-dodecyl-β-D-maltoside)-solubilised KcsA channel was immobilised on an Au electrode through mutated cysteine residues at the C-terminal end of the channel (161C-KcsA). The four cysteine residues securely fixed the channel in an upright orientation on the Au surface (Fig. 4) [12, 19]. At neutral pH, the activation gate is closed, and the SEIRA spectrum of 161C-KcsA was

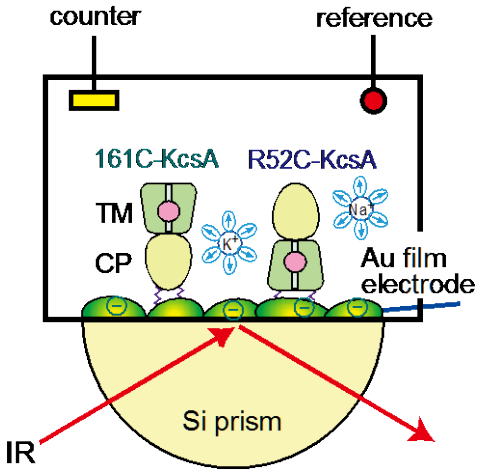

Figure 4. Experimental set up for surface-enhanced IR absorption spectroscopy to study the structural changes of KcsA on Au electrode induced by changes in the electrode potential. A cysteine mutation was introduced into the C-terminal end of KcsA (161C-KcsA) or into the extracellular loop of KcsA (R52C-KcsA). Four cysteine residues in the tetrameric channel react with the Au electrode, and the channel was fixed upright in either orientation. TM: transmembrane; CP: cytoplasmic. Reprinted from Chemical Physics, 419, Yamakata, A., et al., Structural changes of the KcsA potassium channel upon application of the electrode potential studied by surface-enhanced IR absorption spectroscopy, 224-228, Copyright (2013), with permission from Elsevier.

measured without applying the potential (Fig. 5). The number of attached KcsA molecules on the Au surface was sufficiently low that the channels were not doubly overlaid on the surface as demonstrated by atomic force microscopy[19]. In 50 mM Na^+ containing bathing solution (pH = 7.5), strong absorption peaks appeared at 1668 and 1550 cm^{-1}, which were assigned to amide-I and -II of the protein backbone vibration of KcsA, respectively[32]. A weak absorption peak appeared at 1400 cm^{-1}, which is the OCO symmetric stretching mode of carboxylate groups.

3.2 Spectral changes of adsorbed KcsA by application of the electrode potential

We then focused on the structural changes of KcsA upon the application of the electrode potential: potential-dependent SEIRA spectra of KcsA were observed in the presence of 50 mM Na^+ or K^+ at pH=7.5. In this experiment, the reference SEIRA spectrum was obtained by applying +200 mV to the Au electrode relative to the Ag/AgCl reference electrode, and the sample SEIRA spectra were measured at +100, 0, and -100 mV vs. Ag/AgCl electrode. The difference spectra were obtained by subtracting the reference spectra measured at +200 mV from the sample spectra measured at varying electrode potentials (Fig. 6A). In these difference spectra, three peaks were observed at 1660, 1550, and 1405 cm^{-1}, and by shifting the applied potential toward negative values, their intensity increased. These bands were present on the original KcsA adsorbed on the Au electrode and are ascribed to the amide-I and –II of the protein backbone vibration and the OCO symmetric stretching vibration. Thus, the structure of the adsorbed KcsA changes upon applying negative potentials.

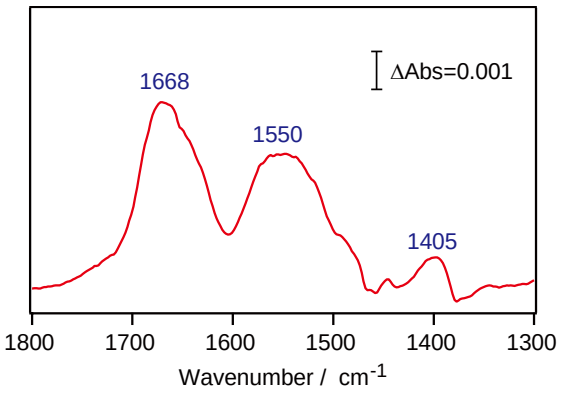

Figure 5. SEIRA spectrum of 161C mutant KcsA immobilized on Au electrode. The spectrum was measured in 50 mM Na^+ containing phosphate buffer (pH=7.5). The reference SEIRA spectrum was measured in the absence of adsorbed KcsA. Reprinted from Chemical Physics, 419, Yamakata, A., et al., Structural changes of the KcsA potassium channel upon application of the electrode potential studied by surface-enhanced IR absorption spectroscopy, 224-228, Copyright (2013), with permission from Elsevier.

Three peaks at 1660, 1550, and 1405 cm^{-1} were observed in either K^+ (red curves) or Na^+ (blue curves) solution, and their intensity increased with the negative potential sweep. On the other hand, a new peak appeared at 1565 cm^{-1} in the K^+ solution, and the band intensity at 1405 cm^{-1} increased slightly compared with that in Na^+ solution. These two peaks are ascribed to the asymmetric and symmetric OCO bands of carboxylate groups, respectively [32, 33]. The origin of the K^+-specific peaks will be discussed in the Section 3.4.

For better understanding of the specific interaction of K^+ with adsorbed KcsA molecules, the R52C mutant KcsA was used. Arginine 52 is located at the tip of the extracellular loop (Fig. 1); thus, replacing it with cysteine (R52C-KcsA) renders the channel fixed in the reverse orientation compared with 161C-KcsA on the Au surface (Fig. 4). The potential-dependent structural change of R52C-KcsA was also examined, as shown in Fig. 6B. Similar spectral changes were observed compared with 161C-KcsA, where the absorption intensities of the amide-I and -II bands increased at negative potentials. Furthermore, K^+-induced specific changes in the carboxylate bands increased their intensity at 1565 and 1405 cm^{-1} at negative potentials, which were observed for both oriented channels. These results seemed to be counter-intuitive at the first glance, because the voltage-dependent conformational changes generally occur with monotonically increasing or decreasing tendency, such as depolarization-activated or hyperpolarization-activated gating. Given the opposite orientations, why not is the potential dependency reversed?

3.3 The electric field-induced structural changes of KcsA

To understand the difference spectra for both orientations, the spectra in the K^+ solution for 161C-KcsA and R52C-KcsA are superimposed, as shown in Fig. 7. The peak intensities of the typical bands were enhanced at negative potentials, and this tendency was shared in both orientations. Slight changes were observed in the vibrational frequencies of the amide-I and -II bands (1660 and 1550 cm^{-1} for 161C-KcsA and 1642 and 1542 cm^{-1} for R52C-KcsA) for the oppositely oriented channels. Also, the predominant peaks for the asymmetric and symmetric OCO bands at 1565 and 1405 cm^{-1} were substantially more enhanced in the R52C mutant than those in the 161C mutant for all electrode potentials.

In these experiments with applied potential, the electric field is steep in the electric double layer formed between the Au electrode surface and the hydrated cations at the interface, and then gradually decays far beyond (Fig. 9). The typical thickness of the electric double layer is a few nm at the experimental ionic strength and hence partly covers the longitudinal

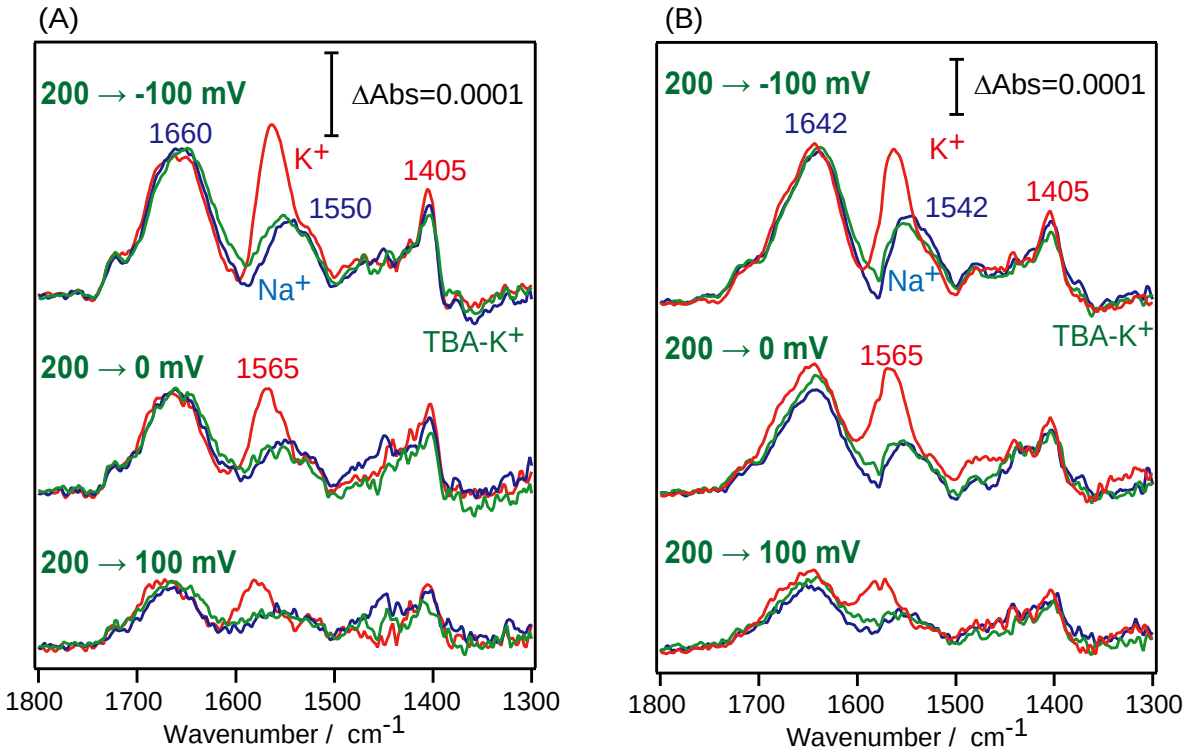

Figure 6. Difference SEIRA spectra. Difference SEIRA spectra of the mutant KcsA channels for 161C (**A**) and R52C (**B**). The KcsA channels are immobilized on an Au electrode. These SEIRA spectra were obtained by the subtractively normalized interfacial Fourier transform infrared spectroscopy (SNIFTIRS) method, in which 1000 interferograms were alternatively accumulated at the sample potentials (100, 0, and -100 mV vs. Ag/AgCl) and reference potential (200 mV vs. Ag/AgCl). After repeating this process 10 times, the accumulated interferograms were Fourier transformed and the spectrum calculated. The spectra were measured in the presence of 50 mM Na^+ (blue) or K^+ (red) solution at pH=7.5. The green curves were measured in the presence of 50 mM K^+, but with a channel blocker (TBA). TBA was loaded into the central cavity at acidic pH (=4.0) and then trapped when the pH was returned to neutral (=7.5). After sufficient rinsing, K^+ buffer (50 mM K^+ at pH = 7.5) was again introduced into the electrochemical IR cell, and the SEIRA spectra were measured. Reprinted from Chemical Physics, 419, Yamakata, A., et al., Structural changes of the KcsA potassium channel upon application of the electrode potential studied by surface-enhanced IR absorption spectroscopy, 224-228, Copyright (2013), with permission from Elsevier.

length of the KcsA channel (~10 nm). Note that the polarity of the electrode potential for the upside-down channels (R52C mutant) defined here is opposite to that used for the membrane potential in biological membranes, in which negative membrane potentials refer to the intracellular potential relative to the extracellular reference potential.

First, we discuss the origin of the potential-dependent structural change of KcsA, detected in the spectra at the amide-I and –II signals observed at 1660 and 1550 cm^{-1}. The amplitude of the peaks increased at negative potentials in both orientations. Thus, the results indicate that the channel responded to the absolute electric field rather than a polarity-specific manner. In the case of the KcsA channel, the voltage-dependent inactivation is more enhanced at negative membrane potentials. Thus, the bipolar voltage-dependent spectral changes observed here (the similar voltage dependence for both orientations) are not likely generated from the voltage-dependent gating conformational changes. The KcsA protein has net positive charges ([Arg + Lys = 19; His = 5; Asp + Glu = 13]), which are accumulated at the CP domain. At neutral pH, the four-helix bundle constitutes the CP domain, and large conformational changes of the CP domain are not feasible. In either the channel orientation, the distance of the centre of the charges of KcsA from the surface and/or the orientation angle of the channel on the surface should be changed in the electric field. Here, we hypothesise an alternative mechanism of the voltage dependence. In solubilised KcsA, the M0 helices are relatively freely movable[16] because of the lack of the interaction with the membrane lipids. Thus, it is likely that, with two positively charged residues, M0 helices change their orientation along the electric field (Fig. 8).

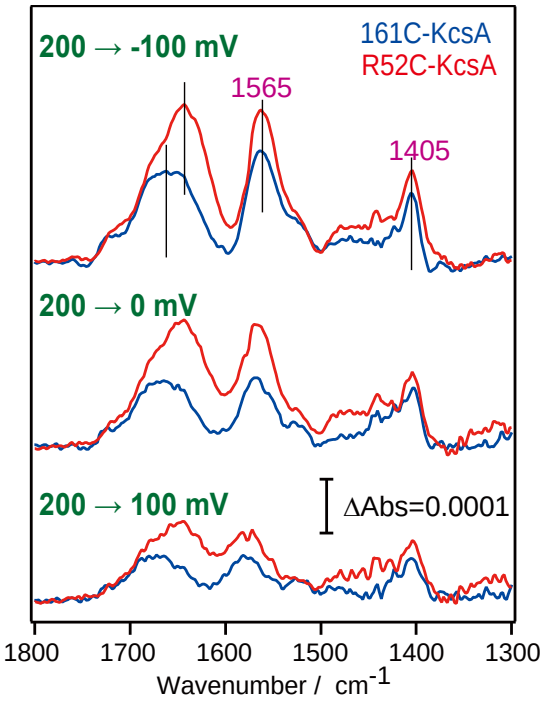

Figure 7. Comparison of K^+-induced structural change in KcsA on Au electrode. Potential difference SEIRA spectra of 161C-KcsA (blue) and R52C-KcsA (red) immobilized on an Au electrode, collected in 50 mM K^+ solution, are shown. These spectra were reconstructed from Figs. 4 and 5. Reprinted from Chemical Physics, 419, Yamakata, A., et al., Structural changes of the KcsA potassium channel upon application of the electrode potential studied by surface-enhanced IR absorption spectroscopy, 224-228, Copyright (2013), with permission from Elsevier.

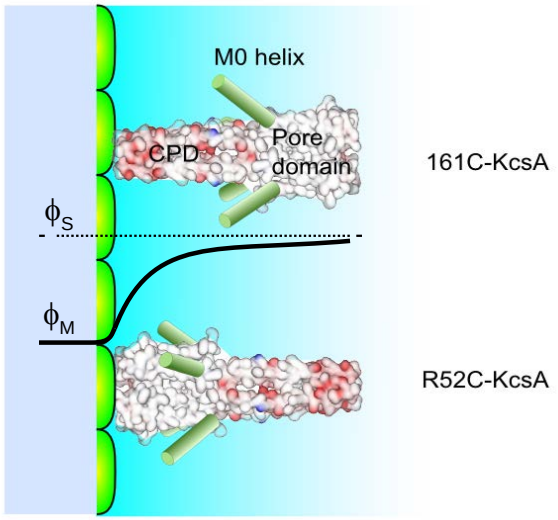

Figure 8. A hypothetical structural change upon application of an electric field. The cycteine residues at the end of the CP domain (161C-KcsA) react with Au surface and fixed the upright orientation (upper). Fixing the channel at the extracellular loop (R52C-KcsA) render the transmembrane domain closely located to the Au surface. In the solubilized KcsA channel, the M0 helices are allowed to move freely, and due to their net positive charges, the M0 helices change their orientation according to the electric field. This motion can account for the observed spectral changes for both oriented channels. The potential profile at the interface is shown. The values ϕ_S and ϕ_M represent the potentials of the solution and the metal electrode, respectively.

3.4 The K^+-induced structural changes of KcsA

Upon applying a negative potential, cations in the electrolyte solution build up near the Au electrode surface. Thus, the effects of the potential application to the electrode are doubled. Not only is the electric field applied near the surface but also the cations accumulate or deplete in the vicinity of the electrode surface.

In the K channel, K^+-specific structural changes occur in the selectivity filter such that the selectivity filter maintains the conductive conformation at high K^+ concentration but turns into the collapsed, non-conductive conformation at low K^+ or in the absence of K^+ (Fig. 2). The crystal structure revealed that the charged residues, E71 and D80, in the back of the pore-lining backbone structure form a hydrogen-bonded structure when K^+ concentration is low (Fig. 10). Additionally, Cordero-Morales et al. suggested that the E71 residues change their configuration according to the electric field[31]. At a positive potential, the local K^+ concentration is low, most likely well below several mM, causing the selectivity filter to adopt the collapsed conformation. When a negative potential is applied, the local K^+ concentration is accumulated and exceeds the concentration range for the transition (5-30 mM[13], 18 mM[22], 10-40 mM[34]) from the collapsed to the open conformation. Thus, it is most plausible that the K^+ accumulation imposed on the attached KcsA channels render the selectivity filter open at negative potential.

Both the electric field and the K^+ accumulation are prominent near the surface, and the SEIRA effect is also stronger in the vicinity of the surface. Thus, the conformational changes and the signals from them are preferentially recorded from the local structure close to the surface. In the upside-down oriented channel (R52C mutant), the transmembrane pore domain is located closer to the surface than in the 161C mutant. Thus, the augmented K^+-specific effect on the OCO bands for the R52C mutant upon the application of the electrode potential should represent the changes in the selectivity filter.

It is well known that Asp and Glu give different vibrations at 1574-1579 cm^{-1} and 1556-1560 cm^{-1}, respectively [32, 33]. Thus, the observed 1565 cm^{-1} band most likely originated from asymmetric OCO vibrations of the glutamate residue, E71.

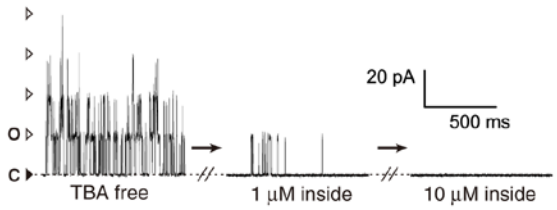

Figure 9. Blocking of the KcsA channel by TBA. The single-channel currents measured in the planar lipid bilayer method. TBA was added to the cytoplasmic space, and the channel current was blocked completely at 10 μM. Reprinted from J. Biol. Chem., 281, 28379-28386, 2006, Iwamoto, M. et al., Surface structure and its dynamic rearrangements of the KcsA Potassium channel upon gating and tetrabutylammonium blocking.

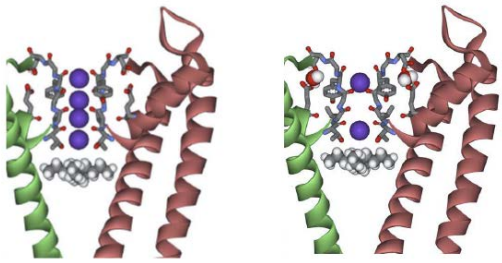

Figure 10. The structure of the selectivity filter with bound TBA at high and low K^+ concentrations. TBA molecule is demonstrated with a space-filling model attached at the bottom of the selectivity filter. There are two charged residues, E71 and D80, around the selectivity filter. PDB code: 2HVK and 2HVL.

3.5 The effect of a channel blocker, TBA, on the SEIRA spectra

To further examine the contribution of the selectivity filter to the K^+- and potential-dependent changes in the SEIRA spectra, the effect of a channel blocker, tetrabutylammonium (TBA), was studied. TBA blocks the K^+ current at low concentration when added to the intracellular solution (Fig. 9)[35]. The structure of the KcsA channel cocrystallised with TBA revealed that TBA binds at the inner entrance of the selectivity filter (Fig. 10)[34].

The surface-attached KcsA channel was treated with TBA, and residual TBA-solution was rinsed off with the buffer solution. At neutral pH, the activation gate is closed, preventing the escape of TBA from the cavity space, and TBA is thus kept bound at the end of the selectivity filter. The potential-dependent SEIRA spectral change revealed that even in K^+-containing solution, the spectral pattern was almost identical to the pattern observed in the Na^+ solution (Fig. 7, green): the amide-I and -II bands (1655 and 1540 cm^{-1}) increased in intensity at negative potentials, but the carboxylate bands (1565 and 1400 cm^{-1}) did not increase even in the presence of 50 mM K^+. Therefore, at negative potentials, accumulated K^+ near the electrode could not cause the collapsed filter to adopt the conductive conformation. It is likely that the bound TBA froze the structure of the selectivity filter in the collapsed conformation. We have previously reported that bound TBA locked the activation gate open at acidic pH[12], and here we revealed that TBA kept the selectivity filter collapsed even at high K^+ concentration.

4. Concluding remarks and future perspectives

ATR-SEIRA coupled with an electrochemical system is a powerful method for examining membrane proteins under electric fields. Using this method, we have examined the KcsA potassium channel to elucidate its voltage-dependent structural changes. Certain backbone structural changes occur upon applying negative potentials, but the specific location of the changes remains speculative. Additional studies using other mutants with the M0 helix deleted and cytoplasmic domain-truncated ones are necessary. On the other hand, the OCO signals can be assigned to the structural changes of the selectivity filter based on these experimental results, such as the different spectra in K^+ and Na^+ solutions, the orientation-dependent augmentation of the signal, and the effect of TBA. The potential applied to the Au electrode induced changes in the concentration of K^+ in the vicinity of the electrode, and physiologically well-known structural changes relating to the inactivation were detected by SEIRA.

The KcsA channel has been used in the study of the structure-function relationship of ion channels. Its simple structure, the extensive crystallographic and functional data available and its stability render KcsA a useful prototypical channel for various types of spectroscopic studies. Here, we introduced the electrochemical system in this SEIRA by fixing the KcsA channel with either orientation, which allows examination of the spectral change upon potential changes and local concentration changes. This method opens a new study approach for membrane proteins.

The results presented in this review are obtained at neutral pH, where the channel is in the resting state. At acidic pH, the pH-dependent activation gate opens, accompanied by global twisting conformational changes. Recently, coupling of the activation gating and the inactivation gating has been suggested. Thus, voltage-dependent SEIRA spectra at acidic pH would reveal the conformation of the activation gate and the activation gate-related inactivation gating, which are the targets of future study.

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References

1. B. Hille, in *Ion Channels of Excitable Membranes*, Sinauer Associates Inc., Sunderland, MA, USA, 2001, 3rd edn.
2. F. M. Ashcroft, in *Ion Channels and Disease*. Academic Press, London, 2000.
3. D. A. Doyle, J. M. Cabral, R. A. Pfuetzner, A. L. Kuo, J. M. Gulbis, S. L. Cohen, B. T. Chait and R. MacKinnon, *Science*, 1998, 280, 69-77.
4. S. Oiki, in *Gating Dynamics of the Potassium Channel Pore. Egelman E., Ed. Comprehensive Biophysics, Vol 6, Channel Proteins*, Academic Press, Oxford, 2012, pp. 31–67.
5. M. Osawa, *Bull. Chem. Soc. Jpn.*, 1997, 70, 2861-2880.
6. J. O. M. Bockris, A. K. N. Reddy and M. Gamboa-Aldeco, *Modern Electrochemistry*, Kluwer Academic/ Plenum Publishers, New York, 2nd edn., 2000.
7. K. Ataka and J. Heberle, *J. Am. Chem. Soc.*, 2004, 126, 9445-9457.
8. X. Jiang, E. Zaitseva, M. Schmidt, F. Siebert, M. Engelhard, R. Schlesinger, K. Ataka, R. Vogel and J. Heberle, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, 105, 12113-12117.
9. H. Krassen, S. Stripp, G. von Abendroth, K. Ataka, T. Happe and J. Heberle, *J. Biotechnol.*, 2009, 142, 3-9.
10. J. Kozuch, C. Weichbrodt, D. Millo, K. Giller, S. Becker, P. Hildebrandt and C. Steinem, Phys.Chem.Chem.Phys., 2014, 16, 9546-9555.
11. S. Oiki, *J. Physiol.* 2015, 593, 2553-2573.
12. A. Yamakata, H. Shimizu, M. Osawa and S. Oiki, *Chem. Phys.*, 2013, 419, 224-228.
13. Y. Zhou, J. H. Morais-Cabral, A. Kaufman and R. MacKinnon, *Nature*, 2001, 414, 43-48.
14. S. Uysal, V. Vasquez, V. Tereshko, K. Esaki, F. A. Fellouse, S. S. Sidhu, S. Koide, E. Perozo and A. Kossiakoff, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, 106, 6644-6649.
15. M. Iwamoto and S. Oiki, *J. Neurosci.*, 2011, 31, 12180-12188.
16. M. Iwamoto and S. Oiki, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, 110, 749-754.
17. D. M. Cortes and E. Perozo, *Biochemistry*, 1997, 36, 10343-10352.
18. J. H. Chill, J. M. Louis, C. Miller and A. Bax, *Protein Sci.*, 2006, 15, 684-698.
19. H. Shimizu, M. Iwamoto, T. Konno, A. Nihei, Y. C. Sasaki and S. Oiki, *Cell*, 2008, 132, 67-78.
20. S. Imai, M. Osawa, K. Mita, S. Toyonaga, A. Machiyama, T. Ueda, K. Takeuchi, S. Oiki and I. Shimada, *J. Biol. Chem.*, 2012, 287, 39634-39641.
21. J. A. Encinar, M. L. Molina, J. A. Poveda, F. N. Barrera, M. L. Renart, A. M. Fernandez and J. M. Gonzalez-Ros, *FEBS letters*, 2005, 579, 5199-5204.
22. Y. Furutani, H. Shimizu, Y. Asai, T. Fukuda, S. Oiki and H. Kandori, *J. Phys. Chem. Lett.*, 2012, 3, 3806-3810.
23. E. Perozo, D. M. Cortes and L. G. Cuello, *Nat. Struct. Bio.*, 1998, 5, 459-469.
24. Y. F. Zhou, J. H. Morais-Cabral, A. Kaufman and R. MacKinnon, *Nature*, 2001, 414, 43-48.
25. L. G. Cuello, V. Jogini, D. M. Cortes and E. Perozo, *Nature*, 2010, 466, 203-U273.
26. A. Sumino, T. Sumikama, M. Iwamoto, T. Dewa and S. Oiki, *Scientific Reports*, 2013, 3.
27. A. Sumino, D. Yamamoto, M. Iwamoto, T. Dewa and S. Oiki, *J. Phys. Chem. Lett.*, 2014, 5, 578-584.
28. A. N. Thompson, I. Kim, T. D. Panosian, T. M. Iverson, T. W. Allen and C. M. Nimigean, *Nat. Struct. Mol. Biol.*, 2009, 16, 1317-U1143.
29. S. Phongphanphanee, N. Yoshida, S.Oiki and F. Hirata, *Pure Appl. Chem.*, 2014, 86, 97-104.
30. J. Lopezbarneo, T. Hoshi, S. H. Heinemann and R. W. Aldrich, *Receptor. Channel.*, 1993, 1, 61-71.
31. J. F. Cordero-Morales, L. G. Cuello and E. Perozo, *Nat. Struct. Mol. Biol.*, 2006, 13, 319-322.
32. K. Nakamoto, *Infrared and Raman Spectra of Inorganic and Coordination Compounds Part A&B*, Wiley, New Jersey, 2009.
33. A. Barth and C. Zscherp, *Q. Rev. Biophys.*, 2002, 35, 369-430.
34. S. Yohannan, Y. Hu and Y. Zhou, *J. Mol. Biol.*, 2007, 366, 806-814.
35. M. Zhou, J. H. Morais-Cabral, S. Mann and R. MacKinnon, *Nature*, 2001, 411, 657-661.