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N-terminal insertion of alamethicin in channel formation studied using its covalent dimer N-terminally linked by disulfide bond

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Abstract

Alamethicin is supposed to form helix-bundle-type channels by inserting the N terminus into bilayer lipid membranes under sufficient voltages. The N-terminal insertion has been studied with an alamethicin dimer (di-alm) N-terminally linked by a disulfide bond and by the asymmetric addition of dithiothreitol (DTT) and tetrathionate (TT) to the membrane. When di-alm was added to the *cis*-side membrane, it forms long-lasting channels with the lifetime τ of about 100 ms at *cis*-positive voltages. The lifetime was reduced to a few milliseconds by addition of DTT to the *cis*-side membrane, indicating that most of the channels were formed by the monomers (alm-SH) that resulted from the cleavage of the disulfide bond in di-alm. The succeeding addition of TT to the *trans*-side produced channels of $\tau = 10-20$ ms besides the channels of alm-SH. The results suggested that TT reacted with the N-terminal thiol group of alm-SH located at the *trans*-side of the membrane to alter the lifetime. The N-terminal insertion of alamethicin helices by voltage activation, therefore, was confirmed.

Keywords: Alamethicin; Planar lipid bilayer; Single-channel recording; Voltage-gated channel; Helix-bundle; Macroscopic current–voltage relation

1. Introduction

Alamethicin, an antibiotic peptide produced by *Trichoderma viride*, forms voltage-gated ion channels in bilayer lipid membranes. The macroscopic current of alamethicin-doped membranes showed a steep increase at *cis*-positive voltages when alamethicin was added to the *cis*-side solution, and in the single-channel recordings, multi-conductance states were observed (for reviews, see Refs. [1–5]). The electrical properties of the channels suggest that alamethicin helices orient perpendicular to the membrane surface by inserting the N termini under sufficient voltages and assemble into a "barrel-stave" or "helix-bundle" structure [6,7]. The multi-conductance behavior is supposed to be due to transient changes in the number of helices per channel. Several spectroscopic techniques have been used to study the orientation and conformation of alamethicin in the membranes [2], but there are relatively few reports on the voltage-induced structural changes [8]. Under the circumstances, we need more direct evidence to judge the validity of the channel formation model.

In this paper, we attempted to confirm the N-terminal insertion by use of an alamethicin dimer (di-alm) N-terminally linked by a disulfide bond and by asymmetric addition of dithiothreitol (DTT) and tetrathionate, $S_4O_6^{2-}$ (TT). In a previous paper, we found that di-alm formed long-lasting channels and that the tethering did not interfere with the normal channel formation [9]. The sequence of di-alm is shown in Fig. 1. DTT can cleave the disulfide bond in di-alm to provide the monomers (alm-SH) and TT can react with the thiol group of alm-SH according to the equation [10]:

$$\text{peptide}-\text{SH}+\text{S}_4\text{O}_6^{2-} \rightarrow \text{peptide}-\text{S}-\text{S}_2\text{O}_3^{-}+\text{S}_2\text{O}_3^{2-}+\text{H}^+$$

Because this reaction is inhibited by DDT and TT is impermeable to lipid bilayers, it would be possible to detect

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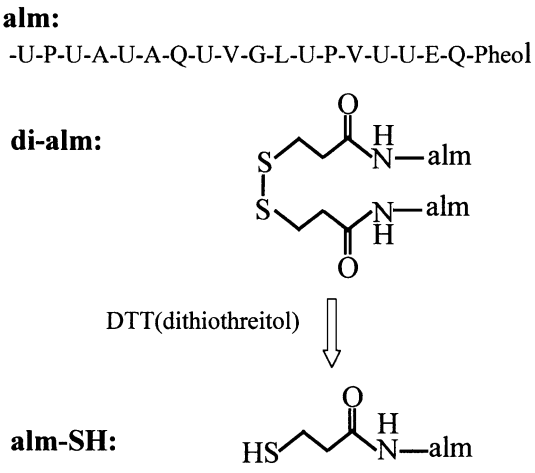

Fig. 1. Covalent alamethicin dimer N-terminally linked with a disulfide bond and its cleavage with dithiothreitol (DTT).

the N-terminal insertion of alm-SH by asymmetric addition of TT and DDT.

2. Materials and methods

2.1. Peptides and reagents

A covalent dimer of alamethicin Rf30 that was N-terminally linked by a disulfide bond was synthesized in a previous paper [9]. Dithiothreitol (DDT) and sodium tetrathionate (TT) were purchased from Wako Pure Chemical Industries, and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (diphy-PC) from Avanti Polar Lipids.

2.2. Channel current measurements

Macroscopic and microscopic current measurements were carried out using planar lipid bilayers of diphy-PC in 1 M KCl buffered with 10 mM Tris (pH 8.4–8.7) as described previously [9,11]. Peptides of a final concentration of 0.1–10 nM were added to one side (*cis*-side) of the aqueous solutions. The opposite side (*trans*-side) was virtually grounded for current measurements. For macroscopic current measurements, triangular wave voltages of 10 mHz were used. Microscopic current fluctuations were measured by applying dc voltages. All measurements were made at 25 ± 1 °C.

3. Results

3.1. Macroscopic and microscopic properties of di-alm and alm-SH channels

When di-alm was added to the *cis*-side solution, an exponential current developed at *cis*-positive voltages (Fig. 2A). The current loci were different between the ascending and descending limbs of the voltage ramp, namely, marked hysteresis was observed. On the other hand, when DTT was added to the *cis*-side solution with di-alm, the increase in current was much steeper than that for di-alm alone and the degree of hysteresis was extremely reduced. The $I-V$ relation was similar to that obtained for alamethicin. This indicated that DTT cleaved the disulfide bond in di-alm to produce the monomers (alm-SH), whose channel opening and closing were in quick response to voltage changes. The degree of hysteresis is related to the time constant of the current response to voltage jumps, or the lifetime of channel bursts in which several kinds of conductance states appear transiently [12].

Microscopic current measurements of di-alm and alm-SH showed multi-level conductance states (Fig. 2B and C). The lifetimes of the open channels were very different from each other. For both data, the histogram of the duration of open channels was fitted by an exponential decay function to

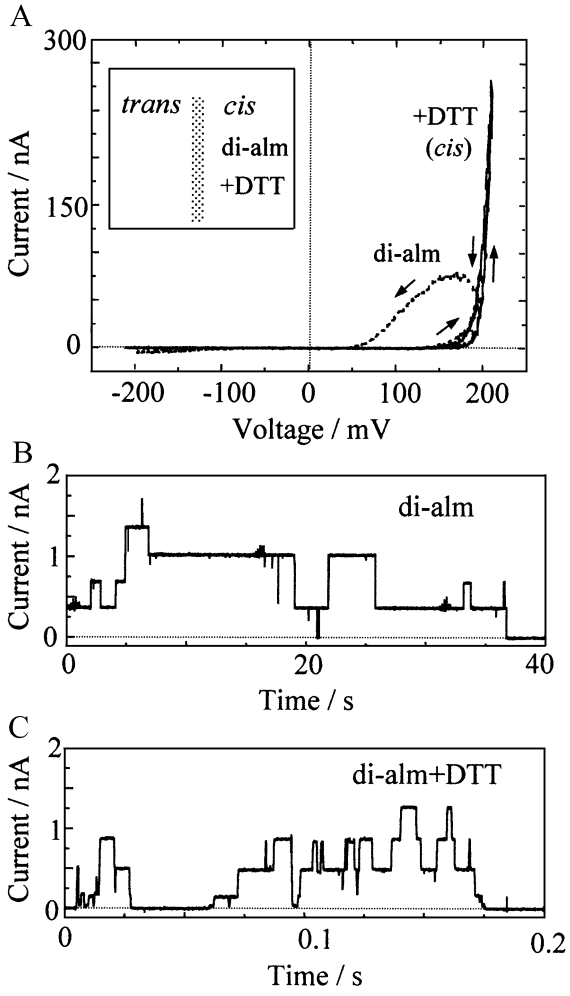

Fig. 2. Ion channel currents observed when di-alm was added to the *cis*-side solution with or without 14 mM DTT. (A) Macroscopic current–voltage relations and (B and C) microscopic current fluctuations measured at voltages of (B) 200 mV and (C) 206 mV.

estimate the time constant or the mean lifetime (Fig. 3A and B). The mean lifetimes were about 100 ms for di-alm and 2–3 ms for alm-SH.

3.2. Effects of DTT added to trans-side

The macroscopic $I-V$ relation observed when di-alm was added to the *cis*-side was modified by addition of DTT to the *trans*-side (Fig. 4). The modification of the $I-V$ curve appeared within a few minutes after the DTT addition. The macroscopic current showed a steep increase in the ascending limb of the voltage ramp like that of alm-SH channels, whereas, in the descending limb of the voltage ramp, a steep drop followed by a slow decrease was found (Fig. 4A). The slow decrease was similar to that for di-alm channels. This indicates that the current includes both the contributions of

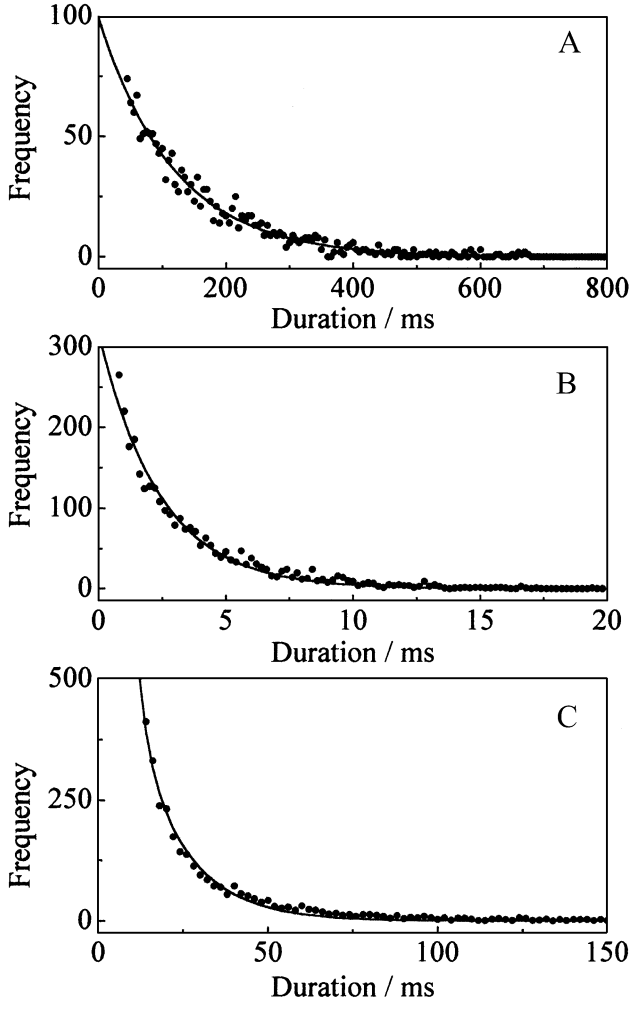

Fig. 3. Estimation of mean lifetimes from histograms of open channel duration obtained for the conductance states of 1.5–2.0 nS. The data of (A) di-alm (*cis*) and (B) di-alm (*cis*) with DDT (*cis*) were fitted by an exponential decay function of a single time constant, and the data of (C) di-alm (*cis*) with DTT (*cis*) and TT (*trans*) by that of two time constants. The time constants estimated were (A) 120 ms, (B) 2.4 ms and (C) 3 and 15 ms.

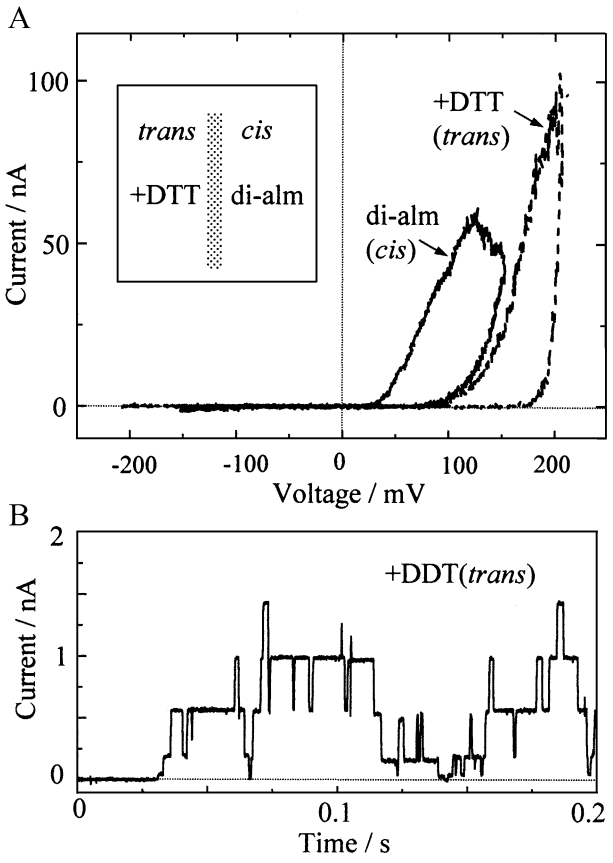

Fig. 4. Effects of DTT in the *trans*-side solution on (A) macroscopic current–voltage relations and (B) microscopic current fluctuations measured at 267 mV. di-alm was added to the *cis*-side and 14 mM DTT to the *trans*-side.

di-alm and alm-SH channels. Indeed, we found similar current fluctuations to that of alm-SH in addition to that of di-alm in the single-channel recordings (Fig. 4B).

Because DTT permeates cell membranes [13] and is soluble in organic solvents such as ethanol and chloroform, the results are insufficient to claim that DTT reacts with the N-terminal disulfide bond of di-alm at the *trans*-side. However, the quick modification of channel properties by the addition of DTT to the *trans*-side is suggestive of the cleavage of the N-terminal disulfide located at the *trans*-side interface of the membrane. To obtain clear evidence for the N-terminal insertion, we performed experiments with TT, which is impermeable to lipid bilayers and reacts with thiol groups, in the next subsection.

3.3. Effect of TT added to trans-side

After confirmation of the channel formation by alm-SH in the presence of di-alm and DTT at the *cis*-side, sodium tetrathionate was added to the *trans*-side. Fig. 5A shows the macroscopic $I-V$ relations. The current increased steeply above a critical voltage in the ascending limb of the voltage ramp in a similar manner to that of alm-SH, whereas, in the descending limb of the voltage ramp, the current slowly

decreased. The *I–V* relation was different from those of di-alm and alm-SH. The single-channel recordings showed long-lasting channels in addition to channels similar to alm-SH channels (Fig. 5B). The lifetime of the long-lasting channels was about 10–20 ms (Fig. 3C). The single-channel conductance was also modified by the TT addition as shown in the conductance histograms (Fig. 6). For TT-treated channels, the probabilities of low-conductance states decreased compared with alm-SH channels.

Although TT is not permeable to lipid bilayers, it is difficult to rule out the possibility that TT ions permeate through the channels. However, even if TT ions migrate to the *cis*-side, there is little possibility that they react with the thiol of alm-SH at the *cis*-side because the reaction is inhibited by DTT that is present in large excess in the *cis*-side solution. Hence, it is reasonable to interpret the results as follows. In the ascending limb of the voltage ramp, alm-SH forms ion channels in the membrane by inserting the N terminus. During the channel opening, TT reacts with the thiol groups of alm-SH at the *trans*-side and introduces a charged group to the N terminus, which prevents the N terminus to return to the *cis*-side of the membrane in the descending limb of the voltage ramp. This delays the channel closing and causes hysteresis in the macroscopic *I–V* curve and prolongs the lifetime of open channels in the single-channel recordings.

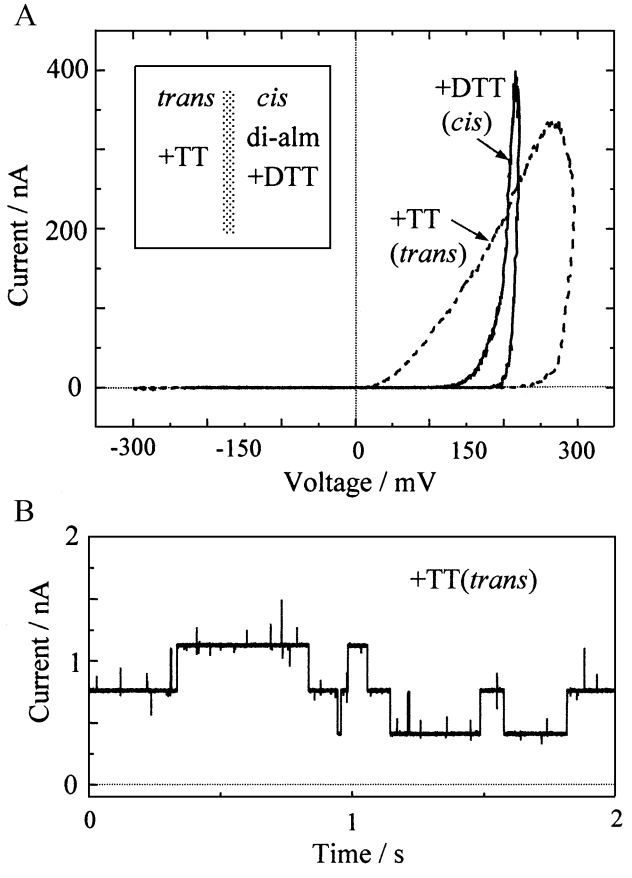

Fig. 5. Effects of TT added to the *trans*-side solution on (A) macroscopic current–voltage relations and (B) microscopic current fluctuations measured at 215 mV. di-alm and 14 mM DTT were added to the *cis*-side solution and 14 mM TT to the *trans*-side solution.

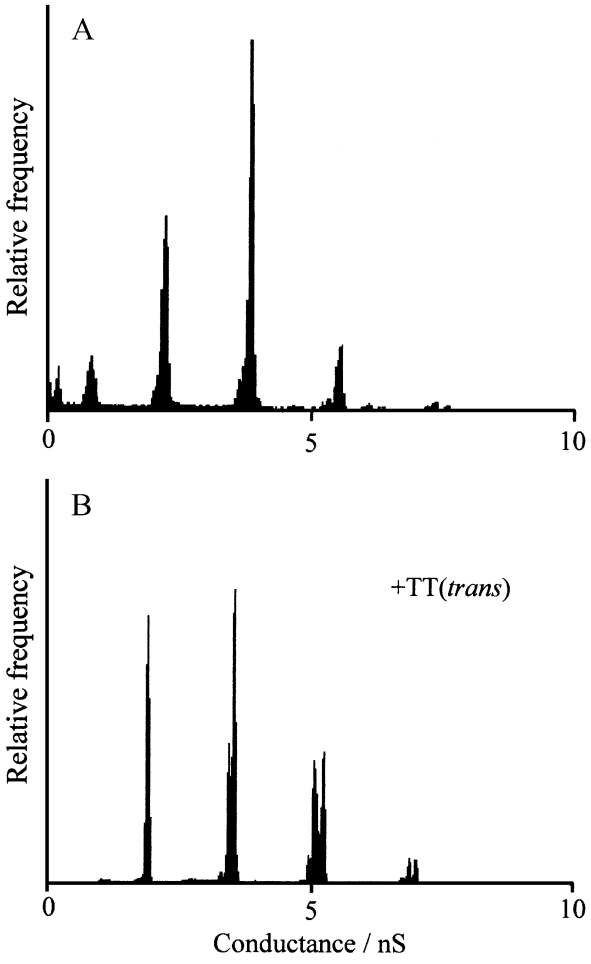

Fig. 6. Conductance histograms of current fluctuations for di-alm in the *cis*-side solution containing 14 mM DTT (A) without and (B) with 14 mM TT in the *trans*-side solution.

4. Discussion

Alamethicin is supposed to form bundles of parallel helices in the membrane by inserting the N termini under sufficient voltages. In the helix-bundles, the N termini and C termini locate at the negative and positive voltage sides of the membrane, respectively. This model is suggested from the macroscopic current–voltage relations of the membranes asymmetrically doped to alamethicin and its analogues [14]. It is also supported by the fact that single channels of covalent alamethicin dimers show intrinsic rectification, which is caused by the macro dipoles of the parallel helices [9,15]. In the present study, we confirmed the N-terminal insertion using the alamethicin dimer N-terminally linked by a disulfide bond

together with the asymmetric additions of DTT and TT to the membrane.

In the current fluctuations of alamethicin channels, the lifetime of the open duration depends on the rate of the transition to the adjacent conductance states through the process that a helix moves away from or joins into the helix-bundle. Hence, the lifetime might be related to the lateral diffusion of helices in the membrane and their tangential movement such that the N-termini return to the *cis*-side across the hydrophobic core of the membrane. The lifetime prolonged by tethering alamethicin molecules [9,15–17] may be mainly interpreted in terms of the slow lateral diffusion of tethered peptides in the membrane. On the other hand, the lifetime prolonged by the *trans*-side addition of TT (which reacts with the thiol of alm-SH to provide the N terminus a negative charge) could be due to preventing the N termini from returning to the *cis*-side across the hydrophobic core.

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