Chemical Physics 419 (2013) 78–83

Contents lists available at SciVerse ScienceDirect

Chemical Physics

journal homepage: www.elsevier.com/locate/chemphys

Control of phospholipid flip-flop by transmembrane peptides

Masanori Kaihara [a], Hiroyuki Nakao [a], Hirokazu Yokoyama [a], Hitoshi Endo [b], Yasushi Ishihama [a], Tetsurou Handa [c], Minoru Nakano [d,*]

[a] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[b] Quantum Beam Science Directorate, Japan Atomic Energy Agency, Tokai, Ibaraki 319-1195, Japan
[c] Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, 3500-3 Minami-Tamagaki-cho, Suzuka, Mie 513-8670, Japan
[d] Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

ARTICLE INFO

Article history:
Available online 7 January 2013

Keywords:
Phospholipid
Flip-flop
Transmembrane peptide
Small-angle neutron scattering
Fluorescence
Dithionite assay

ABSTRACT

We designed three types of transmembrane model peptides whose sequence originates from a frequently used model peptide KALP23, and we investigated their effects on phospholipid flip-flop. Time-resolved small-angle neutron scattering and a dithionite fluorescent quenching assay demonstrated that TMP-L, which has a fully hydrophobic transmembrane region, did not enhance phospholipid flip-flop, whereas TMP-K and TMP-E, which have Lys and Glu, respectively, in the center of their transmembrane regions, enhanced phospholipid flip-flop. Introduction of polar residues in the membrane-spanning helices is considered to produce a locally polar region and enable the lipid head group to interact with the polar side-chain inside the bilayers, thereby reducing the activation energy for the flip-flop. A bioinformatics approach revealed that acidic and basic residues account for 4.5% of the central region of the transmembrane domain in human ER membrane proteins. Therefore, polar residues in ER membrane proteins are considered to provide flippase-like activity.

1. Introduction

Transbilayer movement of phospholipids between the inner and outer leaflet is called flip-flop and is essential for cell growth and development. The plasma membrane of cells retains an asymmetric lipid distribution, where phosphatidylserine and phosphatidylethanolamine exclusively appear in the cytosolic leaflet through the action of aminophospholipid translocases, and phosphatidylcholine (PC) and sphingolipids are preferably located in the extracellular leaflet [1]. This asymmetry is disrupted in apoptosis, and the consequent exposure of phosphatidylserine to the cell surface is recognized by macrophages, which leads to phagocytosis [2]. Scramblase transfers phospholipids bi-directionally in a manner that is dependent on the Ca^{2+} concentration, and its activity is involved in apoptosis and blood coagulation processes because it externalizes phosphatidylserine [3–5].

In contrast, the endoplasmic reticulum (ER), which is considered to be a biogenic membrane (i.e., a membrane that can synthesize lipids), maintains membrane symmetry although most of the lipids are synthesized on the cytosolic leaflet. Newly synthesized lipids in the ER are therefore considered to flip rapidly into the luminal side with a $t_{1/2}$ ranging from a few seconds to a few minutes. Because the spontaneous flip-flop of PC, which is a major phospholipid, is known to occur at a very low rate ($t_{1/2}$ of hours to days [6–8]), phospholipid flip-flop in the ER may be governed by membrane proteins called flippases [9]. The conceivable flippase activity in the biogenic membranes is phospholipid-nonspecific, ATP-independent, and bi-directional [10,11]. Although several extensive studies have attempted to identify ER flippases, their activities remain to be elucidated [12].

Recently, it was suggested that flippase activity found in rod cells could be attributed to opsin, which is a G protein-coupled receptor [13]. This finding points out the possibility that membrane proteins that have other specific functions can display flippase-like activity. Indeed, Kol et al. [14] reported that the flip-flop of a fluorescent lipid, 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoglycerol)] (C_6NBD-PG), was induced by transmembrane peptides [15] in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles. However, DOPC membranes have higher fluidity than general biological membrane models such as those formed from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), which may also influence the dynamics of the probes. We have previously shown by time-resolved small-angle neutron scattering (SANS) that KALP23, which is a transmembrane peptide used by Kol et al., does not induce flip-flop of POPC [7]; this finding suggests that the mere presence of a transmembrane helix does not induce phospholipid flip-flop, and other additional factors are necessary for flip-flop.

* Corresponding author. Tel.: +81 76 434 7565; fax: +81 76 434 7568.
E-mail address: mnakano@pha.u-toyama.ac.jp (M. Nakano).

0301-0104/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chemphys.2012.12.041

In this study, we used peptides with their amino acid sequences modified from that of KALP23 by replacing an amino acid in the center of transmembrane hydrophobic sequence (Leu) with a hydrophilic amino acid (Lys or Glu), and we investigated the effects of the replacement on phospholipid flip-flop by using large unilamellar vesicles (LUVs) composed of POPC.

2. Materials and methods

2.1. Materials

POPC, 1-palmitoyl(d_{31})-2-oleoyl-*sn*-glycero-3-phosphocholine (d_{31}-POPC), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (C_6NBD-PC), and C_6NBD-PG were purchased from Avanti (Alabaster, AL, USA). D_2O was obtained from Sigma-Aldrich (St. Louis, MO, USA).

The amino acid sequences of the transmembrane model peptides (TMPs) used in this study (TMP-L, TMP-K, and TMP-E) are shown in Table 1. TMP-L and TMP-K were synthesized by Fmoc solid-phase synthesis and purified by HPLC with a reverse-phase C18 HPLC column (Waters, Milford, MA, USA), using a gradient from 70% A, 30% B to 40% A, 60% B and from 60% A, 40% B to 30% A, 70% B, respectively, where A is 0.1% trifluoroacetic acid in water and B is 0.1% trifluoroacetic acid in acetonitrile. TMP-E was purchased from Bonac Corporation (Fukuoka, Japan). These peptides were characterized by HPLC and MALDI-TOF MS (AXIMA-CFR plus; Shimadzu, Kyoto, Japan), which confirmed that the purity was greater than 90%.

2.2. LUV preparation

The solvent used in this study was Tris-buffered saline (10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 0.01% NaN_3, pH 7.4). For the SANS experiment, Tris-buffered saline containing 30 vol.% D_2O was used. Lipids were hydrated with the buffer, and the mixture was repeatedly freeze–thawed and extruded through a polycarbonate membrane with a pore size of 100 nm using LiposoFast (Avestin, Ottawa, Canada). Peptide-containing LUVs were prepared by mixing the peptides with lipids prior to hydration. The concentration of phosphatidylcholine was determined using an enzymatic assay kit for choline (Wako, Osaka, Japan).

2.3. Quenching of tryptophan fluorescence by acrylamide

POPC LUVs (2.5 mM) containing TMPs (0.4 mol% of total lipids) were titrated with 1.5 M acrylamide, and the tryptophan fluorescence intensity was determined at excitation/emission wavelengths of 285/325 nm on a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). Data were analyzed according to the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of acrylamide with a concentration of [Q], respectively, and K_{sv} is the Stern–Volmer constant. As a control, a quenching experiment was also conducted against 10 μM amino acid tryptophan in the presence of peptide-free POPC LUVs (2.5 mM).

2.4. Quenching of tryptophan fluorescence by n-AS

To 1 mM POPC LUVs containing TMPs (0.1 mol% of total lipids), 500 μM *n*-(9-anthroyloxy)stearic acid (*n*-AS, $n = 2$, 6, and 12) in methanol was added to a final concentration of 0.5 mol%, and the mixtures were incubated for 1 h at 25 °C. The fluorescence intensity of tryptophan was determined in the absence (F_0) and presence (F) of *n*-AS at excitation/emission wavelengths of 285/325 nm.

Table 1
Amino acid sequences of TMPs.

Peptide	Sequence
TMP-L	Ac-GKKLALALALALWLALALALKKA-NH_2
TMP-K	Ac-GKKLALALALAKWLALALALKKA-NH_2
TMP-E	Ac-GKKLALALALAEWLALALALKKA-NH_2

2.5. Fluorescence emission spectrum and steady-state fluorescence anisotropy of tryptophan

LUVs containing TMPs (0.4 mol% of total lipids) were prepared. The emission spectrum of tryptophan was recorded at an excitation wavelength of 285 nm. Steady-state fluorescence anisotropy (r_S) of tryptophan was measured at excitation/emission wavelengths of 285/325 nm, and r_S was calculated from the following equation:

$$r_S = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2\,GI_{VH}} \quad (2)$$

where I_{VV} and I_{VH} are the intensities of vertically and horizontally polarized fluorescent light, respectively, when the excitation light is vertically polarized. G represents the compensating factor for the anisotropy sensitivity of the instrument, which is expressed as $G = I_{HV}/I_{HH}$, where I_{HV} and I_{HH} represent the intensities of vertically and horizontally polarized light, respectively, when the excitation light is horizontally polarized.

2.6. Time-resolved SANS

LUVs (30 mM) containing TMPs (0.4 mol% of total lipids) were prepared using POPC (H-LUV) or d_{31}-POPC (D-LUV). SANS measurement were performed by SANS-U of the Institute for Solid State Physics, the University of Tokyo, at research reactor JRR-3, Tokai, Japan. The wavelength (λ) of the neutron source was 7 Å ($\Delta\lambda/\lambda = 10\%$), and the sample-to-detector distance was set to 4 m. Samples were measured in quartz cells (Nippon Silica Glass, Tokyo) with a pass length of 2 mm. Each measurement was started immediately after mixing of an equivalent volume of D-LUV and H-LUV at 37 °C in the presence of 1 mM methyl-β-cyclodextrin (MβCD). Whole counts collected by detector for 10 min were accumulated in time-resolved measurements. The count rate for the solvent was then subtracted. The normalized contrast, $\Delta\rho(t)/\Delta\rho(0)$, was calculated from the following equation:

$$\Delta\rho(t)/\Delta\rho(0) = \left(\sqrt{I(t)} - \sqrt{I(\infty)}\right) \Big/ \left(\sqrt{I(0)} - \sqrt{I(\infty)}\right) \quad (3)$$

where $I(t)$ is the count rate at time t after LUV mixing. $I(\infty)$ is the count rate from LUVs consisting of a 1:1 mixture of POPC and d_{31}-POPC, and $I(0)$ is the average count rate from D- and H-LUVs. MβCD catalyzes the interparticle transfer of phospholipids [7] and the value of $\Delta\rho(t)/\Delta\rho(0)$ ideally reaches to 0 if the flip-flop occurs and to 0.5 if it does not. This value, however, seems to be influenced by the sample preparation (presence of multilamellar species) and the instrumental setting. We therefore used LUVs without TMPs as a negative control.

2.7. Flip assay with NBD-lipids

To produce asymmetrically fluorescence-labeled LUVs, a methanol solution of C_6NBD-PC or C_6NBD-PG (0.5 mol% of lipids) was added to LUVs containing TMPs (0.1 mol% of total lipids for C_6NBD-PC and 0.025 mol% for C_6NBD-PG). After incubation at