Single Channel Analysis of Ion Transport across Membranes Containing Gramicidin A and KAT1 Channels

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2016

Contents

General Introduction

The charge transport across biomembranes play significant roles on various biofunctions such as aspiration, metabolism, photosynthesis, neurotransmission, *etc*. The energy conversion systems in living bodies consist of a series of the charge transports (ion transports and electron transfers) and have been investigated.[1-8] However, these reaction mechanisms have not been revealed in detail.

It is generally explained that the ion transport across biomembranes is mainly classified into three types as shown in Fig. 1; (i) an ion transport through an ion channel and/or a pump, (ii) an ion transport by a carrier compound and (iii) a direct transport of a hydrophobic ion across lipid bilayers. According to the conventional theory, it is difficult to understand these ion transport mechanisms because the imbalance of the electric charge occurs inside and outside the cell. In recent years, it has been elucidated that not only a hydrophobic ion or an ion associated with a carrier compound but also its counter ion is distributed from aqueous phases to the membrane and that both ions are transported by applying the membrane potential.[9,10] Taking these results and the electroneutrality principle into consideration, it is expected to reconstruct the understanding of ion transport mechanisms. On the other hand, the ion transport through the ion channel is usually recognized as the bloodstream of the ion transport in living bodies, and specific ions seems to be transported through the channel pore. Since the influence on the channel transport of the counter ion was predictable, the author investigated it and considered the transport processes by use of ion channels.

The electrophysiological properties of a single ion channel have been investigated

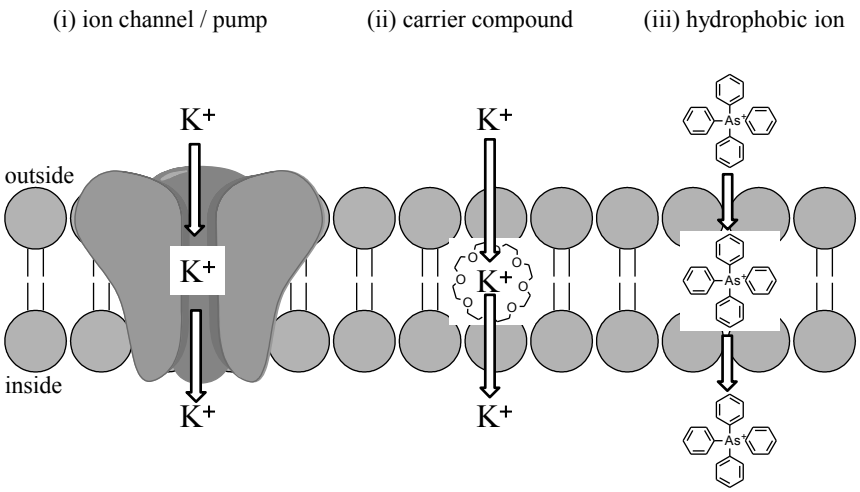

Fig. 1 Three types of the ion transport

by the patch clamp technique.[11-13] However, many kinds of molecules such as lipids, proteins, hydrophobic redox reagents, *etc*. coexist within the cell system, and they seem to affect channel properties such as single channel conductance, life-time, ion-selectivity, *etc*.[14-16] The planar bilayer lipid membrane has been utilized as artificial membranes in order to exclude the effects of their contaminants.[17-19] This method allows investigators to easily set and they can control the ionic composition of the solutions bathing both faces of the channel.[10,19]

In chapter 1, the author proposed an expression mechanism of the ion-selectivity of the Gramicidin A (GA) channel based on the analysis of single channel properties. Gramicidin A is a linear pentadecapeptide isolated from *Bacillus brevis* and is well known to form a simplest single ion channel within the membrane phase.

In chapter 2, the author evaluated the diffusion coefficients of K^+ and Cl^- within the GA channel by varying the concentration ratio of KCl in two aqueous phases. This evaluation was conducted by the non-linear least square regression analysis based on the proposed model. It was revealed that K^+ and Cl^- were distributed to the membrane

concurrently but the diffusion coefficient of K^+ within the membrane phase was different from that of Cl^-.

In chapter 3, the author measured single channel currents of KAT1 channel expressed in the planar bilayer lipid membrane. KAT1 channel is not a simple channel like the GA channel and is a typical voltage-gated K^+ channel from *Arabidopsis thaliana*. By considering the effect of the counter ions, the detailed ion transport mechanism through KAT1 channel was elucidated.

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Chapter 1

Ion Transport Model and Selectivity of Gramicidin A Channel

INTRODUCTION

Ion transport from one aqueous phase (W1) to another (W2) across a bilayer lipid membrane (BLM), which is one of the simplest biomembrane models, has been generally utilized in order to interpret mechanisms of ion transport across biomembranes.[1] Many studies have been performed on ion transport in the presence of ionophores, such as ion channels, ion channel-forming peptides, complex-forming agents and hydrophobic ions in BLM systems.[2-8] It is generally recognized that ion transport currents are generated by only the transport of ions associated with their transporters.

Gramicidin A (GA) is a linear pentadecapeptide isolated from *Bacillus brevis*, and exhibits antibiotic activity.[9] It is well known that two GA molecules form a single ion channel within the BLM phase and that small monovalent cations such as alkali metal ions and small neutral molecules such as water, urea, etc. can easily permeate through the pore of the GA channel.[10] The influence of the counter anions on the transport of cations has not been considered.[11-13] In this mechanism, however, the electroneutrality within the BLM is not considered. In addition, the author's group found that GA serves as not only a channel-type transporter but also a carrier-type transporter.[14] Since GA molecules can combine with some alkali cations as carrier compounds, these cations are distributed to the BLM with a counter anion. Then, the antiport of K^+ and Cl^- across the BLM was observed by applying the potential difference across the BLM after GA molecules were added to the cell system.

It was pointed out that the magnitude of ion transport current across the BLM depended on the hydrophobicity of the counter anion coexisting with the objective cation.[14-16] Watanabe *et al*. and Cohen investigated the effect of coexisting anions on the cation permeability by use of liposomes containing GA and proposed the possibility of penetration of not only cations but also anions at the same time in the presence of GA.[17,18] The electrochemical behavior of a single channel of GA and the influence of the counter anion on the transport of a monovalent cation has not yet been investigated.

In this chapter, the influence of ionic properties on the facilitated ion transport across BLM containing GA was investigated by recording the current fluctuation. Taking into account the distribution of both the alkali metal cation and its counter anion and the relation between the pore size of the GA channel and the ionic diameter of the transport ions, the author proposes a new ion transport model.

EXPERIMENTAL PROCEDURES

Chemicals

The lipids used to form BLM were lecithin (Wako Pure Chemical Ind., Ltd.) and cholesterol (Kanto Chemical Co., Inc.). Gramicidin A was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of reagent grade and used without further purification.

Preparation of BLMs

The electrochemical cell used for the experiments was essentially the same as that used in previous reports.[14-16] The cell has two aqueous compartments separated by a

tetrafluoroethylene resin sheet (Du Pont-Mitsui Fluorochemical Co., Ltd.) with a thickness of 0.2 mm. The compartments were filled with 15 mL of an aqueous solution. The aqueous solution contained 20 mM Bis-Tris buffer (pH 7.0) and 0.1 M 1:1 electrolyte salt. The BLM was obtained as a black lipid membrane by brushing a BLM-forming solution on a 1-mm diameter aperture created on a tetrafluoroethylene resin sheet. Gramicidin A was dissolved in ethanol at a concentration of about 10^{-3} M, and this solution served as a stock solution. An adequate volume of the ethanol solution was transferred to a 1-mL flask. After ethanol was evaporated, BLM-forming solution was prepared by dissolving the residual GA, 10 mg of lecithin and 5 mg of cholesterol into *n*-decane in the 1-mL flask. The amount of GA in the BLM was estimated from the molar ratio of BLM components in the forming solution. Cholesterol was added to obtain stable BLMs. The formation of BLM was confirmed by capacitance measurements and microscopic observations of light reflection from the membrane surface.

Single-channel recordings

The ion transport current between W1 and W2 across BLM, $i_{W1\text{-}W2}$, was recorded by applying a constant potential difference between W1 and W2; $E_{W1\text{-}W2}$. $E_{W1\text{-}W2}$ was applied through two Ag|AgCl electrodes, RE1 and RE2, soaked in W1 and W2, respectively, and is defined as the potential of RE1 referred to RE2. In this work, the positive current is defined as being due to the transports of positively charged species from W1 to W2 and those of negatively charged species from W2 to W1; $i_{W1\text{-}W2}$ was measured by two platinum wire electrodes. The current was sampled every 10^{-3} s and a low-pass filter with cut-off frequency of 10 Hz was used.

All electrochemical measurements were performed in a Faraday cage at room

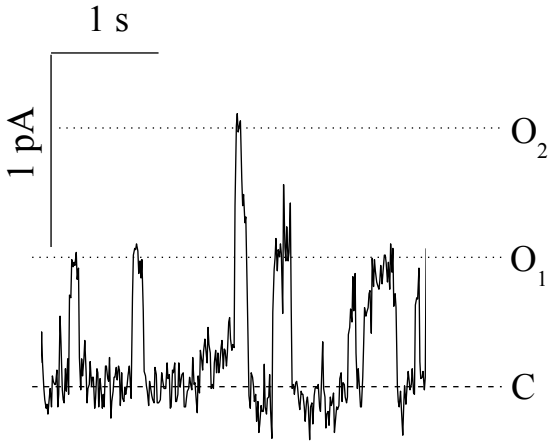

Fig. 1 A single-channel recording for GA in the presence of 0.1 M KCl. The applied potential was +50 mV. Concentration of GA in the BLM-forming *n*-decane solution: 10^{-7} M. C indicates the closed state and O the open state.

temperature using a four-electrode potentiostat Model HA-1010mM1A (Hokuto Denko Co.) and an A/D converter Model GL500 (Graphtec Co.).

RESULTS AND DISCUSSION

Single-channel current of a gramicidin A-channel between two aqueous phases containing alkali metal chlorides

In the absence of GA in BLM, the conductance of BLM ranged from 10 to 30 pS. The mean area of BLM was about 3×10^{-3} cm^2. These BLMs were stable for a few

hours, and no conductance fluctuation was observed in the range of $E_{W1\text{-}W2}$ from −120 to +120 mV.

Figure 1 shows a typical trace of $i_{W1\text{-}W2}$ in the presence of GA. It was obtained for BLM containing GA between W1 and W2 containing 0.1 M KCl as a supporting electrolyte. In this case, the concentration of GA in the BLM-forming *n*-decane solution was 10^{-7} M, and the applied $E_{W1\text{-}W2}$ was +120 mV. The dashed line (C) represents the closed state of the GA channel, and refers to the baseline. In the present case, a step-like current fluctuation was observed, and it related to the opening and closing of the GA channel. One or two channels were served (O_1 or O_2). The single channel current was evaluated as the difference between the mean value of the current flowing in the case of the opening state, O_1, and that of the baseline current by analyzing current-amplitude histograms of single-channel activities observed at each $E_{W1\text{-}W2}$. From the data obtained at $E_{W1\text{-}W2}$ ranging from +40 to +120 mV, single-channel currents were practically proportional to $E_{W1\text{-}W2}$.

As shown in Fig. 2, the single-channel current depends on the cation species and the selectivity is similar to those reported by other authors.[11-13,19,20] The single-channel current increased with an increase in the ionic diameter when the ionic diameter was smaller than about 0.30 nm. It is thought that this trend was caused by stabilization of the cation within the pore of the GA channel due to the association of the cation with GA.[13,19,20] On the other hand, several research groups reported that the GA channel was blocked by quarternary ammonium ions, such as tetramethylammonium ion (TMA^+), tetraethylammounium ion (TEA^+), tetrabutylammonium ion (TBA^+).[13,21-23] Table 1 indicates the ionic radius and hydration

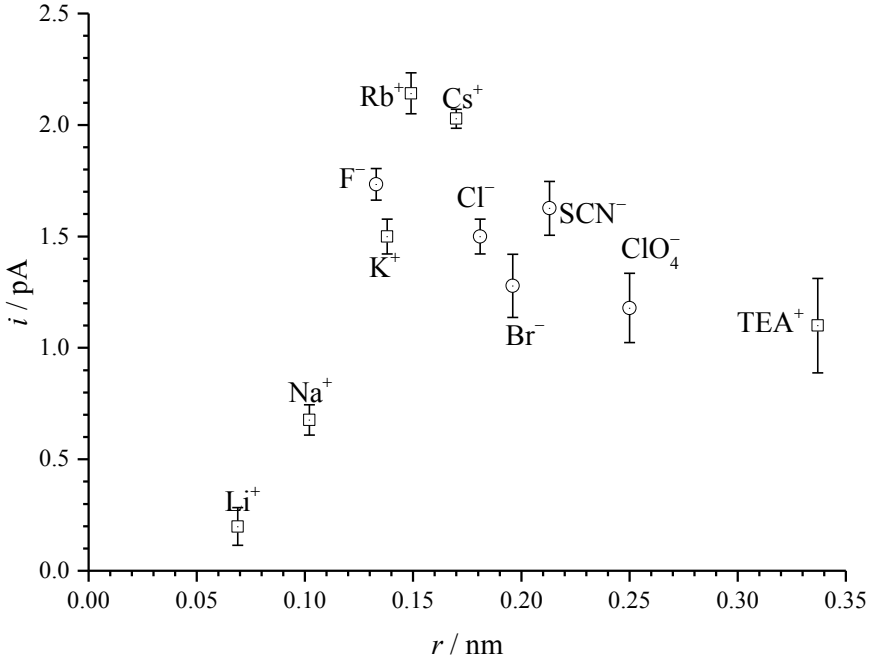

Fig. 2 The dependence of single-channel currents at +120mV on the ionic radii of ions. All cations (open square) were used as chloride salts and all anions (open circle) were used as potassium salts dissolved in 20 mM Bis-Tris buffer (pH 7.0). Concentration of GA in the BLM-forming *n*-decane solution: 10^{-7} M.

Table 1 Ionic radius, *r*, of ion and the standard molar Gibbs free energy of the hydration of ion[24]

Cation	Ionic radii	Hydration energy	Anion	Ionic radii	Hydration energy
	r/nm	ΔG°_{hyd} / kJ mol^{-1}		*r*/nm	ΔG°_{hyd} / kJ mol^{-1}
Li^+	0.069	–475	F^-	0.133	–465
Na^+	0.102	–365	Cl^-	0.181	–340
K^+	0.138	–295	Br^-	0.196	–315
Rb^+	0.149	–275	SCN^-	0.213	–280
Cs^+	0.170	–250	ClO_4^-	0.250	–205
TMA^+	0.280	–160			
TEA^+	0.337	0			
TBA^+	0.413	–			

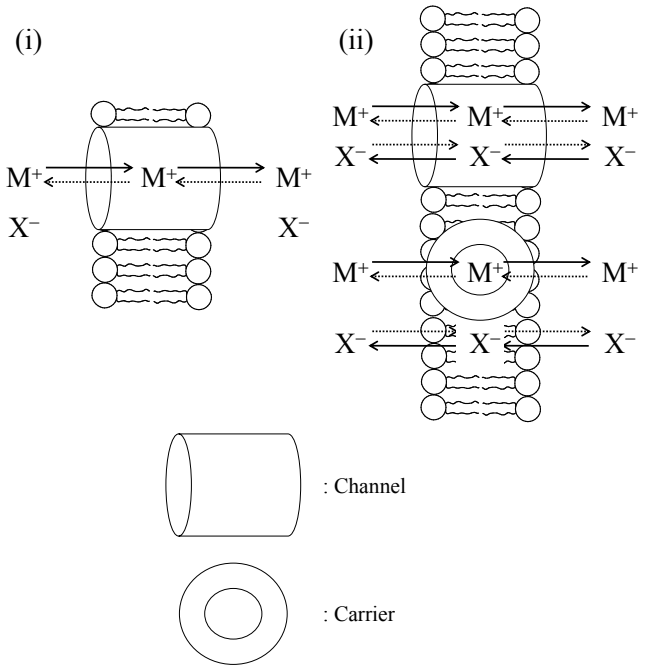

Fig. 3 Schematic model of ion transport mechamisms in the presence of GA. M^+: cation, X^-: anion. (i) conventional model and (ii) proposed model.

energy of various ions.[24] Taking into account the fact that the radius of these quarternary ammonium ions was much larger than the pore size of the GA channel (i.d. 0.38 nm), [9,13,23] the author can conclude that the GA channel exhibits the cation selectivity mentioned above on monovalent cations.

Single-channel current of gramicidin A-channel between two aqueous phases containing potassium salts

Figure 3(i) indicates the conventional ion-transport mechanism in the presence of GA channels.[9,10] It was frequently mentioned that the GA channel served as a cation-selective transporter without any anion transport. On the other hand, some research groups reported on the dependence of counter anions on the permeability of electrolytes through the GA channels by the use of liposomal membranes.[17,18] Then, the author also

revealed by cyclic voltammetry previously that the current density for the transport of K^+ across the BLM containing GA increased with an increase in the hydrophobicity of the counter anion.[15] The current density at a given $E_{W1\text{-}W2}$ was relative to the square of the concentration of GA in the BLM when the ionic diameter of the counter anion was smaller than the pore size of the GA channel. This property was in agreement with that of the channel-transport model.[13,23] However, the current density at a given $E_{W1\text{-}W2}$ was proportional to the concentration of GA in the BLM when the ionic diameter of the counter anion was larger than the pore size of the GA channel. Therefore, it was thought that the ion-transport current was attributable to the ion transport across the lipid layer site facilitated by GA as a carrier compound of K^+.[15]

The single channel current of the GA channel between two aqueous phases containing various potassium salts was evaluated. The result indicated that the single-channel current gradually decreased with increasing the diameter of the counter anion, when the diameter of anionic species was larger than the inner diameter of the channel pore, as shown in Fig. 2. The single-channel current obtained in the case of Cl^- was almost identical to that in the case of F^-. It is contemplated that the magnitude of the ion-transport current may depend on the amounts of transferring ions, K^+ and the counter anion, in the BLM and on the diffusion coefficients of the ions. Therefore, the author will discuss the ion transport of a single-charged cation, M^+, and that of a single-charged anion (counter ion), X^-, across the BLM in the presence of the same concentration of MX in W1 and W2, as presented here in,

$$\boldsymbol{c}_i^{W1,o} = \boldsymbol{c}_i^{W2,o} = \boldsymbol{c}_i \,, \tag{1}$$

where c_i is the concentration of one ion (i) in W1 and W2, $c_i^{W1,o}$ is the concentration of i in the neighborhood of the W1 | BLM interface in W1 and $c_i^{W2,o}$ is the concentration of i

in the neighborhood of the BLM | W2 interface in W2. Under the Goldman assumption,[25,26] the flux of i, J_i, is given as

$$\boldsymbol{J}_{\mathrm{i}} = -\frac{\boldsymbol{D}_{\mathrm{i}} \boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RTd}} \Delta \boldsymbol{E} \left\{ \frac{\boldsymbol{c}_{\mathrm{i}}^{\mathrm{W2,in}} \exp\left(\frac{\boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RT}} \Delta \boldsymbol{E} \right) - \boldsymbol{c}_{\mathrm{i}}^{\mathrm{W1,in}}}{\exp\left(\frac{\boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RT}} \Delta \boldsymbol{E} \right) - 1} \right\}, \tag{2}$$

where D_i is the diffusion coefficient of i in the BLM, z_i is the charge of i, F is Faraday constant, R is the gas constant, T is the temperature in K, d is the thickness of the BLM, ΔE is potential difference between W1 and W2, $c_i^{W1,in}$ is the concentration of i in the neighborhood of the W1 | BLM interface in the BLM and $c_i^{W2,in}$ is the concentration of i in the neighborhood of the BLM | W2 interface in the BLM. The distribution coefficient of one ion (i), β_i, is defined as

$$\boldsymbol{\beta}_{\mathrm{i}} = \frac{\boldsymbol{c}_{\mathrm{i}}^{\mathrm{W1,in}}}{\boldsymbol{c}_{\mathrm{i}}^{\mathrm{W1,o}}} = \frac{\boldsymbol{c}_{\mathrm{i}}^{\mathrm{W2,in}}}{\boldsymbol{c}_{\mathrm{i}}^{\mathrm{W2,o}}}. \tag{3}$$

By using Eqs. (1) and (3), Eq. (2) can be rewritten as

$$\boldsymbol{J}_{\mathrm{i}} = -\frac{\boldsymbol{c}^{\circ}{}_{\mathrm{i}} \boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RTd}} \Delta \boldsymbol{E} \boldsymbol{\beta}_{\mathrm{i}} \boldsymbol{D}_{\mathrm{i}}. \tag{4}$$

The current density of one ion (i), j_i, is given by

$$\boldsymbol{j}_{\mathrm{i}} = -\frac{\boldsymbol{c}^{\circ}{}_{\mathrm{i}} \boldsymbol{z}_{\mathrm{i}}^{2} \boldsymbol{F}^{2}}{\boldsymbol{RTd}} \Delta \boldsymbol{E} \boldsymbol{\beta}_{\mathrm{i}} \boldsymbol{D}_{\mathrm{i}}. \tag{5}$$

There are two ion species, M^+ and X^-, in the present ion transport system. Taking into account the electroneutrality within each phase, β_{M^+} is equal to β_{X^-},

$$\boldsymbol{\beta}_{\mathrm{M}^+} = \boldsymbol{\beta}_{\mathrm{X}^-} = \boldsymbol{\beta}. \tag{6}$$

Therefore, the observed ion transport current density, j_{total}, is equal to the sum of j_{M^+}

and j_{X^-},

$$\boldsymbol{j}_{\text{total}} = -\frac{\boldsymbol{c}^{\circ}\boldsymbol{F}^2}{\boldsymbol{RTd}}\Delta\boldsymbol{E}\boldsymbol{\beta}\left(\boldsymbol{D}_{M^+} + \boldsymbol{D}_{X^-}\right) \tag{7}$$

Then, the distribution coefficient, β, is expressed as Eq. (8) based on the distribution of ions at an aqueous | organic interface,[27]

$$\ln \boldsymbol{\beta} = -\frac{\Delta\boldsymbol{G}^{\circ}_{\text{tr,M}^+} + \Delta\boldsymbol{G}^{\circ}_{\text{tr,X}^-}}{2\boldsymbol{RT}}, \tag{8}$$

where $\Delta G^{\circ}_{\text{tr,i}}$ is the standard molar Gibbs energy of transfer of i from the aqueous phase to the BLM. The $\Delta G^{\circ}_{\text{tr,i}}$ value, however, is not evaluated exactly, because the BLM is too thin to measure the ion concentration. Therefore, the author may utilize the hydration energy, $\Delta G^{\circ}_{\text{hydi}}$, which is the energy required for dehydration of the ion as a measure of $\Delta G^{\circ}_{\text{tr,i}}$ by considering the fact that $\Delta G^{\circ}_{\text{hydi}}$ is proportional to $\Delta G^{\circ}_{\text{tr,i}}$ from W to organic solvents, such as nitrobenzene, 1,2-dichroloethane.[28,29] Accordingly, Eq. (8) can be rewritten as

$$\ln \boldsymbol{\beta} = -\frac{1}{2\boldsymbol{RT}}\left\{\left(\boldsymbol{A}_{+}\Delta\boldsymbol{G}^{\circ}_{\text{hyd,M}^+} + \boldsymbol{A}_{-}\Delta\boldsymbol{G}^{\circ}_{\text{hyd,X}^-}\right) + \left(\boldsymbol{B}_{+} + \boldsymbol{B}_{-}\right)\right\} \tag{9}$$

Here, A_+, A_-, B_+ and B_- are specific coefficients that are dependent on the solvent species. Equations (7) and (9) indicate that the magnitude of j_{total} at a given ΔE depends on both the diffusion coefficients of not only the counter anion, but also the cation and the distribution coefficients, and is proportional to ΔE. In addition, not only the counter anion, but also the alkali metal cation can hardly distribute from the aqueous phase to the BLM, because the counter anion cannot enter the channel pore when the ionic diameter is still larger than the pore size of the GA channel. In addition, the counter anion can hardly move within the channel pore. It is reasonable to suppose that a decrease in both the

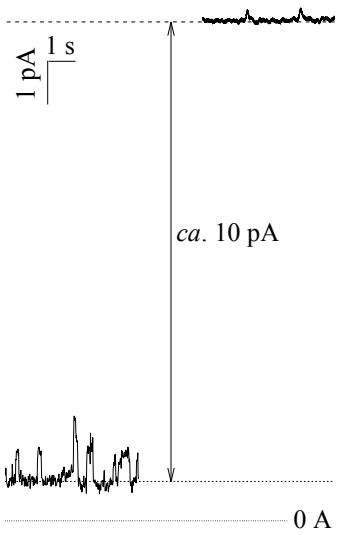

Fig. 4 Single-channel recordings in the presence of GA. Supporting electrolyte in W1 and W2: 0.1 M KCl (left) and 0.1 M $KClO_4$ (right).

amount of the electrolytes within the channel pore and the diffusion coefficient through the channel pore causes a decrease in the magnitude of the single-channel current. Therefore, it seems reasonable to conclude that large anions, such as ClO_4^- and SCN^-, reduce the ion permeation of not only the counter anion, but also the alkali metal cation through the GA channel. In practice, the magnitude of the single-channel current and the open-time channel probability decreased, as shown in Fig. 4, when $KClO_4$ was used instead of KCl as an electrolyte. Similarly, it seems reasonable to assume that TMA^+, TEA^+ and TBA^+ reduced the ion permeation through the GA channel. When the ionic diameter is smaller than the pore size of the GA channel, it is expected that β increases with an increase in the ionic diameter by considering $\Delta G^\circ_{\mathrm{hydj}}$ and the stabilization of the cation by association with GA. Since it can be assumed that j_{total} mainly depends on β in this case, j_{total} might increase with an increase in the ionic diameter. On the other hand, the baseline current rose by about 10 pA when $KClO_4$ or KSCN was used instead of KCl

as a supporting electrolyte. The magnitude of the baseline shift was in proportion to the concentration of GA within the BLM. By considering the data reported previously,[15] this result indicates that ClO_4^- and SCN^- facilitated the transport of K^+ across the lipid layer site by the addition of GA to the BLM. It is found from the result that GA served as a carrier compound of K^+, as shown in Fig. 3(ii).

Ion transport mechanism through gramicidin A-channel

Figure 3(ii) indicates a new mechanism of the ion transport. It consists of two transportation routes. One is ion transport across the lipid bilayer site. The other is ion transport through the pore of the GA channel, which is related to the single-channel current.

The amounts of transport ions across the lipid layer site are related to the height of the current baseline, as mentioned above. It is clear that the amounts of transport ions depend on the Gibbs free energies of the electrolyte ions and on the complex formation energy between the alkali metal cation and the GA molecule, based on the ion transport mechanism reported in a previous work.[15] Therefore, when an anion such as ClO_4^- and SCN^-, of which diameter is larger than the pore size of the GA channel, is used as the supporting electrolyte, the height of the current baseline increased.

The magnitude of the single-channel current increased with increasing the diameter of the transport ion, when the diameter of cationic species was smaller than the inner diameter of the channel pore. It seems reasonable to assume that the selectivity for ion permeation results from both the hydrophobicity of the transport ion and stabilization due to the interaction between the cation and the GA molecules. The magnitude of the single-channel current, however, decreased with an increase in the diameter of the

transport ion, when the diameter of the transport ion, such as TEA^+, TBA^+, ClO_4^-, SCN^-, *etc*. was larger than the inner diameter of the channel pore. It is quite likely that the ion of which the diameter is larger than the diameter of the GA channel pore closes the channel gateway and hardly moves within the channel pore.

SUMMARY

Ion transport from one aqueous to another across bilayer lipid membranes (BLM) containing gramicidin A (GA) was investigated by recording current fluctuations, when various alkali metal chlorides and potassium salts were used as supporting electrolytes. The magnitude of the single-channel current at a given membrane potential depended on not only cationic species, but also on anionic species, and then it decreased with an increase in the diameter of the anion when the diameter of the anion was larger than the pore size of the GA channel. The baseline of the recording current, however, increased with an increase in the diameter of the anion, and its height depended on the concentration of GA in the BLM. The results indicate that GA serves as not only a channel-forming compound, but also as a carrier compound in the BLM.

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Chapter 2

Analysis of the Ion Transport Current through a Single Channel

INTRODUCTION

The electrophysiological properties of a single ion channel have been investigated by the patch clamp technique.[1-3] However, many kinds of molecules coexist within cell systems and affect channel properties such as single channel conductance, life-time, permselectivity, *etc*.[4-6] The planar bilayer lipid membrane (BLM) has been used as artificial membranes in order to exclude the effects of their contaminants.[7-9] Since the electrochemical cell system using the BLM is convenient to structurally control the ionic composition of two aqueous phases and lipid constituents, it is easy to evaluate the permeability of all electrolyte ions and the influence of lipid components on the ion transport.[9,10]

Gramicidin A (GA) is a linear pentadecapeptide isolated from *Bacillus brevis* and is exhibiting antibiotic activity.[11,12] It is well known that two GA molecules form a single ion channel within the BLM phase and that small monovalent cations such as alkali metal ions and small neutral molecules such as water, urea, *etc*. can easily permeate through the pore of the GA channel.[13-16] In addition, the author's group found that GA serves as not only a channel-type transporter but also a carrier-type transporter.[17] Since GA molecules can combine with some alkali cations as carrier compounds, these cations are distributed to the BLM with a counter anion. Then, the antiport of K^+ and Cl^- across the BLM was observed by applying the potential difference across the BLM after GA molecules were added to the cell system. On the other hand, Cohen investigated the effect

of coexisting anions on the cation permeability by use of liposomes containing GA and proposed the possibility of penetration of not only cations but also anions at the same time in the presence of GA.[18] Although Eisenman, *et al*. reported also the influence of coexisting counter ions on permeability coefficient of the target transporting ion,[19] the antiport of the target cation and the counter anion was not assumed. The author's group pointed out that the magnitude of the single channel current became maximum when the ionic radius of not only the cation but also the anion was similar to the channel pore radius. The author proposed then the antiport of the target cation and the counter anion on the GA channel by analyzing the single channel conductance.

In this chapter, the influence of GA on the transport of electrolyte ions across bilayer lipid membranes is investigated by considering the electroneutrality and the mass balance in the whole phases. The expression mechanism of the permeselectivity is proposed based on the permeability of K^+ and Cl^-. By varying the concentration ratio of the objective electrolyte, KCl, in two aqueous phases, the diffusion coefficients of K^+ and Cl^- within the GA channel were evaluated in detail.

EXPERIMENTAL PROCEDURES

Chemicals

The lipids used were lecithin (Wako Pure Chem., Ind., Ltd.) and cholesterol (Sigma–Aldrich Co., Ltd.). GA was purchased from Sigma Chemical Co. All other reagents were of reagent grade and used without further purification.

Measurements of single channel currents

The electrochemical cell used for the BLM system was the same as that used in

previous works.[17] Two aqueous phases (W1 and W2) were filled with 15 mL of aqueous solutions and were separated with a 0.2-mm thick tetrafluoroethylene resin sheet (Mitsui Fluorochemical Co.). The BLM-forming solution was prepared by dissolving 10 mg of lecithin and 2.5 mg of cholesterol into *n*-decane in a 1-mL flask. The BLMs were obtained as black lipid membranes by brushing the *n*-decane solution of the lipids on a 1.0-mm diameter aperture created on the tetrafluoroethylene resin sheet. The formation of BLMs was confirmed by capacitance measurements and microscopic observations of the internal reflection from the membrane surface. The aqueous solutions contained 20 mM Bis-Tris buffer (pH 7.0) and x × 0.1 M KCl.

The single channel current between W1 and W2 across the BLM ($i_{W1\text{-}W2}$) was recorded when applying a constant potential difference between W1 and W2 ($E_{W2\text{-}W1}$). $E_{W2\text{-}W1}$ was applied by two Ag|AgCl electrodes (RE1 and RE2) that were soaked in W1 and W2, respectively, and $i_{W1\text{-}W2}$ was measured by two platinum wire electrodes (CE1 and CE2). The current was sampled at 1 kHz and a low-pass filter with cut off frequency of 10 Hz was used. The positive current is defined as the current due to the transports of positively charged species from W1 to W2 and to those of negatively charged species from W2 to W1.

The electrochemical cell was placed in a Faraday cage during the measurements in order to decrease background electric noises. All electrochemical measurements were performed using a potentiostat/galvanostat Model HA-1010mM1A (Hokuto Denko Co.) and an A/D converter Model mini LOGGER GL900 (Graphtec Co.) at 25 ± 1 °C.

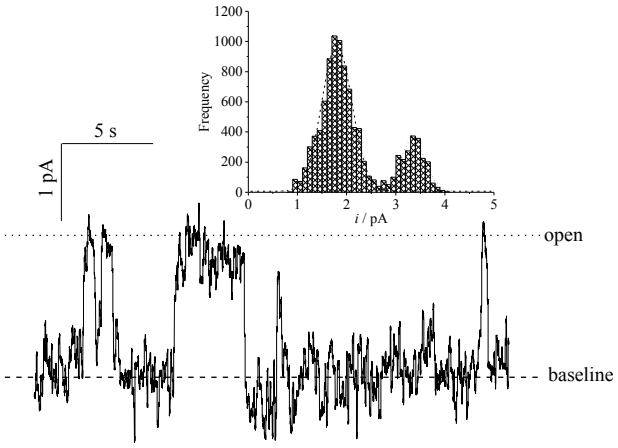

Fig. 1 Current fluctuation across the BLM between W1 and W2 in the presence of GA. Concentration of KCl in W1 and W2: 0.3 M. $T = 298 \pm 3$ K. The inset shows a typical histogram of the current fluctuation for 10 s.

RESULTS AND DISCUSSION

Single channel current of GA channel

In the absence of GA in the cell system, the capacitance of the BLM was 0.6 ± 0.1 μF cm^{-2}. The thickness of BLM was estimated at 6 ± 1 nm. The mean value of the area of the BLM was about 3×10^{-3} cm^2 by microscopic observation. The BLMs obtained were stable for a few hours, and no conductance fluctuation caused by any ion transporters was observed even when $E_{W1\text{-}W2}$ was applied in the potential range from −120 mV to +120 mV.

Figure 1 shows a typical fluctuation of $i_{W1\text{-}W2}$ between W1 and W2 containing 0.3 M KCl in the presence of GA. In order to decrease the change in the concentration of GA within the BLM, the concentration of GA in the BLM-forming *n*-decane solution was

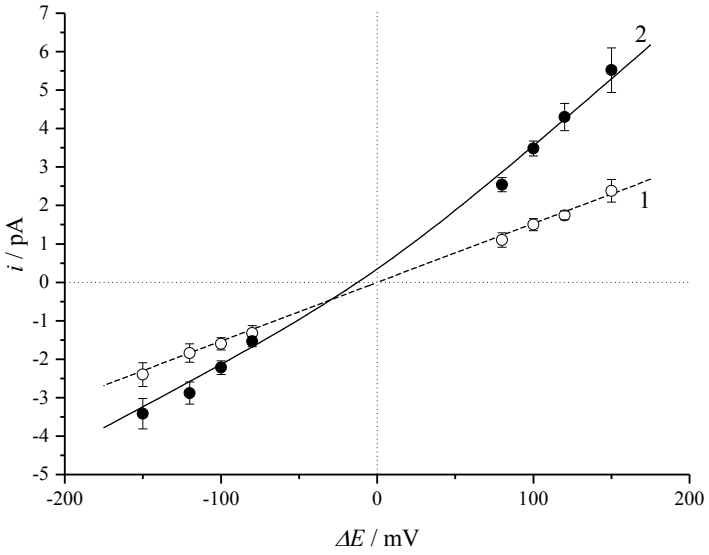

Fig. 2 Dependence of the single channel current on the membrane potential. The concentration of KCl in W1 and W2: 0.3 M (○). The concentration of KCl in W1 and W2: 1.0 M (W1) and 0.3 M (W2) (●). T = 298 ± 1 K. Curves 1 and 2: calculated curves fitted to the experimental results.

10^{-7} M and the concentration of GA in W1 and W2 was 10^{-9} M. In this case, the applied $E_{W2\text{-}W1}$ was +100 mV. The dashed line (C) represents a baseline and means that the GA channel is closed state. Then, a step-like current fluctuation was observed and it related to the opening state of the GA channel (O). The single channel current was evaluated as the difference between the mean value of the current flowing in the case of the opening state O and that of the baseline by analyzing current-amplitude histograms of single channel activities, as shown by the inset of Fig. 1. From the data obtained at $E_{W2\text{-}W1}$ ranging from +40 mV to +120 mV, the single channel current was practically proportional to $E_{W2\text{-}W1}$, as indicated by open circles (○) in Fig. 2. Similarly, the dependence of the single channel current on $E_{W2\text{-}W1}$ was equivalent in the potential region between –40 mV and –120 mV. In addition, the magnitude of the single channel current at a given $E_{W2\text{-}W1}$

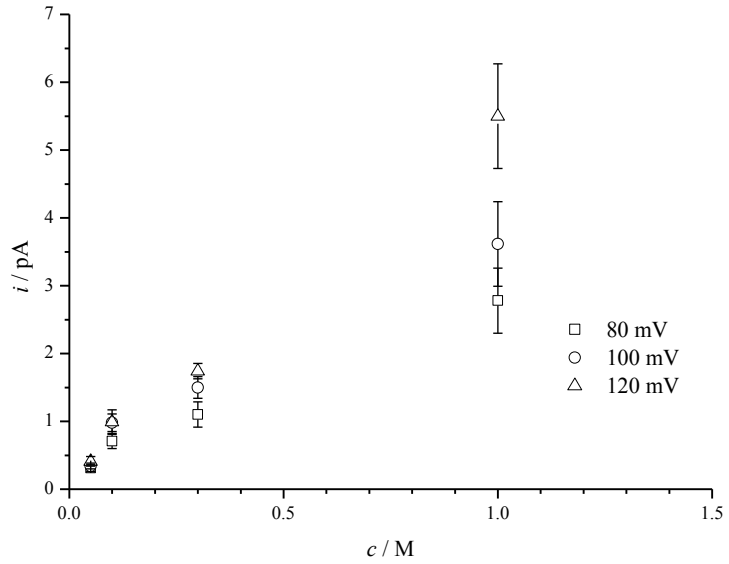

Fig. 3 Concentration dependence of the single channel current. Applied membrane potential: 80 mV, 100 mV and 120 mV.

was in proportion to the concentration of KCl in W1 and W2, as represented in Fig. 3. Thus, the single channel conductance standardized by the concentration of KCl was evaluated as 50 ± 5 pS M^{-1} (mean ± SD). The value of GA channel conductance obtained is close to those determined by the other groups.[20-22] Markham *et al*., Hirano *et al*. and Busath *et al*. reported the conductances as 29 ± 1, 73 ± 6 and 45.9 ± 0.4 pS M^{-1}, respectively. As described in chapter 1, the single channel current is composed of both the transport of cation and that of anion. In the present case, the positive current is caused by both transport of K^+ from W2 to W1 and that of Cl^- from W1 to W2, as shown in Fig. 4. On the contrary, the negative current occurs because of transport of K^+ from W1 to W2 and that of Cl^- from W2 to W1, when the negative potential is applied as $E_{W1\text{-}W2}$.

Transport of electrolyte ions in the case of an asymmetrical ionic composition cell system

The single channel current in the presence of 0.3 M KCl in W1 and W2 is

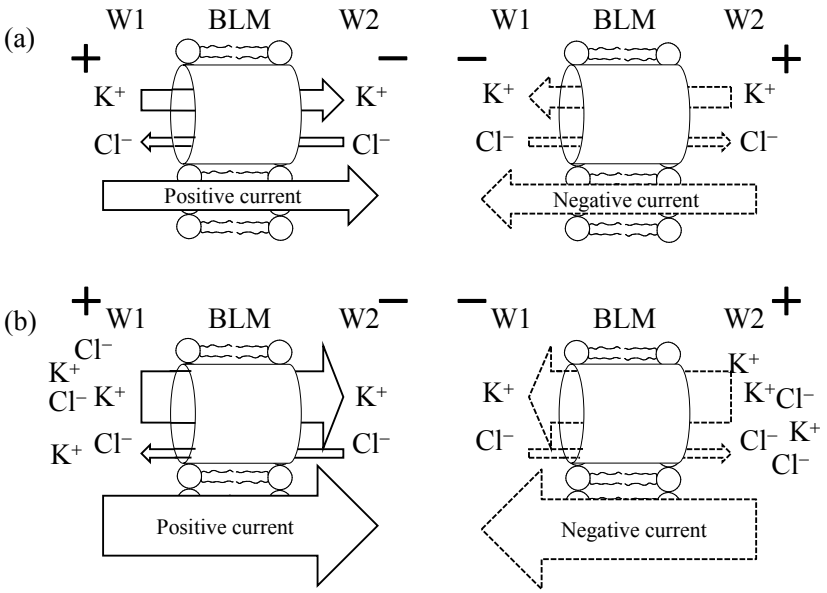

Fig. 4 Conceptional scheme on ion transport through the GA channel. Concentration of KCl: (a) W1 and W2: 0.3 M KCl, (b) W1: 1.0 M KCl, W2: 0.3 M.

proportional to the magnitude of $E_{W2\text{-}W1}$, as mentioned above. On the other hand, the single channel current between W1 containing 0.3 M KCl and W2 containing 1.0 M KCl in the presence of GA was also recorded, as represented by filled circles (●) in Fig. 2. An asymmetrical potential-dependent characteristic of the single channel current was observed about the origin (0 V, 0 A). The single channel current increased with an increase in the absolute value of $E_{W2\text{-}W1}$ ($|E_{W2\text{-}W1}|$). It is thought that the asymmetric behavior is generated by the difference between the cation selectivity and the anion selectivity, as shown in Fig. 2. By considering the direction of the ion transfer current, the magnitude of the single channel current in the positive potential region increases with an increase of the concentration of K^+ in W1 (0.3 M → 1.0 M). Therefore, the increment of the single channel current corresponds to the increment of the positive current caused by the transfer of K^+ from W1 to W2. On the other hand, the increase in the single channel current in the negative potential region is attributed to the increment of the current caused by the

transfer of Cl^- from W1 to W2 (0.3 M → 1.0 M). By the way, the distribution process might be affected by the electrical charge of the BLM phase or the channel pore phase. In the present case, not only K^+ but also Cl^- seems to be distributed simultaneously from the aqueous phase to the GA channel pore phase,[17] because the GA channel doesn't have any charged functional groups.[23] Accordingly, the ratio of the diffusion coefficient of K^+ (D_{K+}) within the channel pore phase to that of Cl^- (D_{Cl-}) can be evaluated by assuming that the distribution coefficient of K^+ between the aqueous phase and the GA channel pore (β_{K+}) is equivalent to that of Cl^- (β_{Cl-}) and is constant.

Permeability of electrolyte ions on the single channel of GA

As mentioned above, it is thought that the magnitude of the positive current in the region of the positive potential depends on both the amount of K^+ transferred from W2 to W1 and that of Cl^- transferred from W1 to W2. Similarly, the negative current in the negative potential region is derived from the sum of the amount of K^+ transferred from W1 to W2 and that of Cl^- transferred from W2 to W1. According to the Goldman assumption,[24,25] the flux of an ion i (J_i,) is given as Eq. (1).

$$\boldsymbol{J}_{\mathrm{i}} = -\frac{\boldsymbol{D}_{\mathrm{i}} \boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RTd}} \Delta \boldsymbol{E} \left\{ \frac{\boldsymbol{c}_{\mathrm{i}}^{\mathrm{BLM2}} \exp\left(\frac{\boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RT}} \Delta \boldsymbol{E} \right) - \boldsymbol{c}_{\mathrm{i}}^{\mathrm{BLM1}}}{\exp\left(\frac{\boldsymbol{z}_{\mathrm{i}} \boldsymbol{F}}{\boldsymbol{RT}} \Delta \boldsymbol{E} \right) - 1} \right\} \tag{1}$$

where D_i is the diffusion coefficient of i within the BLM, z_i is the charge of i, F is Faraday constant, R is gas constant, T is the temperature, d is thickness of the BLM, ΔE is potential difference between W1 and W2 (the membrane potential), c_i^{BLM1} is concentration of i at the neighborhood of the W1|BLM interface in the BLM and c_i^{BLM2} is concentration of i at the neighborhood of the BLM|W2 interface in the BLM. Assuming the distribution

coefficient is constant at both W1|BLM and BLM|W2 interfaces, the distribution coefficient of i (β_i) is defined as Eq. (2).

$$\beta_i = \frac{c_i^{W1}}{c_i^{BLM1}} = \frac{c_i^{W2}}{c_i^{BLM2}} \tag{2}$$

By using Eq. (2), Eq. (1) can be rewritten as Eq. (3).

$$J_i = -\frac{D_i z_i F}{RTd} \Delta E \beta_i \left\{ \frac{c_i^{W2} \exp\left(\frac{z_i F}{RT} \Delta E\right) - c_i^{W1}}{\exp\left(\frac{z_i F}{RT} \Delta E\right) - 1} \right\} \tag{3}$$

Since the magnitude of the single channel current due to the transfer of i (i_i) is in proportion to J_i ($i_i = z_i F S J_i$), Eq. (3) can be converted to Eq. (4). Here, S is the area of the GA channel pore.

$$i_i = -\frac{D_i z_i^2 F^2 S}{RTd} \Delta E \beta_i \left\{ \frac{c_i^{W2} \exp\left(\frac{z_i F}{RT} \Delta E\right) - c_i^{W1}}{\exp\left(\frac{z_i F}{RT} \Delta E\right) - 1} \right\}$$

(4)

Only two ion species, K^+ and Cl^-, existed in the present system. Then, the observed ion transport current, i_{total}, is equal to the sum of i_{K^+} and i_{Cl^-}.

$$i_{total} = -\frac{c^\circ F^2}{RTd} \Delta E \beta \left(D_{K^+} + D_{Cl^-} \right)$$

(5)

On the other hand, the distribution coefficient of KCl (β) is expressed as Eq. (6) based on the distribution of ions at aqueous|BLM interface.[10]

$$\ln \beta = -\frac{\Delta G^\circ_{tr,K^+} + \Delta G^\circ_{tr,Cl^-}}{2RT} \tag{6}$$

where $\Delta G^\circ_{tr,i}$ is the standard molar Gibbs energy of transfer of i from aqueous phase to the BLM. It is difficult to evaluate the $\Delta G^\circ_{tr,i}$ value exactly, because the BLM is too thin

to measure the ion concentration in it. Incidentally, the author can evaluate the ratio of D_{M^+} to D_{Cl^-} by comparing the data of open circles with those of filled circles of Fig. 2. Then, Eq. (5) is transformed to Eq. (7) by using the ratio of D_{M^+} to D_{Cl^-} (□).

$$i_{total} = -\frac{c^\circ F^2}{RTd} \Delta E \beta D_{Cl^-} (1+\alpha) \tag{7}$$

Curve 1 of Fig. 2 indicates the fitting curve on the potential dependence of the single channel current of the GA channel by use of Eq. (7) when W1 and W2 contain 0.3 M KCl. Next, curve 2 of Fig. 2 is obtained by fitting Eq. (7) to results observed when W1 and W2 contained 1 M KCl and 0.3 M KCl, respectively. When the author assumes α = 2.6, the calculated curve is in fair agreement with experimental results. As expessed in Fig. 4, it is clear that the ion transport across the BLM in the presence of GA is generated by the antiport of K^+ and Cl^-. Thus, the single channel currents of the GA channel were analyzed in the present work, and it is expected the author can comprehend the channel properties more than before.

SUMMARY

Ion transport through a single channel of gramicidin A (GA) within the bilayer lipid membrane (BLM) between two aqueous phases (W1 and W2) was analyzed based on the electroneutrality principle. The single channel current increased in proportion to the magnitude of the applied membrane potential and was also dependent on the permeability coefficients of electrolyte ions (K^+ and Cl^-). By varying the ratio of the concentration of KCl in W1 to that in W2, the ratio of the diffusion coefficient of K^+ in the BLM to that of Cl^- in the BLM could be evaluated.

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Chapter 3

Ion Transport across Membranes Containing KAT1 Potassium Channel

INTRODUCTION

KAT1 channel, a voltage-gated K^+ channel from *Arabidopsis thaliana*,[1,2] is expressed in guard cells and has been suggested to have a key role in stomatal opening by osmoregulation. It consists of four identical subunits and belongs to the family of inward-rectifying *Shaker*-type channels. The electrophysiological properties of KAT1 channels that were expressed in the heterologous systems such as oocytes of *Xenopus laevis*, insect cells and yeast have been investigated by the patch clamp technique.[3,4] The planar BLM method using an artificial membrane can have a number of advantages for the determination of the biophysical properties of the channel,[5] as compared to the patch clamp technique using a natural membrane. This method allows the investigator to easily set and strictly control the ionic composition of the solutions bathing both faces of the channel protein, which are essential for the comprehensive characterization of the ion-channel conduction and selectivity. This method has been applied in this work by making the BLM area smaller in order to diminish the electrical noise.

It has been reported that monovalent cations such as Rb^+, Cs^+ and tetraethylammonium ion (TEA^+) inhibited KAT1 channel under a given range of applied membrane potential values.[6, 7] This inhibition has been explained as a channel blocker. On the other hand, the author has shown an example that the magnitude of the single channel current at a given membrane potential decreased on the dependence of the species

of the counter anions. The influence of the coexisting anions on the single channel current of KAT1 channel has not yet been evaluated. However, the author has presented a wealth of detailed evidence for the similar anion transport in gramicidin A channel which is a well-known cation selective channel.[8-10] The single channel current of gramicidin A is changed by the replacement of Cl^-, which is a counter anion of K^+, with other anions.[11]

In this chapter, single channel recordings of KAT1 channel in the planar BLM system were analyzed in order to diminish the effect of these interfering substance and to elucidate the detailed ion transport mechanism by considering the effect of the counter ions.

EXPERIMENTAL PROCEDURES

Reagents and chemicals

The lipids used to form a planar BLM were L-α-phosphatidylcholine (Sigma-Aldrich, Co.) and cholesterol (Sigma-Aldrich, Co.).

All other reagents were of reagent grade and used without further purification.

Preparation of BLMs

The electrochemical cell used for the electrochemical measurements with the BLM system was essentially the same as that used in the previous works.[11-15] Two aqueous phases (W1 and W2), which were filled with about 400 μL of aqueous solution respectively, were separated by a tetrafluoroethylene resin sheet (Du Pont-Mitsui Fluorochemical Co., Ltd.). The aqueous solution contained 20 mM Bis-Tris buffer components (pH 7.0) and a given concentration of 1:1 electrolyte salt. The BLM was

obtained as a black lipid membrane by brushing a BLM-forming solution on a *ca*. 0.6-mm diameter aperture created on the tetrafluoroethylene resin sheet. The BLM-forming solution was prepared by dissolving 10 mg of L-α-phosphatidylcholine and 5 mg of cholesterol into *n*-decane in a 1-mL flask. The formation of BLMs was confirmed by capacitance measurements and microscopic observations of internal reflections from membrane surface.

Expression of KAT1 channel

The details of expression and purification of KAT1 channel were written in elsewhere.[16] The channel protein was solubilized from the membrane fraction of the cultured cell. KAT1 solution also contained 5 mM *n*-dodecyl-β-D-maltopyranoside (DDM; Affymetrix, Inc. Anatrace Products), NaCl (<400 mM), 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Wako Pure Chemical Industries, Ltd.), 1 mM phenylmethylsulfonyl fluoride (PMSF; Tokyo Chemical Industry Co., Ltd.), 1 mM ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid (EDTA; Dojindo Molecular Technologies, Inc.), 2 μg mL^{-1} L-α-phosphatidylcholine and 1% protease inhibitor cocktail (Nacalai Tesque, Inc.) in 50 mM sodium phosphate buffer (pH 7.4).

Reconstitution of KAT1 channel

KAT1 was reconstituted into the preformed L-α-phosphatidylcholine liposome by the freeze-thaw method,[5,16-18] described as below. Fifty milligrams of L-α-phosphatidylcholine was dissolved in an adequate amount of chloroform, and it was placed in a 300 mL brown colored round-bottom flask. Chloroform was removed by a rotary evaporator to obtain a thin lipid film on the inside surface of the flask. The lipid

film was suspended in 5 mL of a 20 mM Tris buffer solution (pH 7.0) containing 300 mM sucrose and an electrolyte such as KCl, KF, KBr, KSCN, $KClO_4$ and tetraethylammonium chloride. The lipid suspension was frozen at −70°C in an ethanol bath and was thawed at room temperature. These procedures were repeated several times. Then, the unilamellar liposome was prepared from the lipid suspension by using the Mini-Extruder (Avanti Polar Lipids, Inc., Catalog No. 610000). The diameter of the liposome was approximately 100 nm, when the pore size of the filter of the extruder is around 100 nm. The liposome solution obtained was mixed with the purified KAT1 solution until the concentration of lipids became twenty times as high as that of the purified KAT1 solution. The mixture was also frozen at −70°C in an ethanol bath and was thawed at room temperature. The freeze-thawing process was repeated several times.

After the formation of the planar BLM in the electrochemical cell, an adequate volume of the liposome solution containing KAT1 was added to W2 by stirring to promote the fusion of the proteoliposomes with the BLM.

Recordings of single channel currents

The ion transport current between W1 and W2 across the BLM (I) was recorded when applying a constant potential difference between W1 and W2 ($E_{W2\text{-}W1}$). $E_{W2\text{-}W1}$ was applied through two Ag|AgCl electrodes (RE1 and RE2) which were soaked in W1 and W2, respectively, and I was measured by two platinum wire electrodes (CE1 and CE2). The current was sampled at 1 kHz and a low-pass filter with cut off frequency of 10 Hz

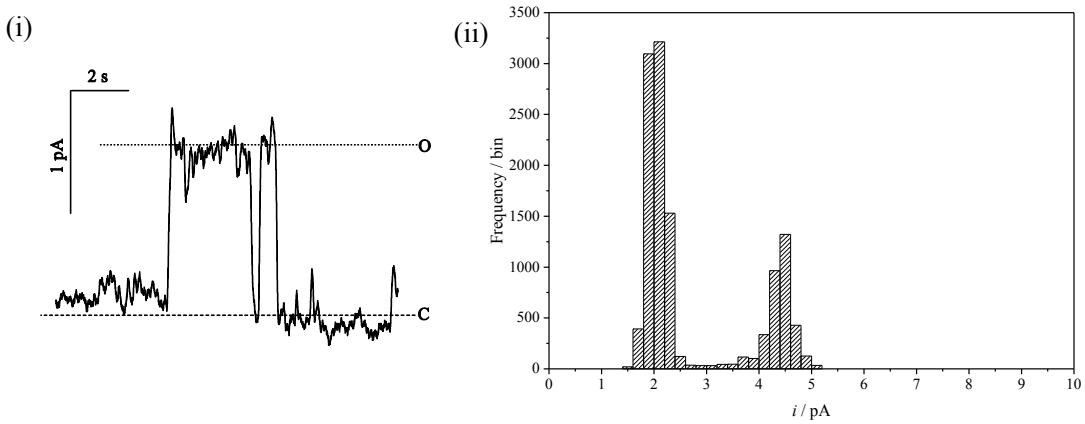

Fig. 1 (i) Single channel recording of KAT1 channel in 0.3 M KCl. The applied potential was +0.1 V. C indicates the closed state and O the open state. (ii) Amplitude histogram constructed from the data for measurement at +0.1 V.

was used. In this work, the positive current is defined as the current due to the transports of positively charged species from W1 to W2 and to those of negatively charged species from W2 to W1.

The electrochemical cell was placed in a Faraday cage in order to decrease background electric noises. All electrochemical measurements were performed using a potentiostat/galvanostat Model HA-1010mM1A (Hokuto Denko Co.) and an A/D converter Model mini LOGGER GL900 (Graphtec Co.) at room temperature.

RESULTS AND DISCUSSION

Single channel currents through KAT1 channel

The conductance of the BLMs ranged from 10 to 30 pS before the addition of the proteoliposomes containing KAT1 channels. The average value of the BLM area was about 3×10^{-3} cm^2 and these BLMs were stable for a few hours. Current fluctuation was

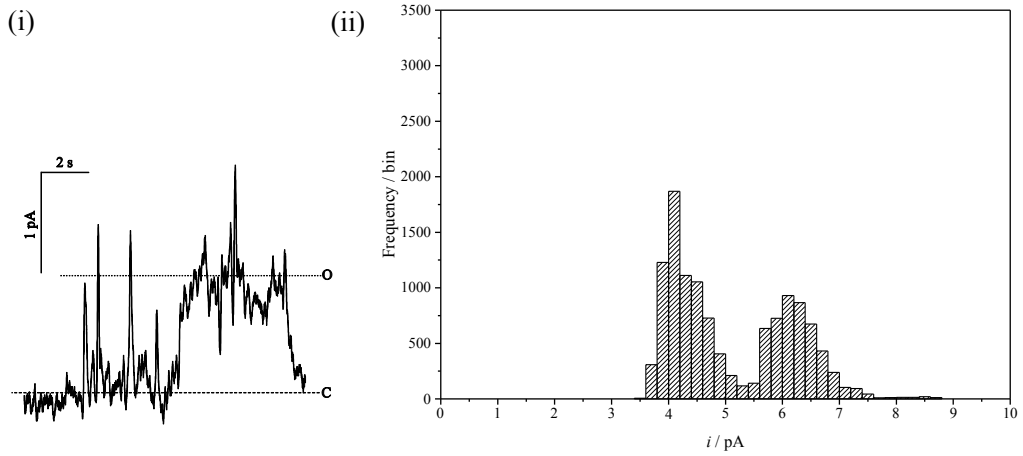

Fig. 2 (i) Single channel recording of KAT1 channel in 0.3 M KSCN. The applied potential was +0.1 V. C indicates the closed state and O the open state. (ii) Amplitude histogram constructed from the data for measurement at +0.1 V.

not observed even when $E_{W1\text{-}W2}$ was applied.

The proteoliposomes containing KAT1 were added to W2, and the aqueous phases were stirred for 30 s. After the fusion of the proteoliposomes with the planar BLM, the current fluctuation between W1 and W2 appeared, as shown in Fig. 1 (i), where the broken line expresses the level of the closed (C), and the dotted line represents the open state of one channel (O), respectively. Their data were obtained when 0.3 M KCl were used as an electrolyte solution in W1 and W2. It is true that the residual detergent (DDM) and other chemicals (TCEP, PMSF, EDTA and protease inhibitor cocktail) remained in the proteoliposome solution, but any current fluctuations were not caused on the addition of the same amounts of the detergent and other chemicals. Based on this result, it is reasonably concluded that KAT1 channel is reconstituted in the BLM and is responsible for the current fluctuation.

Current-amplitude histogram of single channel activity was constructed from the data observed at 100 mV (Fig. 1 (ii)). In this histogram there appeared two peaks which

Table 1 Conductance ratio of KAT1 channel in the potassium salt solution and ionic radii of anion species[25]

	Cl^-	F^-	Br^-	SCN^-	ClO_4^-
% Cl^- single channel conductance	100	87 ± 11	94 ± 9	83 ± 9	55 ± 19
ionic radius / nm	0.181	0.133	0.196	0.213	0.250

represent open and closed states. By analyzing the histogram, the single channel conductance standardized by the concentration of KCl was evaluated as *ca*. 47 ± 2 pS M^{-1}. In this case, the value of KAT1 channel conductance obtained by the BLM method is close to those determined by the whole-cell patch clump analyses. Hedrich *et al*. and Brüggemann *et al*. reported the conductances as 50 pS M^{-1} and 57 ± 2 pS M^{-1}, respectively.[19,20] These values were converted in comparison with our value. When 0.3 M KSCN was used instead of KCl as an aqueous electrolyte solution in W1 and W2, characteristic current fluctuations, for example, needle-shaped waveforms of rapid flickering between open and closed states, were observed, as shown in Fig. 2 (i). Analyzing the histogram in a similar manner as in the case of 0.3 M KCl, the single channel conductance was evaluated as 39 ± 4 pS M^{-1} (Fig. 2 (ii)). The decrease of the single channel current, which is equivalent to that of the number of transferring ions, resulted from replacing Cl^- with SCN^- as anion species. It is noted that the open channel probability and the open channel time per a single channel cannot be discussed here, because of the unknown number of KAT1 channels in the BLM system.

Effect of counter ions on the magnitude of the single channel current

It has been reported that KAT1 channel is a cation-selective channel and can

Table 2 Conductance ratio of KAT1 channel in the chloride salt solution and ionic radii of cation species[6,25]

	K^+	Li^+	Na^+	NH_4^+	Rb^+	Cs^+
% K^+ single channel conductance	100	6 ± 3	7 ± 8	30 ± 12	28 ± 13	9 ± 11
ionic radius / nm	0.138	0.069	0.102	0.148	0.149	0.170

permeate K^+ selectively.[6,7] Although the effect of the coexisting anions on the ion permeation has not yet been evaluated, some differences in the single channel conductance were observed by replacing anion species, as summarized in Table 1. Table 1 shows single channel conductances of KAT1 channel in 0.3 M potassium salt solutions with various counter anions (KCl, KF, KBr and KSCN) and in 0.1 M $KClO_4$ aqueous solution. The maximum value was obtained when Cl^- was used as the counter anion. The single channel conductance decreased with an increase of the ionic radius of the coexisting anions except F^-. The conductance in the case of F^- is smaller than that of Cl^-. These results mean that the coexisting anions also have a role in the ion transports through KAT1 channels, and indicate the existence of the anion-selectivity of KAT1 channel. As shown in Fig. 2 (i), a lot of needle-shaped waveforms (open time: 10-100 ms) were observed when using SCN^- and ClO_4^-. The open channel probability could not be analyzed because it was impossible to evaluate the number of KAT1 channels.

Table 2 shows the conductance ratio of various cations to K^+.[6] The characteristics indicate that KAT1 channel has a permeation selectivity for K^+ and other ions behave as channel blockers. The behavior in the case of anions, however, is less noticeable than that in the case of cations. Taking into account the fact that the radii of anions were much larger than K^+ radius, it is reasonable that K^+ is predominantly transported through KAT1

channel. This is consistent with the conventional notion that KAT1 channel serves as K^+-selective channel.

On the other hand, it is well-known that gramicidin A channel serves as a cation selective channel.[8-10] The author has reported the effect of the coexisting anions on the ion transport through gramicidin A channel.in chapter 1. Effect of the coexisting counter ions was also mentioned by Watanabe *et al*. and Cohen.[21,22]

Since the ion transport across the BLM involves the ion distribution process and the ion diffusion process, the pearmeability coefficient, P (m s^{-1}), is expressed by Eq.(1).[23]

$$P = \frac{\beta D}{d} \qquad (1)$$

β, D and d represent the permeability coefficitent, the distribution coefficient between the aqueous phase and the BLM phase, the diffusion coefficient within the BLM (m^2 s^{-1}), and the thickness of the BLM (m). Assuming that the thickness of the BLM is constant, the permeability coefficient depends on the distribution coefficient and the diffusion coefficient. The distribution coefficient can be expressed as Eq. (2) based on the results of the voltammetry for the ion transfer between two immiscible electrolyte solutions,[24]

$$\ln \beta = -\frac{\Delta G^{\circ}_{\mathrm{tr,\,M^+}} + \Delta G^{\circ}_{\mathrm{tr,\,X^-}}}{2RT}, \qquad (2)$$

where $\Delta G^{\circ}_{\mathrm{tr,\,i}}$ is the standard molar Gibbs energy of transfer of the ion (i) from the aqueous phase to the BLM phase, R the gas constant and T the absolute temperature. This equation means that distribution coefficient depends on the standard molar Gibbs energies of transfer of both cations and anions. The transfer energies of both cations and anions from the aqueous phase to the BLM phase or the channel pore phase, however, are impossible to be evaluated exactly. Using hydration energies as indications for these transfer energies, the hydration energies are known to be ordinary proportional to their

ionic radii when ions are monovalent.[25] Therefore, it can be expected that the distribution coefficient increases with an increase of the ionic radius. Although the distribution is affected by the electrical charge of the BLM phase or the channel pore phase, not only cations but also the coexisting anions are thought to be distributed simultaneously from the aqueous phase to the BLM phase in the case of gramicidin A channel, because it is a non-charge channel.[26] Since the pore site of KAT1 channel is also regarded as charge-free,[27] similar distribution is assumed. On the other hand, the diffusion coefficient usually decreases with an increase of the ionic radius.[28] In addition, it is understandable that the pore size of the channel limits the ion permeation. Considering that the ion permeation depends on the distribution, the diffusion and the pore size, the expression mechanism of the ion selectivity can be understandable.

SUMMARY

Ion transports from one aqueous phase to another across bilayer lipid membranes (BLM) containing KAT1 potassium channel were investigated by recording current fluctuations. KAT1 channel is a voltage-gated K^+ channel from *Arabidopsis thaliana* and has been suggested to have a key role in stomatal opening by osmoregulation in guard cells. Although KAT1 channel is a K^+-specific transporter, the species of the counter anions significantly affected the level of the single channel current, which suggested that KAT1 also transported these anions. When various potassium salts were used as electrolytes, the single channel conductance decreased with an increase of the ionic radius of the coexisting anions except for F^-. This indicates the existence of selective permeation for anions, but the anion-selectivity is less noticeable than the cation-selectivity.

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Conclusions

The ion transport across biomembranes play significant roles on various biofunctions. In this doctoral thesis, the ion transport through the ion channel is investigated and a new transport model taking the electroneutrality principle into consideration is proposed.

In chapter 1, a new mechanism for ion transport across BLM in the presence of GA is proposed. The magnitude of the single channel current at a given membrane potential depended on not only the cationic species, but also the anionic species. It decreased with an increase in the diameter of the anion when the diameter of the anion was larger than that of the GA channel. The baseline of the current recordings, however, increased with an increase in the diameter of the anion. The results indicate that the facilitated ion transport by GA consists of ion transport across the lipid bilayer site and that though the channel pore. By evaluating the influence of both the distribution and the diffusion individually, it is revealed that a pair of cation and anion distribute from aqueous phase to the BLM or the GA channel and the amounts of ions distributed depend on the relation between the ion size and the pore size of the GA channel. Therefore, it is also proved that the ion selectivity of the GA channel is expressed by both the distribution and the diffusion processes.

In chapter 2, the single channel current of the GA channel was analyzed by varying the concentration of the objective electrolyte (KCl) in W1 and W2. Based on the present analytical method, the ratio of diffusion coefficients of electrolyte ions, not only the objective ion but also the counter ion, within the channel pore can be evaluated. This

analysis method is promising to evaluate transport properties of channel compounds in detail.

It has been reported that KAT1 channel is a K^+-selective ion channel, however, in chapter 3, it has been revealed that KAT1 channel also transports counter anions such as Cl^-, Br^-, SCN^- etc. From the point of view of ionic radius or hydration energy, both anions and cations exhibited a similar tendency in the ion selectivity of KAT1 channel. Due to the dependence of the counter anions on the single channel current, KAT1 channel should have the anion selectivity in the same way as gramicidin A channel. These characteristics can be understood based on the distribution and the diffusion of both cations and anions and on the pore size of the channel.

On the ion transport across membranes containing the GA and KAT1 channels, not only a target ion but also its counter ion is distributed from aqueous phases to the membrane and that both ions are transported by applying the membrane potential. The antiport model of the target cation and the counter anion on the channel is proposed. Hence the author could conclude that it is necessary to analyze the behavior and the role of not only a target ion but also a counter ion in the ion channel study.

Acknowledgements

This research was conducted at laboratory of Bio-Analytical and Physical Chemistry in Division of Applied Life Sciences, Graduated School of Agriculture, Kyoto University.

The author expresses his sincere and heartfelt gratitude to Dr. Kenji Kano, Professor of Kyoto University, for his continuous guidance, criticisms, and encouragement throughout the author's doctoral study. The author sincerely appreciates the research guidance and valuable discussion of Dr. Osamu Shirai, Associate Professor of Kyoto University. The author would like to thank Dr. Seiya Tsujimura, Associate Professor of Tsukuba University, for his advices and a number of critical comments on this study. The author thanks Dr. Yuki Kitazumi, Assistant Professor of Kyoto University, for his helpful supports and comments. Also, the author is very grateful to Mr. Katsumi Hamamoto, Researcher of Kyoto University, for his kind suggestions and technical assistance.

The author is grateful to Dr. Takao Hibi, Professor of Fukui Prefectural University and Ms. Shiho Aoki, the member of Hibi's laboratory, for supplying KAT1 samples and advices. The author also thanks to Dr. Yuzuru Tozawa, Professor of Saitama University, for supplying KAT1 and comments.

The author gratefully thanks to the members of "Maku-Group" in Kano's laboratory, Dr. Shunsuke Ozaki, Mr. Jun Ohnishi, Mr. Keisuke Sasakura, Mr. Noriyoshi Ueya, Mr. Takafumi Yamauchi, Mr. Kei Hichiri, Mr. Yuki Kushida, Mr. Hiroki Kagohashi, Mr. Masahiro Takeshige and Mr. Keisuke Kimura for their kind advices and

encouragement. Also special thanks to Dr. Yung-Fu Wang, Dr. Maiko Goda-Tsutsumi, Dr. Ryoko Santo, Dr. Stefano Freguia, Dr. Shiue-Lin Li, Mr. Kenji Ishibashi, Ms. Yuko Kamitaka-Miura, Dr. Jun Fukuda, Mr. Ryosuke Yamada, Mr. Tsunetoshi Samukawa, Ms. Akiko Nishina, Mr. Masaki Masuda, Ms. Mizue Wanibuchi, Dr. Tatsuo Noda, Dr. Eiji Ueyama, Mr. Ryota Kontani, Mr. Kenta Sagara, Ms. Yumi Matsuda, Dr. Chi-Hua Nieh, Mr. Masafumi Asahi, Mr. Tsukasa Inoue, Mr. Yu Sugimoto, Mr. Itaru Asano, Dr. Shota Kawai, Ms. Yuko Kikuchi, Ms. Michiko Shinmura, Mr. Yasuyuki Hamano, Ms. Sae Hamada, Mr. Akihiro Maruyama, Mr. Daichi Mori, Mr. Noriyuki Yamazaki, Mr. Hajime Yamada, Mr. Keisei So, Mr. Rui Hamamoto, Ms. Kanako Chimori, Mr. Kento Sakai, Mr. Yoshinari Takano, Mr. Issei Hirata, Mr. Masahiro Hori, Mr. Yutaka Takeuchi, Mr. Yuya Hibino, Mr. Masaki Motoike and Ms. Toshie Koyama for their kind supports in the author's laboratory life.

The author also wishes to thank professors, doctors and friends who he has ever seen in conferences and seminars for their advice, kindness and encouragement.

Finally, the author would like to express his greatest thanks to his family for all their help and encouragement.

Shintaro Kubota

Himeji

March 2016

List of publications

Kubota, S., Ozaki, S., Onishi, J., Kano, K., and Shirai, O.,

"Selectivity on Ion Transport across Bilayer Lipid Membranes in the Presence of Gramicidin A.",

Anal. Sci., **2009**, *25*, 189.

(Chapter 1)

Kubota, S., Shirai, O., Hibi, T., Tozawa, Y., and Kano, K.,

"Effect of Counter Ions on the Transport Ccurrent across Membranes Containing KAT1 Potassium Channel.",

Anal. Sci., **2013**, *29*, 161.

(Chapter 3)

Kubota, S., Shirai, O., Kitazumi Y., and Kano, K.,

"Analysis of Ion Transport through a Single Channel of Gramicidin A in Bilayer Lipid Membranes.",

Anal. Sci. *Accepted.*

(Chapter 2)