Biochimica et Biophysica Acta, 1064 (1991) 256–266

ADONIS 000527369100195L

BBAMEM 75218

Formation of ion channels in planar lipid bilayer membranes by synthetic basic peptides

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(Received 18 December 1990)

Key words: Synthetic peptide; Peptide, basic; Ion channel; Planar bilayer; Antibacterial activity

We made use of a planar lipid bilayer system to examine the action of synthetic basic peptides which model the prepiece moiety of mitochondrial protein precursors and have antibacterial activity against Gram-positive bacteria. The sequences of the peptides used were as follows: Ac-(Ala-Arg-Leu)$_3$-NHCH$_3$ (3_3), Ac-(Leu-Ala-Arg-Leu)$_2$-NHCH$_3$ (4_2), Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (4_3), Ac-(Leu-Leu-Ala-Arg-Leu)$_2$-NHCH$_3$ (5_2). These peptides interacted differently with planar lipid bilayer membranes and membrane conductance increased by the formation of ion channels. The effects of the peptides on the macroscopic current-increase and on the probability of channel formation, at the single channel level were in the order of $4_3 > 4_2 \approx 5_2 \gg 3_3$, a finding which correlates with the antibacterial activity of these peptides. The micromolar (μM) order concentration at which the channel was formed resembles that causing antibacterial activity. Thus, the peptide antibacterial activity may occur through an increase in ion permeability of the bacterial membrane. The single-channel properties were investigated in detail using 4_3, the peptide with the highest ion channel-forming activity. Many types of channels were observed with respect to conductance (2–750 pS) and voltage dependency of gating. However, the channels were all cation-selective. These results suggest that the ion channels formed by peptide 4_3 may be able to take on a variety of conformations and/or assembly.

Introduction

There are reports of various kinds of peptide with antibacterial action and the ability to form ion channels in the membrane; e.g., gramicidin A [1], alamethicin [2], diphtheria toxin [3], botulinum toxin [4], tetanus toxin [5], melittin [6], mastoparan [7], colicin A [8], cecropin [9], δ-toxin [10], magainin I [11], and defensin [12].

Techniques used to detect ion channel activities include, patch-clamp, tip-dip, and planar bilayer membrane approaches [13]. The planar bilayer membrane technique is useful for examining properties of ion channel proteins in biomembrane vesicles by reconstituting them from vesicles to planar lipid bilayer membranes [13]. In addition, the technique has been widely used to detect channel-forming activity of a variety of peptides [1–12,14–16].

Some synthetic model peptides of the prepiece moieties of mitochondrial protein precursors were found to have antibacterial activity against Gram-positive bacteria [17]. The antibacterial activity paralleled both the content of the α-helical structure of the peptide in liposomal membranes and the ability to cause leakage of carboxyfluorescein from liposomes [17].

To elucidate mechanisms involved in the appearance of antibacterial activity in the presence of synthetic basic model peptides, we made use of the planar bilayer membrane technique to search for ion-channel forming potential. Properties of the single ion channels were also given attention.

Materials and Methods

Materials. Four kinds of peptide, Ac-(Ala-Arg-Leu)$_3$-NHCH$_3$ (3_3), Ac-(Leu-Ala-Arg-Leu)$_2$-NHCH$_3$ (4_2), Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (4_3), Ac-(Leu-Leu-Ala-Arg-Leu)$_2$-NHCH$_3$ (5_2), were synthesized, as

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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described [17]. Soy bean phospholipids (asolectin), Type II-S from Sigma, were purified by acetone-washing [18]. Diphytanoyl phosphatidylcholine was obtained from Avanti Polar-Lipids Inc. Other reagents were of analytical grade and were used without further purification.

Formation of planar bilayer membrane. The planar lipid bilayers were formed by the folding method originally described by Takagi et al. [19] and modified by Montal and Mueller [20]. This procedure involved the use of a Teflon chamber with two compartments (each about 1.5 ml in internal volume) separated by a Teflon septum (25 μm thick) with an aperture 200 μm in diameter. A small amount (15–20 μl) of asolectin or diphytanoyl phosphatidylcholine solution in *n*-hexane (10 mg/ml) was placed on the surface of an electrolyte solution (0.5 ml in each compartment) in the chamber. A lipid monolayer was formed spontaneously at the air/water interface after evaporation of the solvent in a few minutes. The water level in each compartment was then raised over the aperture, where the lipid bilayer subsequently formed.

Measurement of membrane current. A small amount of a methanolic solution of the peptide was added to one compartment of the chamber, which was defined as the *cis* compartment. The *cis* solution was continuously stirred with a magnetic stirrer under applied membrane potential (usually +60 mV, the potential at the *cis* compartment with respect to the *trans* compartment, which is held at virtual ground) until current fluctuation occurred. The current across the membrane was measured with a hand-made current/voltage converter (bandwidth 800 Hz) and was displayed on both a digital storage oscilloscope (DSS5020A, Kikusui Electronics, Kawasaki, Japan) and on a chart recorder (R61VL, Rikadenki, Tokyo, Japan). The data were accumulated using videotape recorder (SL-HF3, Sony, Tokyo, Japan) after A/D conversion with a modified digital audio processor (PCM-501ES, Sony) [21]. Selected parts of the recorded data were transcribed on a pen oscillograph (WR3101, Graphtec, Tokyo, Japan: frequency response 120 Hz) through a programmable filter (FV-664, NF Electronic Instruments, Yokohama, Japan). They were also digitized with a 12-bit A/D converter (PCN-2198, Neolog Electronics, Tokyo, Japan) and analyzed using a micro-computer (PC9801VM, NEC, Tokyo, Japan).

Results

Change in membrane conductance caused by model peptides

Fig. 1 shows the rapid increase in the membrane current, under voltage-clamp conditions and after peptide 4_3 at different concentrations had been added to the *cis* compartment of the chamber. Among the four peptides used, peptide 4_3 has the most potent in antibacterial activity [17] and also in causing leakage from

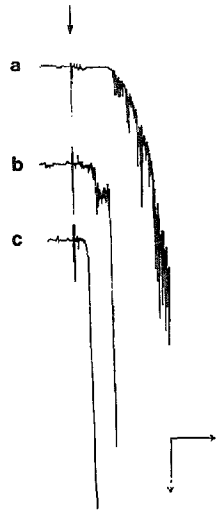

Fig. 1. Time course of the membrane current after addition of 4_3 at different concentrations. The planar bilayer membrane was made of asolectin. Small amounts of a methanolic solution of 4_3 (1 mg/ml) were added at the arrow to the *cis* compartment of the chamber. The final concentration of 4_3 in the *cis* compartment was (a) 5.8 μM, (b) 11.7 μM, and (c) 29.2 μM, respectively. The holding potential at the *cis* compartment was kept at +60 mV (the *trans* compartment being defined 0 mV). The composition of the solution in both compartments was 100 mM KCl and 10 mM Tris-Hepes (pH 7.0). The horizontal scale is 2 min and the vertical one is 10 pA.

liposomes [17]. The concentration used exceeded that required to exhibit antibacterial activity (Table I). As shown in Fig. 1, the increase in the membrane current depended on the concentration of peptide 4_3. At the highest concentration (29.2 μM), the current began to increase within 20 seconds after the addition of 4_3 to the *cis* compartment of the chamber. Soon the current exceeded the detection range of our current measuring system, which corresponded to the membrane rupture. At lower concentrations, the lag time of the current-increase was longer and the current-increase was less steep. The phenomena we observed were rate processes and equilibrium was not reached, thus the lag time and the steepness of the current-increase were varied from run to run. The traces shown were chosen as typical examples.

Fig. 2 shows the effect of four kinds of the peptides on the membrane current, the concentration used was

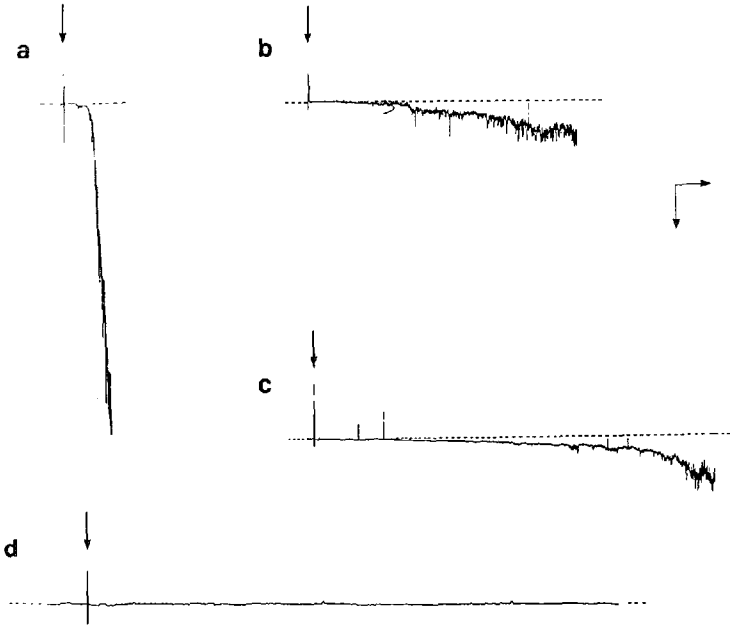

Fig. 2. Time course of the membrane current after addition of four kinds of peptide. The planar bilayer was made of asolectin. Methanolic solution of 4_3 (a), 5_2 (b), 4_2 (C), and 3_3 (d) were added to the *cis* compartment to a final concentration of 11.7 μM. The membrane potential was held at +60 mV. The composition of the solution was symmetric (100 mM KCl, 10 mM Tris-Hepes (pH 7.0)). The horizontal scale is 2 min for all traces. The vertical scale is 100 pA for (a)–(c) and 10 pA for (d).

TABLE I

Correlation between probability of channel appearance and antibacterial activity of the basic model peptides

The probability is expressed as a fraction of the number of runs in which channel activity was observed within 30 min after addition of each peptide to the *cis* compartment of the chamber. Experiments were performed under different conditions I–IV: I, symmetric 100 mM NaCl, 2 mM Mops-Tris (pH 7.0), sample 3.5 μM; II, symmetric 100 mM KCl, 0.2 mM Mops-Tris (pH 7.0), sample 5.0 μg/ml; III, symmetric 1.0 M KCl, 2 mM Mops-Tris (pH 7.0), sample 5.0 μg/ml; IV, *cis*: 333 mM KCl, 2 mM Mops-Tris (pH 7.0)//*trans*: 100 mM KCl, 0.2 mM Mops-Tris (pH 7.0), sample 5 μg/ml. The antibacterial activity is reproduced from Ref. 17 and is shown as the minimum inhibitory concentration.

Peptide	Channel appearance probability					Minimum inhibitory concn. (μg/ml)	
	I	II	III	IV	Total	*S. aureus*	*B. subtilis*
3_3	1/8	0/3	0/1	–	0.08 (1/12)	>100	>100
4_2	3/9	2/3	–	–	0.42 (5/12)	25	25
4_3	5/9	3/5	3/3	12/15	0.72 (23/32)	6.25	3.13
5_2	2/8	3/6	–	–	0.36 (5/14)	25	12.5

11.7 μM. Peptide 4_3 was most effective for a rapid increase in membrane current and 3_3 was without effect. The effectiveness of the four kinds of peptide was judged to be in the order of $4_3 > 4_2 \approx 5_2 \gg 3_3$.

At low concentration range, and one which was similar to that causing antibacterial activity, we measured current fluctuation which occurred within 30 min after adding the four kinds of the peptides. The success rate in observing the current fluctuation differed among the four peptides used. The ion channel-forming activities of the peptides were compared quantitatively in terms of the probability of detecting membrane current fluctuation. As an index of the ion channel-forming activity of the peptide, we used the ratio of the number of runs in which membrane current fluctuation was observed within 30 min after the addition of each peptide to the total number of runs, under the same experimental conditions. The highest channel-forming

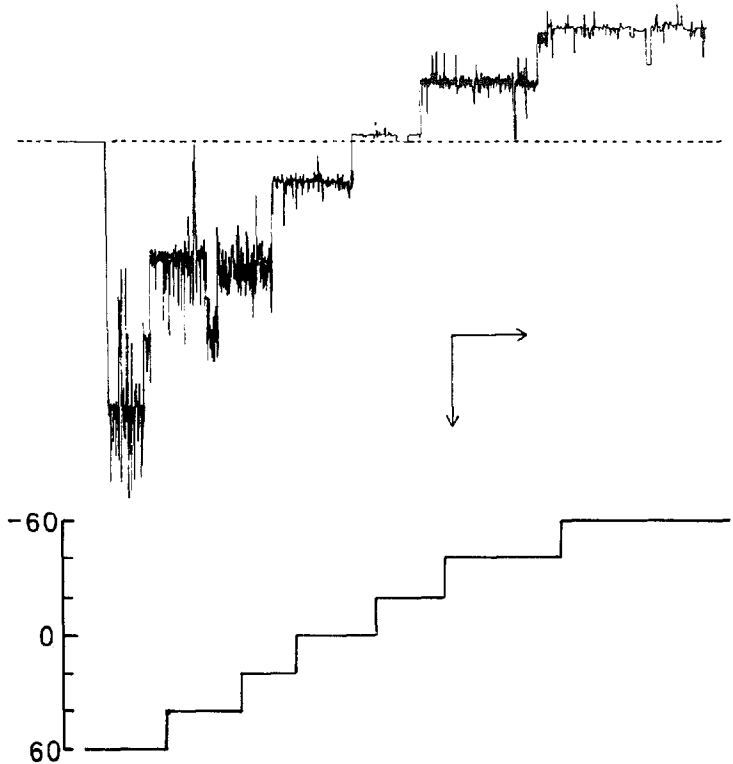

Fig. 3. A current trace obtained by the incorporation of 4_3 into the asolectin bilayer. The peptide 4_3 (final 3.5 μM) was added to the *cis* compartment. The holding potential is shown below the current trace. The composition of the solution was asymmetric (*cis*: 333 mM KCl, 3.3 mM Tris-Mops (pH 7.0)//*trans*: 100 mM KCl, 1.0 mM Tris-Mops (pH 7.0)). The vertical scale is 20 pA and the horizontal scale is 1 min.

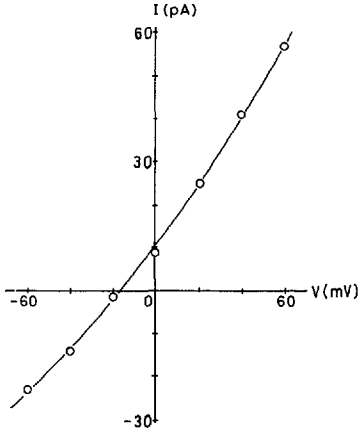

Fig. 4. A current-voltage relationship of the channel shown in Fig. 3. The conductance around 0 mV was about 680 pS and the reversal potential was −17 mV.

activity was observed for peptide 4_3, in both KCl and NaCl solutions. The channel-forming activity of the four peptides occurred in the order of $4_3 > 4_2 \approx 5_2 \gg 3_3$, as summarized in Table I. This order is the same as that obtained for the rapid current-increase at high concentration of the peptides (Fig. 2) and is the same as the order of the antibacterial activity of these peptides [17] (see Table I).

Single-channel behavior of ion channels formed by the peptide 4_3

The single-channel properties of ion channels formed by peptide 4_3 could be examined in detail, because this peptide had the highest probability of channel formation among the four peptides examined. This peptide exhibited several different types of single-channel behavior. Fig. 3 shows a current trace of a channel most frequently observed in asymmetric KCl solutions (*cis*: 333 mM// *trans*: 100 mM) using asolectin as a membrane forming lipid. This channel existed mostly in its open state and was independent of membrane potentials, under the conditions described in the figure legend. A current-voltage plot (Fig. 4) shows a slight curvature and that the single-channel conductance was about 680 pS measured around 0 mV. The curved current-voltage relationship was derived from the asymmetric ion concentration between the membrane. The reversal potential was −17 mV and from this value the ion permeability ratio between K and Cl (P_K: P_{Cl}) was calculated to be 1:0.26, according to the Goldman-Hodgkin-Katz equation.

Similar single-channel behavior was observed when diphytanoyl phosphatidylcholine was used for the membrane lipid (Fig. 5). Three open channels with the same conductance were seen in the trace. A current-voltage plot (Fig. 6) revealed the single-channel conductance of about 520 pS obtained around 0 mV and the reversal potential of −11 mV, which corresponds to P_K: $P_{Cl} = 1$: 0.44.

Another type of channel with a rapid, voltage-dependent gating was also observed. A current trace of this channel is shown in Fig. 7. Fig. 8 shows the amplitude histogram corresponding to the current fluctuations shown in Fig. 7. A current-voltage plot shows that the single-channel conductance was 39 pS and the reversal potential −12.0 mV (Fig. 9A). The ion permeability ratio between K and Cl (P_K: P_{Cl}) was 1: 0.40. The open-channel probability (P_o) was calculated as the ratio of the area of the open-channel peak to the whole area, the values being 0.261 at 59 mV, 0.124 at 40 mV, and 0.0536 at 20 mV. Gating of the channel was voltage-dependent (higher open-channel probability at more positive voltage) as shown in Fig. 9B.

The rate constant for the channel closing (k_1) and opening (k_2) was calculated from the open and closed time histograms, as shown in Fig. 10. The rate constants k_1 and k_2 at 59 mV were 5.71 s^{-1} and 1.93 s^{-1}, respectively. The rate constants at the other holding potentials were not obtainable because the open probabilities at such potentials were too small. The mean open time (t_o) and closed time (t_c) were $t_o = 1/k_1$ and $t_c = 1/k_2$, respectively, so that the open probability could be written as $P_o' = t_o/(t_o + t_c)$. The value for P_o' was 0.253 at 59 mV, which is in good agreement with $P_o = 0.261$ deduced from the amplitude histogram. These values for rate constants, mean lifetime and open probabilities are summarized in Table II.

Other channels different from those described above were also observed. The single-channel conductance, voltage dependency of the gating, and ion selectivity of some of these channels were examined and the findings are listed in Table III. The channel conductance ranged from several pS to about 750 pS. The channels given in Table III can be separated into voltage-dependent and voltage-independent ones. However, the reversal potential was negative in all cases, hence the ion selectivity of these channels is $K^+ > Cl^-$.

Discussion

We obtained evidence that four kinds of synthetic peptides interacted with planar lipid bilayer mem-

branes, causing an increase in the membrane conductance by the formation of ion channels. The effects of the peptides as related to the macroscopic current-increase and the probability of channel formation at the single channel level were in the order of $4_3 > 4_2 \approx 5_2 \gg 3_3$, a finding which correlates with the antibacterial

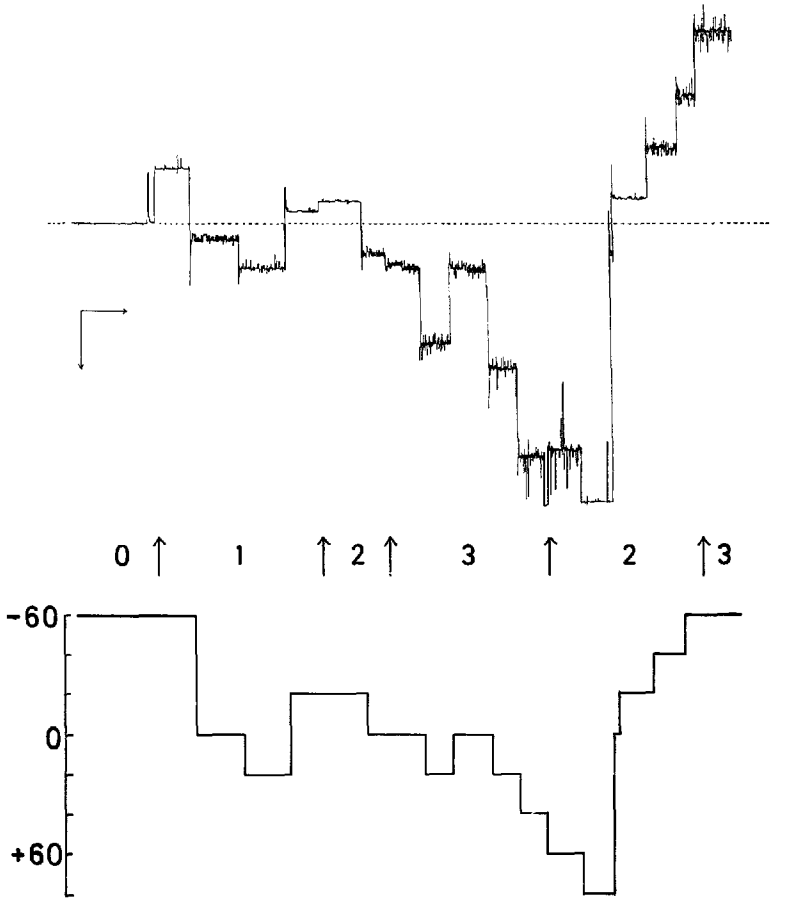

Fig. 5. A current trace obtained by the incorporation of 4_3 into diphytanoyl phosphatidylcholine bilayer. The concentration of 4_3 at the *cis* compartment was 3.5 μM. The composition of the solution was asymmetric (*cis*: 333 mM KCl, 3.3 mM Tris-Mops (pH 7.0)//*trans*: 100 mM KCl, 1.0 mM Tris-Mops (pH 7.0)). The holding potential is shown below the current trace. The vertical scale is 20 pA and the horizontal scale is 1 min. At the arrows the membrane conductance changed discretely. The numerals between the arrows indicate the number of open channels.

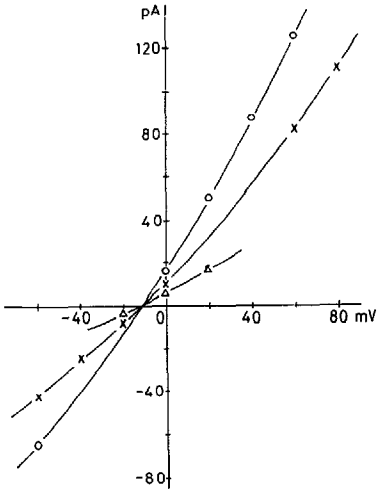

Fig. 6. A current-voltage relationship of the channel shown in Fig. 5. Opening of one (△), two (×), and three (○) channels is shown. The single-channel conductance was 520 pS and the reversal potential was −11 mV.

activity of these peptides. The concentration at which the channel was formed was close to that causing antibacterial activity. Therefore, the peptide antibacterial activity may occur through an increase in ion permeability of the membrane.

At a concentration of about 3 μM, peptide 4_3 formed a single ion channel. This concentration is higher than that in case of gramicidin A (nM order or less) [1,22], but is similar to that for melittin (about 1 μM) [6]. It is unlikely that the channels observed here were derived from contaminants in the sample because we used purified synthetic peptides and because the probabilities for the channel formation among the four peptides differed. Since the four peptides were synthesized using identical procedures, similar amounts of contaminants, if any, would exist and would produce a similar probability for channel formation.

In a previous study [17], we found that the antibacterial activities of these peptides closely correlated with their content of the α-helix. A spectroscopic study suggested that peptide 4_4 which was related to 4_3 interacted with liposomes in a manner so that they were in parallel on the surface of the bilayer [23]. Since the planar bilayer technique used in the present study is sensitive enough to detect even single-channel activity, it is likely that we observed only a small portion of the α-helices existing perpendicular to the membrane plane and forming a *trans*-membrane ion channel, as discussed below.

The thickness of the hydrophobic part of the lipid bilayer (approx. 3 nm) requires that the channel be at least 3 nm long and 20 amino acid residues are required. The number of amino acid residues in the peptides we studied were 8, 9, 10 and 12 for 4_2, 3_3, 5_2 and 4_3, respectively. If we take into account the modification of both termini in these peptides, the effective number of residues may be 10, 11, 12 and 14, respectively. These values are too small to allow the membrane to be spanned with one molecule.

Since the peptides are too short to span the bilayer, formation of the ion channel can be interpreted in the following manner: (1) The 'channel' is actually a distortion of the bilayer caused by the peptide partly penetrating the interior of the bilayer. (2) The channel is formed by a dimer, as has been postulated in case of gramicidin A. Although the active structure of the *trans*-membrane channel of gramicidin A has not been established, it probably is either a head-to-head dimer or intertwiched double helices [24–26]. (3) The channel is formed by an oligomeric cluster of the dimeric peptides as illustrated in Fig. 11. The unit constituting the cluster should be at least a dimer, for the present peptides. The structure of the N- and C-termini of these peptides may contribute to formation of the head to tail dimer structure by inter-terminal hydrogen bonding. A well examined peptide forming this type of channel structure is alamethicin, although the unit is a single peptide, not a dimer. Alamethicin, a 20-amino-acid peptide, has been reported to form voltage-gated ion channels [2,27,28], each thought to consist of 8–12 molecules [29].

Model 2 can probably be ruled out because the single-channel conductance most frequently observed is too large for this type of channel where the ions presumably permeate through the inside of the helix. The constant level of single-channel conductance, as shown in Figs. 3 and 5, suggests that model 1 is also unlikely. However, the rapid current fluctuation with an unfixed conductance level observed in some experiments (data not shown) may be caused by this mode of action. Thus, model 3 seems to be the most probable. According to recent investigations of the primary structure of some ion channel proteins, these compounds seem to be composed of bundles of α-helices that assemble to form ion channels [30–32]. Therefore, a channel structure formed from an oligomeric cluster of α-helices may be more general in nature than other structures. If it were feasible to measure the concentration dependence of the

macroscopic steady conductance using the present peptides, then one could determine the number of peptide molecules required to form the ion channel. Such experiments could not be done because the planar lipid bilayer ruptured before the current reached equilibrium (Fig. 1). However, the rate of current-increase at the initial stage of the conductance change seems to depend on the peptide concentration of more than the first order, hence several peptide molecules probably contribute to formation of structure of the channel.

It has been reported that *Staphylococcus aureus* δ-toxin forms ion channels [10]. The properties of the δ-toxin are similar to those of the synthetic peptide molecules studied here; they take an α-helical conformation primarily in the presence of phospholipid micelles or membranes and the α-helix is amphipathic (one lateral side hydrophilic and the other side hydrophobic: see Fig. 11). A hexameric cluster model of the δ-toxin channel has been proposed [10] and the structure resembles the barrel-stave model of the alamethicin channel [27]. Most of the peptide molecules investigated in the present study take on an α-helical structure in the membrane [17]. Based on analogy with the δ-toxin, a hypothetical structure of the channel formed by the

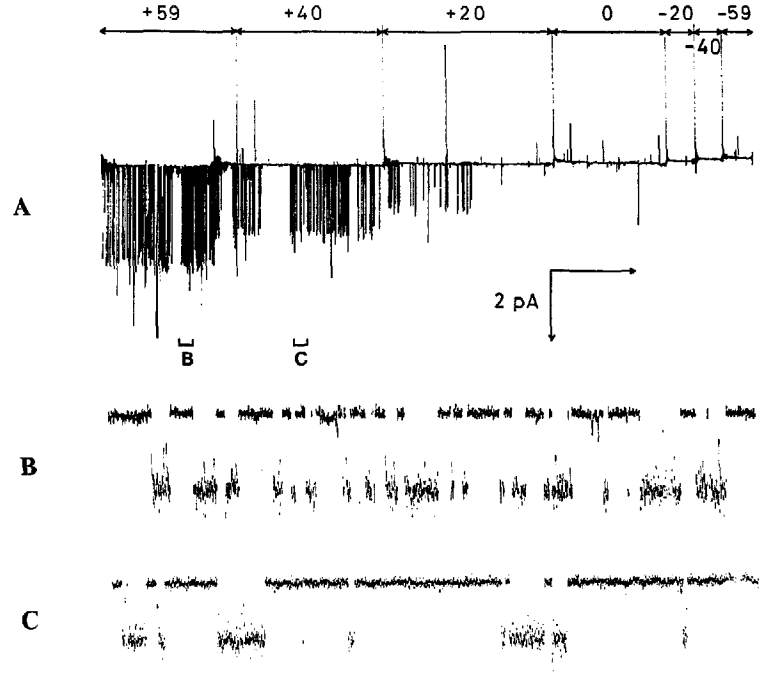

Fig. 7. (A) A current trace of another type of channel obtained by the incorporation of 4_3 into the lipid bilayer in asymmetric KCl solutions (*cis*: 333 mM,// *trans*: 100 mM). The holding potentials are shown at the top of the trace. The downward current deflections are the channel openings at positive potentials. The brief upward current deflections seen between the times of changing membrane potential do not have a square-wave shape characteristic to channel events, in the expanded scale (data not shown); these probably represent noise. The vertical scale is 2 pA and the horizontal scale is 1 min. (B)(C) Expanded traces of the B and C portion of trace A. The holding potential was +59 mV for (B) and +40 mV for (C). Downward current deflection corresponds to the channel opening. The vertical bar is 2 pA and the horizontal bar is 1.2 s.

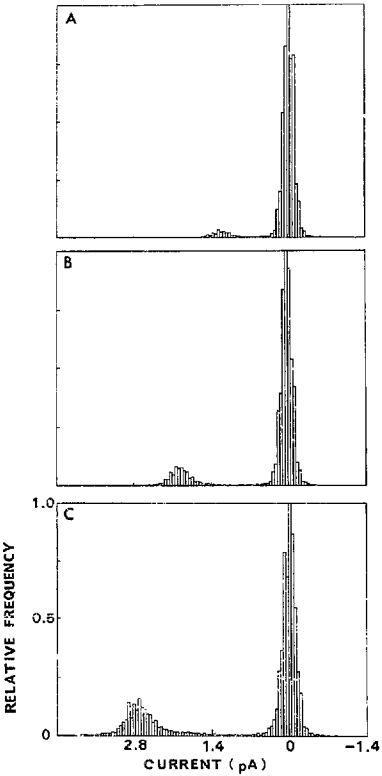

Fig. 8. Amplitude histogram corresponding to the channel current shown in Fig. 7 at holding potentials of +20 mV (A), +40 mV (B), and +59 mV (C). The ordinate indicates the relative period of time for which the current shown in the abscissa was recorded.

present model peptide is shown in Fig. 11. It is constituted of an oligomeric bundle (e.g. hexameric bundle) of α-helices.

The presence of arginine residues gives the peptide positive charges, which lie on one side of the α-helix (Fig. 11). The hydrophilic side of the helix bearing the arginine residues should face the channel pore, and anion selectivity would be expected from this structural feature. Surprisingly, however, all the channels were

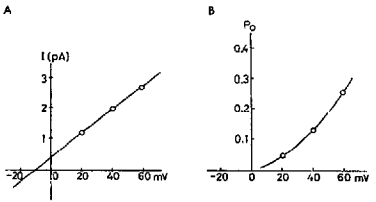

Fig. 9. Current-voltage relationship (A) and voltage dependency of the open probability (B) of the 4_2 channel, the trace of which is shown in Fig. 7. The reversal potential (V_{rev}) was −12.0 mV. The open probability at 0 mV will be almost zero, as seen from the trace in Fig. 7.

found to be cation selective (Table III). Gramicidin A, which forms a highly cation-selective channel, has no acidic amino acid residues. In addition, it has been

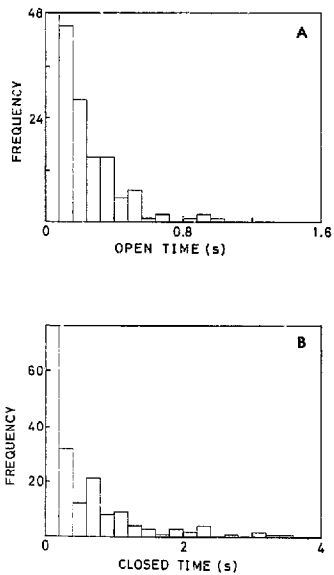

Fig. 10. Lifetime histogram of the channel corresponding to the current traces shown in Fig. 7 at a holding potential of +59 mV. (A) Open time histogram and (B) closed time histogram. The ordinate represents the number of events at each bin. The rate constants, k_1 and k_2 in Table II were obtained from the slopes of semi-logarithmic plots of these data.

TABLE II

Parameters of gating dynamics of the channel formed by 4_3 in asymmetric KCl concentrations

The corresponding current trace, amplitude histogram, and lifetime histogram are shown in Figs. 7, 8, and 10. The definitions of k_1, k_2, t_o, t_c, P_o and P_o' are as follows: k_1 and k_2 are the rate constants for channel closing and opening, and t_o and t_c are the mean open and closed times. The open probability was calculated by two different methods, P_o from the amplitude histogram (Fig. 8) and P_o' from the mean open and closed times (see text).

Potential (mV)	Current (pA)	k_1 (s^{-1})	k_2 (s^{-1})	t_o (ms)	t_c (ms)	P_o	P_o'
+59	2.7	5.71	1.93	175	518	0.254	0.253
+40	2.0	–	–	–	–	0.130	–
+20	1.2	–	–	–	–	0.049	–

TABLE III

Properties of 4_3 channels observed in asymmetric KCl solutions

The *cis* compartment contained 333 mM KCl and 0.67 or 3.3 mM Mops-Tris (pH 7.0). The *trans* compartment contained 100 mM KCl and 0.2 or 1.0 mM Mops-Tris (pH 7.0).

Conductance (pS)	Voltage dependency of P_o	V_{rev} (mV)	Ion selectivity ($P_K : P_{Cl}$)
680–750	no	−17 to −23	1:0.13–0.26
217	+ < −	−2	1:0.88
55	no	−24	1:0.11
39–40	+ > −	−12 to −17	1:0.26–0.40
25–38	no	–	$P_K > P_{Cl}$ [a]
1.7	+ > −	–	–

[a] This was determined by the direction of the current at zero membrane potential. V_{rev} was not obtained because many levels of conductance were present.

reported that a positively charged segment of the Na-channel polypeptide forms cation-selective channels [33]. Therefore, the acidity (or basicity) of the peptides may not necessarily be related to the ion selectivity of a channel formed from peptides. At present, we have no proper explanation for the cation selectivity of the channel. The nature of the bilayer lipids with an overall negative charge [21] could not explain the cation selectivity because the same ion selectivity was observed even when a neutral phospholipid, diphytanoyl phosphatidylcholine, was used (Fig. 6).

The oligomeric bundle of head-to-tail dimers of α-helices requires many peptide molecules (for example 12 molecules for a hexameric bundle) (Fig. 11). We ob-

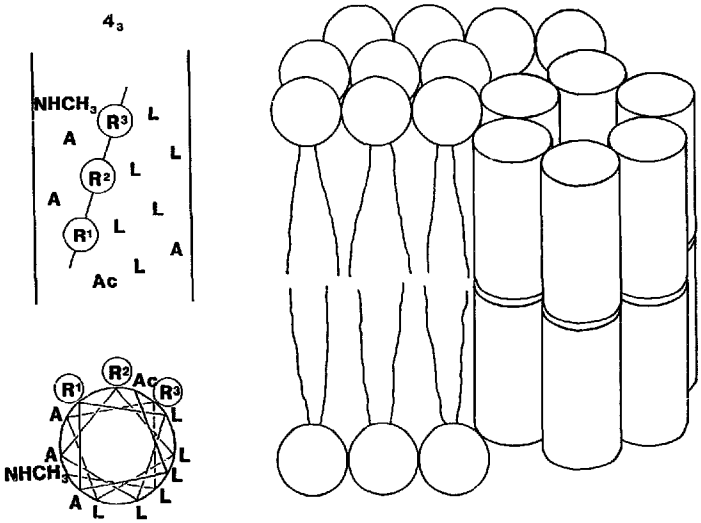

Fig. 11. A hypothetical structure of the channel formed by peptide 4_3. The channel is drawn as the aggregate of 6-dimeric peptides in a lipid bilayer. Shown on the left are the α-helical net and wheel of peptide 4_3. One-letter symbols of amino acids are used: A, alanine; L, leucine; R, arginine.

served a variety of channel types with respect to conductance and voltage dependency of gating (Table III), thus the structure of the channel formed with this peptide is not restricted to a certain conformation and/or assembly.

In general, the ion-channel proteins of biomembranes are large-molecular-weight polypeptides which are apparently composed of pore, ion-selective filter, and gate structures. It is interesting that some of the channels studied here showed a voltage dependency, despite the simple peptide structure (Fig. 9B, Table III). The mechanism of this voltage dependency and ion selectivity remains the subject of ongoing study.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research No. 63771918 (to K.A.) and Nos. 01460269 and 01659002 (to Y.K.) from the Ministry of Education, Science, and Culture, Japan. We are indebted to Dr. H. Kijima of Nagoya University and Dr. K. Kuba of Saga Medical College for the computer programs used in data analysis. We thank Naohide Hirashima for assistance with data analysis.

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