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Ion-channels formed by hypelcins, antibiotic peptides, in planar bilayer lipid membranes

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Abstract

Ion-channel properties of native hypelcins (HP) A-I, A-V and B-V isolated from *Hypocrea peltata* and a synthetic analog, HP-A-Pheol, were studied in planar bilayer lipid membranes by a single-channel recording technique. The native and synthetic hypelcins formed ion-channels with three conductance levels for 3 mole dm^{-3} KCl: ≤ 0.09 nS at 225 mV (level 0, only detectable at voltages above 200 mV), ≈ 0.6 nS at 150 mV (level 1, most common level) and ≈ 3 nS at 150 mV (level 2). The effects of the C-terminal aminoalcohol on the channel properties were examined with HP-A-I, HP-A-V and HP-A-Pheol, whose C-termini are leucinol (Leuol), isoleucinol (Ileol) and phenylalaninol (Pheol), respectively. The substitution of Pheol for Leuol and Ileol prolonged the open channel lifetime. A comparison of HP-A-V (Gln18) and HP-B-V (Glu18) indicated that the carboxyl group at position 18 increased both the open channel lifetime and the magnitude of unitary channel conductance at each conductance level. The pores of level 1 showed poor ion-selectivity for K^+ over Cl^-. The selectivity order of alkali metal cations was $Rb \geq Cs \geq K > Na > Li$ for level 1 and $Cs > Rb > K > Na > Li$ for level 0. The unitary current-voltage characteristics showed non-linear relationships, which were simulated by a Nernst-Planck approach with a simple barrier model.

Keywords: Ion-channel; Hypelcin; Single channel measurement; Bilayer lipid membrane; Ion selectivity; Peptaibol

1. Introduction

Hypelcin, isolated from *Hypocrea peltata*, is a mixture of several peptides with similar amino acid sequences, which have been determined by Fujita and Matsuura [1–6]. These are hydrophobic peptides of 20 amino acid residues that are rich in α-aminoisobutyric acid (Aib, U) and that have an acetylated N-terminus and an aminoalcohol at the C-terminus. Hypelcins are, therefore, regarded as a family of peptaibols like alamethicin [7,8] that is a well-known ion-channel forming peptide [9–13].

Hypelcins were found to inhibit the growth of various fungi and bacteria [1,2] and to uncouple oxidative phosphorylation in mitochondria [14]. These biological activities may result from increased ion permeability of the membranes. Matsuzaki et al. demonstrated that hypelcins induced the leakage of calcein (a fluorescent dye) entrapped in phosphatidylcholine (PC) vesicles [15]. The leakage could be explained in terms of the perturbation or disordering of bilayer lipid membranes, because hypelcins simultaneously induced the fusion of PC small unilamellar

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Table 1

		1	9	12	20
HP-A-I	Ac-	U-P-U-A-U-U-Q-U-	*L*-U-G-	*U*-U-P-V-U-U-Q-Q-	*Leuol*
HP-A-V	Ac-	U-P-U-A-U-U-Q-U-	*L*-U-G-	*U*-U-P-V-U-U-Q-Q-	*Ileol*
HP-A-Pheol	Ac-	U-P-U-A-U-U-Q-U-	*L*-U-G-	*U*-U-P-V-U-U-Q-Q-	Pheol
HP-B-V	Ac-	U-P-U-A-U-U-Q-U-	*L*-U-G-	*U*-U-P-V-U-U-E-Q-	*Ileol*
Alm Rf50	Ac-	U-P-U-A-U-U-Q-U-	V-U-G-	L-U-P-V-U-U-Q-Q-	Pheol
Alm Rf30	Ac-	U-P-U-A-U-U-Q-U-	V-U-G-	L-U-P-V-U-U-E-Q-	Pheol

U: α-aminoisobutyric acid.
Leuol, leucinol; Ileol, isoleucinol; Pheol, phenylalaninol.
The amino acid residues of hypelcins that differ from those of alamethicin are in italics.

vesicles [16]. However, there has been no evidence that hypelcins form a defined ion-channel structure in membranes.

Thus, the purpose of this study was to confirm the ion-channel formation of hypelcins in planar bilayer lipid membranes and to characterize the ion-channels, using a single-channel recording technique. We examined four kinds of hypelcins (HP-A-I, HP-A-V, HP-B-V and HP-A-V-Pheol), whose sequences are the same as that of alamethicin (Alm Rf50 and Alm Rf30) except for the amino acid residues at positions 9 and 12 and the C-terminal aminoalcohol (see Table 1). Comparison among HP-A-I, HP-A-V and HP-A-V-Pheol, which have different C-terminal aminoalcohols, enabled us to elucidate the role of the C-terminal aminoalcohol in the ion-channel formation. The effect of the carboxyl group with a negative charge at position 18 on the ion-channel properties was also examined by comparing HP-A-V (Q18) and HP-B-V (E18). The current-voltage relationship and ion selectivity of the ion-channels at the single-channel level are discussed.

2. Materials and methods

2.1. Preparation of peptides

In this study we examined three native hypelcins (HP-A-I, HP-A-V and HP-B-V) and a synthetic analog (HP-A-Pheol). The native hypelcins were isolated from *Hypoclea peltata* and purified by reversed-phase HPLC as described previously [4,6]. HP-A-Pheol was synthesized by the solution-phase method and purified by reversed-phase HPLC. As controls, we used alamethicin Rf50 (Alm Rf50) synthesized by Akagi and Nishino (Kyushu Institute of Technology) and alamethicin (Alm (Sigma)) purchased from Sigma, whose main component is alamethicin Rf30 (Alm Rf30).

2.2. Single-channel recording with planar bilayer lipid membranes

Planar bilayer lipid membranes were created on a hole with a diameter of about 100 μm in a thin (25 μm) sheet of Teflon between two chambers by the monolayer folding technique [17,18]. The Teflon sheet surrounding the hole was pretreated with a drop of 0.5% hexadecane in hexane to generate a hydrophobic environment for bilayer formation. Monolayers were formed by applying a few μl of 10 g dm^{-3} diphytanoyl PC (purchased from Avanti Polar Lipids) in hexane to the surfaces of aqueous solutions in both the chambers below the hole. A bilayer is formed across the hole by successively raising the solution levels over the hole. An aliquot of 10 mg dm^{-3} peptide in ethanol is added to the electrolyte solution on one side of the membrane (*cis*-side) to give a final concentration of 1–100 μg dm^{-3}.

A pair of Ag-AgCl electrodes were used for current measurements. The *cis*-side and *trans*-side electrodes were connected to a DC voltage source and to the virtual ground of a homemade current amplifier, respectively. Hence, a positive voltage means that the *cis*-side is positive relative to the *trans*-side and a positive current means that cations flow from the *cis*-side to the *trans*-side. The output voltages of the current amplifier (amplification, 10^9 V/A) were recorded at sampling frequency of 50 kHz with a DR-F2a digital recorder (TEAC Corporation). All measurements were performed at 25°C.

The applied voltage is equal to the membrane potential $\Delta\varphi_m$ with a symmetric solution because the electrode potentials of the two electrodes cancel each other out. Correction for the electrode potentials is, however, requisite with an asymmetric solution, and is made by subtracting the electrode potential difference $\Delta\varphi_{el}$ from the applied voltage V.

$$\Delta\varphi_m = V - \Delta\varphi_{el}, \quad (1)$$

$$\Delta\varphi_{el} = \varphi_{cis} - \varphi_{trans} = \frac{RT}{F}\ln\frac{a_{trans}}{a_{cis}}, \quad (2)$$

where φ_{cis} and φ_{trans} are the electrode potentials of the *cis*- and *trans*-side electrodes, respectively, and a_{cis} and a_{trans} are the activities of chloride ions of the *cis*- and *trans*-side solutions, respectively.

3. Results

3.1. Single-channel recordings

Current fluctuations were measured with diphytanoyl PC bilayer membranes exposed to HP-A-I, HP-A-V, HP-B-V and HP-A-Pheol in 3 mol dm^{-3} chloride salts of alkali metals. Fig. 1 shows examples of HP-A-V and HP-V-B measured in 3 mole dm^{-3} KCl, together with those of Alm Rf50 and Alm (Sigma) as controls. The data of HP-A-I and HP-A-Pheol (not shown) were very similar to those of HP-A-V. All the hypelcins showed current fluctuations characteristic of ion-channels, i.e., current pulses with discrete conductance levels occurred randomly. The current fluctuations were observed only at positive voltages. These results provide strong evidence that the hypelcins form ion-channels like alamethicin. However, as can be seen from the conductance histograms (Fig. 1, right), the hypelcins have only a few conductance levels, i.e., a major conductance level (level 1) and a minor one (level 2), in contrast to alamethicin that showed more than three conductance levels.

In addition to levels 1 and 2, when the applied voltage exceeded about 200 mV, a lower conductance level (level 0) was detectable in 3 mol dm^{-3} chloride salts of alkali metals for all hypelcins. Fig. 2 shows an example of HP-B-V measured in 3 mol dm^{-3} CsCl. This conductance level is similar to the lowest

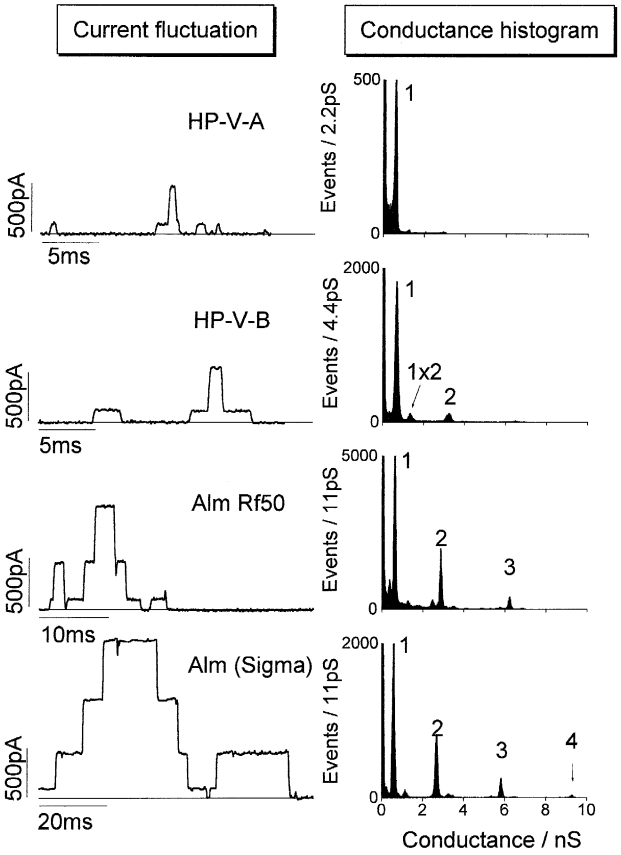

Fig. 1. Single-channel current recordings. Current fluctuations were measured in 3 mol dm^{-3} KCl for HP-A-V, HP-B-V, Alm Rf50 and Alm (Sigma). The peptide concentrations and the applied voltages are: 10 μg dm^{-3}, 164 mV for HP-A-V; 7.5 μg dm^{-3}, 170 mV for HP-B-V; 120 μg dm^{-3}, 173 mV for Alm Rf50; 2 μg dm^{-3}, 176 mV for Alm (Sigma). The conductance histograms, which are so-called 'all-points' histograms, were calculated from the current fluctuation data. The figures in the conductance histograms indicate conductance level numbers.

level reported for alamethicin by Hanke and Boheim [19]. Consequently, hypelcins form ion-channels with three conductance levels in total. In the subsequent studies we confined our attention to the pores of the most common conductance level (level 1).

The mean lifetime of channel opening was estimated for level 1 by fitting a single exponential decay function to the duration histograms. The estimated values of the mean lifetime in 3 mol dm^{-3} KCl were as follows: 0.8 ms for HP-A-I, 0.7 ms for HP-A-V, 1.2 ms for HP-A-Pheol and 1.1 ms for HP-B-V. These results suggest that the substitution of phenylalaninol (HP-A-Pheol) for leucinol (HP-A-I)

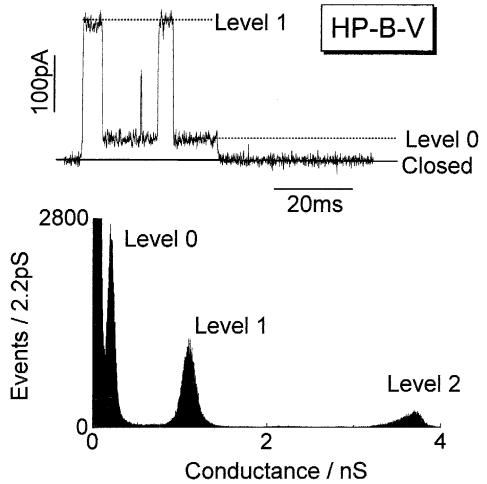

Fig. 2. The lowest conductance state (level 0) for HP-B-V measured at 225 mV in 3 mol dm^{-3} CsCl.

or isoleucinol (HP-A-V) at the C-terminal prolonged the open lifetime. Further, a comparison of HP-A-V (Q18) and HP-B-V (E18) indicates that the carboxyl group with a negative charge at position 18 is also important for prolongation of the open lifetime.

3.2. Unitary current-voltage characteristics

Fig. 3 shows the unitary current I as a function of applied voltage V for HP-A-V; a non-ohmic relationship is clearly seen. Similar non-ohmic properties were reported for alamethicin by Eisenberg et al. [9] and Gordon and Haydon [10], although the nonlinearity was not discussed in detail in their papers.

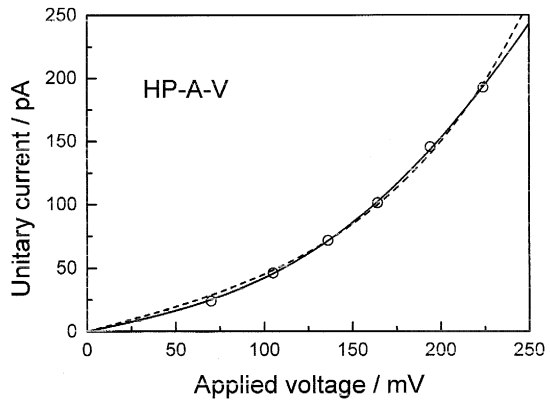

Fig. 3. Unitary current-voltage characteristic of level 1 pore of HP-A-V in 3 mole dm^{-3} KCl. The broken line was calculated from $I = \alpha \sinh \beta V$ with $\alpha = 34.2$ pA and $\beta = 0.0109$ mV^{-1}, and the solid line from Eq. (3) with B = 277 pA and $\phi = 3.48$.

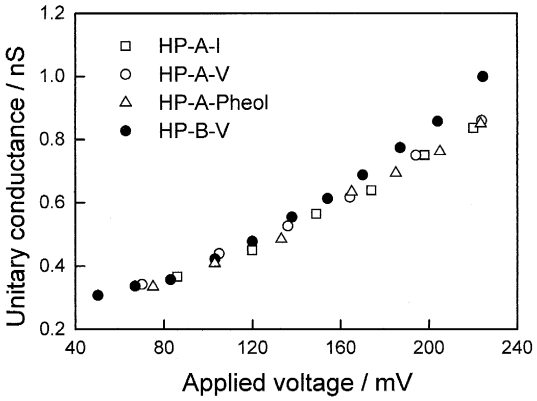

Fig. 4. Voltage dependence of unitary conductance for level 1 pores of HP-A-I, HP-A-V, HP-A-Pheol and HP-B-V.

The I-V curves were approximately expressed by a hyperbolic sine function, i.e., $I = \alpha \sinh \beta V$, where α and β are parameters to be adjusted. The non-ohmic I-V characteristic means that the unitary conductance also depends on applied voltage, as shown in Fig. 4. In Fig. 4, it should be noted that the negatively charged HP-B-V (E18) showed higher conductance values than the other uncharged hypelcins with Q18 at voltages above about 150 mV.

Fig. 5 shows the unitary conductance for Level 1 as a function of the KCl concentration of the bathing solution. The unitary conductance increased with increasing KCl concentration and there was no saturation at least up to 3 mol dm^{-3}, as in the case of alamethicin [9].

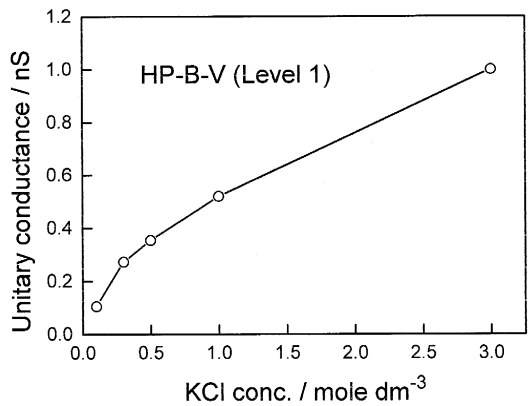

Fig. 5. KCl concentration dependence of unitary conductance at 225 mV for level 1 pore of HP-B-V.

3.3. Ion-selectivity

The ion selectivity for the pore of Level 1 between K^+ and Cl^- was examined by comparing the unitary conductances measured at different symmetric and asymmetric concentrations (in mol dm^{-3}, *cis*/*trans*): 3/3, 3/1, 1/3, and 1/1. Since the channel is formed only at positive voltages (*cis*-positive), if the pore were fully K^+-selective, the unitary conductance G_{3-1} for 3(*cis*)/1(*trans*) and G_{1-3} for 1(*cis*)/3(*trans*) would coincide with the unitary conductance G_{3-3} for 3(*cis*)/3(*trans*) and G_{1-1} for 1(*cis*)/1(*trans*), respectively. If there were no selectivity between K^+ and Cl^-, G_{3-1} would lie between G_{3-3} and G_{1-1}, and be equal to G_{1-3}. As can be seen in Fig. 6, both the HP-A-V and HP-B-V channels showed poor selectivity for K^+ over Cl^-.

The order of selectivity for alkali metal cations was examined by comparing the unitary conductances with 3 mol dm^{-3} symmetric solutions of KCl, NaCl, LiCl, RbCl, and CsCl at a fixed voltage. The selectivity order for HP-B-V was Rb ≈ Cs ≥ K > Na > Li, which is close to the order of ionic mobility in solution, whereas the selectivity order for HP-A-V (Rb > Cs ≥ K > Na > Li) was slightly different.

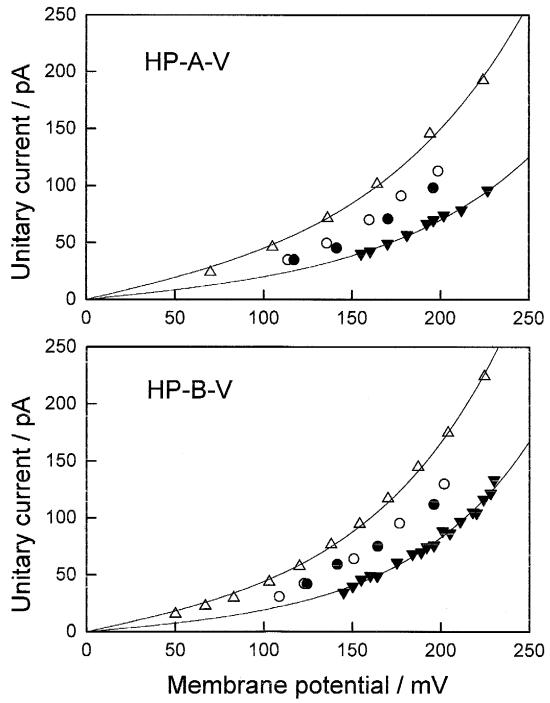

Fig. 6. Unitary current-voltage curves for level 1 of HP-A-V and HP-B-V measured at symmetric and asymmetric KCl concentrations (in mol dm^{-3}, *cis*/*trans*): △, 3/3; ○, 3/1; ●, 1/3; ▼, 1/1. The solid curves were calculated from $I = \alpha \sinh \beta V$ with the best-fit parameters.

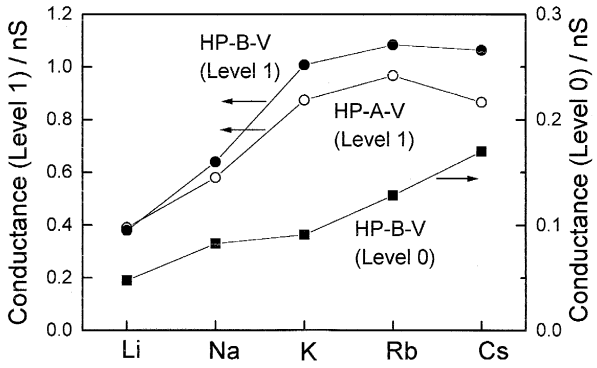

Fig. 7. Unitary conductance of level 0 and level 1 for HP-A-V and HP-B-V obtained at 225 mV with 3 mol dm^{-3} symmetric solutions of LiCl, NaCl, KCl, RbCl and CsCl.

On the other hand, the selectivity order for the pore of Level 0 of HP-B-V (E18) was Cs > Rb > K > Na > Li at 225 mV. For uncharged hypelcins (HP-A-I, HP-A-V and HP-A-Pheol), however, the selectivity order could not be determined because their conductance values were very small, and close to the limitation of the measurements. The selectivity order for HP-B-V is different from that for the lowest conductance state of the alamethicin Rf30 (E18) (see Fig. 7) [19].

4. Discussion

4.1. Ion-channel formation

In this study we demonstrated that hypelcins form ion-channels in planar bilayer lipid membranes with a few conductance levels of ≤ 0.09 nS (level 0, at 225 mV), ≈ 0.6 nS (level 1, at 150 mV) and ≈ 3 nS (level 2, at 150 mV) in 3 mol dm^{-3} KCl. Level 1 was the most common conductance level and level 0 was detectable only at voltages above 200 mV. The number of conductance levels of hypelcins was less than that of alamethicins Rf30 and Rf50 [9–13] and was not affected by replacement of the C-terminal Ileol and Leuol by Pheol. Since the sequence difference between HP-A-Pheol and alamethicin Rf50 is

only in the two hydrophobic residues at positions 9 and 12 (see Table 1), the high conductance levels (corresponding to large pores) found for alamethicin could be stabilized by the hydrophobic residues, which may interact with lipids or hydrophobic residues of the adjacent helices.

The C-terminal substitution of Pheol for Leuol and Ileol did not alter the multiplicity of conductance levels, but increased the open channel lifetime. A similar C-terminal effect was reported for trichorzianins by Duclohier et al. [20], i.e., trichorzianin B-IIIc (Trpol) formed ion-channels with a longer open lifetime than trichorzianin B-VII (Pheol). However, Molle et al. [21] found that C-terminal residues (Phe-NH_2, Pheol, Trp-NH_2) did not influence the open lifetime of channels of synthetic alamethicin analogs in which all Aib residues were substituted by leucine residues. The role of the C-terminal aminoalcohol in ion-channel formation is still controversial.

According to a simple macroscopic cylindrical model [12,13], the alamethicin channels of the lowest (level 0) and the next lowest conductance level (level 1) correspond to bundles of three and four peptides, respectively. Recently, this has been directly confirmed with cyclic template-assembled alamethicins [22]. Since the ion-channel behavior of hypelcins is similar to that of alamethicin, the major conductance level (level 1) of hypelcins may correspond to the pore made up of a bundle of four peptide helices.

The replacement of Q18 by E18 prolonged the lifetime of the open channels and increased the magnitude of the unitary conductance at each level. A similar tendency was found for trichocellins A (Q18) and B (E18) (Wada S., Iida A., Asami K., Tachikawa E. and Fujita T., unpublished data) and for trichorzianin A-VII (Q18) and B-VII (E18) [20]. The carboxyl group at position 18 may stabilize the ion-channel structure and the repulsion between negative charges of E18 of parallel helices in a bundle could expand the interhelical distance to make the pore larger.

4.2. I-V characteristics

The unitary current-voltage curves of hypelcin channels showed non-ohmic characteristics. Usually, ion transport through membranes has been expressed by the Nernst-Planck continuum model and the rate-theory model. We attempted to interpret the non-ohmic I-V curves in terms of the Nernst-Planck continuum model that provides a simple description of the current due to the ion flux through a pore in terms of parameters such as diffusion coefficient D, the effective cross-sectional area A of the pore, and the energy profile of the ion in the pore. We assumed a constant field and a simple triangular profile as the energy barrier in a pore of length d. We confine our discussion to the conductance level 1 of the hypelcin channels in a symmetric KCl solution of concentration c. Since there was poor selectivity between K^+ and Cl^-, the current for K^+ (I_K) is almost equal to that for Cl^- (I_{Cl}). Thus, following the derivation described by Hall et al. [23] and Hladkey [24], the total current is obtained as:

$$I = I_K + I_{Cl} = B\frac{\sinh(U/2)}{S} \tag{3}$$

where $B = 8AFDc/d$, $U = VF/RT$, R = gas constant, F = Faraday constant, T = absolute temperature, V is the voltage at the *cis*-side and S is a function of U and the height ϕ of the triangular barrier that is a dimensionless variable expressed in RT unit. The quantity S is given by

$$S = \frac{\exp\phi - \exp(U/2)}{\phi - U/2} + \frac{\exp\phi - \exp(-U/2)}{\phi + U/2}. \tag{4}$$

Eq. (3) provided a much better simulation of the I-V curve shown in Fig. 3 than did a hyperbolic sine function ($I = \alpha \sinh \beta V$)). The height of the triangular barrier was estimated to be 3.5RT (2.1 kcal/mol), which is somewhat lower than the image energy calculated for an ion in an aqueous cylindrical pore in a lipid membrane [25–27]. For more detailed discussion of the I-V curve, it is necessary to have a knowledge of the potential energy profile in the ion-channel which would be obtained by sophisticated computer calculation [28,29].

4.3. Ion-selectivity

The ion-selectivity of peptaibol channels has been little studied at the single-channel level. In general, ion-selectivity can be determined from channel conductances and more quantitatively from reversal potentials. However, the reversal potential method is

difficult to apply to single channels whose formation is strongly voltage-dependent, except in the case of long-lived channels, for which Kienker et al. succeeded in measuring the reversal potentials at the single-channel level using fast voltage pulses and ramps [30,31]. For the hypelcin channels with short open lifetimes, we employed the conductance method.

The pore of level 1 showed poor selectivity for K^+ over Cl^- in both cases of HP-A-V (uncharged) and HP-B-V (negatively charged). Hall et al. examined the ion-selectivity of alamethicin channels at the macroscopic level and found that they were slightly cation-selective in KCl solutions and that the negative charge at position 18 was not responsible for the cation selectivity [32].

The order of selectivity for alkali metal cations at level 1 was slightly affected by the negative charge of E18, being similar to the order of the ionic mobility in aqueous solution. This suggests that the hydrated ions move through the pore in the same manner as in aqueous solution. However, the pore of level 0 discriminated among K, Rb and Cs, which have the same mobility in aqueous solution, indicating a short-range interaction of the ions with the wall of the narrow pore.

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