Further Characterization of the Cation Channel of a Yeast Vacuolar Membrane in a Planar Lipid Bilayer

Masayuki Sato, Manabu Tanifuji and Michiki Kasai

Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan

ABSTRACT. A voltage-dependent and Ca²⁺-activated cation channel recently found in the vacuolar membrane of the yeast *Saccharomyces cerevisiae* was incorporated into planar lipid bilayers and further characterized in macroscopic and single channel levels. Single channel conductances for various cations were in the order: \( \text{NH}_4^+ > K^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ > \text{Li}^+ \), and were nearly consistent with the order of permeability ratio estimated from reversal potentials determined by macroscopic measurement. Up to 6 mM of Ca²⁺ added to the cis (cytoplasmic) side opened the channel, but higher concentrations closed the channel without affecting the single channel conductance. Ba²⁺ closed the channel without opening. Further, large anions such as gluconate closed the channel from the cis side. In addition to the above channel, a small cation-selective channel of about 40 pS was found.

Vacuoles in plants and fungi occupy 25-90% of the cell volume and have essential and dynamic physiological functions (1): they act as storage compartments and lytic compartments (6). For the function of storage, the uptake of large amount of inorganic ions, amino acids, or organic acids into the vacuoles is required. This uptake generates the osmotic pressure differences across the vacuolar membrane. Furthermore, an acidic environment inside the vacuoles, which is essential for the lytic activity, is maintained by an H⁺ influx driven by an H⁺-ATPase. The H⁺ influx generates not only a concentration gradient of proton, but also an interior positive membrane potential difference. Several investigators predicted the existence of ion transport systems on vacuolar membranes which balance these osmotic and electrochemical potential differences (7).

In the preceding papers, we found a cation selective channel in vacuolar membranes of yeast (15), and the gating properties of the channel were studied in a planar lipid bilayer system (13). The results indicated that the channel had two types of gate operating independently, which had different rate constants and opposite voltage dependence. When the voltage of the cis side (the side to which the vesicles were added) was kept more negative, the fast responding gate opened and the slow responding gate closed. The fast gate was locked in the open state when DIDS, a disulfonic stilbene derivative, was added from the cis side, and the slow gate opened when about 1 mM Ca²⁺ was present on the cis side.

In this research in order to characterize the channel further, firstly, the selectivity properties of the channel were extensively studied. The single channel conductances

\* Present address: National Institute for Physiological Sciences, Okazaki 444, Japan.
for various cations were compared with the permeability ratio estimated from reversal potentials determined by macroscopic measurement. Secondly, the effects of Ca\textsuperscript{2+} and Ba\textsuperscript{2+} on single and macroscopic conductances were studied. It was found that these ions affected only the gating properties of the channel, but not the single channel conductance. Further, the effect of large anions on the single channel conductance was studied. In the course of these experiments, the existence of a small cation channel was found. Some properties of the channel were also analyzed. From these results, the physiological role of these channels was discussed.

**MATERIAL AND METHODS**

**Materials.** Vacuolar membrane vesicles from yeasts of the haploid strain of *Saccharomyces cerevisiae* X2180-1A, which was supplied by Mr. Y. Wada, were prepared as described (11). These were resuspended at 10–20 mg protein/ml in 0.4 M sucrose, 5 mM HEPES-Tris, pH 7.2 and stored at −80°C. The preparation was thawed at room temperature before use. Asolectin (Type II-S) was purchased from Sigma Chemical Co., U.S.A. Other reagents were commercial products of analytical grade.

**Electrical.** Planar bilayer was formed from asolectin solution (14.7 mg/ml in n-decane), vesicles were fused into the bilayer membrane, and the electrical conductance of the membrane was measured as described previously (13). The solution used was composed of 300 mM KCl buffered with 5 mM HEPES-Tris, pH 7.2, unless otherwise specified. The number of channels incorporated into planar bilayers was controlled by the amount of vesicles added to the solution. The macroscopic measurement was carried out when 10–50 channels were incorporated into the membrane, and the single channel current fluctuation was observed when one or two channels were incorporated. The *cis* side of the membrane was defined as the side to which the vesicles were added, and the *trans* side was defined as the opposite side. The voltage across the membrane was defined with respect to the *trans* side. Experiments were carried out at room temperature, 25±2°C.

**RESULTS**

**Single channel conductance.** When a small amount of vacuolar vesicles (about 3 μl) was added to the *cis* side of lipid bilayer, the current fluctuation shown in Fig. 1a and b was observed, as described previously (13, 15). The single channel currents were plotted in Fig. 1 as a function of holding potentials. The currents changed linearly with applied voltages and the single channel conductance was determined as 407 pS in 300 mM KCl from the slope.

**Ion selectivity.** When the *trans* solution in Fig. 1 was changed from 300 mM KCl to 100 mM, the reversal potential moved to −22.9 mV. The permeability ratio of Cl\textsuperscript{−} over K\textsuperscript{+} was 0.09 as calculated by using the Goldman equation. This result is consistent with the previous observation that the channel is cation selective (15). To characterize the ion selectivity further, single current fluctuations in various cations were measured (Fig. 2). Single channel conductances are listed in Table 1 and compared with the permeability ratio estimated from reversal potentials (15). The following selectivity order was obtained from the single channel conductance: NH\textsubscript{4}\textsuperscript{+} > K\textsuperscript{+} > Rb\textsuperscript{+} > Cs\textsuperscript{+} > Na\textsuperscript{+} > Li\textsuperscript{+}, which corresponds to Eisenman’s VIII series (12); the order Cs\textsuperscript{+} > K\textsuperscript{+} > Na\textsuperscript{+} > Li\textsuperscript{+}, which corresponds to Eisenman’s X or XI (12), was obtained from the permeability ratio. These two series resemble each other although they are not exactly the same.
Conductance-concentration relationship. Figure 3 shows the K\(^+\) concentration dependence of K\(^+\) conductance. Single channel currents were measured at symmetrical K\(^+\) concentrations except that the cis solution contained 1 mM CaCl\(_2\). The conductances were obtained from the slope of I-V relationships between -50 mV and +50 mV at each K\(^+\) concentration. The data could be fitted to a simple satura-
tion curve:

\[ \gamma = \frac{\gamma_m}{1 + K_i/K^+} \]  

(1)

where \( \gamma \) is the single channel conductance, \( \gamma_m \) is the maximum conductance and \( K_i \) is the apparent dissociation constant for \( K^+ \). Their values obtained by the curve fitting were: \( K_i = 85 \) mM and \( \gamma_m = 508 \) pS. A good fitting with Eq. 1 also suggests that the channel is a single-ion-filled one, that is, the channel can accommodate only one ion at a time.

\textbf{Ca}^{2+} \textbf{dependence.} In the previous paper (5), we showed that the channel required a few mM of \( \text{Ca}^{2+} \) from the \textit{cis} side to open. In this paper the effect of \( \text{Ca}^{2+} \)

<table>
<thead>
<tr>
<th>Ion(X)</th>
<th>( \gamma_\text{x} ) (pS)</th>
<th>( \gamma_\text{x}/\gamma_\text{K} )</th>
<th>( P_\text{x}/P_\text{K} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl(^-)</td>
<td>110</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td>Li(^+)</td>
<td>230</td>
<td>0.51</td>
<td>0.72</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>450</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>K(^+)</td>
<td>420</td>
<td>0.93</td>
<td>1.00</td>
</tr>
<tr>
<td>Rb(^+)</td>
<td>390</td>
<td>0.87</td>
<td>1.05</td>
</tr>
<tr>
<td>Cs(^+)</td>
<td>470</td>
<td>1.04</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1. ION SELECTIVITY

Single channel conductances, \( \gamma_\text{x} \), were determined from the difference between the maximum and minimum conductance levels as in Fig. 2. The ratios of single channel conductances, \( \gamma_\text{x}/\gamma_\text{K} \), were calculated from the average values of single channel conductances. Permeability ratios, \( P_\text{x}/P_\text{K} \), were determined according to the Goldman equation from the reversal potentials measured at asymmetric ionic compositions. The conditions were 300 mM KCl on the \textit{cis} side and 100 mM KCl on the \textit{trans} side for the determination of Cl\(^-\) permeability, and 300 mM KCl on the \textit{cis} side and 300 mM XCl on the \textit{trans} side for the determination of cation permeabilities. In every case the \textit{cis} solution contained 1 mM CaCl\(_2\) and 5 mM HEPES-Tris, pH 7.2 and the \textit{trans} solution contained 5 mM HEPES-Tris, pH 7.2.
on the channel was studied in more detail. Figure 4A shows the Ca\(^{2+}\) concentration dependence of macroscopic conductance. In this experiment, at first the cis side was perfused with the same solution as the trans side, 300 mM KCl and 15 mM HEPES-Tris (pH 7.2). Then, the same concentrations of CaCl\(_2\) were added to both sides and macroscopic conductances were determined at -10 mV. Relative conductance indicates the value normalized to the conductance in the solution containing 1 mM CaCl\(_2\). (B) Single channel fluctuations. Single channel fluctuations were recorded under the same conditions as in A except that only one vesicle was fused to lipid bilayer membrane. Ca\(^{2+}\) concentrations are shown by each trace. The arrows show the zero current levels. Holdings potential was -10 mV.

Effects of Ba\(^{2+}\). In order to characterize the effect of Ca\(^{2+}\) on gating properties of the channel, Ba\(^{2+}\) was applied. Figure 5A shows the effect of Ba\(^{2+}\) on macroscopic conductance. Addition of Ba\(^{2+}\) caused closure of the channel which had been opened in the presence of 1 mM Ca\(^{2+}\). Addition of Ba\(^{2+}\) alone, in the absence of Ca\(^{2+}\), did not open the channel. Figure 5B shows the effect of Ba\(^{2+}\) on single channel fluctuations. Ba\(^{2+}\) seems not to affect the single channel conductance and decreased only the open channel probability. Ba\(^{2+}\) seems to bind to the closing site of the gate instead of Ca\(^{2+}\) in Fig. 4A.

Anion contribution to the channel. It was found that no current fluctuation could be observed when the aqueous solution contained 300 mM K-gluconate, although the channel was highly permeable for K\(^{+}\). To exclude the possibility that occurrence of fusion events might be decreased by the change of ionic composition, the cis side was perfused from KCl to K-gluconate after the current fluctuation was observed. As the perfusion proceeded, the single channel current first increased
Fig. 5. Effect of Ba\(^{2+}\) concentrations. Experiments similar to Fig. 4 were carried out. (A) Macroscopic conductance. After perfusion of the cis side with 300 mM KCl and 15 mM HEPES-Tris (pH 7.2), the same concentrations of BaCl\(_2\) were added to both sides in addition to 1 mM CaCl\(_2\), and then macroscopic conductances were determined at \(-10\) mV. Relative conductance indicates the value normalized to the conductance in the absence of BaCl\(_2\). (B) Single channel fluctuations. Single channel fluctuations were recorded under the same condition as in Fig. B except that only one vesicle was fused to the lipid bilayer membrane. Ba\(^{2+}\) concentrations are shown by each trace. The arrows show the zero current levels. Holding potential was \(-10\) mV.

Fig. 6. Effect of anion exchange on single channel current. Initially 300 mM KCl and 5 mM HEPES-Tris (pH 7.2) were contained in both sides of the membrane, and 1 mM CaCl\(_2\) in the cis side. The arrow shows the point when perfusion of the cis side begun with 300 mM K-gluconate, 5 mM HEPES-tris (pH 7.2) and 1 mM CaCl\(_2\). The arrow heads show noise caused by the perfusion. The horizontal arrows show the zero current levels. Holding potential was \(+10\) mV.
slightly and the current fluctuation disappeared finally, as shown in Fig. 6. Similar phenomena were observed when K$_2$SO$_4$, K-glutamate and K-glucronate were used instead of K-gluconate, but were not as remarkable as these observed for K-gluconate. The result indicates that the channel activity depends on the anion pre-

Fig. 7. Appearance of a small channel. At point a, the voltage was turned to +30 mV. The point b indicates an appearance of a small channel. At point c, 20 μl of 20 mM DIDS (final concentration 0.1 mM) was added to the cis solution. Aqueous solutions were the same as in Fig. 1. Holding potential is shown under the trace. Channel opening was upward for negative voltage and downward for positive voltage. Arrows indicate the zero-current level.

Fig. 8. Properties of the small channel. (A) The trace was obtained from the same membrane shown in Fig. 7. Holding potential is shown under the trace. Channel opening was upward for negative voltages and downward for positive voltages. Arrows indicate zero-current level. (B) single channel currents of the small channel. A unitary current was measured at different potentials: (●) under symmetrical conditions in 0.3 M KCl on both sides as in A, or (▲) under asymmetrical condition in 0.1 M KCl on the cis-side and 0.3 M KCl on the trans-side. Fifteen mM HEPES/Tris (pH 7.2) and 1 mM CaCl$_2$ were present on both sides in all cases.
sent in the aqueous solution. The gating mechanism of the channel may be affected by the anion.

A small channel observed in the same membrane. Sometimes, a small channel fluctuation was observed when the large channel (the channel we discussed above) was closed. Figure 7 shows an example of the small channel. At the beginning, a current fluctuation of the large channel can be seen. When the voltage was turned to +30 mV from −30 mV (point a), a small channel hidden by the current fluctuation of the large channel appeared (point b). Since this current fluctuation was not always observed together with the large channel and in some experiments nothing but this small channel was observed, we can conclude that this is another type of channel coexisting in the same membrane preparation. The gating properties were different from those of the large channel, so it was easily distinguished. At point c, DIDS was found to inhibit this small fluctuation.

This small channel was studied in more detail (Fig. 8). Current fluctuations at different voltages were shown in Fig. 8A. From the I-V relationships of the single channel current, the single channel conductance was estimated to be about 40 pS, as shown in Fig. 8B. Under asymmetric ionic conditions, in which the cis-side was 0.1 M KCl and the trans-side was 0.3 M KCl, the single channel conductance was slightly smaller (29 pS) than that under symmetrical conditions; this may be due to the difference of the ionic concentrations. The reversal potential was shifted to −13 mV. This result indicates that the small channel is a cation-selective channel. Using the Goldman equation, we calculated the permeability ratio of K+ to Cl− as 2.9. Further, current fluctuations shown in a, b, and c (Fig. 8A) suggest that the open channel probability decreased as the applied voltage became negative. The figures also shows that the large channel becomes open at lower voltage than +10 mV (shown in d) and it becomes difficult to discriminate the small channel.

DISCUSSION

The ion selectivity of the channel of the yeast vacuolar membrane was studied by measuring single channel conductance in various kinds of cations, and the results were compared with the permeability ratio obtained from reversal potential in macroscopic measurement. The selectivity followed Eisenman's VIII series for single channel conductance and X or XI series for permeability ratio (12). Although some discrepancies are seen between the two types of experiments, these series suggest the weak interaction of the permeant cations with anionic sites in the channel pore at any event. Low affinity of the binding site for K+(K1=83 mM) is also consistent with the above interpretation. In the previous paper (13, 15), we showed that the channel has two types of gates operating independently, which have different rate constants and opposite voltage dependence. The fast gate (F-gate) was locked in the open state when DIDS was added to the cis side, and the slow gate (S-gate) was opened when about 1 mM Ca2+ was present on the cis side. The effect of Ca2+ on the voltage dependence of the S-gate was studied in the previous paper (13) and was analyzed by assuming that the shift of voltage dependence along the voltage axis to the left direction takes place with increase of Ca2+. Such a shift of voltage dependence was observed in other Ca2+-gated K+ channels (5, 8, 10, 16) and was interpreted in terms of voltage-dependent Ca2+ binding to the sites in the channel pore. Since the behavior of the S-gate is similar to that of the Ca2+-gated K+ channels in other tissues, we studied the effect of divalent cations extensively in this
paper. Our result showed that there exist two types of Ca$^{2+}$ binding sites in the Ca$^{2+}$ sensitive gate: the opening site and closing site. When Ca$^{2+}$ concentration is lower than 6 mM, Ca$^{2+}$ binds to the opening gate, but at higher concentrations Ca$^{2+}$ binds to the closing site. Ba$^{2+}$ could replace the effect of closing, but not that of opening.

As far as the physiological significance of the channel is concerned, it is not clear because the channel required a few mM Ca$^{2+}$ to open from the cis side, the cytoplasmic side. This concentration is too high compared with the physiological one, and the channel could not open under the physiological condition. However, the fact that there is some similarity between the present channel and the voltage-gated ion channel in the vacuolar membrane of higher plants regulated by cytoplasmic calcium (4) suggests the physiological role of the present channel. It is possible that the Ca$^{2+}$ dependence may change with lipid composition as found in Ca$^{2+}$-gated K$^+$ channel of muscle sarcolemma (9) or that vacuolar membrane potential may be largely negative as postulated. The fact that the gating of the channel is regulated by organic anions also suggests its physiological significance. Further analysis is required.

Finally, we found a small cation-selective channel in the same preparation. This might be a contamination from the channel of plasma membrane recently found by using the patch clamp technique (2, 3), although some differences are seen between them. Further analysis has not yet been conducted.

**Acknowledgments.** This work was supported in part by a Grant-in-Aid to M.K. from the ministry of Education, Science and Culture of Japan. We thank Mr. Y. Wada (Department of Biology, Faculty of Science, University of Tokyo) for his useful comments and for supplying the vacuolar membrane vesicles.

**REFERENCES**

11. **Onsumi, Y. and Y. Anraku.** Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 256, 2079–2082,


(Received for publication, June 9, 1989 and in revised form, August 4, 1989)