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Selective Interaction of Synthetic Antimicrobial Peptides Derived from Sapecin B with Lipid Bilayers[1]

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By measuring carboxyfluorescein leakage from liposomes and the increase in membrane current through planar lipid bilayer membranes, we examined the capacities of a series of low-molecular-weight cationic amphiphilic peptides derived from the α-helix domain of sapecin B for membrane-perturbation and ion-channel formation. Some of these peptides strongly interact with membranes containing acidic phospholipids and phosphatidylethanolamine, with a very negative potential, which are characteristic of the *Escherichia coli* membrane, in parallel with their antimicrobial activity. In contrast, they do not interact with membranes which predominantly contain choline phospholipids and cholesterol in their outer leaflets, with a slightly negative potential, all of which are characteristic of eukaryotic membranes, thereby providing a molecular basis for their selective toxicity. Membranes doped with these peptides are as permeable to inorganic phosphates as to chloride ions and are far more permeable to cations. The loss of inorganic phosphates may damage bacterial cells due to rapid depletion of cytoplasmic ATP. Examination of the structure-activity relationships of a series of derived peptides in their interaction with a model of the *E. coli* membrane confirmed the necessity of cationic amphiphilicity for the peptides to attack the bacterial membrane and to exhibit antimicrobial activity.

Key words: amphiphilicity, antimicrobial peptide, liposome, planar bilayer, selective toxicity.

Sapecin is an antimicrobial peptide which can be induced in the larval haemolymph of *Sarcophaga peregrina*, in response to bacterial challenge or injury (*1*). Sapecin B is one of two sapecin homologues isolated from the culture medium of the embryonic cell line, NIH-Sape-4 (*2*). The active moiety of sapecin B consists of 11 amino acid residues, and shows more potent antimicrobial activity toward Gram-negative bacteria than the entire sapecin B molecule (*3*). Recently, peptides derived from the active moiety were systematically synthesized for further functional optimization, which revealed that terminal basic motifs and internal oligo-leucine sequences play important roles in this antimicrobial activity (Table I) (*4*).

Previously, using a series of well-defined model peptides with a cationic amphiphilic structure in both aqueous and hydrophobic environments, we found strong correlations among antimicrobial activity, carboxyfluorescein leakage from liposomes, and ion-channel formation in planar lipid bilayer membranes (*5-9*). There have also been various reports of natural peptides with small molecular weights (less than 5,000) which show both antimicrobial activity and the ability to form ion-channels in phospholipid bilayer membranes, *e.g.*, gramicidin A (*10*), alamethicin (*11*), mellitin (*12*), mastoparan (*13*), colicin A (*14*), cecropin (*15*), magainin I (*16*), magainin II (*17*), and defensin (*18*). The perturbation and subsequent ion-channel formation in bacterial membranes has been assumed to be fatal for bacterial cells because it induces the efflux and influx of electrolytes, and abolishes any membrane potential (*19, 20*). Therefore, since the derived peptides have cationic amphiphilic properties similar to those of some of the above model and natural peptides, their antimicrobial activity may be due to perturbation and ion-channel formation in bacterial membranes.

On the other hand, it has been demonstrated that one of these derived peptides does not show significant haemolytic activity ($KLKL_5KLK\text{-}NH_2$, peptide II; Table I) (*4*). It may be useful to elucidate the cause of this selective toxicity to bacteria. Furthermore, since the derivative peptides, including peptide II, are not expected to induce immune responses because of their low molecular weights, they are attractive from the viewpoint of the development of therapeutic drugs. Other peptides with selective toxicity have also been reported, *e.g.*, cecropins (*21*), magainins (*22*), and dermaseptins (*23*). Since the derivative peptides are thought to attack bacterial membranes as part of their antimicrobial activity, the selective toxicity may arise from differences in the properties of the cell membranes of bacterial and eukaryotic cells. For example, human and

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Abbreviations: PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PG, L-α-phosphatidyl-DL-glycerol; PS, L-α-phosphatidyl-L-serine; CL, cardiolipin; Chol, cholesterol; Sph, sphingomyelin.

other mammalian erythrocyte membranes consist primarily of phosphatidylcholine (PC), sphingomyelin (Sph), phosphatidylethanolamine (PE), phosphatidylserine (PS) (*24*), and abundant cholesterol (Chol) (*25*). The phospholipids are distributed asymmetrically: choline phospholipids are mostly located in the outer leaflets of plasma membranes, whereas amino phospholipids are mainly found in the inner leaflets (*26*). On the other hand, bacterial membranes characteristically contain phosphatidylglycerol (PG) and cardiolipin (CL), and completely lack cholesterol (*27, 28*). Acidic phospholipids have been confirmed to be present in the outer leaflets (*29*). Furthermore, the bacterial membrane potential (typically more negative than −100 mV) (*30*) is far greater than those (−9 mV) (*31*) of human and other mammalian erythrocytes. Thus, differences in the lipid composition of cell membranes and in membrane potentials may be responsible for the selective toxicity to bacteria.

Therefore, in the present study, we examined the membrane-perturbing ability of the derived peptides by measuring the leakage of a fluorescent dye, carboxyfluorescein, entrapped in liposomes composed of various lipids. This should enable us to examine selective interactions of the peptides with lipid bilayers. Furthermore, we also examined the effects of these peptides on the ion-permeability of planar lipid bilayer membranes. This should allow us to obtain more detailed information regarding selective interactions, particularly ion selectivity, the effects of membrane potentials, and the effects of asymmetric distribution of lipids. This is the first thorough examination of membrane selectivity using the planar lipid bilayer technique. The mode of action of the peptides on lipid bilayers and the mechanism of bacterial death are discussed based on the results of these experiments.

MATERIALS AND METHODS

Materials—Seven peptides were synthesized by the solid-phase method with a peptide synthesizer (model PSSM-8; Shimadzu, Tokyo). They were purified by high-performance liquid chromatography on a Syncropac RP-P (C18) reverse-phase column to homogeneity. Their amino acid sequences were confirmed using a protein sequencer (Shimadzu PPSQ-10). Egg yolk PC, egg yolk PE, bovine brain PS, egg yolk PG, bovine brain Sph, bovine heart CL, synthetic 1-palmitoyl-2-oleoyl PC, synthetic 1-palmitoyl-2-oleoyl PE, and Chol were purchased from Sigma (St. Louis, MO, USA). Carboxyfluorescein was purchased from Lambda (Graz, Austria). Phospholipids were quantified by phosphorus analysis using a phospholipid test reagent (Wako Pure Chemicals, Osaka). Hepes was supplied by Dojindo (Kumamoto). All other reagents were purchased from Wako Pure Chemicals. They were of analytical grade and used without further purification.

Preparation of Liposomes Encapsulating Carboxyfluorescein—Lipid films were obtained by evaporating chloroform solutions of lipids (20 mg). The lipids were then hydrated in a buffer (1.5 ml) containing carboxyfluorescein at a high concentration (50 mM carboxyfluorescein, 20 mM Hepes-Tris, pH 7.4) by vortex-mixing for 10 min at room temperature. Each suspension was sonicated for 30 min at room temperature using a Branson Sonifier cell-disrupter 200. Liposomes encapsulating carboxyfluorescein were subjected to gel filtration (Sepharose 4B, 1×20 cm) in a buffer (100 mM NaCl, 20 mM Hepes-Tris, pH 7.4). Two milliliter fractions were collected and the small unilamellar vesicles (SUV) in the seventh fraction were used for measuring leakage (*32*). The lipid concentration of the seventh fraction was about 2.8 mM and varied with the lipid composition.

Leakage of Liposome Contents—Buffer (100 mM NaCl, 20 mM Hepes-Tris, pH 7.4) was added to the liposome suspension of the seventh fraction so that the final concentration of lipids was 70 μM. A peptide solution in doubly-distilled water (2 mg/ml) was added to the diluted liposomal suspension (2 ml). Carboxyfluorescein was excited at 470 nm and the emission at 515 nm was monitored with a fluorescence spectrophotometer (model F-2000; Hitachi, Tokyo). After measurement, Triton X-100 (final concentration, 0.5% v/v) was added to completely release the dye from the liposomes. The percentage of dye leakage (PDL) induced by the peptides was evaluated using the equation, $PDL = [(F - F_0)/(F_t - F_0)] \times 100$, where F is the fluorescence intensity after the addition of the peptides, and F_0 and F_t, respectively, the fluorescence intensity without the peptides and after the addition of Triton X-100.

Formation of Planar Lipid Bilayer Membranes—Planar lipid bilayer membranes were formed by the folding method originally described by Takagi *et al.* (*33*), and modified by Montal and Mueller (*34*). A chamber with two separate compartments was made using Teflon. The compartments were referred to as *cis* and *trans*, and sample peptides were added to the former. A thin Teflon septum (50 μm thick) with an aperture of 150 μm in diameter separated the two compartments. To each compartment was added 0.5 ml of a buffer (KCl buffer) consisting of 100 mM KCl and 10 mM Hepes-Tris (pH 7.4). Ten microliters of a lipid solution in an organic solvent (10 mg/ml) was dropped into each compartment. The organic solvents used for the lipid solutions were *n*-hexane for PE, PC, PS, PG, and CL, chloroform for Sph, and petroleum ether for Chol. After the solvent had been evaporated off over several minutes, a lipid monolayer was formed on the surface of the buffer in each compartment. A planar lipid bilayer then formed spontaneously at the aperture when the air-water interface was raised by injecting 0.7 ml of the KCl buffer into the bottom of each compartment. Lipid bilayers with a resistance of less than 200 GΩ were not used in the experiments.

Measurement of the Membrane Current—Five minutes after the formation of the bilayer membrane, the KCl buffer in the *cis* compartment was stirred with a magnetic stirrer for another 5 min to confirm that the conductance of the membrane did not spontaneously increase with stirring alone. An appropriate amount of a peptide dissolved in doubly-distilled water (2 mg/ml) was then added to the *cis* compartment (final concentrations, 4.2-66.7 μg/ml). The membrane potential was defined as that of the *trans* compartment with respect to that of the *cis* compartment. The membrane current was measured with a current-voltage (I-V) converter (bandwidth 1.6 kHz) constructed by us using a Burr-Brown OPA 104CM operational amplifier and a 10-GΩ feedback resistor (Victoreen, Cleveland, OH, USA). After I-V conversion, the signals were filtered with a low-pass filter when necessary and monitored with a digital storage oscilloscope (DSS5020A; Kikusui Elec-

tronics, Kawasaki). The data were stored using a videotape recorder (SL-HF3; Sony, Tokyo) after A-D conversion with a modified digital audio processor (PCM-501ES; Sony). The stored data were retrieved, and displayed on a chart recorder (R61; Rikadenki, Tokyo) and/or analyzed with a personal computer (386/20N; Hewlett-Packard, Sunnyvale, CA, USA).

RESULTS

Carboxyfluorescein Leakage from Liposomes Induced by Peptides—Carboxy-fluorescein was released from sonicated lipid vesicles by the addition of peptides. The time course of the increase in fluorescence intensity, which is a measure of the amount of carboxyfluorescein released, is shown in Fig. 1A for the case of peptide $KLKL_5KLK\text{-}NH_2$ (II) applied to vesicles composed of PE : PG (7 : 3). A plateau was attained about 1 min after addition of the peptide. Figure 1B shows the result of a similar experiment for vesicles composed of PC : PG (7 : 3) challenged with the same peptide. Based on these results, fluorescence intensity was measured 3 min after the addition of various peptides throughout the following experiments.

Figures 2 and 3 show PDL from liposomes at 3 min with different lipid compositions. These results reflect the membrane-perturbing ability of peptides depending on the lipid composition. Figure 2A shows that peptide II with potent antimicrobial activity toward both Gram-positive and -negative bacteria perturbs acidic membranes [PS, PE : PG : CL (7 : 2 : 1), and PC : PG : CL (7 : 2 : 1)] very strongly. In contrast, the peptide has little effect on neutral membranes [PC, Sph, and PC : Sph : Chol (3 : 3 : 2)]. These findings suggest that acidic phospholipids such as PS, PG, and CL are necessary for membranes to be perturbed by the peptide. The contribution of each of these acidic phospholipids to the susceptibility to the peptide was examined further. Figure 2B shows PDL after the application of the peptide to membrane vesicles composed of 70% PE and 30% PS, 30% PG or 20% PG+10% CL. Membrane perturbation is not particularly specific to any of the acidic phospholipids, although a membrane which contains CL is slightly more perturbed than others. The same results were obtained with peptides $RLKL_5RLK\text{-}NH_2$ (I) and $KLKL_5KLK\text{-}NH_2$ D-form (III).

In marked contrast to the results with peptides I, II, and III, peptides with weaker antimicrobial activity [$HLHL_5HLH\text{-}NH_2$ (IV), $KLKL_5\text{-}NH_2$ (VI), and $KKL_5KK\text{-}NH_2$ (VII)] interacted weakly to moderately with membranes of all compositions, as shown in Fig. 3, A, B, and C. The most prominent difference between these two groups of peptides lies in their membrane-perturbing ability in vesicles composed of PE : PG : CL (7 : 2 : 1). The potency of the antimicrobial activity of the peptides (Table I) parallels their membrane-perturbing ability as to a membrane of this composition. Since this particular composition is a model of the *E. coli* cell membrane, these results suggest that the peptides target the bacterial membrane.

It is worth noting again that peptides I, II, and III perturb neutral membranes [PC, Sph, PC : Sph : Chol (3 : 3 : 2), and PE : PC (1 : 1)] only slightly. This accounts for the selective toxicity of the peptides. Peptide II has been shown to exhibit no haemolytic activity despite its strong antimicrobial activity (*4*). Peptides I and III also showed no haemolytic activity (data not shown). These findings are consistent with the fact that the plasma membranes of eukaryotic cells are mainly composed of neutral lipids such as PC, Sph, PE, and Chol (*24, 36-42*).

In addition, it should be pointed out that peptide II, which has potent antimicrobial activity, perturbs a membrane of PE : PG (7 : 3) more strongly than one of PC : PG (7 : 3) (Fig. 1, A and B). Accordingly, Fig. 2A indicates that a membrane consisting of PE : PG : CL (7 : 2 : 1) is more susceptible to peptide II than is one consisting of PC : PG : CL (7 : 2 : 1). Thus, this peptide more strongly perturbs membranes containing egg yolk PE than ones containing egg yolk PC, which are both neutral phospholipids that comprise 70% of the phospholipid mixture. This indicates that the peptide discriminates between PE and PC. This may be significant with regard to selective toxicity because PC is a representative component of eukaryotic cell membranes, whereas PE is a major component of Gram-negative bacteria (*24, 27, 36-42*). To clarify whether this discrimination is actually due to differences in the phospholipid headgroups, experiments were carried out using synthetic

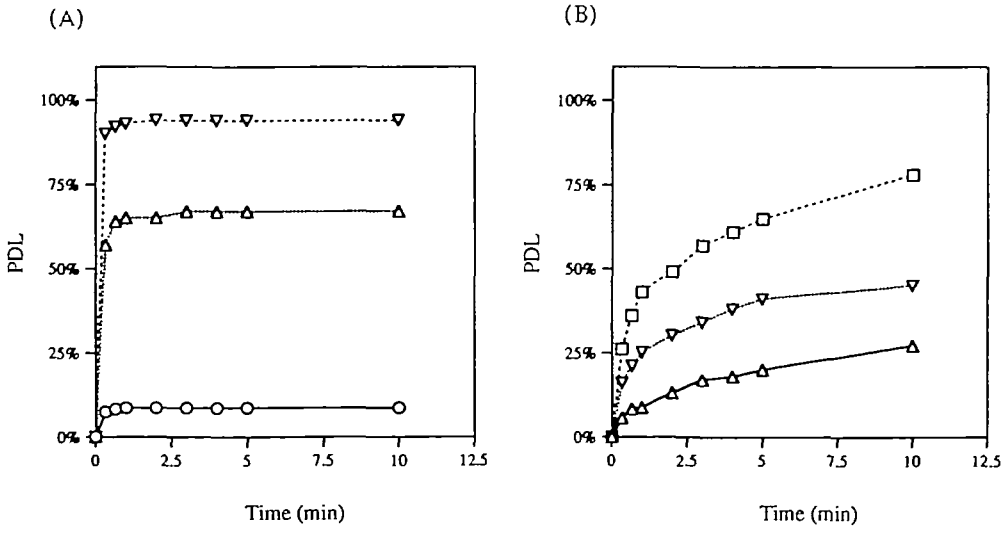

Fig. 1. Time course of carboxyfluorescein leakage from liposomes induced by peptide II ($KLKL_5KLK\text{-}NH_2$). Abscissa, time (min). Ordinate, percentage of dye leakage, $PDL=(F-F_0)/(F_t-F_0)\times100$. The lipid compositions of liposomes were (A) PE : PG (7 : 3), and (B) PC : PG (7 : 3). The final concentrations of the peptide were: ○, 1 μg/ml; △, 5 μg/ml; ▽, 10 μg/ml; □, 25 μg/ml.

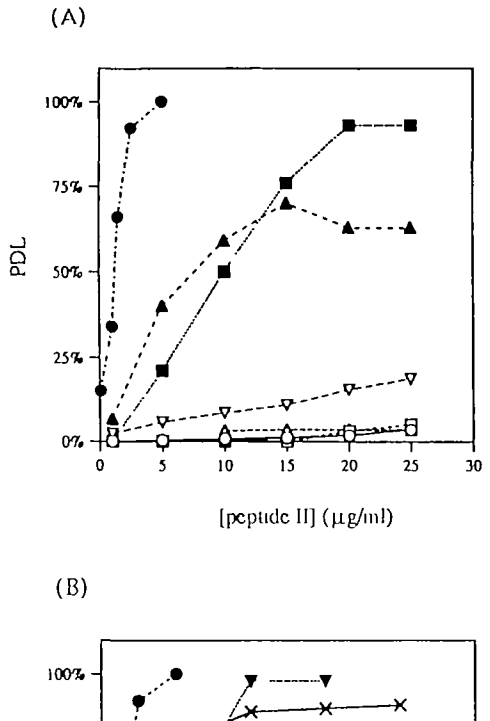

Fig. 2. **Carboxyfluorescein leakage from liposomes with different lipid compositions challenged by peptide II with potent antimicrobial activity.** Abscissa, peptide concentrations (μg/ml). Ordinate, percentage of dye leakage, PDL=$(F-F_0)/(F_t-F_0)\times 100$. The lipid compositions of vesicle membranes were: ●, PE : PG : CL (7 : 2 : 1); ▲, PC : PG : CL (7 : 2 : 1); ×, PE : PS (7 : 3); ▼, PE : PG (7 : 3); ■, PS; ○, PC; ▽, Sph; □, PC : Sph : Chol (3 : 3 : 2); △, PE : PC (1 : 1).

1-palmitoyl-2-oleoyl-PE and -PC, which differ only in their headgroups. As shown in Fig. 4, the result indicates that the difference between the headgroups of PE and PC gives rise to different susceptibilities to peptide II, and essentially the same result was obtained with peptide III. This result confirms that the discrimination by peptides II and III is due to the difference between the headgroups of PE and PC. Therefore, this result strongly suggests that discrimination between PE and PC by the peptides contributes to selective toxicity.

Membrane Current Caused by the Incorporation of Peptides into a Planar Lipid Bilayer—Figure 5 shows an example of the macroscopic membrane current caused by peptide II incorporated into a planar lipid bilayer composed of a mixture of PE, PG, and CL (7 : 2 : 1), which resembles the composition of the *E. coli* membrane. When the peptide was added to the *cis* compartment at a membrane potential of −100 mV, a significant membrane current was detected in about 4 min and then gradually increased. This increase in the membrane current accelerated with time and membrane disruption finally occurred. No significant current was detected without the addition of the peptide: the conductance of the lipid membrane was less than 5 pS, which is the background noise level. Therefore, the emergence of a membrane current is definitely due to the addition of the peptide. Examination of the current trace at a greater time-resolution did not reveal any typical single-channel currents with discrete conductance levels. This suggests that the membrane current should be considered to pass through very short-lived ion-channels.

In many other experimental runs, membrane currents increased to a plateau level, about which they randomly fluctuated, and did not proceed to membrane disruption during the observation period of about 30 min. In other cases, no membrane currents were observed and the conductance remained below 5 pS. Similar phenomena were observed with all of the peptides listed in Table I.

Dependence of the Membrane Current on the Peptide Concentration—The length of time from the addition of a peptide to the emergence of a membrane current may be an index of the ability of the peptide to produce membrane perturbation and disruption. Table II shows the latency periods with the addition of various doses of the peptides. With all of the peptides studied, the latency decreased with an increase in dose, which again supports the notion that the membrane current is due to the incorporation of peptides into the lipid bilayer membrane.

Comparison of Tables I and II reveals that the greater the antimicrobial activity, the shorter the latency of the emergence of the membrane current. Thus, there appears to be a direct relationship between the antimicrobial activity of the peptides and their ability to enhance membrane permeability. This result also suggests that the peptides target the bacterial membrane.

Dependence of the Membrane Current on the Membrane Potential—Figure 6A shows the membrane currents at various membrane potentials after a significant current was obtained by the application of peptide I. At potentials of −80 mV or less, the conductance increased with time, while it decreased after the potential had been changed to a corresponding positive value. A similar tendency was observed for peptide II, as shown in Fig. 6B. The current increased so that the membrane was ultimately disrupted only at negative potentials. Membrane disruption did not occur at positive membrane potentials. These findings clearly demonstrate that the increase in membrane current, *i.e.*, the incorporation of peptides into a phospholipid bilayer, strongly depends on the membrane potential.

In contrast, the membrane conductance remained fairly constant with some fluctuations with peptide II (Fig. 6C). Rectification is apparent from the I-V curve shown in Fig. 6D. This behavior was frequently observed with peptide II, but rarely seen with peptide I.

The fact that membrane conductance is increased at negative potentials reasonably explains the action of these peptides on bacterial membranes, which show a negative potential as deep as −200 mV (*35*).

Ion Selectivity—We examined the dependence of pep-

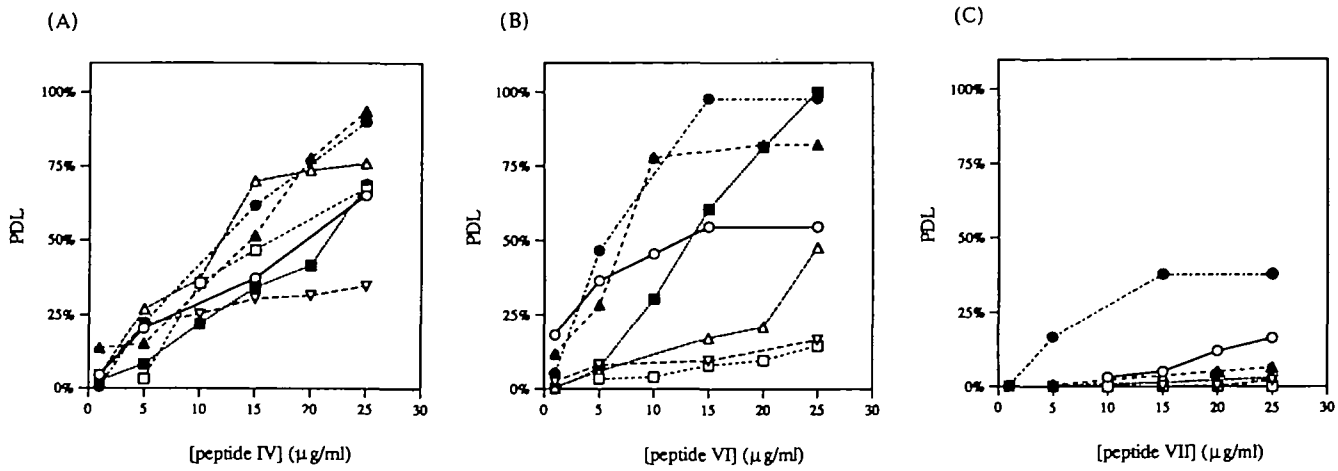

Fig. 3. **Carboxyfluorescein leakage from liposomes with different lipid compositions challenged by peptides with weaker antimicrobial activity.** Abscissa, peptide concentrations (μg/ml). Ordinate, percentage of dye leakage, $PDL=(F-F_0)/(F_t-F_0)\times100$. (A) peptide IV ($HLHL_5HLH\text{-}NH_2$); (B) peptide VI ($KLKL_6\text{-}NH_2$); (C) peptide VII ($KKL_5KK\text{-}NH_2$). The lipid compositions of vesicle membranes were: ●, PE : PG : CL (7 : 2 : 1); ▲, PC : PG : CL (7 : 2 : 1); ■, PS; ○, PC; ▽, Sph; □, PC : Sph : Chol (3 : 3 : 2); △, PE : PC (1 : 1).

TABLE I. **Dye release ability and antimicrobial activity of peptides.**

Peptide	EC_{50}[a]	*S. aureus*[b]	*E. coli*[c]	MRSA[d]
I. $RLKLLLLLRLK\text{-}NH_2$	0.04	1	20	30
II. $KLKLLLLLKLK\text{-}NH_2$	1	2	20	30
III. $KLKLLLLLKLK\text{-}NH_2$ (D-form)	2.5	2	20	15
IV. $HLHLLLLLHLH\text{-}NH_2$	11.5	>16	>35	>35
V. $KLLLLLK\text{-}NH_2$	ND[e]	>16	>35	>35
VI. $KLKLLLLL\text{-}NH_2$	5.5	8	20	>35
VII. $KKLLLLLKK\text{-}NH_2$	ND[e]	>16	>35	>35

[a]50% effective concentrations of peptides for carboxyfluorescein leakage in 3 min from liposomes composed of PE : PG : CL (7 : 2 : 1), a model membrane of *E. coli*. Data are from Fig. 2. [b c d]Minimum inhibitory concentrations of peptides (μg/ml) for *S. aureus* (Gram-positive), *E. coli* (Gram-negative), and MRSA (methicillin-resistant *S. aureus*, Gram-positive). Data are from Ref. *4*. [e]Not determined.

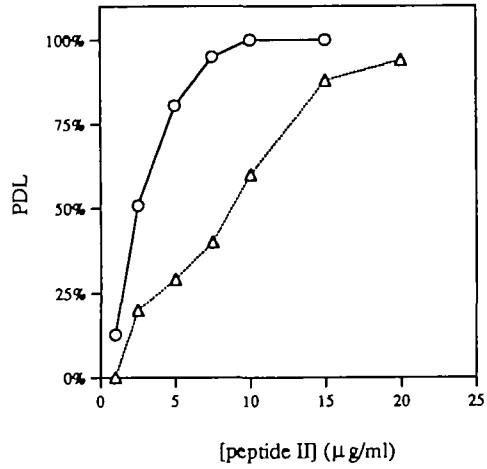

Fig. 4. **Carboxyfluorescein leakage induced by peptide II from liposomes made of a phospholipid mixture consisting of 70% synthetic PE or PC.** Abscissa, peptide concentrations (μg/ml). Ordinate, percentage of dye leakage, $PDL=(F-F_0)/(F_t-F_0)\times100$. ○, 1-palmitoyl-2-oleoyl-PE : PG : CL (7 : 2 : 1); △, 1-palmitoyl-2-oleoyl-PC : PG : CL (7 : 2 : 1).

tide-induced currents on the ion species in the solution. Table III shows the permeability coefficients for ions relative to that for potassium ions with membranes composed of PE : PG : CL (7 : 2 : 1) doped with the three peptides (I, II, and III). These values were calculated from the observed reversal potentials using the Goldman-Hodgkin-Katz equation (the GHK equation). Since the membrane used here contained 30% of acidic phospholipids, its surface was negatively charged in the *trans* compartment while the negative charge may be canceled in the *cis* compartment by the positively charged peptide molecules added. Thus, in the GHK equation, we substituted the sum of the observed reversal potential and surface potential in the *trans* compartment calculated with the Gouy-Chapman equation for the membrane potential, and used salt concentrations adjusted according to the Boltzmann relation for the *trans* compartment.

The results indicate that these peptides induce a strongly anion-selective current, which is consistent with the fact that the peptides are positively charged. It is worth noting that peptide II makes a membrane far more permeable to inorganic phosphates than to cations. A rapid outflow of inorganic phosphate ions may occur from bacterial cells due to highly negative membrane potentials, which could lead to the rapid depletion of intracellular ATP.

Dependence of the Membrane Current on the Compositions of Planar Lipid Bilayer Membranes under Voltage Clamp Conditions—We measured membrane currents caused by the action of peptide II with planar membranes of various lipid compositions under voltage clamp conditions. Some membranes have asymmetric lipid compositions in which the lipid compositions of the "outer" and "inner" leaflets of the bilayer, which face the *cis* and *trans* compartments, respectively, are different.

Table IV summarizes the results obtained with various membrane compositions at membrane potentials of −40 and −100 mV. Membranes at −100 mV appear to be disrupted more frequently than ones at −40 mV, and only membranes E and H can be disrupted at −40 mV. This

TABLE II. **Delay before the emergence of a membrane current (min).**

Peptide	66.7 μg/ml	16.7 μg/ml	4.2 μg/ml
I. RLKLLLLLRLK-NH_2	6.3±7.82 (n=7)	16 (n=2)	
		>30 (n=3)	>30 (n=4)
II. KLKLLLLLKLK-NH_2	2.8±3.15 (n=7)	3.67±4.48 (n=3)	4 (n=2)
		>30 (n=2)	>30 (n=3)
III. KLKLLLLLKLK-NH_2 (D-form)	3.5±4.42 (n=5)	5.25±3.01 (n=4)	5.5 (n=2)
		>30 (n=1)	>30 (n=3)
IV. HLHLLLLLHLH-NH_2	12.2±12.3 (n=3)	20.5 (n=1)	
	>30 (n=2)	>30 (n=4)	>30 (n=5)
V. KLLLLLK-NH_2	3.5 (n=1)	28 (n=1)	
	>30 (n=4)	>30 (n=4)	>30 (n=5)

Enhanced conductance of more than 10 pS was seen only after the indicated periods of time. The indicated periods represent the averages of trials performed under identical conditions (mean±SD, n=number of trials). The recording conditions were the same as those in Fig. 5 except that the current signals were filtered using a 5-Hz low-pass filter.

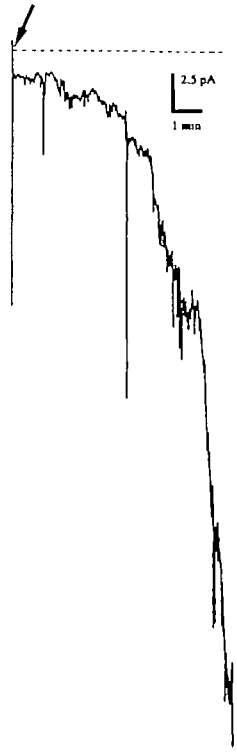

Fig. 5. **A trace of the membrane current caused by the incorporation of peptide II into a planar lipid membrane consisting of a mixture of phospholipids** [PE : PG : CL (7 : 2 : 1)]. The composition of the solution in both compartments was 100 mM KCl and 10 mM Hepes-Tris (pH 7.4). Peptide II (final concentration, 33 μg/ml) was added to the *cis* compartment of the chamber. The *cis* compartment was then stirred for about 4 min under a membrane potential of −100 mV to detect a significant current using a 5-Hz low-pass filter (4-pole, Bessel-type). Stirring was then discontinued (shown by arrow) and the membrane current was recorded without filtering. The dotted line indicates the baseline (0 pA).

clearly indicates that a more-negative potential enhances membrane perturbation, and that the membrane potential is a critical factor in a peptide's action on a membrane. With regard to the lipid composition, only membranes F and G were not disrupted at −100 mV, and membranes F, G, O, and P did not allow the emergence of significant currents at −40 mV. A common feature of these four robust membranes is that they contain a considerable amount of Chol (30-43%). Since membrane N, which contains 23% Chol, allows the induction of currents, the presence of a large amount of Chol (higher than 30%) appears to be necessary to resist the attack of the peptide. However, the fact that membranes E, H, and I are perturbed by the peptides even at −40 mV despite a large amount of Chol (43-50%) suggests that the choline phospholipids, PC and/or Sph, are necessary in addition to Chol. Although substitution between PC and Sph seems to be possible, the total amount of choline phospholipids must be fairly high (greater than 43%). In membrane P, although the outer leaflet satisfies these conditions, the inner leaflet is predominantly occupied by amino phospholipids (PE and PS). This finding suggests that choline phospholipids and Chol only need to exist in the outer leaflets of bilayers to protect against the action of a peptide. These results, taken together, indicate that an abundance of choline phospholipids and Chol in the outer leaflet is critical for resisting the attack of a peptide.

DISCUSSION

Correlation between Membrane-Perturbing Ability and Selective Toxicity of the Peptides—In the present study, liposome experiments revealed that synthetic peptides derived from the active moiety of sapecin B interact with lipid bilayers in a selective manner. These peptides perturb a model of the *E. coli* membrane in parallel with the potency of their antimicrobial activity, which strongly suggests that they target the bacterial membrane. Peptides with potent antimicrobial activity interacted with acidic membranes very specifically. Since these peptides do not exhibit haemolytic activity, this observation may be explained by the difference between bacterial and eukaryotic cell membranes. The ability of the peptides to discriminate between PE and PC may also play a significant role in the selective toxicity.

Planar bilayer experiments showed that the peptides can induce a strong anion-selective current through an *E. coli* model membrane. The ability of the peptides to induce a current again parallels the potency of their antimicrobial activity. This further confirms the finding in the liposome experiments that the peptides target the bacterial membrane. The voltage-dependency of the current can also be used to explain the action of the peptides on bacterial membranes with a very negative potential, *i.e.* a more-negative potential facilitates a membrane current and membrane disruption. Examination of the dependence of the membrane current on the lipid composition showed that the molecular basis of the selective toxicity of peptide II

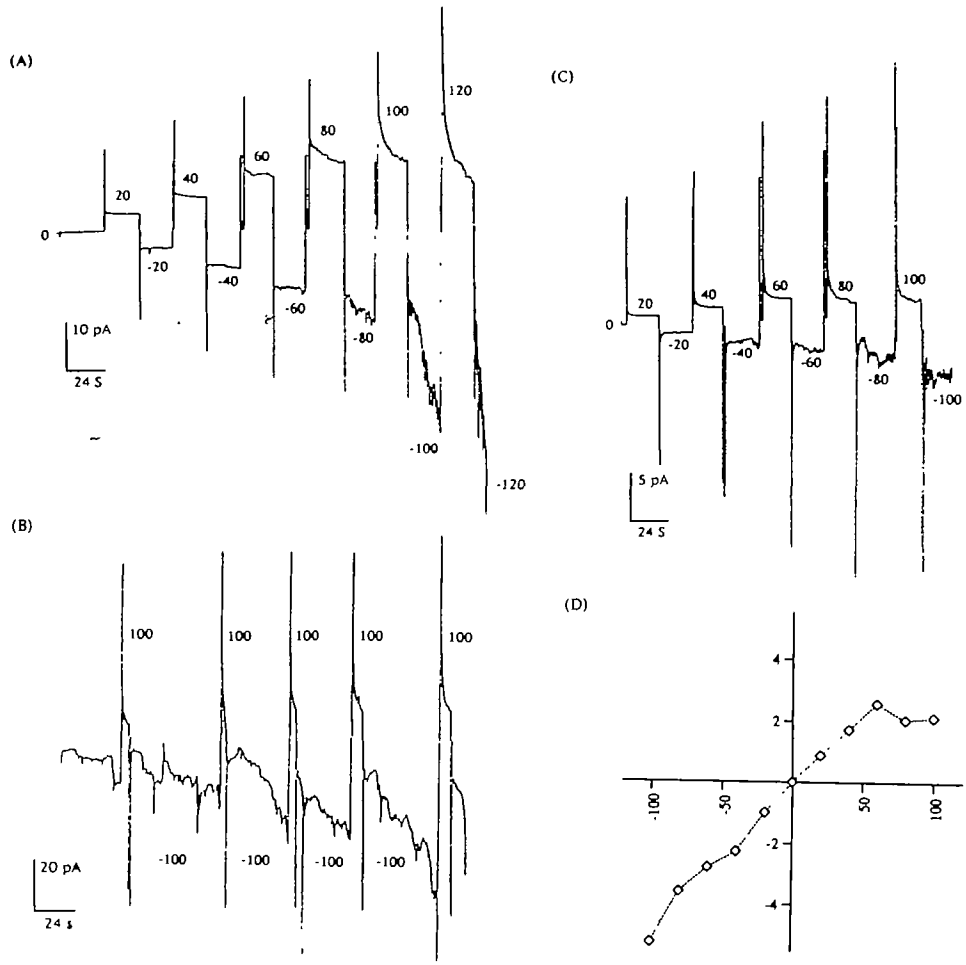

Fig. 6. **Peptide-induced membrane currents under various membrane potentials.** Peptides were added to the *cis* compartment to a final concentration of 66.7 μg/ml. After a significant current was observed under −100 mV with continuous stirring of the *cis* compartment, the membrane current was recorded under the membrane potentials indicated, without either stirring or low-pass filtration. The lipid composition of the membrane was PE : PG : CL (7 : 2 : 1). The composition of the solution in both compartments was KCl 100 mM and Hepes-Tris 10 mM (pH 7.4). *The numbers on the current* trace indicate the membrane potentials in mV. (A) Peptide I. (B, C) Peptide II. (D) I-V relation of the current trace shown in (C). Data were obtained just before membrane potentials were switched; *i.e.* in quasi-stationary states. Abscissa, membrane potential (mV). Ordinate, membrane current (pA).

involves differences in membrane properties, *i.e.* the conditions suitable for resisting the peptide are: (i) a membrane potential of less negative than −40 mV, and (ii) an abundance of choline phospholipids and Chol in the outer leaflets of bilayer membranes. Both conditions are completely satisfied in erythrocytes, whereas bacterial plasma membranes consist of PE, PG, and CL, and show a much deeper membrane potential (more negative than −100 mV). This is also consistent with the results of the liposome experiments.

Enhancement of Ion-Permeability and Membrane Disruption—In the present study, observation of ion-channels with discrete conductance level was initially expected because a cationic amphiphilic peptide with a similar molecular weight (12 amino-acid residues) had been reported to form ion-channels in a planar lipid bilayer (*5*), and also because a correlation between antimicrobial activity and ion channel-forming ability had been shown with various other peptides (*6-18, 43-47*). However, no typical ion-channel currents with discrete levels were observed, and gradual enhancement of ion-permeability, represented by the emergence of erratic currents, was observed (Figs. 5 and 6). This suggests that the ion-channels formed are so short-lived that discrete conductance could not be detected with the time-resolution of the present methods. This result is qualitatively similar to in the case of mellitin in oxidized Chol (*48*), although the magnitude of the macroscopic current was, in many trials, less than that with mellitin, and sudden disruption of membranes was observed more often even when a small conductance level (less than 100 pS) was obtained. Since membrane disruption of a planar lipid bilayer is often observed with channel-forming antimicrobial peptides (*18, 43-47*), both membrane disruption and ion-channel formation are correlated with antimicrobial activity. The capacity for membrane disruption was well represented by the carboxyfluorescein leakage observed in liposome experiments (Figs. 1-4).

Mode of Action (1): Lipid Composition—The phospholipid composition is one of the determining factors of a peptide attack. In this respect, the liposome experiments clearly showed that peptides with potent antimicrobial activity (I, II, and III) specifically interacted with acidic membranes (Fig. 2). Since they do not induce dye leakage from liposomes composed of neutral phospholipids, electrostatic attractions must be involved in the interactions of these cationic peptides with acidic membranes. Therefore, negative charges on the headgroups of the acidic bilayers appear to play an important role in promoting adsorption and subsequent incorporation of the peptides. The effect of negative charges has also been seen with magainins (*49, 50*): interaction with acidic phospholipids was decisively

TABLE III. **Permeability coefficients relative to that for K^+.**

Peptide	Permeability coefficient			
	Na^+	Cl^-	Pi	K^+
I	1.96	14.97	ND[b]	1
	(17.3±0.78 mV, $n=3$)	(16.2±0.34 mV, $n=3$)		
II	1.32	11.67	15.19[a]	1
	(7.05±0.47 mV, $n=4$)	(12.2±0.57 mV, $n=4$)	(−16.6±1.54 mV, $n=3$)	
III	1.62	16.39	ND[b]	1
	(12.3±5.10 mV, $n=3$)	(17.5±9.00 mV, $n=3$)		

Reversal potentials (mean±SD) and the total number of trials (n) are indicated in parentheses. All of the solutions contained 10 mM Hepes-Tris (pH 7.4), except for the NaPi solution (pH 7.0). For Na^+ to K^+, the reversal potential was measured with *cis* 100 mM NaCl and *trans* 100 mM KCl; for K^+ to Cl^-, *cis* 100 mM KCl and *trans* 500 mM KCl; and for Cl^- to Pi, *cis* 100 mM NaCl and *trans* 100 mM NaPi. The lipid composition of the planar bilayer membranes was PE : PG :CL (7 : 2 : 1). [a]The relative permeability coefficient for P_i was determined as follows: The sum of the observed reversal potential and surface potential calculated with the Gouy-Chapman equation was used in the Goldman-Hodgkin-Katz equation which satisfies a zero current condition, $I_{Cl}+I_{Pi^{2-}}+I_{Pi^-}=0$, where $I_{Cl}=-P_{Cl}F^2(E+\phi)/RT\{C_{Cl}/(1-\exp(-F(E+\phi)/RT))\}$, $I_{Pi^{2-}}=-4P_{Pi^{2-}}F^2(E+\phi)/RT\{-C_{Pi^{2-}}\exp(-2F(E+\phi)/RT)/(1-\exp(-2F(E+\phi)/RT))\}$ and $I_{Pi^-}=-P_{Pi^-}F^2(E+\phi)/RT\{-C_{Pi^-}\exp(-F(E+\phi)/RT)/(1-\exp(-F(E+\phi)/RT))\}$. Assuming that $C_{Cl}=100$ mM, $C_{Pi^{2-}}$ and $C_{Pi^-}=33.3$ mM, this equation can be simplified to, $3P_{Cl}-4P_{Pi^{2-}}[\exp(-2FE/RT)/\{1+\exp(-F(E+\phi)/RT)\}]-P_{Pi^-}\exp(-FE/RT)=0$, using the Boltzmann relation. Substituting −16.6 mV for the reversal potential and −77 mV for the surface potential gives $266P_{Cl}-33.3P_{Pi^{2-}}-171P_{Pi^-}=0$. Assuming $33.3P_{Pi^{2-}}+171P_{Pi^-}=204.3P_{Pi}$, $P_{Pi}/P_{Cl}=1.302$ and hence $P_{Pi}/P_K=15.19$. E: reversal potential, ϕ: surface potential in the *trans* compartment, Pi: inorganic phosphates (HPO_4^{2-}, $H_2PO_4^-$). $P_{Pi^{2-}}$, P_{Pi^-}, P_{Pi}, P_{Cl}, P_K: permeability coefficients for HPO_4^{2-}, $H_2PO_4^-$, inorganic phosphates (HPO_4^{2-} and $H_2PO_4^-$), Cl^-, or K^+, $C_{Pi^{2-}}$, C_{Pi^-}, C_{Cl}: concentrations of HPO_4^{2-}, $H_2PO_4^-$, or Cl^-, $I_{Pi^{2-}}$, I_{Pi^-}, I_{Cl}: membrane currents carried by HPO_4^{2-}, $H_2PO_4^-$, or Cl^-. F, R, T: Faraday (96,500 coulombs/equivalent), universal gas constant (8.31 J/K), and temperature (298 K). [b]Not determined.

TABLE IV. **Membrane selectivity with peptide II.**

Membrane	Lipid composition (% w/w)							−40 mV				−100 mV			
	PE	PG	CL	PS	Sph	PC	Chol	−	+	++	n	−	+	++	n
A[a]	70	20	10					1	3	0	4	2	6	2	10
B	50			50				3	2	0	5	0	2	3	5
C	50				50			1	4	0	5	2	0	3	5
D	50					50		3	2	0	5	0	6	1	7
E	50						50	1	3	1	5	3	0	4	7
F	14.3				42.9		42.9	6	0	0	6	4	1	0	5
G	14.3					42.9	42.9	5	0	0	5	4	2	0	6
H	14.3	42.9					42.9	2	3	1	6				
I	14.3		42.9				42.9	1	2	2	5				
J	50	50										1	1	3	5
K	50		50									0	3	2	5
L	50	25	25									1	4	3	8
M	100											2	1	4	7
N[b](outer)	8.9				26.5	41.6	23.0			3	2	0	5		
(inner)	35.4			22.9	5.7	13.1	22.9								
O[c](outer)	14.3				42.9		42.9		5	0	0	5			
(inner)	13.0			8.7	39.1		39.1								
P[d](outer)	8.1				24.2	37.9	29.8		4	0	0	4			
(inner)	32.1			20.7	5.2	11.9	30.1								

Peptide II ($KLKL_5KLK\text{-}NH_2$) was added to the *cis* compartment, and the current signal was passed through a low-pass filter (5 Hz) for 30 min in each trial with continuous stirring of the *cis* compartment. The membrane potential indicates that of the *trans* compartment relative to that of the *cis* compartment. The composition of the solution was symmetric (100 mM KCl, 10 mM Hepes-Tris, pH 7.4). The final concentrations of the peptide: 41.7 μg/ml for (C), (D), (E), (F), (G), (J), (K), (L), and (M) under −40 mV, and 16.7 μg/ml for all other conditions. On the right side of the table, the data are classified into three groups: −, trivial conductance of less than 10 pS was observed within 30 min; +, DC currents greater than 10 pS were observed within 30 min; ++, membrane disruption occurred within 30 min. n=the total number of trials with each lipid composition. [a]Corresponds to the lipid composition of the *E. coli* membrane. [b c d]"Outer" refers to the leaflet of the planar bilayer which faces the *cis* compartment. "Inner" refers to the leaflet which faces the *trans* compartment. [b d]The composition (*24*) and asymmetric distribution (*26*) of phospholipids resemble those of a human erythrocyte.

important in magainin-induced leakage of calcein entrapped in liposomes.

On the other hand, PE, a neutral phospholipid, inhibits the action of magainin II (*51*). This is in contrast to the results with our peptides. Choline phospholipids in the outer leaflet are necessary for resisting the attack of peptide II, whereas membranes composed mainly of PE are easily affected by the peptide in planar lipid bilayers (Table IV). A similar preferential action for PE was also observed in the experiments shown in Figs. 1 and 2, and was confirmed in Fig. 4, where PE and PC with the same acyl chains were used. These results indicate that the headgroups are crucial, and also support the notion that the incorporation of peptides into a membrane is a rate-determining step. The initial target of peptides is the outer leaflet of the membrane, and PC and PE have different headgroups (methyl groups and hydrogens, respectively) which reside at the surface of the bilayer. In addition, since the phase-transition point of PC is lower than that of PE with the same acyl chains, we should expect that membranes containing PC are more fluid than ones containing PE at a given temperature. Therefore, the inhibitory effect

of PC cannot be explained in terms of the hardness of the membrane. Thus, the steric hindrance due to the three methyl groups of PC may play a critical role in obstructing the incorporation of peptides.

Furthermore, membranes containing Chol as well as choline phospholipids are more resistant to attack by peptide II (Table IV). Chol generally makes membranes in a liquid-crystalline state harder (*52*). Therefore, the fluidity of a membrane may also be an important factor: incorporation of the peptide into a membrane, which is a rate-determining step, will be suppressed with harder membranes containing Chol. The same conclusion has been reported previously with cecropins (*15*).

Mode of Action (2): Membrane Potential—A negative membrane potential is also an important factor because a greater negative potential induces a membrane current and causes membrane disruption regardless of the lipid composition (Table IV). In addition, since there have been several reports of peptides which do not affect depolarized membranes (*19, 43-45, 47*), a negative potential may be important for cationic amphiphilic peptides of this type to affect bilayer membranes. From a physico-chemical point of view, a negative membrane potential may facilitate the incorporation of cationic peptides into the hydrophobic core of bilayer membranes by electrophoresis. This notion is supported by the voltage-dependency shown in Fig. 6, which indicates that the more negative the membrane potential, the faster the increase in conductance, and the more positive the membrane potential, the faster the decrease in conductance. Therefore, the cationic properties of peptides contribute not only to electrostatic interactions with bacterial acidic membranes, but also to their incorporation into the hydrophobic core of the membranes.

Amphiphilicity of Peptides—For a better understanding of antimicrobial activity and selective toxicity, the structural aspects of the peptides can be discussed. Based on the results in Figs. 2 and 3, the affinity for acidic membranes depends on the hydrophilicity of the peptides. Peptides I, II, and III are more hydrophilic than peptides IV and VI, and the more hydrophilic the peptide, the more specifically it interacts with acidic lipid bilayers. Conversely, the more hydrophobic a peptide is, the more indiscriminately it interacts with lipid bilayers. Therefore, more-hydrophilic peptides seem to be desirable for this purpose. However, extremely hydrophilic peptides are not distributed in the membrane phase. In fact, the most hydrophilic peptide in Figs. 2 and 3, peptide VII, does not interact with lipid bilayers. These results suggest that an amphiphilic structure is crucial for the desired interaction, that the optimal amphiphilicity for selective toxicity can be estimated by quantifying the hydrophilicity.

Mechanism of Bacterial Death—The influx of protons and sodium, and the efflux of potassium and other electrolytes are anticipated to be physiological effects of enhanced ion-permeability in bacterial cells because there are steep concentration gradients of these electrolytes across bacterial membranes (*19, 20*). This leads to the assumption that the bacteria should be affected, since elimination of the membrane potential and the proton gradient, in principle, should adversely affect ATP synthesis. However, it has been shown experimentally using *E. coli* cells that the addition of a protonophore (TCS, 3,3,4′,5-tetrachlorosalicylanilide) does not lead to significant depletion of cytoplasmic ATP, whereas the application of colicins and phage T5, which are channel-forming peptides, produces drastic cytoplasmic ATP depletion within a few minutes (*53*). This suggests that the degeneration of ATP synthesis alone cannot explain the cytoplasmic ATP depletion caused by channel-forming peptides.

The addition of peptide II to *E. coli* rapidly decreased the ATP level in a few minutes, similar to colicins and phage T5 (*54*). This rapid decrease clearly paralleled the time course of bacterial death. This indicates that depletion of the ATP pool is definitely related to a fatal event in bacteria, and results from some mechanism other than the degeneration of ATP synthesis. The outflow of ATP itself is also not the mechanism because a decrease in the total amount of ATP was observed in both the cytoplasm and the buffer solution. Even if ATP flows out from the cytoplasm, the total amount does not change. Along these lines, it should be noted that inorganic phosphates can pass through a doped membrane very rapidly (Table III). The physiological consequence of the outflow of inorganic phosphates from the cytoplasm is the promotion of the reaction, $ATP+H_2O \rightarrow ADP+Pi$ (Pi: $HPO_4^{2-}/H_2PO_4^-$). BF_0F_1 ATPase does not seem to be essential for this reaction because if it is significantly active, application of a protonophore can give rise to rapid hydrolysis of ATP, which is the inverse of ATP synthesis. Therefore, this reaction may be catalyzed by various other ATPases (*53*). It is possible that the loss of inorganic phosphates is responsible for the rapid ATP depletion and bacterial death.

In addition, the surface activity of the peptides may disrupt bacterial membranes, and thereby cause bacterial lysis. Since the peptides strongly perturb liposomes and often disrupt the planar lipid bilayer, surface activity could be another factor in bacterial death. However, the cell structure is maintained because a cytoplasmic enzyme, β-galactosidase, does not leak out even after attack by peptide II (*54*). This clearly indicates that the cell membrane is essentially maintained after a peptide's action. Therefore, surface activity may not play an important role in bacterial lysis. Instead, the outflow of inorganic phosphates facilitated by the peptide seems to be a lethal event in bacteria.

Conclusion—In the present study, we examined the capacity for membrane-perturbation and ion-channel formation of peptides derived from the active moiety of sapecin B by examining carboxyfluorescein leakage from liposomes and by the planar lipid bilayer technique. Both studies showed that the peptides target bacterial membranes, and that the selective toxicity of the peptides arises from differences between bacterial and eukaryotic cell membranes. This latter finding is an interesting example of how membrane properties affect the activity of peptides in lipid bilayers. This strongly suggests that biological membranes with different properties can modulate the activity of physiological ion-channels in various ways. Since membrane-targeting peptides with selective toxicity are rare, and are possible sources for drugs, the present study may contribute to the development of therapeutic drugs.

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