Interaction with Phospholipid Bilayers, Ion Channel Formation, and Antimicrobial Activity of Basic Amphipathic \( \alpha \)-Helical Model Peptides of Various Chain Lengths*

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Basic amphipathic \( \alpha \)-helical peptides Ac-(Leu-Ala-Arg-Leu)\(_{3-4}\)-NH\(_2\) (43 or 44) and H-(Leu-Ala-Arg-Leu)\(_{2-3}\)-(Leu-Arg-Ala-Leu)\(_{2-3}\)-OH (45 or 46) were synthesized and studied in terms of their interactions with phospholipid membranes, biological activity, and ion channel-forming ability. CD study of the peptides showed that they form \( \alpha \)-helical structures in the presence of phospholipid liposomes and thus they have amphipathic distribution of the side chains along the axis of the helix. A leakage study of carboxyfluorescein encapsulated in phospholipid vesicles indicated that the peptides possess a highly potent ability to perturb the membrane structure. Membrane current measurements using the planar lipid bilayer technique revealed that the peptide 44, which was long enough to span the lipid bilayer in the \( \alpha \)-helical structure, formed cation-selective ion channels at a concentration of 0.5 \( \mu \)M in a planar diphytanoylphosphatidylcholine bilayer. In contrast, other shorter peptides failed to form discrete and stable channels though they occasionally induced an increase in the membrane current with erratic conductance levels. The probability of detecting a conductance increase was in the order of 44 > 45 > 46 > 43, which corresponds to the order of the peptide chain lengths. Furthermore, 44 but not 46 showed an antimicrobial activity against both Gram-positive and -negative bacteria. The structure of ion channels formed by 44 and the relationship between the peptide chain length and biological activity of the synthetic peptides are discussed.

Ion channels have various important biological functions, e.g. transmission of information in the nervous system, excitation of muscle and secretory cells, toxic and antimicrobial activities, and others. Recently it has been reported (1–7) that amphipathic peptides can form ion channels in terms of self-aggregation with hydrophilic residues facing inward to form the channel wall and with hydrophobic side chains contacting the phospholipid molecules in the interior of the bilayers. We previously reported (8) that a basic model peptide 43 (Fig. 1) containing a repeat sequence of the tetrapeptide unit, Leu-Ala-Arg-Leu, could form an amphipathic \( \alpha \)-helical structure. It has one lateral hydrophilic side and a hydrophobic side (Fig. 2) in the presence of phospholipid liposomes. In addition, 44 was found to form voltage-gated and cation-selective ion channels in planar lipid bilayer membranes (9, 10). The amphipathic property and ion channel-forming activity of 44 were closely correlated to antimicrobial activity (10). The peptides appeared to assemble to form a transmembrane impermeable channel. An oligomeric bundle of head-to-tail dimers was considered as a channel model, because 44 is too short to span a lipid bilayer with one molecule. However, it formed a variety of channels with respect to conductance and voltage dependency of gating. The channels or assemblies formed are not uniform in terms of conformation. These results prompted us to study the relationship between the peptide chain length and channel-forming ability in lipid bilayers.

In the present study, the new peptides 45 and 46 were designed and synthesized, and 44, designed previously (8) was also used for channel-forming activity assay as its chain length was between 45 and 46 (Fig. 1). The numbers of amino acid residues in the peptides are 12, 16, 20, and 24 for 43, 44, 45, and 46, respectively. Peptides 43 and 44 have chain lengths capable of spanning a lipid bilayer barely or sufficiently, respectively (11). The C-terminal-elongated sequences of 43 and 45 have repeated structures of the unit Leu-Arg-Ala-Leu instead of the Leu-Ala-Arg-Leu in 44, which may allow formation of an \( \alpha \)-helical structure with amphipathic properties (Fig. 2). The highly basic amphipathic structure of 45 may resemble the S\(_2\) segment in sodium ion channel protein which has ion channel-forming activity (3).

In order to investigate the relationship between the peptide chain length and biological activity using these designed peptides, we examined their structures, interaction with lipid bilayers, ion channel-forming activity in the bilayers, and antimicrobial activity.

**EXPERIMENTAL PROCEDURES**

Materials—DPPC and DPPG were purchased from Sigma. DiphytPC was obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Carboxyfluorescein from Eastman Kodak Co. was purified by recrystallization from ethanol. All other reagents were of the highest analytical grade.

**Synthesis of the Peptides**—All the peptides were synthesized by the solution method. Peptides 43 and 46 were synthesized as reported previously (8). Synthetic routes for peptides 43 and 44 are shown in Fig. 3. Tetrapeptide \( \alpha \) was prepared stepwise from the C terminus by the active ester method. After the cleavage of the Boc or benzyl group, the fragments obtained were coupled by the BOC-HOBt method (12).

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1 The abbreviations used are: DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidyglycerol; DiphytPC, diphytanoylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinopropanesulfonic acid.
to give a C-terminal fragment 2 or 3. The N-terminal fragment 4 was similarly synthesized. Fragment 4 was coupled with 2 or 3 followed by removal of all protecting groups with anhydrous HF (13) in the presence of anisole as a scavenger to give the final product 45 or 46, respectively. Each free peptide obtained was passed through a Sephadex G-15 column (3 x 150 cm) with 30% AcOH, and the fractions containing the peptide were collected and lyophilized. The crude peptides were purified by HPLC on a YMC-Pack D-ODS-5 preparative column (25 x 200 mm) using the following solvent system: A: 2-propanol; B: 50 mM triethylammonium phosphate buffer (pH 2.5), followed by desalting with a Sephadex G-15 column (3 x 100 cm) with 30% AcOH. The purity of the peptides was confirmed by HPLC on a Hitachi ODS 3056 self-packed column (4.6 x 250 mm) using the solvent system described above. Amino acid analyses were performed on a Hitachi Model 835 amino acid analyzer and the results were as follows: 4s, Leu 1.90, Ala 1.00, Arg 0.93; 4e, Leu 1.93, Ala 1.00, Arg 0.94 in amino acid ratios.

Preparation of Phospholipid Liposomes—A mixture of uni- and multilamellar vesicles was prepared for CD measurement as follows. Phospholipid (20 mg) was dissolved in CHCl3 (2 ml) and dried by a stream of N2 gas. The dried lipid was hydrated in 3 ml of 5 mM Hepes buffer (pH 7.4) with repeated vortex mixing at 50 °C for 30 min. The suspension was sonicated at 50 °C for 1 h using a TOTMY SEIKO ultrasonic disiputer Model UR-200P and diluted to 25 ml with the same buffer (lipid concentration ~1 mM).

Unilamellar vesicles trapping carboxyfluorescein were prepared as described previously (14). The dried lipid was hydrated in 2.0 ml of 0.1 M NaCl/5 mM Hepes buffer (pH 7.4) containing 100 mM carboxyfluorescein. The mixture of uni- and multilamellar vesicles trapping carboxyfluorescein was gel-filtrated through a Sepharose 4B column (1 x 20 cm) in 0.1 M NaCl/5 mM Hepes buffer (pH 7.4). The separated small unilamellar vesicles were used in carboxyfluorescein leakage measurements.

CD Measurement—CD spectra were recorded on a JASCO J-600 spectropolarimeter using quartz cell of 1-mm pathlength. Peptide concentrations in solution were determined on the basis of the amino acid analysis data. Spectra in Hepes buffer (pH 7.4) were measured at a peptide concentration of 100 μM. Spectra in the presence of phospholipid liposomes were measured at a peptide concentration of 10 μM in 5 mM Hepes buffer (pH 7.4) in the presence of 0.9 mM vesicles. All measurements were carried out at 25 °C. The CD data were expressed as mean residue ellipticities. The peptide conformations were evaluated by the three structures of α-helix, β-structure, and random coil according to the Greenfield and Fasman method (13).

Carboxyfluorescein Leakage—The dye-leakage experiment was carried out according to the procedure of Weinstein et al. (16) with a minor modification (14). To 2 ml of 0.1 M NaCl/5 mM Hepes buffer (pH 7.4) in a cuvette was added 50 μl of vesicles containing 100 mM carboxyfluorescein to give a final concentration of 20 μM lipid. The cuvette was placed in a heated (25 °C) cuvette holder in the fluorometer for 5 min until the temperature was equilibrated. Then, to the mixture was added 20 μl of an appropriate dilution of the peptides in Hepes buffer. Fluorescence spectra were recorded on a JASCO FP-550A spectrofluorophotometer at 22 °C. The fluorescence intensity data were collected 5 min after the addition of the peptides. Carboxyfluorescein was excited at 470 nm, and emission at 540 nm was monitored with a Toshiba V9-50 filter which cuts off below a wavelength of 505 nm. To determine the fluorescence intensity for 100% dye release, 10 μl of Triton X-100 (20% in Hepes buffer) was added to dissolve the vesicles. The percentage of dye release caused by the peptides was evaluated by the equation, 100 X (F - Fo)/(Fmax - Fo), where F is the fluorescence intensity achieved by the peptides, Fmax and Fo are the intensities observed without the peptides and after Triton X-100 treatment, respectively.

Antimicrobial Assay—The antimicrobial assay was carried out by the serial dilution method in solution (17).

Formation of Planar Bilayer and Measurement of Membrane Current—The planar lipid bilayers were prepared by the folding method as described previously (10). Peptides were dissolved in methanol, and an aliquot was added to one side (cis side) of a preformed bilayer with stirring. A membrane potential was applied and the current was measured using Ag/AgCl electrodes which were connected to both compartments through saturated KCl and agar bridges. Membrane potential (usually ~60 mV, the potential of the trans compartment being defined as zero) was held until current fluctuation was observed. The current passing through the membrane was transduced into an analog voltage signal by an in-house current-to-voltage converter (DS5502A, Kikusui Electronics, Kawasaki) and a chart recorder (R61VL, Rikadenki, Tokyo). The data were recorded on videotape with a videotape recorder after A/D conversion with a modified digital audio processor (PCM-501ES, Sony) and analyzed off-line by a personal computer (8039801VM, NEC, Tokyo, Japan).

RESULTS

CD Study—CD spectra of peptides 4s, 4e, and 4 in Hepes buffer (pH 7.4) and in the presence of liposomes are shown in Fig. 4. In the buffer, peptides 4s and 4e exhibited a weak
band at 218–222 nm representative of an α-helix and a negative band near 205 nm representative of a random coil and the helix. On the other hand, 4c showed strong negative bands at 208 and 222 nm characteristic of an α-helix. A chain length of 4e allowed the peptide to form a more stable α-helical structure in the buffer, which may cause a more preferential self-association of 4e through the interactions between the hydrophobic regions of the amphipathic α-helical structure, when compared with 4f and 4g.

In the presence of acidic liposomes (DPPC–DPPG, 3:1), all the peptides exhibited strong negative bands at 208 and 222 nm. Addition of liposomes induced the increase in the α-helical content of 4f and 4g. However, the CD spectra of 4f in Hepes buffer and in the presence of DPPC–DPPG (3:1) liposomes were nearly identical. The CD spectra of 4f and 4g in DiphytPC also were the same as those in acidic liposomes (data not shown). The helical contents of 4f and 4g analyzed by the CD spectra according to Greenfield and Fasman (15) were about 60% in the presence of DPPC–DPPG (3:1) and DiphytPC liposomes.

Carboxyfluorescein Leakage Measurement—The effects of the peptides on membrane permeability were investigated on the basis of release of carboxyfluorescein encapsulated in phospholipid vesicles. Profiles of the dye leakage from DPPC–DPPG (3:1) vesicles by the action of peptides 4f, 4g, and 4h are shown in Fig. 5. All peptides caused an almost complete release of vesosomal contents at 1.5 μM. The leakage induced by 4f was nearly the same as that by 4g. In contrast, 4h showed a distinctive efflux of entrapped carboxyfluorescein in the low concentration range when compared with 4f and 4g. Peptide 4f had the same leakage ability as 4g as previously reported (8). These results indicated that all the peptides forming the amphipathic α-helical structure in the presence of the phospholipid bilayer perturb the model membrane. Above all, the longest peptide 4h showed the highest ability to perturb the membrane.

Antimicrobial Activity—The results of the antimicrobial assay of the peptides are listed in Table I in terms of minimum inhibitory concentration of growth (MIC). Compared with the activity of the mother peptide 4f, activities of peptides 4g and 4h against the Gram-positive bacteria, Staphylococcus aureus and Bacillus subtilis were negligible and low, respectively. It is noteworthy that 4e with 24 amino acid residues revealed an antimicrobial activity against Gram-negative bacteria, Escherichia coli and Shigella sonnei although relatively weak, while 4f and 4g were inactive against them. Peptide 4h, showing antimicrobial activity against both types of bacteria, may act on the bacterial membrane with a different mode from that of 4f.

Formation of Ion Channels—The solvent-free planar bilayer method, originally described by Takagi et al. (19) and developed by Montal and Müller (20), was applied, to determine whether the model peptides formed ion channels in a phospholipid bilayer membrane. Previously, we found that peptide 4h had formed ion channels in soybean phospholipid (asolectin) or DiphytPC planar bilayers (10). In the present study, we used a DiphytPC planar bilayer, which is neutral in net charge and known to form stable bilayer membranes (21). Asolcetin was found to be inappropriate for the present study, since peptide 4h disrupted its bilayer. The conductance of the planar lipid bilayer before the addition of the peptides was less than 3 picoeimens, indicating that the lipid bilayer greatly impeded the diffusion of ions between aqueous compartments. The addition of the peptides to the cis compartment caused an increase in the membrane conductance (Fig. 6). Two modes of the conductance increase were observed. Type 1, a discrete conductance level, is characteristic of ion channels (upper trace in Fig. 6). Type 2, with an erratic conductance level (lower trace in Fig. 6), may represent a different interaction mode of the peptide with the membrane rather than channel formation, although the possibility of ion channel formation with very low conductance and gating too rapid to be resolved by our methods cannot be ruled out. The
membrane-perturbing ability of these peptides was compared quantitatively in terms of the probability of detecting membrane conductance increase involving both types 1 and 2. As an index of the ability, we used the ratio of the number of runs in which membrane conductance increase was observed within 30 min after the addition of each peptide, to the total number of 10 runs, under the same experimental conditions. The probability of detecting a conductance increase was in the order of \(4_n > 4_1 > 4_4 > 4_4\), with values of 7/10, 6/10, 5/10, and 3/10, for 4n, 4c, 4h, and 4s, respectively. This order is the same as that of the peptide chain lengths. The values of type 1 interaction (channel formation) are 4/10, 0, 0, and 0, for 4n, 4c, 4h, and 4s. At this concentration (0.5 \(\mu\)M), only peptide 4n formed ion channels in the DiphycPC bilayers.

Fig. 7 shows a current trace of multiple ion channels formed by 4n in asymmetric KCl solutions (cis, 194 mM; trans, 100 mM). Various conductance levels and steps (100–1200 picosiemens) were observed, indicating that 4n forms various channel structures. The gating of the channel was voltage-independent. The direction of the current under no membrane potential (0 mV) was from cis to trans, indicating that the channel is cation-selective. Although a precise value for the reversal potential was not obtained because of the presence of various conductance levels, the reversal potential was around –10 mV, which leads to a permeability ratio \(P_K/P_C\) of about 1:0.25.

**DISCUSSION**

The study of the relationship of the chain length and channel-forming ability of amphipathic \(\alpha\)-helical peptides is important regarding the probable conformation of putative intramembrane segments of channel-forming intrinsic membrane proteins (3, 4). In general, it has been estimated that a chain length of at least 20 amino residues in an \(\alpha\)-helical structure is required to span a lipid bilayer (11, 22, 23). In the present investigation, the CD measurement showed that all the peptides studied, regardless of chain lengths, formed \(\alpha\)-helical structures of almost the same helical content in the presence of neutral and acidic liposomes. Therefore, we compared their channel-forming abilities. The current measurement on a planar DiphycPC bilayer showed that 4n formed stable ion channels at a concentration of 0.5 \(\mu\)M. Peptides 4n, 4c, 4h, and 4s, despite having shorter chain lengths than 4n, interacted with the planar bilayer, but failed to form stable ion channels. However, the current increase with an erratic conductance level was observed occasionally for all peptides. The frequency of observation of a membrane conductance increase was in the order 4n, 4c, 4h, 4s, suggesting that the increase in the peptide chain length is correlated with that in the ion permeability of the phospholipid bilayer induced by the peptide. The increase in the permeability by the peptides coincides with the increase of the ability to leak carboxyfluorescein encapsulated in liposomes. It should be noted that only 4n formed stable ion channels in the DiphycPC bilayer membrane at the concentration examined (0.5 \(\mu\)M), indicating that a chain length sufficient to span the lipid bilayer is a requisite for the formation of stable ion channels.

We reported that 4n formed ion channels in the DiphycPC bilayer at a peptide concentration of 4 \(\mu\)M, and that the channel structure might consist of oligomeric bundles of head-to-tail dimers of \(\alpha\)-helices (10). In the present study, 4d did not form an ion channel in the planar DiphycPC bilayer at a concentration of 0.5 \(\mu\)M at which 4n did. Apparently a higher concentration is required for 4d to form oligomeric bundles of head-to-tail dimers.

Step changes in the membrane conductance of varying sizes were observed by the incorporation of 4n into the planar DiphycPC bilayer. Some of the conductance levels were integral multiples of smaller ones. However, it was found that all the conductance levels recorded could not be attributed to integral multiples of the lower levels. These different sizes in channel conductances suggest that the pores formed in the bilayer contain several types of bundles with different numbers of monomers, as observed in the channels of alamethicin (6).

We propose that the transmembrane ion channels formed by 4n are composed of bundles of \(\alpha\)-helices which assemble at the lateral sides and have an ion-conductive pore at the center. Peptide 4n has many positively charged residues, which remain on one lateral side of the \(\alpha\)-helix (see Fig. 2). The hydrophilic side of the helix bearing arginine residues faces the channel pore. Anion selectivity, therefore, is to be expected from this structural feature. The present study revealed, however, that the ion channels formed by 4n were inclined to conduct cations selectively as observed for 4t (10).

Generally, the ion channels formed by basic peptides display anion-selective properties as reported for magainin (2), melittin (24), cecropin (5), defensin (25) and so on. The channels formed by neutral and acidic peptides like gramici-
din A (26), alamethicin (6), δ-toxin (7) and the S₃ segment of sodium-channel protein (5) were reported to have cation selectivity. However, it was recently demonstrated that basic peptides such as the S₃ segment of sodium channel protein (3) the mast cell-degranulating peptide (27) could also form cation-selective ion channels. Particularly, the S₃ segment with 22 amino acid residues, in which there are 7 basic residues each of which are at every third position, possesses a highly amphipathic property, if the 3₁₀-helical structure is assumed. Peptide 4, with 6 basic amino acid residues has also an amphipathic α-helical structure in the lipid bilayer. These findings indicate that an amphipathic structure with repeating units of basic residues can form cation-selective ion channels.

The peptide series studied here are all assumed an amphipathic structure in the presence of lipid bilayer vesicles. Among these peptides, 4 exhibited weak antimicrobial activity against Gram-positive and -negative bacteria, whereas 4 was inactive, suggesting that a chain length capable of spanning the lipid bilayers is effective for antimicrobial activity. Naturally occurring peptides with more than 20 residues such as cecropin (28) and magainin (29), which can form amphipathic α-helical structures, have high antimicrobial activities against Gram-positive and -negative bacteria. These natural peptides can also form ion channels (2, 5). It is probable that 4 acts on the membrane of the Gram-negative and -positive bacteria and forms ion channels, thereby showing antimicrobial activity, whereas 4 and 4, show highly potent activity only against Gram-positive bacteria (8). We cannot offer a good explanation as to why these peptides are active only against Gram-positive bacteria. However, it is understood that the membrane structures of Gram-positive and -negative bacteria are quite different. The protecting groups of N-terminal and C-terminal methylamid in 4 and 4, may have an important role in their interaction with the membrane of Gram-positive bacteria.

Recently, the channel-forming property has been investigated using many kinds of bioactive peptides such as antibiotics and neurotoxins (27, 30). The amphipathic α-helical peptide 4, which has a chain length sufficient to span a lipid bilayer with transmembrane bundles of α-helices, showed the channel-forming and antimicrobial activities. The present study on the channel formed by model peptides may be helpful in the investigation of natural channels in biomembranes.

Acknowledgments—We are deeply grateful to Prof. H. Nishikawa, Fukuoka University, for the CD measurement and Prof. K. Amako and Dr. N. G. Park, Kyushu University, for the antimicrobial assay.

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