ELSEVIER

Biochimica et Biophysica Acta 1467 (2000) 431–443

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

# Flammutoxin, a cytolysin from the edible mushroom *Flammulina velutipes*, forms two different types of voltage-gated channels in lipid bilayer membranes

Gulnora Tadjibaeva [a], Ravshan Sabirov [b], Toshio Tomita [a,*]

[a] *Department of Molecular and Cellular Biology, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981-8555, Japan*

[b] *Department of Cellular and Molecular Physiology, National Institute for Physiological Sciences, Myodaiji, Okazaki 444-8585, Japan*

Received 8 February 2000; received in revised form 11 May 2000; accepted 16 May 2000

**Abstract**

Flammutoxin, a 31-kDa cardiotoxic and cytolytic protein from the edible mushroom *Flammulina velutipes*, has been shown to assemble into a pore-forming annular oligomer with outer and inner diameters of 10 and 5 nm on the target cells [Tomita et al., Biochem. J. 333 (1998) 129–137]. Here we studied electrophysiological properties of flammutoxin channels using planar lipid bilayer technique, and found that flammutoxin formed two types of moderately cation-selective, voltage-gated channels with smaller and larger current amplitudes (1–4.5 pA and 20–30 pA, respectively, at 20 mV) in the lipid bilayers composed of phospholipid and cholesterol. The larger-conductance single channel showed the properties of a wide water-filled pore such as a linear relationship between channel conductance and salt concentration of the bathing solution. The functional diameter of the larger-conductance channel was estimated to be 4–5 nm by measuring the current conductance in the presence of polyethylene glycols of various sizes. In contrast, the smaller-conductance single channels showed a non-linear current to voltage curve and a saturating conductance to increasing salt concentration. These results suggest that the larger-conductance channel of flammutoxin corresponds to the hemolytic pore complex, while the smaller-conductance channel may reflect the intermediate state(s) of the assembling toxin. 

*Keywords:* Flammutoxin; Pore-forming toxin; Planar lipid bilayer; Multiple channel; *Flammulina velutipes*

## 1. Introduction

Several cytolytic proteins have been isolated from both the toxic and the edible mushrooms [1]. Flammutoxin has been isolated as a cardiotoxic and cytolytic 31-kDa protein from the basiocarps of an edible mushroom, *Flammulina velutipes* [2–4]. It causes lysis of mammalian erythrocytes, swelling of Ehrlich ascites tumor cells, and electrocardiographical changes in parenterally administrated animals (although it is not toxic by oral administration) [3]. Our previous study showed that flammutoxin forms a hydrophilic pore with a functional diameter of approximately 5 nm in the cell membrane of human erythrocyte [5]: (i) flammutoxin induced potassium leakage from human erythrocytes and swelling of the cells before lysis, and (ii) flammutoxin-induced hemolysis was entirely suppressed by the addition of polyethylene

* Corresponding author. Fax: +81-22-717-8780; E-mail: tomita@biochem.tohoku.ac.jp

0005-2736/00/$ – see front matter 
PII: S0005-2736(00)00240-6

glycols $\geq 6000$ (with hydrodynamic diameters of $\geq 5$ nm) to the extracellular space, while toxin-induced leakage of intracellular potassium ions was not affected under the same conditions. Biochemical and electron microscopical analyses of the cell-bound flammutoxin also demonstrated the formation of a ring-shaped 180-kDa oligomer with outer and inner diameters of 10 and 5 nm on the cell surface, and the ring-shaped 180-kDa oligomer of flammutoxin was isolated from the cell membrane of lysed human erythrocyte [5]. The hemolytic pore-forming oligomer may be a hexamer of the toxin on the basis of its apparent molecular size. Furthermore, our preliminary experiments showed that flammutoxin permeabilized phospholipid–cholesterol liposomes by forming the pore-forming oligomer of 180 kDa, and that the liposomes consisting of negatively charged phospholipids (i.e. cardiolipin and phosphatidylserine) were more susceptible to the toxin than the liposome consisting of phosphatidylcholine or sphingomyelin (Tomita et al., unpublished results).

In this study, we characterized electrophysiological properties of flammutoxin pore by using the planar lipid bilayer technique, and found an unprecedented ability of flammutoxin to form two types of ion-conducting channels with different current amplitudes. The results showed that both of two flammutoxin channels are voltage-dependent and moderately cation-selective, and that the flammutoxin channel with a larger conductance has a functional diameter of the same size (4–5 nm) as was estimated for the hemolytic pore in the cell membrane of human erythrocyte. The channels with smaller amplitudes might correspond to the intermediate state(s) of toxin assembly on/in the lipid bilayers.

## 2. Materials and methods

### 2.1. Flammutoxin and chemicals

Flammutoxin was purified from the fruiting bodies of *F. velutipes* as described previously [5], and stored at −80°C until use. The purified flammutoxin used in this study showed a single protein band on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis [6]. Protein was determined as described by Bradford [7], using bovine serum albumin as a standard. Asolectin (type IIS, a phosphatidylcholine fraction of soybean extract) was purchased from Sigma Chemical (St. Louis, MO, USA). Cardiolipin from bovine heart was from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Phosphatidylcholine from porcine liver, phosphatidylserine from bovine brain and phosphatidylethanolamine from hen egg yolk were from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ, USA). Phosphatidylcholine from hen egg yolk was from Nippon Oil and Fat Co. (Tokyo, Japan). Polyethylene glycols 300, 400, 1000, 2000, 1540, 4000 and 6000 were from Wako Pure Chemical Industries (Osaka, Japan). Polyethylene glycols 8000 and 10 000 were from Sigma Chemical. Polyethylene glycol 3000 was from Fluka (Neu-Ulm, Germany). Other chemicals used in this study were of analytical grade.

### 2.2. Planar lipid bilayer experiment

The apparatus consisted of two Teflon chambers (capacity 2.5 ml each) connected by an aperture made by electric spark (diameter 0.15–0.20 μm) in a Teflon film (thickness 20 or 50 μm). Planar lipid bilayers were formed by the apposition of two lipid monolayers in the aperture of the Teflon film, as described by Montal and Mueller [8]. Unless otherwise stated, asolectin (a phosphatidylcholine-rich fraction of soybean lipids) was used for preparation of lipid bilayers, because it formed stable lipid membranes with low noise. The lipid membranes had an electric capacitance of 200–400 pF. The two compartments were connected to the amplifier via a pair of Ag–AgCl electrodes (In Vivo Metric, Healdburg, CA, USA). Flammutoxin was added to one side (*cis*-side) of the lipid bilayer. The *trans*-compartment was connected to the virtual ground, and voltage signs are referred to it. The membrane currents were measured with either a CEZ-2300 amplifier (Nihon Kohden, Tokyo, Japan) or Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Capacitance compensation was not used. Voltage protocols were controlled by an i-486-based computer coupled to a DigiData 1200 interface (Axon Instruments). Currents were filtered at 0.1–1 kHz, and sampled at 1–5 kHz. Data acquisition and data analyses were done using pCLAMP software (Axon Instruments) and ASCD software

(Guy Droogmans, Katholic University of Leuven, Louven, Belgium). Bathing solution was 5 mM Tris–HCl buffer (pH 7.5) containing 100 mM KCl, unless otherwise stated. All the experiments were done at room temperature.

Time constants of inactivation of flammutoxin-induced macroscopic current were calculated from the fitting of the traces of the channel inactivation with a single-exponential equation:

$$I = I_{ss} + A \exp(-t/\tau) \quad (1)$$

where $I_{ss}$ is the current in the steady state, $A$ is the amplitude of the inactivating part of the current, and $\tau$ is time constant.

In the measurement of ion selectivity of the channels, the *cis*- and the *trans*-chambers contained 100 mM KCl and 10 mM KCl, respectively, buffered with 5 mM Tris–HCl to pH 7.5. Flammutoxin-induced macroscopic and single-channel currents were measured upon application of the step or the ramp voltages of between −150 mV and +100 mV. The cation transference number ($t_+$) was calculated as follows:

$$t_+ = (E_{rev} - E_{an})/(E_{cat} - E_{an}) \quad (2)$$

where $E_{rev}$ is the measured reversal potential in the presence of the KCl gradient, and $E_{cat}$ and $E_{an}$ are the theoretical Nernst potentials for cations and anions, respectively.

Dose–response results for proton block of single-channel amplitudes were fitted to the equation:

$$y = a + b/(1 + 10^{(pK - pH)n}) \quad (3)$$

where $y$ is the single-channel current or single-channel conductance at a given pH, $pK = -\log(K)$, where $K$ is the apparent dissociation constant for proton, $a$ is a limiting value of $y$ in the completely protonated state, $b$ is a titratable part of $y$, and $n$ is the Hill coefficient.

To estimate the functional diameter of flammutoxin channel, the single-channel conductance was measured in the presence of 20% (w/v) polyethylene glycols of different molecular weights of between 300 and 20000, as described previously [9]. The bathing solution was 5 mM Tris–HCl buffer (pH 7.5) containing 100 mM KCl and one of the polyethylene glycols (20%, w/v). Permeability of each polyethylene glycol through the toxin channel was calculated as the relative change of single-channel conductance normalized to the relative change in the conductivity of the bathing solution, and it is calculated as a formal permeability parameter ($\nu$) as follows:

$$\nu = [(g_0 - g_{20})/g_0]/[(\chi_0 - \chi_{20})/\chi_0] \quad (4)$$

where $g_0$ and $g_{20}$ are the single-channel conductances in the absence and in the presence of 20% (w/v) polyethylene glycol, respectively; $\chi_0$ and $\chi_{20}$ are the conductivities of the bathing solution in the absence and in the presence of 20% (w/v) polyethylene glycols of different sizes, respectively. Calculated values of the permeability parameter ($\nu$) were plotted versus the hydrodynamic diameters of polyethylene glycols [9,10]. Conductivity of the bathing solutions was measured with a conductivity meter Model CM-78 (TOA Electronics Ltd., Tokyo, Japan) at 25°C.

## 3. Results

### 3.1. Flammutoxin forms two types of ion-conducting channels in the lipid bilayers

Flammutoxin-induced current increase across the lipid bilayers of asolectin was measured under the voltage-clamped conditions. When flammutoxin (final concentration of 1 μg/ml) was added to one side of the lipid bilayer, a stepwise increase in ionic current across the asolectin membrane was observed at a holding potential of 20 mV, and it routinely reached a level of several nA within 20–30 min. Fig. 1 illustrates the representative results of the smaller and the larger current jumps across the flammutoxin-treated membranes under the same conditions. Application of flammutoxin to the lipid bilayers normally induced consecutive current steps of the smaller amplitudes after a lag time of approximately 1 min (Fig. 1A), and current jumps of the larger amplitudes took place thereafter (Fig. 1B,C). One or a few current jumps of the smaller amplitude were often found in between two larger current jumps (Fig. 1C). No significant fluctuation in the ionic current was observed before addition of flammutoxin, and the asolectin membrane showed a stable and low electrical background for at least 1 h (results not shown). The larger-conductance channels had a fairly uniform distribution of current ampli-

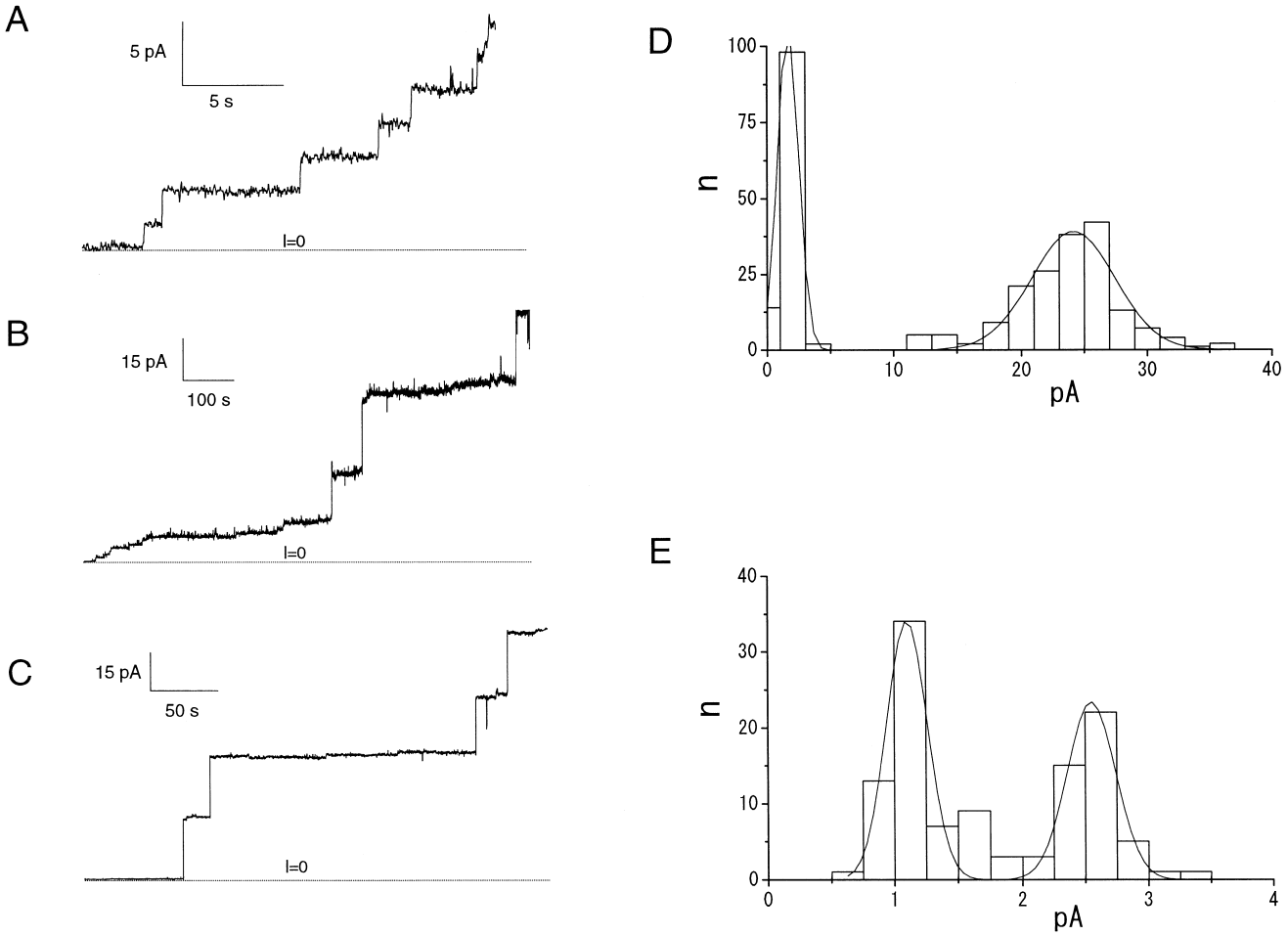


Fig. 1. Flammutoxin-induced stepwise increase in current across asolectin membrane (A–C) and size distribution of the larger and the smaller current amplitudes (D and E). (A–C) After addition of flammutoxin (final concentration of 1 μg/ml), current through the lipid bilayers was monitored at a holding potential of 20 mV, as described in Section 2. The traces are the representative ones illustrating a consecutive appearance of smaller current jumps (A) and appearance of smaller and larger current jumps (B and C). Line $I=0$ represents a zero current level. (D) Toxin-induced smaller and larger current jumps ($n=289$) were randomly collected from 20 independent experiments, and the numbers of the different amplitudes obtained were plotted versus current amplitudes. (E) Frequencies of the appearance of the smaller current jumps ($n=114$) with different magnitudes were re-plotted versus current amplitudes.

tudes with a mean value of $24.1 \pm 3.3$ pA at a holding potential of 20 mV, while the smaller current jumps varied in the range of 1–3 pA under the same conditions (Fig. 1D). These results indicated that flammutoxin formed two types of ion-conducting channels in the lipid bilayer of asolectin, possibly through formation of channels with different molecular architectures and/or with different functional states. Concerning current amplitudes of the smaller-conductance channels, two peaks of current amplitudes at $1.1 \pm 0.2$ pA and $2.6 \pm 0.2$ pA were found in the histogram of size distribution (Fig. 1E). It was also noted that flammutoxin tended to form one of the two smaller current jumps (i.e. 1.1 pA or 2.6 pA) consecutively in between two current jumps of the other magnitude. In this study, the channels with the smaller and the larger amplitudes are tentatively designated 'mini-' and 'maxi-channels', respectively.

'Mini-' and 'maxi-channels' of flammutoxin were also observed using the lipid bilayers consisting of phosphatidylcholine/cholesterol and cardiolipin/cholesterol in a molar ratio of 8:2, while only 'maxi-channels' were detected using the membranes composed of phosphatidylserine/cholesterol and phosphatidylethanolamine/cholesterol in a molar ratio of 8:2 (Table 1). Thus, flammutoxin formed 'maxi-

Table 1
Current amplitudes of flammutoxin channels formed in the membranes of different lipid compositions

| Membrane composition | Current amplitudes of flammutoxin channels (pA) | |
|---|---|---|
| | 'maxi-channels' | 'mini-channels' |
| PC/Chol | 20.9 ± 2.8 | 3.8 ± 0.6 |
| PE/Chol | 20.8 ± 1.0 | ND |
| CL/Chol | 30.1 ± 2.7 | 4.5 ± 0.7 |
| PS/Chol | 29.1 ± 2.3 | ND |

Toxin-induced current jumps were randomly collected from 20–30 independent experiments, and means ± S.D. ($n$ = 50–100) were calculated. PC, phosphatidylcholine; CL, cardiolipin; PS, phosphatidylserine; PE, phosphatidylethanolamine; Chol, cholesterol; ND, not detected.

channels' in the lipid bilayers of different compositions, and the current amplitudes of 'maxi-channels' varied in between 20 and 30 pA, possibly due to the surface charge of the membranes (i.e. negative charge of the phospholipids might attract and accumulate potassium ions on the membrane surface). It should be noted that flammutoxin formed 'mini-channels' not only in the asolectin membrane but also in the membranes of phosphatidylcholine (or a neutral phospholipid) and cardiolipin (or a negatively charged phospholipid), and that the 'mini-channels' have a wide distribution of current amplitudes in the range of 1–4.5 pA under the same conditions (Fig. 1 and Table 1). We used asolectin membrane throughout the following experiments, because of its high stability and low noise in the membrane current.

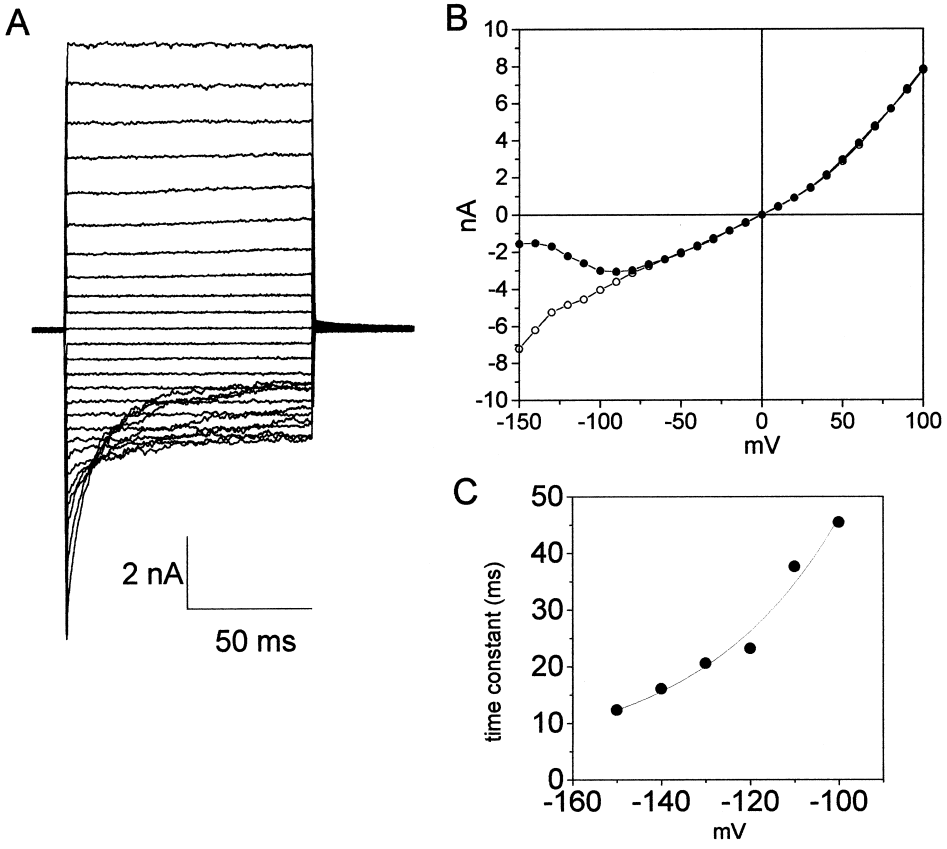


Fig. 2. Kinetics of voltage-dependent inactivation of the flammutoxin-induced macroscopic current upon application of 100-ms step pulses of different voltages (A), instantaneous and steady-state $I$–$V$ relationships (B), and the time constant for the voltage-dependent inactivation of the macroscopic current (C). The applied voltage was stepped from a holding potential of 0 mV to different levels ranging from −150 to +100 mV with 10-mV increments. Macroscopic current was monitored upon elicitation of the toxin-treated lipid bilayer with the 100-ms pulses of the different voltages. Current amplitudes were measured at the beginning and at the end of the 100-ms long voltage pulses (A), and were plotted versus the applied voltages (B). The time constants were calculated for the current traces in the range of −100 to −150 mV as described in Section 2, and were plotted as a function of the test pulse amplitude (C).

### 3.2. Flammutoxin-induced macroscopic current displays voltage-dependent inactivation

Prolonged exposure of asolectin membranes to flammutoxin (final concentration of 1 μg/ml) led to formation of high-conductance membranes without significant increase in current noise, and the macroscopic current across the membranes routinely reached 1–4 nA. To characterize the macroscopic current across the multi-channel asolectin membrane, kinetic response of the membrane was monitored upon application of the 100-ms step pulses from holding 0 mV to high positive and negative potentials (i.e. 0 to +100 mV and 0 to −150 mV). The macroscopic currents across the membrane were stable at positive voltages, and the magnitude of the current was proportional to the applied potential (Fig. 2A). Such stable currents were also observed when low negative potentials of $> -50$ mV were applied to the membrane (Fig. 2A). However, a significant time- and voltage-dependent inactivation of the flammutoxin-induced current was observed upon application of high negative potentials ($< -80$ mV), and the rate and the extent of the inactivation increased with increasing negative potentials (Fig. 2A). Current to voltage (*I–V*) curve of the flammutoxin-induced current was obtained from the results of Fig. 2A. Instantaneous *I–V* relationship for the macroscopic current, which was measured at the beginning of the voltage pulses, was non-linear and asymmetric (Fig. 2B). Steady-state *I–V* curve obtained from the measurements at the end of the 100-ms pulses was indistinguishable from the instantaneous one at positive potentials, while it had a portion with so-called 'negative resistance' (i.e. the current decrease with increasing voltage) at high negative potentials of $< -80$ mV (Fig. 2B). The kinetics of inactivation were fitted to a single-exponential function, and increasing negative potential led to more rapid inactivation of flammutoxin channels with time constants of 45 ms at 100 mV and 12 ms at 150 mV (Fig. 2C). These results suggested that flammutoxin was incorporated into the lipid bilayer of asolectin to form voltage-gated channels with asymmetrical structure(s), and that the gating machinery of the channels was active at high negative voltages. Furthermore, when the multi-channel membranes were exposed to longer (500 ms) step pulses

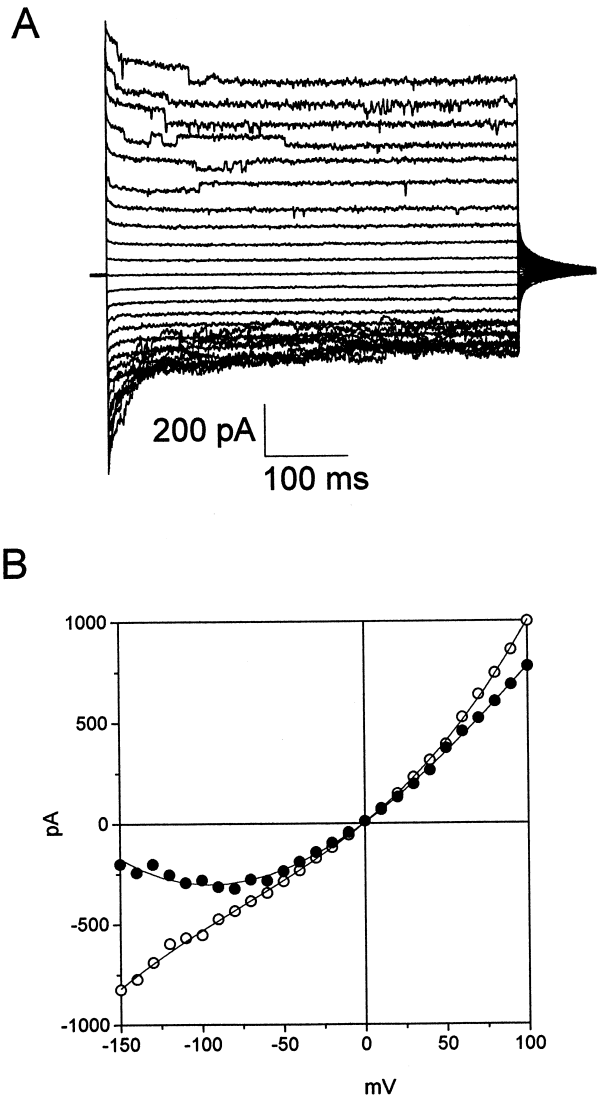


Fig. 3. Time course of voltage-dependent current inactivation of the lipid bilayer carrying a smaller number of flammutoxin channels upon application of longer 500-ms step pulses of different voltages (A), and instantaneous and steady-state *I–V* relationships of the toxin-induced macroscopic current (B). (A) The current was monitored upon elicitation of the toxin-treated membrane with the same step voltages as described in the legend for Fig. 2, except for the pulse period (i.e. 500 ms instead of 100 ms). Single-channel closing events can be detected on the current traces. (B) Current amplitudes measured at the beginning and at the end of the 500-ms long voltage pulses were plotted versus the applied voltages.

of different voltages, voltage- and time-dependent closing of single channels took place at positive voltages of $> +50$ mV as well as at negative voltages of $< -50$ mV (Fig. 3). Thus, flammutoxin channels are inactivated at high positive voltages as well as at high

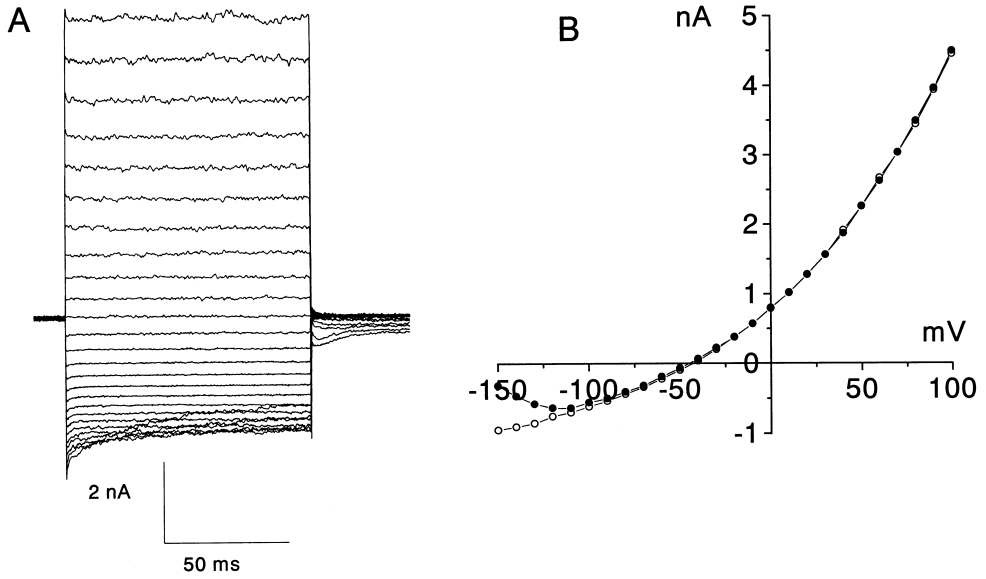


Fig. 4. Kinetic response of flammutoxin-induced macroscopic current to different step voltages in the presence of a KCl gradient (A) and *I–V* curves of the macroscopic current under the conditions (B). KCl concentration was 10-fold different in between *cis*- (100 mM) and *trans*- (10 mM) sides. Macroscopic current was monitored upon elicitation of the toxin-treated lipid bilayer with the 100-ms long pulses of the different voltages, as described in the legend for Fig. 2. Current amplitudes were measured at the beginning and at the end of the 100-ms long voltage pulses.

negative voltages, although positive voltage induced channel closing more slowly than negative voltages.

### 3.3. Flammutoxin forms cation-selective channels

To analyze ionic selectivity of the flammutoxin channels, macroscopic current across the toxin-treated membrane was measured at different voltages in the presence of a 10-fold KCl gradient (i.e. 100 mM KCl in the *cis*-side and 10 mM KCl in the *trans*-side). As shown in Fig. 4, the current was asymmetric and reversed at $-44 \pm 1.2$ mV (mean ± S.D., $n = 15$). Since the *cis*-compartment contained a 10-fold higher concentration of KCl, negative reversal potential indicated the selective current of cations over anions. The calculated cation transference number ($t_+$) was $0.88 \pm 0.03$, indicating that the flammutoxin-treated membrane has a moderate, non-ideal selectivity for cations over anions.

### 3.4. I–V characteristics and ionic selectivity of the 'mini-' and 'maxi-channel' of flammutoxin

*I–V* relationships of the 'mini-channels' were studied in the absence and in the presence of a 10-fold KCl gradient (i.e. 100 mM KCl in the *cis*-side and 10 mM KCl in the *trans*-side). The recordings of the *I–V* characteristics were done by application of a series of the ramp pulses from −100 mV to +100 mV with a rate of 100 mV/s. The initial current background was monitored before addition of flammutoxin, and formation of only a single 'mini-channel' was confirmed by measuring the current levels before and after the recording. The 'mini-channels' showed asymmetric and non-linear *I–V* curves (Fig. 5A), probably due to asymmetric distribution of charged groups along the channel lumen. The presence of a 10-fold KCl gradient shifted the reversal potential from 0 mV to −40 mV (Fig. 5B), indicating a non-ideal cationic selectivity of the 'mini-channels' of flammutoxin. The mean value of the reversal potential ($-40 \pm 0.6$ mV; $n = 108$) gave a cation transference number of $0.84 \pm 0.01$, which was close to the value obtained for macroscopic current (Fig. 5).

*I–V* characteristics of the 'maxi-channel' were studied in the absence or in the presence of a 10-fold KCl gradient, and a representative set of superimposed *I–V* curves from a series of recordings are illustrated in Fig. 6. In contrast to the 'mini-channels', the 'maxi-channel' gave a substantially sym-

metric and linear *I–V* curve (Fig. 6A). The *I–V* curves of the channels gave an averaged reversal potential of $-38.1 \pm 0.96$ mV ($n = 144$) when the 10-fold KCl gradient was present across the lipid bilayer, indicating that the 'maxi-channel' is also a moderate cationic selective channels. The calculated cation transference number ($t_+$) was $0.83 \pm 0.01$. Thus, the ionic selectivities of the 'maxi-channel' as well as the 'mini-channels' seem to contribute equally to the moderate cationic selectivity of the multi-channel membranes. It should be noted that both of the 'mini-' and the 'maxi-channel' of flammutoxin showed closing at high negative and positive potentials in the ramp single-channel recordings (Figs. 5 and 6). Evidently, these single-channel events underlie the voltage-dependent inactivation of the macroscopic currents across the toxin-treated membranes.

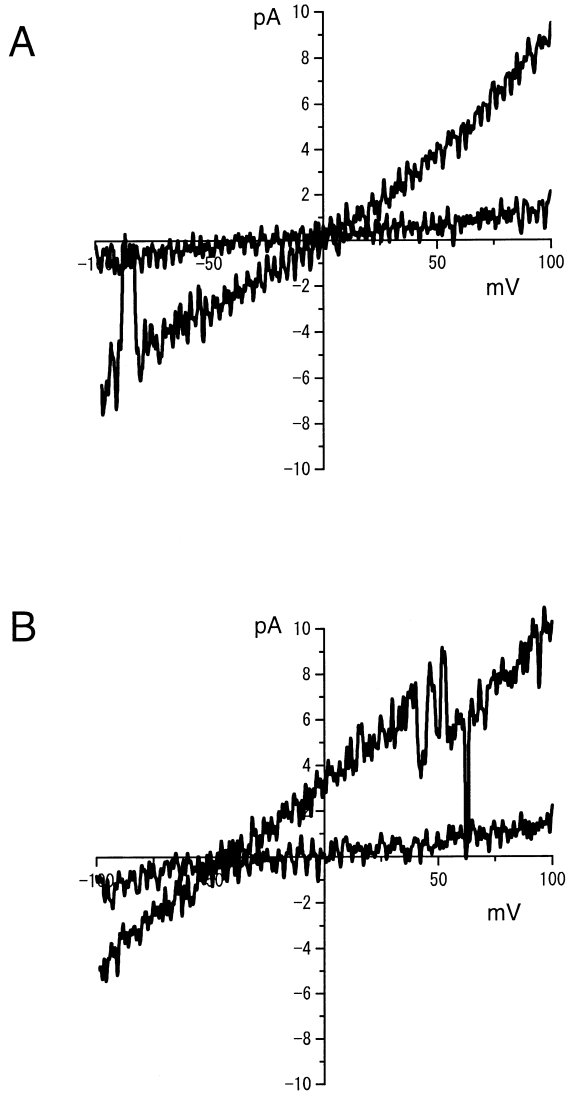


Fig. 5. *I–V* relationships for single 'mini-channel' in the absence (A) or in the presence of a 10-fold KCl gradient (B). The current through a single 'mini-channel' (2.6 pA at 20 mV) was recorded upon application of a series of ramp pulses from −100 mV to +100 mV with a rate of 100 mV/s. Representative 'mini-channel' *I–V* curves are illustrated. Background level of the current across the membrane without flammutoxin is also illustrated for the range of applied voltages.

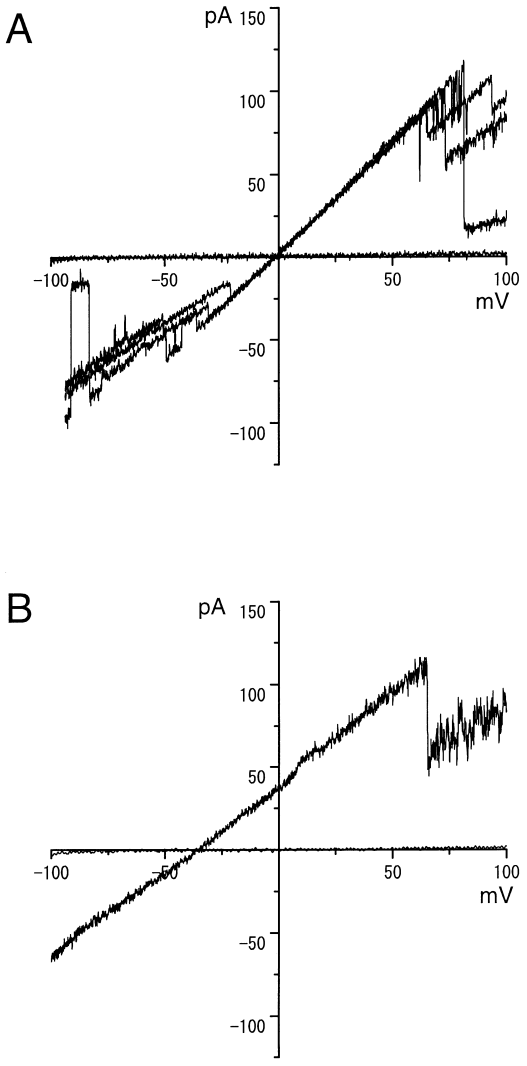


Fig. 6. *I–V* relationships for single 'maxi-channel' in the absence (A) or in the presence of a 10-fold KCl gradient (B). The current through a single 'maxi-channel' (25 pA at 20 mV) was measured as described in the legend for Fig. 5. (A) Three representative *I–V* curves of the 10 consecutive measurements in the absence of the KCl gradient were superimposed. (B) A representative *I–V* curve from the 10 consecutive measurements in the presence of the KCl gradient is illustrated.

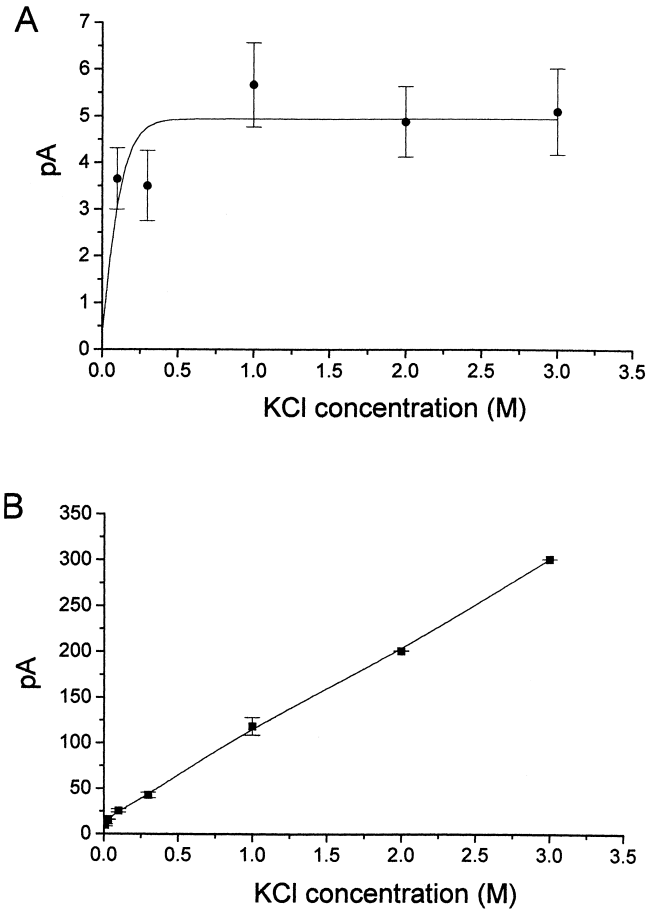


Fig. 7. Relationships between single-channel amplitudes of 'mini-' (A) or 'maxi-channel' (B) and KCl concentration. (A) Current amplitudes of 'mini-channels' were measured at 50 mV in the presence of 0.1, 0.3, 1.0, 2.0 and 3.0 M KCl. (B) Current amplitudes of 'maxi-channels' were measured at 20 mV in the presence of 0.01, 0.03, 0.1, 0.3, 1.0, 2.0 and 3.0 M KCl. Averaged values of current amplitudes for single 'mini-' and 'maxi-channel' ($n = 50$–100) were plotted versus KCl concentrations.

### 3.5. *Effect of salt concentration and pH on the amplitudes of flammutoxin channels*

Current amplitudes of the 'mini-' and the 'maxi-channel' of flammutoxin were measured in the bathing solutions containing 0.01–3 M KCl, and averaged values of the current amplitudes obtained were plotted versus KCl concentrations (Fig. 7). The increase in current amplitude of the 'mini-channels' reached a plateau at a low concentration of KCl ($\leq 0.1$ M), and it did not increase with high concentrations of KCl (up to 3 M; Fig. 7A). In contrast, current amplitude of the 'maxi-channel' increased with increasing KCl concentrations of up to 3 M (Fig. 7B), and its value reached approximately 300 pA under the conditions (20 mV), suggesting that the 'maxi-channel' may have a wide water-filled lumen which allowed passage of potassium and chloride ions.

Current amplitudes of flammutoxin channels were measured at different pHs of between 3.5 and 8.0. Current amplitudes corresponding to the 'maxi-channels' were consistently detected in the pH range, and the frequency of channel formation was not affected by the change in environmental pH (results not shown). The 'maxi-channels' also showed fairly uniform size distributions of the current amplitudes in the pH range (Fig. 8A). However, current amplitude of the 'maxi-channel' was affected by the pH of the bathing solution (Fig. 8C): the channel conductance was pH-insensitive at pH 6–8, and the channel currents gradually decreased in between pH 6 and pH 5, and reached a lower plateau (i.e. mean values of the amplitudes were 20–25 pA at pHs 5.5–8.0

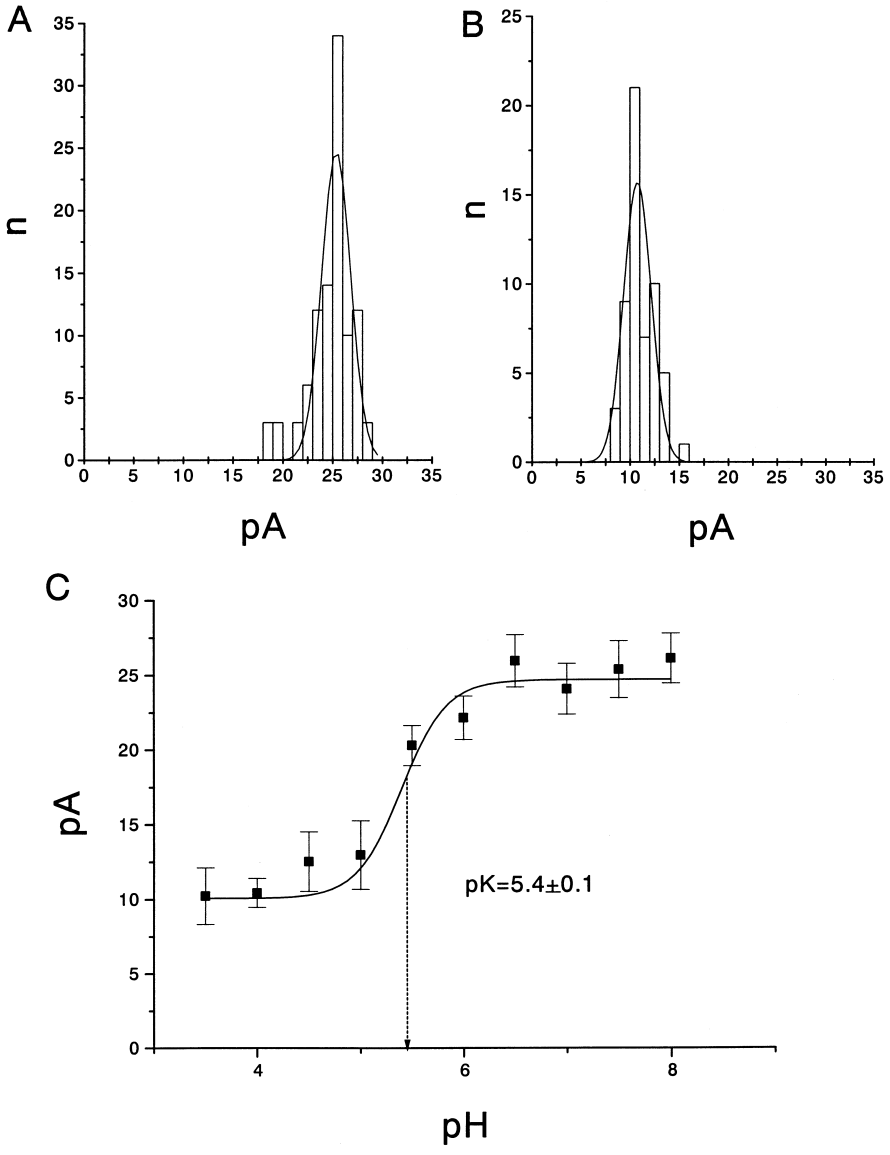


Fig. 8. pH-dependent change in the current amplitude of 'maxi-channel'. Current amplitudes of 'maxi-channels' were measured at different pHs at a holding potential of 20 mV. Size distributions of the current amplitudes measured at pH 7.5 (A) and pH 4.0 (B) are illustrated. The solid curves in the histograms represent Gaussian fits (A and B). The averaged values of current amplitudes obtained ($n = 45$–100) at different pHs were plotted versus pH (C). The solid curve is the best fit to Eq. 3 described in Section 2 (C).

and 10–13 pA at pHs 3.5–5.0, respectively). The titration curve was well fitted to Eq. 2 with a Hill coefficient of $n = 2$, suggesting that binding of at least two protons to the channel would modulate the channel current. The p$K$ value of $5.4 \pm 0.1$ implies involvement of histidine residue(s) and/or acidic amino acid residue(s) in the regulation of the permeation properties of the 'maxi-channel'. In contrast to the 'maxi-channel', the relatively large distribution of 'mini-channel' amplitudes made it difficult to detect the pH-sensitive change of their channel amplitudes.

### 3.6. *Estimation of the functional diameter for the 'maxi-channel'*

To estimate the functional size of the 'maxi-channel', current amplitudes of the channels were recorded in the absence or in the presence of 20% (w/v) polyethylene glycols 300, 400, 1000, 1540,

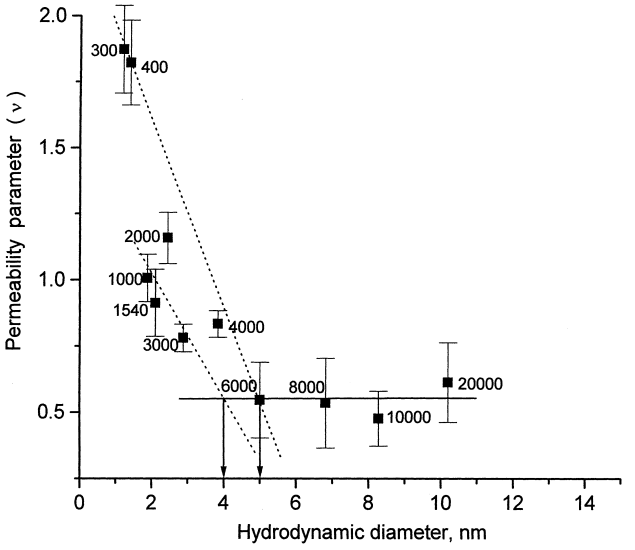


Fig. 9. Estimation of the functional diameter of 'maxi-channel'. Flammutoxin-induced current increase was recorded in the absence or in the presence of 20% (w/v) polyethylene glycols 300, 400, 1000, 1540, 2000, 3000, 4000, 6000, 8000, 10 000 and 20 000. The permeability parameter ($\nu$) for each polyethylene glycol was calculated using Eq. 4 as described in Section 2, and was plotted versus the hydrodynamic diameter of each polyethylene glycol.

2000, 3000, 4000, 6000, 8000, 10 000, and 20 000, as described in Section 2 [9,10]. The method is based on the observation that the single-channel amplitude of wide water-filled pores is decreased by the presence of small non-electrolytes, whereas the channel conductance is not significantly affected by large impermeant non-electrolytes. The permeability parameter ($\nu$) was calculated for each polyethylene glycol as described in Section 2, and it was plotted versus the hydrodynamic diameter of the non-electrolyte. As shown in Fig. 9, when polyethylene glycols 300–4000 were present in the bathing solution, the permeability parameter ($\nu$) for these non-electrolytes showed a decline with increase in their molecular sizes. In contrast, the magnitude of the permeability parameter ($\nu$) was constant in the presence of polyethylene glycols $\geq$6000 (Fig. 9). The declining part is thought to reflect decreasing permeability of the test molecules, while the plateau implies size exclusion of the larger non-electrolytes by the channel. Thus, the functional diameter of the 'maxi-channel' of flammutoxin was estimated to be 4–5 nm (Fig. 9). This value coincides with the functional diameter (i.e. 3.8–5.0 nm) of flammutoxin pore in human erythrocyte membrane, which was estimated by using the osmotic protection experiments using polyethylene glycols of various sizes [5].

## 4. Discussion

In the present study, we showed that flammutoxin forms two types of ion-conducting channels with smaller and larger conductances, or 'mini-' and 'maxi-channel', in the lipid bilayers of different phospholipid compositions. The 'mini-' and the 'maxi-channel' of flammutoxin share some properties such as voltage-sensitive gating and moderate selectivity for cations over anions. However, they show different $I$–$V$ curves and different relationships between current amplitudes and KCl concentrations.

'Maxi-channel' of flammutoxin exhibited a linear increase in current amplitude with increasing concentration of KCl (Fig. 7B), implying that the 'maxi-channel' is a wide water-filled pore. The functional diameter of 'maxi-channel' could be estimated to be 4–5 nm by measuring current amplitude of the channel in the presence of polyethylene glycols with various molecular sizes (Fig. 9). This method is based on the 'filling' of the wide water-filled pore with non-electrolytes, whose hydrated diameters are smaller than that of the pore [9,11–13], and it has been successfully used for determination of the functional diameter of several pore-forming toxins including staphylococcal α-hemolysin [9,11,12], colicin Ia [13] and mitochondrial voltage-dependent anion channels [14]. Polyethylene glycols of similar sizes (i.e. $\geq$ 6000 Da) prevented osmotic lysis of human erythrocytes [5] and failed to reduce the current conductance of 'maxi-channel' (Fig. 9), implying that the hemolytic pore and the 'maxi-channel' of flammutoxin have similar sizes in the two systems. Thus, the 'maxi-channel' may correspond to the ring-shaped toxin oligomer on the lysed erythrocytes. Rather uniform distribution of the current amplitude of 'maxi-channels' in different lipid bilayer membranes may also reflect an ultimate and stable membrane-bound state of flammutoxin, or the ring-shaped oligomeric form which is resistant to the treatment with 2% SDS at room temperatures [5]. The pH-dependency of channel conductance (Fig. 8) implied another structural feature of 'maxi-channel': the 'maxi-channel' has

crucial amino acid residue(s) which can modulate the current amplitude of the channel in a pH-dependent manner. The inflection point for the change in current amplitude (p$K$ 5.4 ± 0.1) implies the involvement of histidine residue(s) in the pH-controlled modulation of channel conductance. Sequencing of the cDNA encoding flammutoxin indicates the presence of four histidine residues (i.e. $His^{110}$, $His^{147}$, $His^{200}$ and $His^{231}$) in flammutoxin, which consists of 251 amino acid residues (manuscript in preparation). Construction of site-directed mutant proteins of flammutoxin, in which histidine residue(s) are replaced with the other amino acid(s), is currently in progress.

Flammutoxin-induced smaller current jumps were collectively designated 'mini-channels', irrespective of the different sizes of their current amplitudes. The 'mini-channels' of flammutoxin exhibited different current amplitudes depending on the lipid composition of the membranes: two different amplitudes (i.e. 1.1 pA and 2.6 pA) were observed using asolectin membrane, and one-sized amplitude (i.e. either 3.7 pA or 4.5 pA) was obtained using phosphatidylcholine/cholesterol or cardiolipin/cholesterol membrane, respectively. The multiple amplitudes of 'mini-channels' might reflect the intermediate states of assembling flammutoxin. When erythrocyte membrane-bound flammutoxin was solubilized with 2% SDS at 20°C and was separated on SDS–polyacrylamide gel electrophoresis, it was recovered mainly as the monomer (31 kDa) and the pore-forming oligomer (180 kDa), and occasionally as a 60-kDa form [5]. The 60-kDa complex, probably dimeric toxin, may not be able to form a hemolytic pore, because it was also formed on the flammutoxin-insensitive horse erythrocytes as well as on the toxin-sensitive human cells [5]. Other toxin complexes of between 60 and 180 kDa may have been dissociated to toxin monomers during solubilization of the cell membranes with SDS, possibly because of their intrinsic lability and/or their sensitivity to SDS. In contrast to the biochemical technique, the electrophysiological technique used in this study contained no use of detergent, and, moreover, it is a superior method for detection of single- and/or oligo-molecular events. Taken together, possible molecular identities of the 'mini-channels' would be transient and unstable intermediate form(s), or monomeric to pentameric forms of flammutoxin. This interpretation may be consistent with the observation: the initial stage of the stepwise increase of toxin-induced macroscopic currents often contained only the 'mini-channels' unless an extremely high concentration of the toxin was used. Concerning different current amplitudes formed by a pore-forming toxin, Belmonte et al. have reported that staphylococcal α-toxin forms a heterogenous population of channels (i.e. smaller and larger current jumps) in planar lipid bilayers, and that the smaller amplitudes were preferentially observed at low temperature [15]. They suggested that the heterogenous current amplitudes may be attributable to aggregation of different numbers of toxin monomers [15]. Furthermore, concerning the 'mini-channel' as an ion-conducting channel, the asymmetric $I$–$V$ curve and the moderate cationic selectivity of the channel would indicate the existence of internal charged groups that can modulate ionic transport through the pore, and the saturating relationship between channel conductance and salt concentration (Fig. 7A) suggests a stronger interaction of permeating ions with channel wall, possibly because of small cross-sectional size of the pore. Incidentally, no 'mini-channel' was detected using phosphatidylserine/cholesterol and phosphatidylethanolamine/cholesterol membranes, although 'maxi-channels' could be formed on/in the membranes. So, 'mini-channels' may be profoundly affected by the lipid composition of the membranes, which may be consistent with the interpretation that 'mini-channels' would reflect the intermediate form(s) of assembling toxin.

Steady-state $I$–$V$ curves for the flammutoxin-induced macroscopic current, which were measured at the end of the 100/500-ms step pulses, had a different shape from that of instantaneous $I$–$V$ curves (Figs. 2B and 3B). A portion of so-called 'negative resistance' was clearly seen in the steady-state $I$–$V$ curves at high negative potentials (Figs. 2B and 3B). Such a phenomenon is generally attributable to the voltage-dependent gating (opening and/or closing) of channels [16]. Single 'mini-' and 'maxi-channels' also displayed closings at high positive potentials, and more profoundly, at high negative potentials (Figs. 5 and 6). So, 'mini-' and 'maxi-channels' of flammutoxin share the voltage-dependent gating characteristics with endogenous ion-selective

channels of cellular plasma membrane. The voltage-dependent gating properties have also been observed for the channels formed by other pore-forming toxins including colicin K [17], staphylococcal α-toxin [18,19] and phallolysin, a cytolysin from the toxin mushroom *Amanita phalloides* [20]. However, it is yet unclear whether the voltage-dependent gating of flammutoxin channels plays a role in the hemolytic action of the channels, because human erythrocytes have very low resting membrane potentials (approximately −10 mV) [21].

## Acknowledgements

The authors are very grateful to Drs. Yoshiyuki Kamio of Tohoku University and Yasunobu Okada of National Institute of Physiological Science for their encouragement and support. We are very much obliged to Drs. Ulrich Seidel and Andre Wiese of Forschungszentrum Borstel (Borstel, Germany) for their effort to detect flammutoxin channels and for their crucial suggestions. We also thank Dr. Oiki of Fukui Medical University (Fukui Prefecture, Japan) for his useful suggestions. G.T. was a recipient of a Japanese Government Scholarship from the Ministry of Education, Science, Sports, and Culture of Japan.

## References

[1] A.W. Bernheimer, B. Rudy, Interactions between membranes and cytolytic peptides, Biochim. Biophys. Acta 864 (1986) 123–141.
[2] J.Y. Lin, Y.J. Lin, C.C. Chen, H.L. Wu, G.Y. Shi, T.W. Jeng, A cytolytic and cardiotoxic protein from the edible mushroom *Flammulina velutipes*, Nature 252 (1974) 235–237.
[3] J.Y. Lin, H.L. Wu, G.Y. Shi, Toxicity of the cardiotoxic protein flammutoxin, isolated from the edible mushroom *Flammulina velutipes*, Toxicon 13 (1975) 323–331.
[4] A.W. Bernheimer, J.D. Oppenheim, Some properties of flammutoxin from the edible mushroom *Flammulina velutipes*, Toxicon 25 (1987) 1145–1152.
[5] T. Tomita, D. Ishikawa, T. Noguchi, E. Katayama, Y. Hashimoto, Assembly of flammutoxin, a cytolitic protein from the edible mushroom *Flammulina velutipes* into a pore-forming ring-shaped oligomer on the target cell, Biochem. J. 333 (1998) 129–137.
[6] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 277 (1970) 680–685.
[7] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[8] M. Montal, P. Mueller, Formation of bimolecular membrane from lipid monolayers and a study of their electrical properties, Proc. Natl. Acad. Sci. USA 69 (1972) 3561–3566.
[9] R.Z. Sabirov, O.V. Krasilnikov, V.I. Ternovsky, P.G. Merzliak, Relation between ionic channel conductance and conductivity of media containing different nonelectrolytes. A novel method of pore size determination, Gen. Physiol. Biophys. 12 (1993) 95–111.
[10] R. Scherrer, P. Gerhardt, Molecular sieving by the *Bacillus megaterium* cell wall and protoplast, J. Bacteriol. 107 (1971) 718–735.
[11] R.Z. Sabirov, O.V. Krasilnikov, V.I. Ternovsky, P.G. Merzliak, The influence of nonelectrolytes on the mobility of ions in bulk solution and inside the channel. Determination of pore radius from electric measurements (in Russian), Biol. Membr. 8 (1991) 280–291.
[12] O.V. Krasilnikov, R.Z. Sabirov, V.I. Ternovsky, P.G. Merzliak, J.N. Muratkhodjaev, A simple method for the determination of the pore radius of ion channels in planar lipid bilayer membranes, FEMS Microbiol. Immunol. 5 (1992) 93–100.
[13] O.V. Krasilnikov, J.B. Da Cruz, L.N. Yuldasheva, W.A. Varanda, R.A. Nogueira, A novel approach to study the geometry of the water lumen of ion channels: colicin Ia channels in planar lipid bilayers, J. Membr. Biol. 161 (1998) 83–92.
[14] C.M. Carneiro, O.V. Krasilnikov, L.N. Yuldasheva, A.C. Campos de Carvalho, R.A. Nogueira, Is the mammalian porin channel, VDAC, a perfect cylinder in the high conductance state?, FEBS Lett. 416 (1997) 187–189.
[15] G. Belmonte, L. Cescatti, B. Ferrari, T. Nicolussi, M. Ropele, G. Menestrina, Pore formation by *Staphylococcus aureus* α-toxin in lipid bilayers. Dependence upon temperature and toxin concentration, Eur. Biophys. J. 14 (1987) 349–358.
[16] B. Hille, Ionic Channels of Excitable Membranes, 2nd edn., Sinauer Associates Inc., Sunderland, MA, 1992.
[17] S.J. Schein, B.L. Kagan, A. Finkelstein, Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membrane, Nature 276 (1978) 159–163.
[18] G.J. Menestrina, Ionic channels formed by *Staphylococcus aureus* α-toxin: voltage-dependent inhibition by divalent and trivalent cations, J. Membr. Biol. 90 (1986) 177–190.
[19] O.V. Krasilnikov, P.G. Merzliak, R.Z. Sabirov, V.I. Ternovskii, R.K. Zaripova, Effect of pH on the potential-dependent functioning of staphylococcal toxin channels in phosphatidylcholine bilayer, Ukr. Biokhim. 60 (1988) 60–66.
[20] H.U. Wilmsen, H. Faulstich, H. Eibl, G. Boheim, Phallolysin, a mushroom toxin, forms proton- and voltage-gated membrane channels, Eur. Biophys. J. 12 (1985) 199–209.
[21] I.B. Zavodnik, A. Piasecka, K. Szosland, M. Bryszewska, Human red blood cell membrane potential and fluidity in glucose solutions, Scand. J. Clin. Lab. Invest. 57 (1997) 59–63.