Asymmetry of Rb⁺ Conduction Emerged under Bi-Ionic Conditions in Epithelial Maxi-K⁺ Channels

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Abstract: K⁺ channels permit more than one ion within their conducting pathway at any given moment and show a saturating single-file behavior. The conduction of Rb⁺ shows an unusual behavior, a so-called “Rb⁺ anomaly,” and it has been used to probe the mechanism of the ion conduction through K⁺-selective channels. Under the bi-ionic condition of K⁺ and Rb⁺, we carried out patch-clamp single-channel current measurements in MaxiK⁺ channels from mouse submandibular acinar cells. Keeping only K⁺ on one side of the membrane while varying fractional Rb⁺ concentration on the opposite, we had a series of current-voltage relationships. It showed a characteristic inflection at which the ion conductance was divided into two components, one ascribed to pure K⁺ conduction and the other to K⁺ and Rb⁺ bi-ionic conduction. By analyzing the latter, we depicted that (1) the bi-ionic conductance showed a characteristic reduction curve as the Rb⁺ fractional concentration increased; (2) Rb⁺ can bind the channel more tightly when it accesses from the outside than from the inside. Thus we conclude that such asymmetry of the Rb⁺ binding determines the pattern of bi-ionic conductance reduction in K-selective channels.

Key words: MaxiK⁺ channels, Rb⁺-induced anomalous conductance, single-channel current.

In an impressive display of unity, K⁺ ions and accompanying water lined in a single file, conduct efficiently through a narrow selective filter in K⁺ channels. Using crystallographic analysis in KcsA channels, MacKinnon and colleagues [1] have evidenced the multi-ion conduction mechanism, conceptually pointed out by many previous papers [2–7]. They have shown that four cation binding sites in the filter, denoted by site-1 to site-4 from outside to inside, evenly accept K⁺ ions. However, when Rb⁺ is used as a probe in the same channel, its occupation is totally different from that of K⁺ [1]; Rb⁺ occupies predominantly at site-1, the outermost binding site in the filter, less dominantly at either site-3 or -4, and scarcely at site-2. Since the filter’s amino acid signature sequence is conserved in every type of K⁺ selective channel [6, 8], its study provides a fundamental structural framework for the known K⁺-selective channels.

The ion conduction, relevant to K⁺ and Rb⁺ bi-ionic conductance, would lead to phenomena commonly referred to as “Rb⁺ anomaly” [7]. Several laboratories have already examined the anomaly in large conductance K⁺ (MaxiK) channels [9–11] and unexceptionally suggest that Rb⁺ is as permeable as K⁺ and is simultaneously able to block the K⁺ conduction. A question arises in the anomaly from the point suggested by crystallography. That is, how does the biased occupancy of Rb⁺ in the filter reflect on the conductance? Accordingly, in the present paper, we re-examined the Rb⁺ anomaly in MaxiK channels by depicting systematically the single channel conductance by varying fractional Rb⁺ concentration under K⁺ and Rb⁺ bi-ionic conditions. This time, however, we deliberately focused on the channel’s asymmetry concerning Rb⁺ access, implying the conductance difference distinguished by the access direction of Rb⁺ either from outside or inside. For this purpose, we carried out experiments by varying fractional concentrations of Rb⁺ on one side of the membrane while keeping a pure K⁺ solution on the opposite side.

MATERIALS AND METHODS

Animals and cell preparation. Male ddy mice (7–8 weeks old), treated in accordance with the Japanese Physiological Society’s Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, were anesthetized with ether and then sacrificed.
by cervical dislocation. The protocol for animal use was approved by the Animal Care and Use Committee of the Tohoku University Graduate School of Medicine. A small piece of the submandibular tissue was digested with two types of enzymes at 37°C as previously described [12]. Briefly, the tissue was treated first with collagenase (200 U/ml; Wako Pure Chemical Industries, Osaka, Japan) for 8 min, minced with fine scissors, treated with trypsin (0.5 mg/ml; Sigma type XI, Sigma-Aldrich, St. Louis, MO, USA) for 5 min, and again with the same collagenase for 4 min. At the end we obtained many small cell clusters consisting of 4–8 submandibular acinar cells, which were rinsed several times with enzyme-free standard external solution and suspended in the same solution until use.

Electrical setup and measurements. Submandibular gland acinar cells are rich in Ca2+-activated large-conductance K+ (MaxiK) channels [10, 13, 14], and they play a steering role in exocrine fluid secretion [15]. We monitored the single MaxiK channel currents with standard patch-clamp techniques under the inside-out patch configuration of the standard patch-clamp techniques under the inside-out patch membranes [16]. The currents, measured with EPC-7 amplifier (List, Darmstadt, Germany), were appropriately low-pass filtered, monitored on an oscilloscope (Kawasaki Electronics, Kawasaki, Japan), and recorded on a digital thermal array-recorder (WR8500, Graphtec, Yokohama, Japan). All the experiments were carried out at room temperature (22°C–24°C).

Solutions. The standard solution in the measurements contained 144 mM monovalent cation-Cl; 2 mM MgCl2; and 10 mM Hepes, where pH was adjusted at 7.2 by NMDG (N-methyl-D(-)-glucamine). The combination of monovalent cations such as K+, Rb+, Na+, Li+, and NMDG (N-methyl-D(-)-glucamine) contained 144 mM monovalent cation-Cl; 2 mM MgCl2; and 10 mM Hepes, where pH was adjusted at 7.2 by NMDG (N-methyl-D(-)-glucamine). The combination of monovalent cations such as K+, Rb+, Na+, Li+, and NMDG for the bi-ionic pair was described in each relevant experiment. The internal solution, the bathing solution in the inside-out configuration of the standard patch-clamp techniques, contained 0.1 mM EGTA without added CaCl2, which fixed pCa at approximately 6.5. This concentration of Ca2+ maintained the channel openings high enough during hyperpolarization and low enough for preventing too many overlapping openings during depolarization.

Origin of an equation used to fit a bi-ionic data-set and its derivation. To estimate bi-ionic results, we used an equation in the Result section. We first comment on its origin and some features briefly. Because of Läuger’s rate theory involving the saturation isotherm [17], Neher proposed an empirical equation for bi-ionic conductance under Na+ and Tl+ bi-ionic conditions in gramicidin channels [18],

\[
g = g_{\text{max}}^K \cdot \frac{A[K]}{1 + A[K] + B[Rb]} + g_{\text{max}}^{Rb} \cdot \frac{B[Rb]}{1 + A[K] + B[Rb]} \tag{1}
\]

where original notations were replaced with our notations for K+ and Rb+ bi-ionic condition. That is, \( g \) stands for the bi-ionic conductance, \( g_{\text{max}}^K \) and \( g_{\text{max}}^{Rb} \), the maximal conductance for K+ and Rb+, respectively; [K] and [Rb], the respective fractional ion concentrations; and \( A \) and \( B \), the binding constants determining the degree of the ion’s contribution, \( A \) for K+-, and \( B \) for Rb+-contributions. The equation was based on a “one-ion” channel rate theory, implying that the channel contains at most one ion in the conduction, and this ion jumps across the energy barriers with rate-constants, and this process is repeated. The binding constants, \( A \) and \( B \), consist of these jumping-rate constants and imply the totality of the channel’s binding affinity to the respective ions, which locate at some distance inside the channel.

The “one-ion” channel model inherently involves a saturating behavior, the concept of which is similar to that of the standard Michaelis-Menten reaction isotherm. Indeed, by dealing with a single ion species (i.e., [Rb] = 0, or [K] = 0) in the equation (1), we know that the equation (1) is to be the same form of the standard Michaelis-Menten isotherm, and the reciprocals of the binding constants, 1/A and 1/B, are identical to the half-saturation constants, \( K_m^K \) and \( K_m^{Rb} \) for K+ and Rb+, respectively. In principle, we can estimate 1/A and 1/B, by measuring \( K_m^K \) and \( K_m^{Rb} \).

Under bi-ionic conditions of K+ and Rb+, the model can be arranged to express the channel exclusively occupied by K+, Rb+, and nothing (vacant channel), the feature of which is represented by a state diagram, such as \( \{ S_{\text{vac}} \} \xlongequal{\text{K+ entry}} \{ S_K \} \xlongequal{\text{Rb+ entry}} \{ S_{KR} \} \), where \( \{ S_{\text{vac}} \} \) represents the vacant channel state, \( \{ S_K \} \) the K+-occupied, and \( \{ S_{KR} \} \) the Rb-occupied channel state. With scheme (2) in mind, we find it not difficult to grasp the apparent meaning of the equation (1). It simply states that the bi-ionic conductance, \( g \), consists of the sum of the two modes, K+- and Rb+-conducting mode, each of which is the product of the maximum conductance multiplied by the state probability of the channel exclusively occupied either by K+ or Rb+.

The derivation of the equation (1) based on the scheme (2) is quite analogous to that of the chemical or enzyme kinetics at equilibrium with the binding constants, \( A \) and \( B \), and the respective ligands, K+ and Rb+. We start with obtaining the probability for either the vacant or a K+- or Rb+-occupied channel state, noted by \( p_{\text{vac}}, p_K, \) and \( p_{Rb} \), respectively, and immediately reach the following three relations:

\[
\begin{align*}
\rho_K &= p_K + p_{Rb} = 1 \\
\rho_K &= p_K \cdot A[K] \\
\rho_{Rb} &= p_{Rb} \cdot B[Rb]
\end{align*}
\]

Combining and rearranging the above three to manifest each of \( p_{\text{vac}}, p_K, \) and \( p_{Rb} \), we obtain:

\[
\begin{align*}
p_{\text{vac}} &= \frac{1}{1 + A[K] + B[Rb]} \\
p_K &= \frac{A[K]}{1 + A[K] + B[Rb]} \\
p_{Rb} &= \frac{B[Rb]}{1 + A[K] + B[Rb]}
\end{align*}
\]
Thus we reach the final formulation of the bi-ionic conductance, \( g \), in terms of the 'one-ion' channel model:

\[
g_{RB} = g_{max} \cdot P_K + g_{max} \cdot P_{RB}
\]

## RESULTS

### Single-channel current-voltage relationship depicting Rb+-induced anomalous behavior of MaxiK channels under bi-ionic conditions

To show the distinctive features of Rb+-induced anomalous currents in MaxiK channels, we evaluated single channel conductance with a variety of bi-ionic pairs of K': Rb+, a permeant cation with which we are concerned, and Na+, Li+, or NMDG+, impermeant cations in comparison. Plotting the single channel current amplitude against membrane voltages, we had a set of current-voltage relationships (\( i/V \)-relationships) in MaxiK channels in combination with these cations (Fig. 1). When keeping only K' on the inside and placing either Na+, Li+, or NMDG+ on the outside, the respective \( i/V \)-relationship equivocally showed (1) outward-going rectification asymptotically approaching the ohmic conductance achieved under K' symmetrical condition; and (2) reversal potential close to the expected K' Nernst potential [9–11, 19]. However, when Rb+ was used instead of these cations, the \( i/V \)-relationship showed a sharp inflection near the reversal potential at which the conductance was divided into two components, a larger one responsible for the normal K' conduction and a smaller one for Rb+ conduction. The feature thus observed under K' and Rb+ bi-ionic conditions has been remarked as "Rb' anomaly" [7, 9–11, 19] and summarized in this way by Hille: "The absolute permeability of Rb+ is minute, since the currents carried by Rb+ are small, but its relative permeability to K' is high since the reversal potential is close to 0 mV" [7]. Thus the anomaly may seem to be elusive, and no precise explanation for its mechanism has so far been provided.

Next we carried out single-channel current measurements by varying fractional Rb+ concentration under bi-ionic K' and Rb+ conditions. We showed a series of \( i/V \)-relationships for Rb+ placed on the outside in Fig. 2 and on the inside in Fig. 3, while keeping only K' on the opposite side. As we increased the fractional Rb+ concentration to 140 mM, from 10, the \( i/V \)-relationship showed increasingly sharper inflections at reversal potentials closer to 0 mV. We obtained two conductance components at the inflection. One component, independent from the changes in Rb+ concentration, showed linear conductance of 240–260 pS, and it was ascribed to the normal K' conduction. The other component, however, showed a variety of conductances in response to the changes in Rb+ concentration, and they were ascribed to K' and Rb+ bi-ionic conduction. Focusing on this bi-ionic conduction, we compared its conductance between two previous cases, one for placing Rb+ on the outside (Fig. 2) and the other for placing it on the inside (Fig. 3). We noticed at this stage that the degree of conductance change (i.e., reduction) was much greater with Rb+ placed on the outside, the details of which we analyze in the next section.

### Rb+-induced conductance reduction under K' and Rb+ bi-ionic conditions

We plotted the two conductance components shown in Figs. 2 and 3 against corresponding fractional Rb+ or K' concentration in Fig. 4 diagrams. Figure 4, A and D depicted the bi-ionic conductance for the outside and inside Rb+, respectively, and Fig. 4, B and C the paired normal K' conductance in comparison. The bi-ionic conductance steadily decreased with increasingly higher Rb+ concen-
tration, and the decrease was much more abrupt when Rb\(^+\) was placed on the outside than on the inside. Thus we noticed that the difference in the side access of Rb\(^+\) to the conductance reduction was present under the bi-ionic condition. To quantify the feature, we adopted a bi-ionic saturation equation by Neher [18], which was represented as the equation (1) in Methods.

To determine the maximal conductance for K\(^+\) and Rb\(^+\) and $g_{K_{\text{max}}}^{\text{Rb}}$, in equation (1) and concomitantly with $K_{\text{m}}^{K_{\text{Rb}}} \text{ and } K_{\text{m}}^{Rb_{\text{Rb}}}$, we carried out single-channel current measurements separately for K\(^+\) and Rb\(^+\) by varying their ion concentrations under symmetrical ion gradients across the membrane. The zero-voltage conductance for each of K\(^+\) and Rb\(^+\), $g_{K_{\text{max}}}^{\text{K}_{\text{Rb}}}$ and $g_{Rb_{\text{max}}}^{\text{Rb}_{\text{Rb}}}$, were 365 pS and 43 pS, respectively, and the half-saturation constants for K\(^+\), $K_{\text{m}}^{K_{\text{Rb}}}$, were 70 mM. That of Rb\(^+\), $K_{\text{m}}^{Rb_{\text{Rb}}}$ was uncertain because the conductance...
was too small to be detected in the low range of Rb⁺ concentration. It may roughly be 30 mM or below.

The bi-ionic conductance reduction in Fig. 4, A and D was best fitted with the equation (1) by inserting the following values: \( g_{\text{max}}^K = 365 \text{ pS}, \ g_{\text{max}}^Rb = 43 \text{ pS}, \ A = 1/70 \text{ mM}^{-1} \), and \( B = 1/15 \text{ mM}^{-1} \) for inside Rb⁺; \( B = 1/4 \text{ mM}^{-1} \) and others the same for outside Rb⁺. In the fitting, we assumed that the value of \( A \) was constant both in Fig. 4, A and D and equal to \( 1/K_n \). The value of \( B \) is 3–4 times larger when Rb⁺ was accessed from the outside rather than from the inside.

**DISCUSSION**

Following a “one-ion” channel model or Neher’s equation [18] based on the rate theory [17], we examined the pattern of K⁺ and Rb⁺ bi-ionic conductance in epithelial MaxiK⁺ channels. Assuming that the channel state was...
exclusively partitioned by K+, Rb+, and vacant-state and following a formal analogy to the enzyme kinetics, we can reach the same expression, the equation (1) for the bi-ionic conductance. Equation (1), inserted with the appropriate parameter values, fitted well to the bi-ionic conductance plotted against the fractional ion concentration (Fig. 4, A and D). The bi-ionic conductance seemed to decrease monotonically in a concave fashion as the fractional Rb+ concentration was increased, and the binding constants for K+ and Rb+, denoted by A and B, respectively, were crucial for steering the reduction pattern.

Approximation by a saturating one-ion channel model

A useful indication of the simplicity of a channel’s conduction process is its “mole-fraction behavior” under bi-ionic conditions. Taking account of this, we can distinguish two classes of saturating ion channels, molded by the rate theory. That is, “one-ion” channel and “multion” channel models, in which the former postulates that the channel contains at most one ion in the conduction, and the latter that it contains more than two at a time. The theory and properties of the one-ion channel are much simpler and fully explored by the previous papers [3, 7, 17, 18]. Under bi-ionic conditions of permeable ion pairs, the bi-ionic conductance in the one-ion channel must vary monotonically with the fractional concentration of the test cations. In contrast, if the bi-ionic conductance shows an extreme, usually a minimum, in the course of its changes, we can decide that the channel is multiion [7]. However, in theory, it is possible that a multi-ion channel shows no such extreme [3]. The channel behavior depicted by the conductance minimum under a bi-ionic condition is collectively remarked as “anomalous mole-fraction behavior” [3, 7]. In reality, as MacKinnon’s group revealed by crystallography, K+-selective channels can accommodate more than two cations at a time [1], and therefore they would show, more or less, the anomalous mole-fraction behavior under bi-ionic conditions of the permeable ion pairs. Eisenman et al. showed the presence of a conductance minimum in the MaxiK-channels from skeletal muscle with the bi-ionic pair of NH4+ and K+ and of NH4+ and Rb+ [11]. Unfortunately, however, they carried out no experiments for the combination of K+ and Rb+. Together, the circumstances indicate that we should interpret the bi-ionic results in K+-channels by the multi-ion channel model. However, by its simplicity the one-ion channel model is advantageous for use to extract essential features of bi-ionic conductance and makes it easier to conceive the physical meaning of the phenomenological parameters, including the binding constants. It is not our intention here to provide a precise full description of the Rb+-anomaly. Instead, we have endeavored to show some causes of channel’s asymmetry with an approximation having a reasonable quantitative accuracy. Thus we have adopted the one-ion model or the equation (1) to interpret the results.

Interpretation of the binding constants in terms of the one-ion channel model

The rate theory describes ion conduction by the combination of the jumping-rate constants across the channel’s energy barriers. The binding constant, denoted by A and B in the present one-ion channel model, consists of such jumping-rate constants summed over in a form of a partition function (if necessary the precise formulation in equations 11, 14, and 20 of [17]). However, at present it is enough to see that A and B denote the channel’s apparent binding affinity, determining the degree of respective ion’s contribution, A for K+ and B for Rb+ contribution. By definition, the larger the value of the constant, the stronger the ion binding. This is analogous to the Michaelis-Menten constant, which reflects the strength of the ligand binding.

One wonders about uncertainty in the selection of values of A in the curve fittings. However, keeping the value of A constant in Fig. 4, A and D would be a reasonable assumption, since from the recent crystallographic result it is evident that K+ can distribute evenly or symmetrically over the four binding-sites in the filter [1]. This argument would be invalid in the case of B, since it has been reported that Rb+ can reside unevenly or asymmetrically over the channel’s cation binding-sites [1]. The value of B was larger than that of A in the present paper. From the above arguments, it indicates that Rb+ binds more tightly to the channel than K+ during the conduction. Moreover, the value of B was much larger with Rb+ accessing from the outside rather than from the inside, indicating that Rb+ binds more tightly to the channel from the outside. It reminds us of the probability of occupancy for Rb+ over the filter of the KcsA channels [1], where the outermost cation binding-site (energy minimum) represents the highest probability. Together, it would be that the outermost energy well is deepest throughout the conducting pathway for Rb+ in MaxiK-channels. Thus we conclude that such asymmetry of the Rb+ binding determines the pattern of bi-ionic conductance reduction in Fig. 4. The K+-channels are flexible enough to change their conduction profile according to a conducting ion species and its access direction. To elucidate the underlying molecular mechanism of such channel flexibility remains for future studies.

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