Effect of a Dynorphin A Analog, E2078, on Phospholipid Membrane Properties

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The effect of the Dynorphin A analog, E2078, on the physicochemical properties of lipid membranes was investigated. E2078 induces the leakage of calcein, especially out of negatively-charged vesicles. The peptide binds to dipalmitoylphosphatidylglycerol sonicated vesicles according to the Langmuir isotherm, with a binding constant of $4.0 \times 10^5 \text{ M}^{-1}$ and a binding-site number of 0.14 per lipid molecule. The leakage seems to occur at a critical binding-site number of approx. 0.025 per lipid molecule. In addition, E2078 increased the membrane fluidity of the acidic lipid bilayer. In conclusion, E2078 interacts with acidic lipids through electrostatic interactions, inducing leakage and increasing membrane fluidity.

Key words Dynorphin A; membrane permeability; lipid charge; binding isotherm

Peptide–lipid interactions have been studied not only to reveal the action mechanisms of bioactive peptides but also to understand the organization principles of biomembranes and lipoproteins composed of lipids and proteins. Many amphiphilic or hydrophobic peptides, both natural and synthetic, are known to interact with lipid membranes, causing changes in the physicochemical properties of the membranes (e.g. the permeability and the phase transition) or inducing the fusion of the membranes.

Dynorphin A (Dyn) is a 17 amino acid opioid peptide (Fig. 1a) containing the sequence of [Leu]enkephaline at its N-terminal.^{1,2)} Recent researchers have focused on the synthesis of the Dyn analog. Yoshino *et al.* have reported that the Dyn analog, E2078, (Fig. 1b) has opioid activity similar to that of Dyn.³⁾ E2078 not only retains opioid receptor selectivity similar to that of Dyn, but also produces a more potent analgesic effect than morphine even when administered subcutaneously into mice.⁴⁾

E2078 is classified as a positively charged peptide. The interaction of positively charged peptides, such as magainin 1⁵⁾ and mastoparan⁶⁾, with phospholipid membranes was investigated. These positively charged peptides are held in amphiphilic helices to interact preferentially with acidic lipids and perturb the membrane functions. It has also been reported that Dyn induces more extensive leakage of contents from phosphatidylserine than from phosphatidylcholine vesicles.⁷⁾ The objective of this study is to clarify the effect of E2078 on the lipid membranes such as the neutral lipid (dipalmitoylphosphatidylcholine, DPPC) and the acidic one (dipalmitoylphosphatidylglycerol, DPPG). The phase transition behavior of these lipids has been widely studied^{8,9)} and we expected that the effect of E2078 on the phase transition of these lipids could be observed more easily than those of other phospholipids. In these systems, we examined the effects of E2078 on membrane permeability, fluidity and gel to liquid crystalline phase transition temperature of these lipids using fluorescence techniques.

MATERIALS AND METHODS

Materials E2078 was obtained from Eisai Chemical Co., Ltd. (Ibaraki, Japan). L- α -Dipalmitoylphosphatidylcholine

(DPPC) and L- α -dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Calcein (3,3'-bis[*N,N*-bis(carboxymethyl)amino-methyl]-fluorescein) was purchased from Dojin (Kumamoto, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Vesicles DPPC and DPPG were dissolved in chloroform. After evaporation of the solvent, 70 mM calcein solution (pH 7.0) was added to give a final lipid concentration of 5 mM. The mixtures were sonicated for 30 min under a stream of nitrogen gas at 50 °C. A probe type sonicator, model UD-200 (Tomy Seiko Co., Ltd., Tokyo, Japan) was used at a power setting of 100 W. Untrapped calcein was removed by gel filtration (Sephadex G-50, 2 cm $\times$ 35 cm column, 5 mM Tris–150 mM NaCl buffer (pH 7.0) was an eluent). The separated vesicular fraction was appropriately diluted with the buffer, then mixed with calcein-free vesicles for obtaining a desired lipid concentration. The lipid concentration was determined by phosphorous analysis.¹⁰⁾

Leakage Measurement The vesicular suspension was mixed with an E2078 buffer solution in a cuvette. The leakage of calcein out of the vesicles was monitored by measuring the fluorescence intensity at 520 nm (excitation at 490 nm) on a Hitachi F-4500 spectrofluorometer.¹¹⁾ The fluorescence intensity corresponding to 100% leakage was determined by the addition of 10% (v/v) Triton X-100 solution (0.1 ml) to 3 ml of the sample. The experiments were carried out at 25 °C.

Effect of E2078 on the Membrane Fluidity and Phase

(a) Dynorphin A

(b) E2078

Fig. 1. Chemical Structure of Dy and Its Analog, E2078

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Fig. 2. Effects of Lipid Charge on the Leakage of Entrapped Calcein from Sonicated Vesicles

Percent leakage is plotted as a function of time. E2078 concentration: 0 μM for DPPC ($\circ$); 50 μM for DPPC ($\bullet$); 0 μM for DPPG ($\triangle$); 50 μM for DPPG ($\blacktriangle$). The lipid concentrations are 200 μM .

Transition Temperature The effects of E2078 on membrane fluidity and gel-to-liquid-crystalline phase transition of the DPPG vesicle were determined using a fluorescence polarization technique (probe: DPH) as reported by Iwamoto *et al.*¹²⁾ DPH was added at 1 mol% of the lipid. All fluorescence measurements were carried out using a Model F-4500 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan) equipped with a thermoregulated cell compartment, Atago Coolnics Model REX-C10 (Atago Co., Ltd., Tokyo, Japan). The degree of polarization (P) was calculated using the following equation:

$$P = (I_{VV} - C_f \cdot I_{VH}) / (I_{VV} + C_f \cdot I_{VH})$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($=I_{HV}/I_{HH}$) is the grating correction factor.

RESULTS AND DISCUSSION

Effect of E2078 on the Membrane Permeability of DPPC and DPPG Vesicles Figure 2 shows the effects of lipid charge on the leakage rate. The addition of 50 μM E2078 to the DPPG vesicles leads to the leakage of entrapped calcein. On the other hand, the leakage did not occur in the DPPC vesicles. The peptide contains two Arg, bearing a net positive charge at pH 7.0. Accordingly, the peptide-lipid electrostatic interactions will play an important role in the observed permeability change of the lipid bilayers. These features of E2078 are similar to Dyn.⁷⁾

We further investigated calcein leakage using the DPPG vesicles to evaluate (1) the affinity of the peptide to the vesicles and (2) the amount of membrane-bound E2078 necessary for the leakage. These factors can be estimated by analyzing both the peptide and lipid concentration dependency of the leakage rate.^{13,14)} Figure 3a shows the dose-response curves for the leakage rate (expressed as % leakage for initial 1 min) at different concentrations. An increase in lipid concentration shifts the curve to the right, suggesting that the binding of E2078 to the vesicles is involved in the leakage process. Thus, we assume that the leakage rate is determined only by the amount of the membrane-bound peptide per lipid

Fig. 3. (a) Dependence of Calcein Leakage Rate on E2078 and Lipid Concentrations for DPPG Sonicated Vesicles

The calcein leakage rate, defined as the percentage of leakage for the initial first minutes, is plotted as a function of $[P]_0$ at different lipid concentrations ($[L]$: $\bullet$, 80 μM ; $\blacktriangle$, 230 μM ; $\blacksquare$, 360 μM).

(b) Estimation of Free and Membrane-Bound E2078 Concentrations at Several Leakage Rates

Three pairs of $[P]_0$ and $[L]$ values, where a given leakage rate was observed were obtained from Fig 3(a). $[P]_0$ is plotted against $[L]$ according to the equation in the text. The free peptide concentration, $[P]_f$, and the amount of membrane-bound peptide concentration, r , were evaluated from the intercept and the slope, respectively. The leakage rate: $\bullet$, 20% $\cdot \text{min}^{-1}$; $\blacktriangle$, 40% $\cdot \text{min}^{-1}$; $\blacksquare$, 60% $\cdot \text{min}^{-1}$.

(r). The amount of r can be connected with the experimental conditions (the total peptide concentration $[P]_0$, and the lipid concentration $[L]$ through a material balance equation:

$$[P]_0 = [P]_f + r[L]$$

where $[P]_f$ stands for the free peptide concentration. The r value and the corresponding $[P]_f$ value can thus be estimated with three sets of $[P]_0$ and $[L]$, obtained from Fig. 3a, at which a given leakage rate is observed. A $[P]_0$ versus $[L]$ plot at any given rate will give a linear relation from which we can estimate $[P]_f$ and r , as Fig. 3b shows. A binding isotherm from the evaluated $[P]_f$ and r values is illustrated in Fig. 4a. The isotherm can be well explained on the basis of the Langmuir equation¹⁵⁾ by using an r_∞ (number of binding sites per lipid) value of 0.14 and a K (binding constant) value of $4.0 \times 10^5 \text{ M}^{-1}$ (the solid line in Fig. 4a). Figure 4b depicts the relationship between the leakage rate and r , suggesting the existence of a critical r value for the leakage at around 0.025. The obtained K ($4.0 \times 10^5 \text{ M}^{-1}$) and r_∞ (0.14) values are similar to those of other peptide-acidic phospholipid systems such as salmon calcitonin-dimyristoylphosphatidylglycerol system ($K = 1.0 \times 10^5 \text{ M}^{-1}$ and $r_\infty = 0.2$)⁷⁾ and magainin 1-bovine brain phosphatidylserine systems ($K = 3.8 \times 10^5 \text{ M}^{-1}$ and $r_\infty = 0.1$).⁵⁾

Fig. 4. (a) Binding Isotherm of E2078 to DPPG Sonicated Vesicles at 25 °C

The solid line is a curve fit according to the Langmuir isotherm with a binding constant of $4.0 \times 10^5 \text{ M}^{-1}$ and a minimum site number of 0.14 per lipid molecule.

(b) Relationship between the Leakage Rate and the Amount of DPPG-Bound E2078

The peptide is expected to change the permeability of the lipid membranes in two steps, *i.e.*, the binding to the membrane and the following perturbation. Accordingly, two determinants for the leakage are (1) the membrane affinity of the peptide and (2) its membrane-perturbing activity. The latter can be expressed as the amount of membrane-bound peptide necessary for the permeability change. We estimated the two factors by an indirect method.^{13,14} This method has two advantages over the direct binding experiments: First, the troublesome bound/free separation procedure is omissible. Second, the binding isotherm (Fig. 3a) shows the saturation of r at the higher $[P]_f$ region. This implies that membrane-bound E2078 is present mainly as a monomer on the assumption that the monomeric peptide is the only species in the aqueous phase.

Effect of E2078 on the Membrane Fluidity and Phase Transition Temperature of DPPG Membranes Effects of E2078 on the membrane fluidity and the gel to liquid-crystalline transition were examined by fluorescence polarization techniques (probe: DPH). Figure 5 shows the fluorescence polarization of DPPG as a function of the molar fraction of E2078, and the phase transition temperature was dependent on the molar fraction of E2078 (X_{E2078}). At $X_{E2078}=0$, the phase transition temperature was 40°C, typical of DPPG membranes.⁹⁾ At $X_{E2078}=0.01$, 0.03 and 0.05, the phase transition temperatures were 38, 37 and 33°C, respectively. These results indicate that E2078 affected the acidic lipid bilayer, and with the increase in X_{E2078} , a more fluid membrane

Fig. 5. Relationship between Temperature and Fluorescence Polarization of DPPG Sonicated Vesicles as a Function of the E2078 Mole Fraction

Mole fraction: ○, 0; ●, 0.01; △, 0.03; ▲, 0.05.

was formed and the phase transition temperature of DPPG membranes decreased.

CONCLUSION

E2078 induces the leakage of calcein, especially out of negatively-charged vesicles, through electrostatic interactions. E2078 binds to DPPG sonicated vesicles according to the Langmuir isotherm with a binding constant of $4.0 \times 10^5 \text{ (M}^{-1}\text{)}$ and a binding-site number of 0.14 per lipid molecule. The leakage seems to occur at a critical binding number of approx. 0.025 per lipid molecule. In addition, E2078 increased the membrane fluidity of the acidic lipid bilayer and decreased the phase transition temperature.

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