Available online at www.sciencedirect.com
SCIENCE DIRECT
Colloids and Surfaces B: Biointerfaces 48 (2006) 148–158
COLLOIDS AND SURFACES B
www.elsevier.com/locate/colsurfb

Investigation of interaction of Leu-enkephalin with lipid membranes

Shaoqian Liu [a,*], Akira Shibata [b], Satoru Ueno [b], Feng Xu [b], Yoshinobu Baba [b], Dejian Jiang [c], Yuanjian Li [c]

[a] College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China
[b] Faculty of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770-8505, Japan
[c] School of Pharmaceutical Sciences, Central South University, Changsha 410083, China

Received 18 August 2005; received in revised form 26 January 2006; accepted 7 February 2006
Available online 6 March 2006

Abstract

Enkephalins are peptides with morphine-like activity. To achieve their biological function, they must be transported from an aqueous phase to the lipid-rich environment of their membrane bound receptor proteins. In our study, zeta potential (ZP) method was used to detect the association of Leu-enkephalin and Leu-enkephalinamide with phospholipid liposomes constituted from egg-phosphatidylcholine (EPC), dioleoyl-phosphatidylethanolamine (DOPE), cholesterol (Chol), sphingomyelin (SM) as well as soybean phospholipid (SBPL). Transfer of the peptides over lipid membranes was examined by electrophysiology technique (ET) and fluorescence spectroscopy (FS), and further confirmed using 4-fluoro-7-nitrobenzofurazan (NBD-F) labeled Leu-enkephalin (NBD-F-enkephalin) with confocal laser scanning microscopy method (CLSM). Results of zeta potential showed that enkephalinamide associated with lipid membranes and gradually saturated on the membranes either hydrophobically or electrostatically or both. Data from electrophysiology technique indicated that Leu-enkephalin could cause transmembrane currents, suggesting the transfer of peptides across lipid membranes. Transfer examined by fluorescence spectroscopy implied that it could be separated into three steps, adsorption, transportation and desorption, which was afterward reaffirmed by confocal laser scanning microscopy. Transfer efficiencies of enkephalin across SBPL, EPC/DOPE, EPC/DOPE/SM, EPC/SM and EPC/Chol lipid bilayer membranes were evaluated with ET and CLSM experiments. Results showed that the addition of either sphingomyelin or cholesterol, or negatively charged lipid in lipid membrane composition could lower the transfer efficiency.

Keywords: Opioid peptide; Transfer; Planar lipid bilayer; Liposome; Electrostatic attraction

1. Introduction

Enkephalins, the first opioid peptide discovered and isolated from pig brain with a primary structure of Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) or Tyr-Gly-Gly-Phe-Met (Met-enkephalin) were termed endogenous morphine-like substances [1]. They are implicated in a wide variety of physiological processes [2]. Circulating enkephalins are believed to play a role in the inflammatory and immune response [3] by acting as neurotransmitters in the central nervous system (CNS). How circulating substances, such as peptides, affect brain functions and behaviors is a classic question that is now still an area of intense research. For example, enkephalins are potential future analgesics as they possess analgesic activity [4].

Opioid peptides demonstrate their biological effects by reacting with their membrane-embedded receptor proteins containing potential sites of association with lipids [5]. The lipid phase of the membranes not only acts as a matrix for the receptors but also is essential for the functionality and the topological arrangement of the receptor proteins [6,7]. Although many studies have been carried out on the interaction of enkephalins and lipid membranes, most of them were focused on the conformation feature of enkephalins upon their binding to receptors or lipid membranes [8–10]. To obtain insight information about interaction between enkephalins and lipid bilayer membranes, we applied zeta potential method to detect the association of the opioid peptides, Leu-enkephalin and Leu-enkephalinamide, with phospholipid liposomes constituted from egg-phosphatidylcholine (EPC), dioleoyl-phosphatidylethanolamine (DOPE), cholesterol (Chol), sphingomyelin (SM) and soybean phospholipid (SBPL). Transfer of the peptides over lipid membranes was examined by electrophysiology technique and fluorescence

* Corresponding author. Tel.: +86 731 4494559.
E-mail address: liushao2001@yahoo.com.cn (S. Liu).

0927-7765/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.colsurfb.2006.02.005

spectroscopy. In addition, NBD-F labeled Leu-enkephalin was used to investigate the transfer of Leu-enkephalin across lipid membranes with confocal laser scanning microscopy. Based on what has been observed from this study, contributions from the electrostatic and hydrophobic interactions to association and transfer processes are determined, factors affecting the relative transfer efficiency of Leu-enkephalin across lipid membranes are ascertained, and a three-step transfer mechanism has been proposed.

2. Materials and methods

2.1. Materials

Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), Leu-enkephalinamide (Tyr-Gly-Gly-Phe-Leu-NH_2), egg-phosphatidylcholine (PC), dioleoyl-phosphatidylethanolamine (DOPE), cholesterol (Chol), sphingomyelin (SM) and soybean phospholipid (SBPL) which contains phosphatidylcholine (40%), phosphatidylethanolamine (30%), phosphatidic acid (15%), cardiolipin (5%), phosphatidylinositol (4%), phosphatidylserine (3%), and others were purchased from Sigma (St. Louis, MO, USA). The NBD-F (4-fluoro-7-nitrobenzofurazan) was obtained from Dojindo Laboratories (Kumamoto, Japan). All peptides and lipids above were used without further purification. Hepes and Tris were purchased from ICN Biomedicals Inc. Chloroform, methanol and hexane were from Kanto Chemical Co., Inc., Japan. Hepes buffer A (10 mM Hepes/100 mM KCl/Tris, pH 7.40) and Hepes buffer B (10 mM Hepes/10 mM KCl/Tris, pH 7.40) were used. Enkephalin and enkephalinamide stock solution (2.4×10^{-3} M) were prepared by dissolving appropriate peptides in Hepes buffer A or buffer B. Solutions of lipids for constructing planar lipid membranes in electrophysiology technique experiments were obtained as following: SBPL solution was 10 mg/1 mL hexane, EPC/DOPE (molar ratio 1:1) 5.24 mg/0.5 mL hexane, EPC/DOPE/SM (weight ratio 3.5:3.5:1.0) 8 mg/0.7 mL $CHCl_3$ + 0.1 mL methanol and EPC/Chol (molar ratio 1:1) 8 mg/0.4 mL $CHCl_3$ + 0.4 mL hexane.

2.2. Labeling of enkephalin

Labeling of enkephalin was performed according to Kazuhiro Imai method [11]. Briefly, 9.9×10^{-6} mol NBD-F (conc. of stock solution in CH_3CN is 0.1 mol/L) was added to 0.5 mL bolic-bolate buffer (50 mmol/L, pH 8.0) dissolved with 5 mg enkephalin (9×10^{-6} mol). The mixture was heated at 60 °C for 1 min under protection from light, followed by cooling in ice water. The solution was then transferred into a dialysis bag (dialysis membrane, size 36 from Wako Pure Chemical Industries, Ltd.) which was immerged in 500 mL buffer B and stirred at room temperature. The buffer was replaced with new one once a day to remove the surplus NBD-F in the mixture. The concentration of enkephalin was determined before and after the labeling by ultraviolet spectrophotometry at 280 nm to get the total concentration of the labeled enkephalin (C^*). To calculate the labeling ratio, concentration of actually labeled enkephalin (C) was measured using ultraviolet spectrophotometry at 470 nm (for enkephalin-NBD-F $\lambda_{excitation} = 470$ nm, $\lambda_{emission} = 530$ nm). $\varepsilon(500\,\text{nm}, \text{NBD-F-enkephalin}) = 2.4 \times 10^4$. The labeling ratio then, inferred by comparing C and C^*, was 0.7.

2.3. Planar lipid bilayer experiment

A piece of thin Teflon film with a hole of 0.017 mm in its center was tightly sandwiched between two Teflon cells. 0.5 mL of Hepes buffer A were added in *cis* and *trans* cells, respectively. The buffers in both cells were connected with amplifier through salt bridges and AgCl electrodes. Planar lipid bilayer membranes of different lipid constitutions as described above were formed on the hole according to Montal M-Mueller P technique [12]. Membrane formation was verified by monitoring membrane capacitance and resistance. After the formation of stable bilayer membranes, Leu-enkephalin or Leu-enkephalinamide was added in *cis* cell, then, −60 to −160 mV voltage was applied on *trans* cell. Under this condition, the peptides could move from *cis* cell to *trans* cell and accordingly cause transmembrane currents if the peptides were able to transport across the bilayer. The signals of transmembrane currents in 160 s duration were monitored by Axopatch 200A/Integrating patch clamp and fed into computer. The data was later copied in floppy disk and analyzed by AxoGraph 4.5 software.

2.4. Liposome preparation

Liposome for zeta potential and fluorescence measurements: chloroform solutions of SBPL, EPC/DOPE (molar ratio 1:1), EPC/Chol (molar ratio 1:1) and chloroform/methanol (volume ratio 9:1) solution of EPC/DOPE/SM (weight ratio 3.5:3.5:1.0) in a round-bottomed flask were evaporated under a gentle nitrogen stream to form a thin lipid film on the flask surface [13]. The film was further dried by the mild nitrogen stream for 2–3 h to remove the remaining solvents. The dried film was then hydrated with Hepes buffer B and stored at room temperature in the dark for 24 h. To achieve uniform size distribution, the resultant liposomes were extruded through a 0.20 μm filter of disposable syringe filter unit, DISMIC-13 cp (Tokyo Roshi Kaisha, Ltd., Japan) for seven times. The liposomes thus prepared were stable for about 1 week in the dark under room temperature.

Giant liposome for CLSM: giant liposomes of EPC/DOPE (molar ratio 1:1), EPC/DOPE/SM (weight ratio 4.2:4.4:1.6), EPC/Chol (molar ratio 1:1) and EPC/SM (weight ratio 7.7:1.7) for confocal laser scanning microscopy experiment were also obtained according to N.M. Jelokhani [13] by putting the lipid solution resulted from the hydration of lipid film with buffer B at room temperature in the dark for 36 h followed by no extrusion instead.

2.5. Zeta potential and particle sizing

The zeta potential is proportional to the surface charge density [14] and thus can be used to monitor the binding of a positively charged peptide to a negatively charged membrane. During an experiment, we first measured the zeta potential for the liposome