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Side Chain Effect on Ion Channel Characters of Aib Rich Peptides

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As models of ion channel proteins and naturally occurring pore-forming peptides, we designed a series of Aib rich peptides [Ac-(Aib-Xxx-Aib-Ala)$_5$-NH$_2$ (Xxx = Lys, Glu, Ser, and Gly: BXBA-20)] to investigate the effects of the side chains of the amino acid residues Lys, Glu, Ser, and Gly on the conformation and electrophysiological properties of ion channels. The conformation of peptides and their affinity for phospholipid membranes were evaluated by CD spectroscopy. Patch-clamp experiments revealed that all BXBA-20 peptides form ion channels in DPhPC bilayers exhibiting clearly resolved transitions between the open and closed states. The channel forming frequency was in the order BKBA-20>BEBA-20>BSBA-20>BGBA-20. In the case of BKBA-20 and BEBA-20, the self-assembled conductive oligomers expressed homogeneous and voltage-independent single channel conductances. In contrast, heterogeneous conductance was observed in BSBA-20 and BGBA-20 ion channels under similar experimental conditions. From these results, we conclude that peptides with a high degree of helical conformation, high amphipathicity, high affinity for lipid membranes, and self-associating characters in vesicles are most suitable for inducing ion channels with a high frequency of occurrence. Moreover, BEBA-20, BSBA-20, and BGBA-20 channels were cation-selective, whereas the BKBA-20 channel was non-selective.

Key words: Aib, channel-forming peptide, frequency of channel formation, ion selectivity.

In ion channel proteins, the structure of the pore region is a fundamental determinant of the ion channel character. For example, the pore region of the acetylcholine receptor channel has several charged and polar residues such as Ser, Glu, and Thr. These residues are assumed to form a ring in the inner helical bundle, which could be a determinant factor for the conductance and selectivity of the channel (*1, 2*). Recently, the core structure of the KcsA K^+ channel (a K^+ channel from *Streptomyces lividas*) was determined by Mackinnon *et al.* They indicated that the main chain carbonyl oxygen atoms of Gly and Tyr in the loop region of the channel, not side chains, are responsible for the potassium selectivity (*3*). On the other hand, comparable structure–function relationships and biophysical properties of ion channel proteins are also encountered in the case of ion channel–forming peptides, such as alamethicin, gramicidin A, δ-toxin, and melittin (for reviews, see Refs. *4–6*). Alamethicin represents a group of peptides with a high content of Aib residues (peptaibols) and contains a Glu residue in its sequence. The presence of Aib residues give stability to the largely α-helical conformation of alamethicin in phospholipid vesicles, and the charged Glu residues may be responsible for the voltage dependence of the pore (*7*).

Information about the function of individual residues in ion channel–forming proteins/peptides is required for understanding the electrophysiological properties of ion channel–forming proteins/peptides. However, the complex conformation of proteins and, to a lesser extent, peptides does not allow a straightforward determination of the contribution of individual amino acid side chains or other necessary structural features to protein function. Several designed channel-forming peptides containing polar/charged residues [*e.g.*, Ser (*8, 9*), Arg (*10*), and Glu (*11*)] have already been reported. However, these studies have used different experimental systems and/or different templates, which makes it difficult to compare the results directly.

In this study, in order to investigate the effects of individual amino acid residues on ion channel characteristics, we have designed and synthesized a series of channel-forming peptides and characterized their ion conductance behaviors. The peptides are simple in amino acid sequence and

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[2] We also measured the CD spectra in the presence of EggPC SUVs instead of DPhPC SUVs; however, smaller different spectra for BXBA-20 were observed under both conditions (unpublished data). These results indicate that the affinity of the peptides for membranes (Fig. 3) is not a special case for DPhPC bilayers.

Abbreviations: Aib (B), 2-aminoisobutyric acid; EggPC, egg yolk L-α-phosphatidylcholine; DPhPC, diphytanoylphosphatidylcholine; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; Rink Amide resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin; RP-HPLC, reversed phase high performance liquid chromatography; SUVs, small unilamellar vesicles; TFE, 2,2,2-trifluoroethanol.

BKBA-20: Ac-(BKBA)$_5$-CONH$_2$

BEBA-20: Ac-(BEBA)$_5$-CONH$_2$

BSBA-20: Ac-(BSBA)$_5$-CONH$_2$

BGBA-20: Ac-(BGBA)$_5$-CONH$_2$

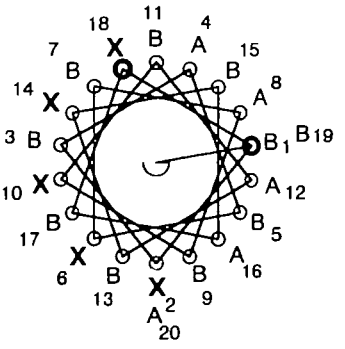

Fig. 1. **The amino acid sequences and the helical-wheel diagram of BXBA-20.** The diagram is shown as a complete α-helix with the view from the N-terminal towards the C-terminal.

contain repetitive units: Ac-(Aib-Xxx-Aib-Ala)$_5$-CONH$_2$, Xxx = Glu, Ser, Gly, and Lys (the BXBA-20 series). We have already used the Lys-containing peptide in a related study (*12*) (Fig. 1). CD and patch clamp methodologies were used to evaluate the conformation and ion channel behavior of the peptides, respectively. All BXBA-20 peptides form ion channels in DPhPC bilayers exhibiting clearly resolved transitions between the open and closed states. On the basis of structure–function relationships in this series of peptides, we have concluded that peptides with a high degree of helical conformation, high amphipathicity, high affinity for lipid membranes, and self-associating character in vesicles are most suitable for inducing ion channels with a high frequency of occurrence. Lys, Glu, Ser, and Gly residues modulate this frequency by changing the peptide conformation and affinity for lipid membranes. Moreover, Glu and Ser residues act as cation selective filters. The Gly-containing peptide showed modest cation selectivity. In contrast, the Lys-containing peptide did not induce anion selectivity and was non-selective.

EXPERIMENTAL PROCEDURES

Materials—*N*-α-Fmoc–protected amino acids, HOBt, HBTU, and Rink Amide resin were obtained from Nova Biochem (Tokyo). DMF and NMP were obtained from Wako Chemicals (Osaka). DPhPC was obtained from Avanti Polar Lipids (Alabaster, AL) as a 50 mg/ml chloroform solution. All other chemicals were obtained from Wako Chemicals, and were of special grade.

Peptide Synthesis—Peptide synthesis was performed as previously reported in detail (*12*). Briefly, the synthesis was carried out manually by a stepwise solid-phase method involving Fmoc-chemisty on Rink Amide resin. The crude products were purified by RP-HPLC and gel-filtration on Bio-Gel P-2. The homogeneities of the purified peptides were confirmed by analytical RP-HPLC and ESI-MS and/or MALDI-TOF MS.

CD Measurement—CD spectra were recorded on a JASCO J-720 spectropolarimeter with cylindrical cells of 0.1–0.01 cm pathlength at room temperature. Eight scans were averaged for each sample, and averaged blank spectra were subtracted. Due to the insolubility of the peptides in water, aqueous solutions, except for BKBA-20, contained EtOH (<1.0%). Peptide concentrations were determined by quantitative amino acid analysis. All equipment was siliconized to prevent the nonspecific adsorption of hydrophobic peptides. The secondary structure contents of BXBA-20 peptides were evaluated by a program using the self-consistent method (*13*).

Preparation of SUVs—SUVs were prepared for CD measurement as follows. Phospholipids were dissolved in $CHCl_3$ and then dried under a stream of nitrogen gas. The lipids were subjected to reduced pressure overnight, and then the resuspended in the appropriate buffer by vortex mixing at 50°C for 10 min. The suspensions were sonicated at 50°C for 10–20 min until a clear solution was obtained, then diluted with the same buffer. The lipid concentration was determined by phosphorus analysis (*14*).

Single-Channel Measurements—Patch-clamp experiments were performed using a pipette-dipping technique, as generally described (*12, 15*). The electrolyte solution was 0.5 M KCl buffered with 5 mM HEPES, pH 7.4. The electrolyte composition was symmetrical for both sides of the DPhPC membrane. Peptide concentrations, when added to patch pipette on the *cis* side, were 100 nM. The ratio by volume of EtOH in solution was as low as 0.05%. The chloroform solutions of DPhPC lipid were mildly evaporated by N_2 flushing. Then the residual lipid was redissolved in hexane (2 mg/ml) before being spread on an aqueous surface. DPhPC hexanic solution was added to the aqueous surface of electrolyte solutions (about 2 μl) in plastic dishes, 3.5 cm in diameter.

The current was measured and voltage was set using an Axopatch 1D patch-clamp amplifier (Axon Instruments). The pipette to which peptide was added is referred to as the *cis* side, and all electrical potentials refer to this side. The other (*trans*) side was electrically grounded through the head-stage. Data were filtered at 4–2 kHz sampled at 41.67 kHz, stored directly on a disk, and analyzed using the program pClamp 6 (Axon Instruments). Patch pipettes were of borosilicate hard-glass type, and pulled through a two-pull method by a pipette-puller (Narishige, Tokyo) to give approximate diameters of 1 μm.

RESULTS

Peptides Design—An interesting approach to evaluating the effects of certain amino acid residues on the structure–function relationships of ion channel–forming peptides involves the host-guest studies. In this approach, functional residues (guests) are introduced into a template (host). However, there have been only a few systematic studies using this method. A reason for this is that extreme changes in the physical character (*e.g.*, conformation and solubility) of the peptide can occur by substitution in a template. For example, in the case of Ac-(Ala-Xxx-Ala-Ala)$_5$-CONH$_2$, Xxx = Lys assumed a random structure in aque-

ous buffers, whereas peptides with substituted Glu and Ser residues formed insoluble aggregates under the same conditions (unpublished data).

We have previously reported that the Aib-containing peptide, Ac-(Aib-Lys-Aib-Ala)$_5$-CONH$_2$, forms a water-soluble helical structure and possesses a high ability to form stable ion channels (*12*). Ac-(Aib-Xxx-Aib-Ala)$_5$-NH$_2$, therefore, was chosen as the template for designing the series of ion channel-forming peptides in this study. By considering the putative functional residues in many naturally occurring ion channel structures (in addition to Lys), Glu, Ser, and Gly were chosen as the guest amino acid residues (Xxx) (Fig. 1). The simple structure of the template facilitates the conformational interpretation of the ion channel forming properties of these peptides.

As shown in the helical wheel projection (Fig. 1), BXBA-20 peptides have an amphipathic character with the hydrophobic region on one side and the hydrophilic region on the opposite side of the peptide helix. According to the quantitative estimation of the amphipathicity of α-helices (*16*), the amphipathicity of BXBA-20 peptides increases with the rising hydrophilicity of the Xxx residues in the following order: BKBA-20>BEBA-20>BSBA-20>BGBA-20.

CD Conformational Studies—The conformations of BXBA-20 peptides in aqueous buffer, TFE, and in the presence of DPhPC liposomes were estimated on the basis of their CD spectra (Fig. 2), and their helical contents were analyzed (Table I). In aqueous buffer, BKBA-20, BEBA-20, and BSBA-20 exhibited double negative bands near 208 and 225 nm, a characteristic of an α-helix conformation (Fig. 2a). On the other hand, BGBA-20 had a negative band at 204 nm, which implies a non-helical structure. The spectra of peptides in the helix-promoting solvent TFE (Fig. 2b) demonstrate that the α-helical conformation was induced in all of the peptides (Fig. 2a). The similarity of the spectra of BKBA-20 and BEBA-20 indicate that these two peptides may possess a comparably high propensity for the formation of helical structures. The ability of BSBA-20 to form helices was lower, and that of BGBA-20 was lower compared with the other three peptides.

Interaction with Lipid Membranes—Concerning the interaction with DPhPC SUVs, clear differences were observed in the peptide series. Figure 3 exhibits the effects of increasing amounts of vesicles on the CD signal $\theta_{\pi\text{-}\pi^*}$ (222–225 nm) of the peptides. The addition of DPhPC vesicles to the peptide solutions caused peptide binding and an increase in the helix content in the case of BKBA-20 and BSBA-20. The concentration of lipid required to achieve the maximum ellipticity was lower for BKBA-20 compared to BSBA-20. In contrast, the spectra for BEBA-20 and BGBA-20 showed little difference compared with the spectra in buffer, suggesting that the binding of the two peptides are weak under these experimental conditions. The affinity of peptides for lipid membranes, therefore, is approximately in the following order: BKBA-20>BSBA-20>BEBA-20–BGBA-20.[2] The spectra of BKBA-20 and BSBA-20 shown in Fig. 2c indicate their membrane-bound conformations, whereas the other two peptides do not show membrane-

TABLE I. **α-Helical contents (%) of peptides under various conditions.**

Environment/Peptide	BKBA-20	BEBA-20	BSBA-20	BGBA-20
Buffer	37	36	21	15
TFE	61	61	49	34
DPhPC SUVs	79	N.D.*	65	N.D.*

Calculations were based on the self-consistent program by Sreerama and Woody (*13*). The helicities of BEBA-20 and BGBA-20 in the membrane-bound state could not be obtained directly because of their weak affinity for lipid membranes under our experimental conditions (see Fig. 3). *Not determined.

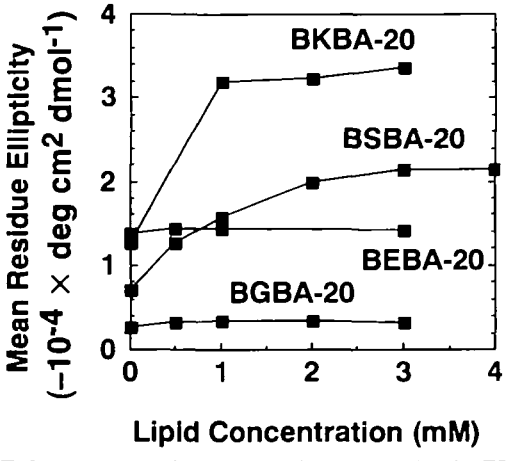

Fig. 3. **Enhancement of mean residue ellipticity in BXBA-20 peptides.** The θ_{225} values of 10 μM peptide solutions (0.5 mM KCl, 5 mM HEPES buffer, pH 7.4) in the presence of DPhPC SUVs at room temperature are plotted as a function of lipid concentration.

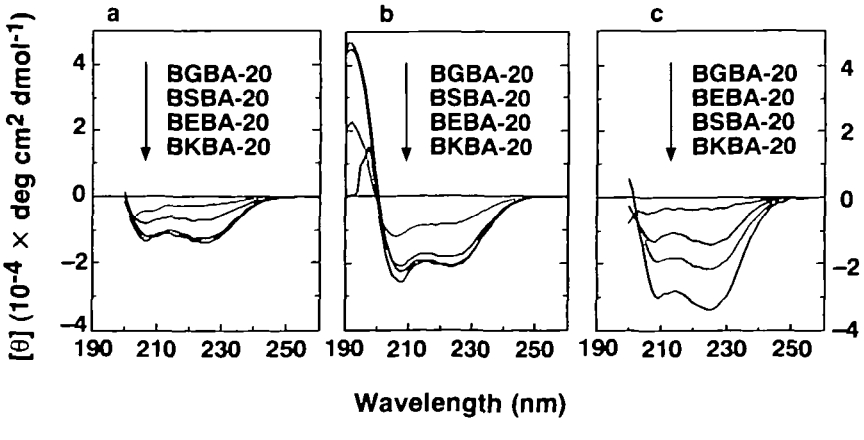

Fig. 2. **CD spectra of BXBA-20 in (a) 0.5 M KCl buffered with 5 mM HEPES (pH 7.4), (b) TFE, and (c) in the presence of DPhPC SUVs [0.5 M KCl buffered with 5 mM HEPES (pH 7.4)].** All peptide concentrations were 10 μM, and the lipid concentrations were 4 mM for BSBA-20 and 3 mM for the other three peptides. All measurements were performed at room temperature.

bound or membrane-incorporated conformations.

The vesicles induced spectral changes not only in the intensity of the $\theta_{n\text{-}\pi^*}$ band, but also in the ratio $\theta_{n\text{-}\pi^*}/\theta_{\pi\text{-}\pi^*}$ (= $\theta_{225}/\theta_{208}$). This ratio was enhanced, compared with the ellipticity ratios in buffer and TFE environments, for both BKBA-20 and BSBA-20. The $\theta_{n\text{-}\pi^*}/\theta_{\pi\text{-}\pi^*}$ values are 1.13 for BKBA-20 and 1.10 for BSBA-20. These values may serve as diagnostics for interhelical interactions (*17*).

Ion Channel Formation—Single-channel currents induced by BXBA-20 peptides were recorded in symmetric 0.5 M KCl solution using a patch-clamp methodology. All BXBA-20 peptides form discrete ion channels in DPhPC bilayers and clearly exhibit resolved transitions between the open and closed states, which make it possible to study the structure–function relationship of the channels. Representative discrete traces for each peptide are shown in Fig. 4.

The channel behavior of BKBA-20 has been described previously (*12*). This peptide forms definite single-state channels at low membrane potentials. However, the same peptides exhibit multi-state channels at potentials above ±100 mV. The most common conductance for BKBA-20 is voltage-independent and the slope of the *I–V* curve is 227 pS (Fig. 6a). On the other hand, BEBA-20 exhibits multi-level opening even when the potential is at –30 mV (Figs. 4b and 5). The number of conductance levels increased with increase in the membrane voltage as shown in Fig. 5. The conductance at the lowest level shows ohmic behavior in the range of a –200 to +200 mV, with a slope of 1,620 pS (Fig. 6b). Other peptides, BSBA-20, and BGBA-20, mainly form single-state openings at potentials <100 mV (Fig. 4). However, heterogeneous conductance patterns were observed for these peptides at each recording, even under the

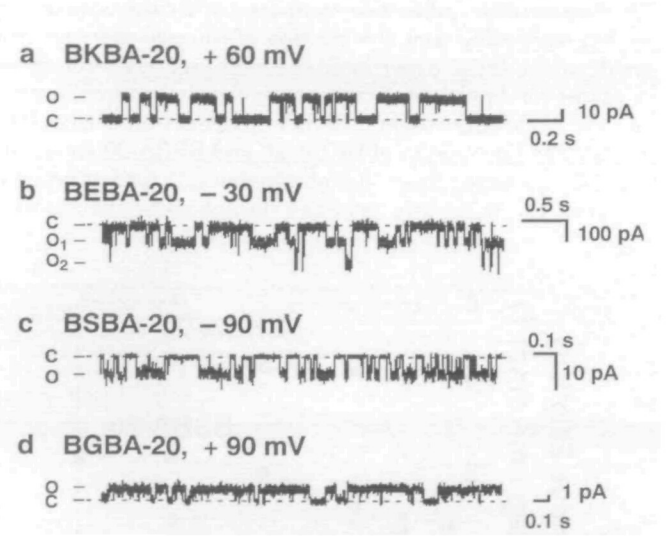

Fig. 4. **Representative single-channel current traces induced by BXBA-20.** (a) BKBA-20: V = + 60 mV, (b) BEBA-20: V = –30 mV, (c) BSBA-20: V = –90 mV, (d) BGBA-20: V = + 90 mV. Peptides (100 nM) were added to the *cis*-side of the DPhPC bilayer, separating symmetrical 0.5 mM KCl (buffered with 5 mM HEPES at pH 7.4) on both sides. The dashed lines indicate the zero current level. The states are indicated (C, O_1, and O_2) as determined from the current amplitude histogram (data not shown).

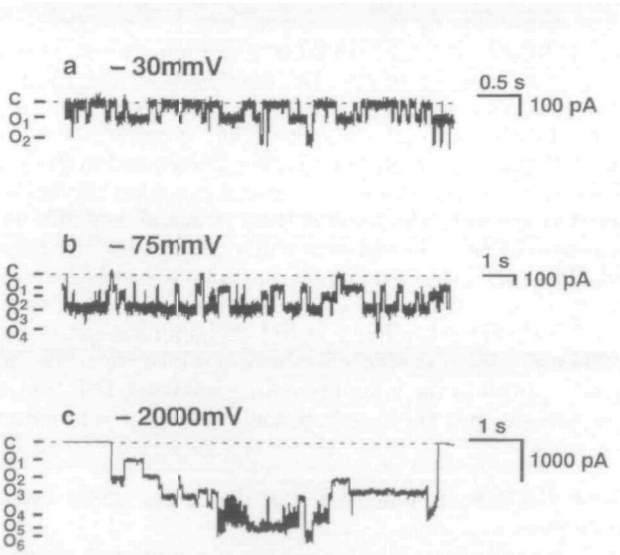

Fig. 5. **Voltage-dependent behavior of BEBA-20 in single-channel experiments at three different applied voltages.** (a) –30 mV, (b) –75 mV, and (c) –200 mV. Conditions were same as described for Fig. 4.

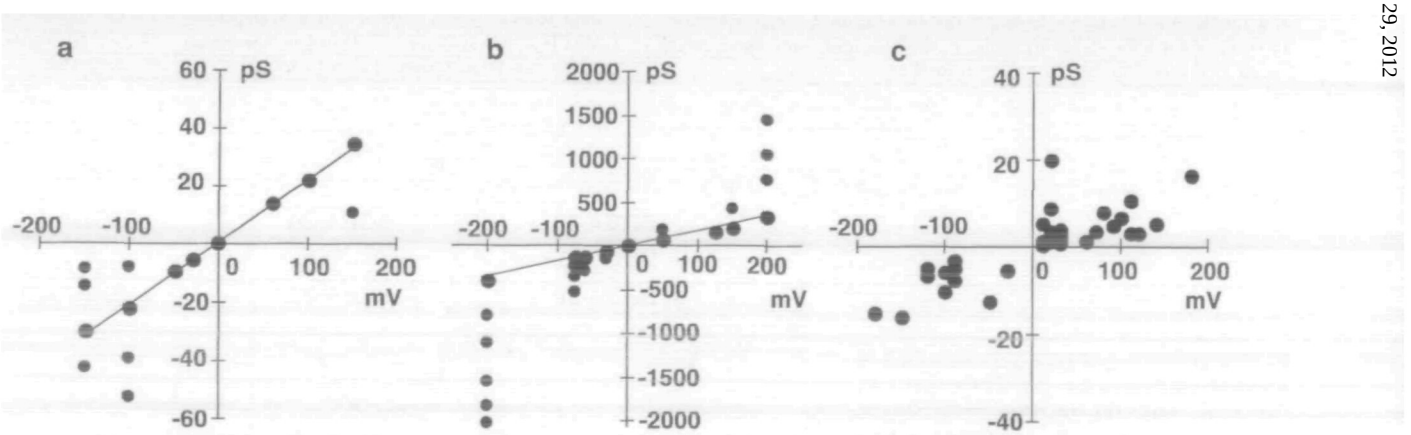

Fig. 6. **The current–voltage relationships of the single-channel trace for (a) BKBA-20, (b) BEBA-20, and (c) BSBA-20.** Each point represents an observation of a single open channel, and current amplitudes were determined from amplitude histograms by the program pClamp 6. The plots include data from more than 10 different experiments. Conditions were the same as described for Fig. 4. The linear lines in Fig. 6a and 6b were fitted by a least-squares program (KaleidaGraph, Synergy Software, Reading, PA) for the main and well reproductive conductance states.

same conditions. For example, as shown in Fig. 7, transitions from one open single-state to another single-state conductance was successively observed during the same recording at a fixed membrane potential.

Frequency of Channel Formation—All of the peptides formed discrete channels as mentioned above. However, except for BKBA-20, erratic and extremely short-lived conductance patterns were also observed under comparable experimental conditions (Fig. 8). To describe the heterogeneity of the frequency (probability) of detecting discrete channels and erratic currents, we expressed the frequency as a ratio of [the number of discrete ion channels observed/the number of erratic current events observed/the number of runs] as shown in Table II. For example, the 5/10/21 for BEBA-20 means that in 21 trials in which both discrete ion channels and erratic current events occurred, the events were observed 5 and 10 times, respectively, within 5 min after the formation of the bilayer. This clearly suggests that BKBA-20 has superior ion channel forming ability, where, BGBA-20 produces mostly unstable erratic ionic currents.

Ion Selectivity of the Discrete Channels—The cation/anion selectivity of BXBA-20 channels was determined by measuring the reversal potential (V_{rev}) in the presence of asymmetric KCl concentrations across the bilayer (0.5 M KCl on the *cis*-side and 0.1 M KCl on the *trans*-side). The application of the Goldman-Hodgkin-Katz equation (*18*) yields to the permeability ratios (Table II). The reversal potential was not observed for BKBA-20 (V_{rev} = 0), which means there is no selectivity between K^+ and Cl^- in this ion channel. Because of the presence of various conductance and short-lived channel events, the V_{rev} of BGBA-20 could not be determined accurately. However, the range of V_{rev} values of BGBA-20 (from −10 to 0 mV) indicated weak anion selectivity (P_K:P_{Cl}<2:1). The Ser- and Glu-containing peptides (BSBA-20 and BEBA-20) showed a preference for cations over anions, as expected from the negative polarity of these side chains.

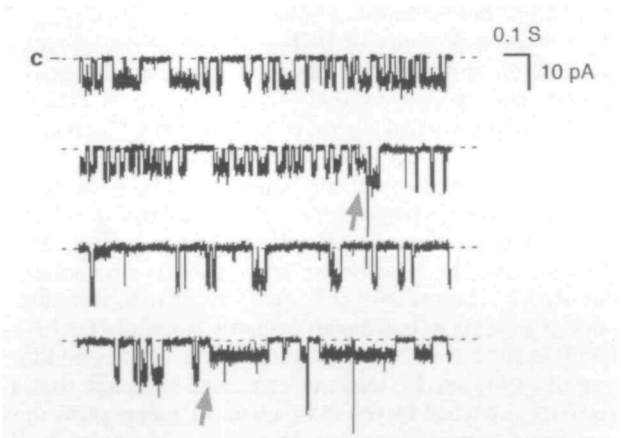

Fig. 7. **Typical conductance transitions during one recording induced by BSBA-20 at a fixed membrane voltage.** Conditions were as for Fig. 4, except that the voltage was −90 mV. Changes in conducting level transitions are indicated by arrows.

DISCUSSION

As described below, BXBA-20 has most of the properties envisioned, such as peptide solubility in water, formation of discrete ion channels in bilayers, and ion selective properties. Our overall success with BXBA-20 enables us to study the relationship between the structure and function of individual residues in ion channel-forming proteins/peptides on one template.

Channel Structure Model—According to the barrel-stave model (*4, 19, 20*), an increase or decrease in the number of incorporated peptides in a pore structure will determine the change in the conduction levels. The number of peptides that participate in a bundle, the stability of the pore, and its ion selectivity would be expected to depend on the individual peptide conformation, affinity for membranes, and peptide–peptide interactions. These factors can explain the ion channel activity of alamethicin (*4, 19, 21*). This model is also applicable for explaining the channel properties of BXBA-20 peptides of this study.

In the case of BKBA-20 and BSBA-20, membrane binding caused CD spectral changes characteristic of an increase in helical secondary structure (*22*). Moreover, the minimum at 225 nm is more pronounced than the minimum at 208 nm ($\theta_{\pi-\pi^*}/\theta_{\pi-\pi^*} > 1$), indicating that both peptides are self-associating in the lipid membrane environment (*17, 23*). The spontaneous binding and subsequent self-association of peptides would act as seeds for the formation of peptide ion channels. Interestingly, despite the lack of (fairly weak) interactions with vesicles (as evaluated by CD), BEBA-20 and BGBA-20 also form ion channels. Thus, the ion channel formation of these peptides seems not to be spontaneous, but to be induced by other factors, such as differences in transmembrane potential. Concomitantly, BEBA-20 exhibited a comparable helicity to that of

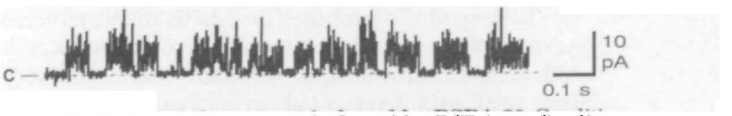

Fig. 8. **Typical erratic current induced by BSBA-20.** Conditions were as for Fig. 4, except that the voltage was +90 mV. The erratic currents by BEBA-20 and BGBA-20 were quite similar, without a stable and constant conductance in the open states.

TABLE II. **Summary of the channel conductance data of BXBA-20 peptides.**

Peptide	BKBA-20	BEBA-20	BSBA-20	BGBA-20
Conductance/pS	227	1,620–10,200	30–200	10–50
Frequency of channel formation[a]	10/0/10	5/10/21	6/27/61	1/15/25
V_{rev}/mV[b]	0	−20.5 ± 4.7	−23.6 ± 7.1	−10–0
Ion selectivity (P_K:P_{Cl})[c]	1:1	3.6:1	4.6:1	<2:1

[a]Frequency of channel formation is expressed as [the number of discrete ion channels observed/the number of erratic current events observed/the number of runs]. [b]V_{rev}: Reversal potential was determined in asymmetric KCl (*cis*: 0.5 mM KCl, *trans*: 0.1 mM KCl), 5 mM HEPES, pH 7.4 solutions. In the case of BEBA-20, the value is for the level 1 opening among the multi-state events. [c]P_K:P_{Cl}: permeability ratios were determined by the Goldman-Hodgkin-Katz equation (*18*).

BKBA-20 in TFE, sometimes considered as a mimic of membrane hydrophobic cores (*24*). This suggests that the membrane-bound state of BEBA-20 may have a similar conformation to BKBA-20. Likewise, the conformation of BGBA-20 in membranes is assumed to be much less helical.

The conductance of BEBA-20 pores shows a resemblance to the multi-state conducting behavior of alamethicin (*4*), which implies structural similarity of the pores. The respective increase or decrease in the conductance of BEBA-20 is possibly caused by the addition or deletion of a single helix. The change in conductance can be explained for BEBA-20 channels with 6 to 13 helical subunits. Likewise, the conductance of 227 pS of BKBA-20 corresponds to a tetrameric bundle of molecules. The other two peptides (BSBA-20 and BGBA-20) exhibited various conductances for their single-state channels. However, the conductance range indicates that the pores are composed mainly of trimeric or tetrameric bundles of peptides. This behavior indicates that BKBA-20 and BEBA-20 have well defined pore structures. In contrast, BSBA-20 and BGBA-20 form non-specific pore structures. Therefore, it seems plausible that the formation of well-defined pore structures is directly related to the high degree of peptide helicity.

Electrostatic repulsive interactions between adjacent BEBA-20 helices (due to the presence of the negatively charged Glu residues) can cause the observed large pore size. Therefore, BEBA-20 pores are partially stabilized and oscillate between multi-state conducting levels. Similar effects (*i.e.* the channel lifetime elongation and conductance magnification) of Glu residues have been reported for hypelcin and trichorzianin by Koide *et al.* (*25*).

The Frequency of Channel Formation—All of the peptides produced ion flux. However, the frequency of the observation of discrete channel events was in the order of BKBA-20>BEBA-20>BSBA-20>BGBA-20 (Table II). When studies are conducted or ion channels formed by peptides in membranes, heterogeneity and poor reproducibility of conductance are sometimes encountered, which can rarely be described by the data. This characteristic behavior of ion channel–forming peptides should receive more attention, and be noted as the ratio of the frequency of channel formation, *vide supra*. We propose that the frequency is related to the following four factors:

(i) The helicity of the peptide: The α-helix dipole moment originates from the particular alignment of the peptide units (3.5 D/unit) in this secondary structure (*26*). Since stiff and long α-helices possess large dipole moments, a stable helix can strongly interact with the applied electric field and stabilize the helix bundle structure in the lipid bilayer (27).

(ii) The self-association of the peptide: The channel is assumed to be a transmembrane peptide bundle. Therefore peptide association is essential for the formation of ion channels.

(iii) The affinity of the peptide for the membrane: The probability of channel formation decreases with the reduction in binding affinity, which can be understood by postulating that the fraction of arranged peptides in a channel is small among the membrane-bound peptides (*28*). In the case of alamethicin, the importance of the above three factors has been reported in different systems (*23, 29, 30*).

(iv) The amphipathicity of the peptide: Helical peptides in the helix-bundle channel are believed to have an interior (lumen face) that is more hydrophilic than its exterior (bilayer face). The capability of forming amphipathic helices is a common feature of several channel-forming peptides (*4, 16*).

The high frequency of channel formation by BKBA-20 is due to its high helical content (Fig. 2c) in vesicles, high amphipathicity, favorable interaction with lipid membranes (Fig. 3), and self-associating properties. The helicity of BEBA-20 is also comparable to that of BKBA-20, as judged from the CD spectra in TFE. However, the weak membrane affinity (Fig. 3) and lower amphipathicity could somehow explain its reduced the channel forming activity. In the case of BGBA-20, the lack of three factors, *i.e.* helical structure, amphipathicity, and affinity for membranes, clearly diminishes the possibility of channel formation, as well as the stability of the formed channels.

Cation/Anion Selectivity—A popular view of the mechanism of ion-selectivity in transmembrane channels is that the side chain structures of the amino acids in the pore region act as filters, selecting ions based on their charge density and size (*31, 32*). In this view, side chains with negative polar density (Glu, Ser, and Thr) are responsible for the cation-selectivity of the channel [*e.g.*, acetylcholine receptor channel (*2*)]. In contrast, Lys, Arg, and His residues are candidates for anion selective filters [*e.g., Staphylococcus aureus* alpha-toxin (*33*), voltage-gated chloride channel (*34*), and PhoE porin (*35*)]. Borisenko *et al.* recently reported that protonation of Lys residues inverts cation/anion selectivity in an alamethicin analog, which also suggests that protonated Lys residues at appropriate positions in a pore act as a determinant of anion selectivity (36).

The cation selectivity of BEBA-20 and BSBA-20 can be explained on the basis of this filter effect of charge density—*i.e.*, the charge on the carboxyl group of Glu side chains and the hydroxyl group on Ser attract cations instead of anions. In the case of the BGBA-20 pore, the exposed carbonyl oxygens of the peptide backbone in the ion pathway would be responsible for a weak cation-selective channel. The cation/anion selectivity of the peptide backbone can also be considered from the thermodynamics point of view: the transfer of K^+ from water to formamide (a model of a peptide backbone) is more favorable (ΔG = –4 kJ/mol) than that of Cl^- (ΔG = +14 kJ/mol) (*37*). In addition, a recent study on K^+ channel structure revealed that K^+ selectivity is caused by the oxygens of the polypeptide main chain, not the side chains (*3*). Therefore, in addition to the side chains of Glu and Ser residues, the main chain of Gly may also act as a cation-selective determinant.

BKBA-20 shows non-selectivity between cations and anions, as opposed to the unexpected result considering the electrostatic ion-charge interactions. A tetrameric pore of BKBA-20 has a net positive charge of 20. This discrepancy of no selectivity or even cation-selectivity of positively charged peptides has been reported for other peptides (*10, 38*). These findings suggest that the presence of positively-charged side chains in the pore alone does not induce anion selectivity. Other factors, *e.g.*, interactions of between cations and anions within the pore, should be considered to explain the origins of anion selectivity of channel-forming peptides (*39*).

In conclusion, our study demonstrates that peptides with

high degrees of helical conformation, high amphipathicity, high affinity for lipid membranes, and self-associating character in vesicles are most suitable for inducing stable ion channels with high frequencies of occurrence. Lys, Glu, Ser, and Gly residues modulate this frequency by changing the peptide conformation, and also the affinity of the peptide for binding to lipid membranes. Concerned with ion selectivity, the side chains of Ser and Glu (and possibly the backbone of the Gly-containing peptide) act as cation-selective filters in the pore structures. The above findings provide useful information to be used in designing more sophisticated ion channel–forming peptides.

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