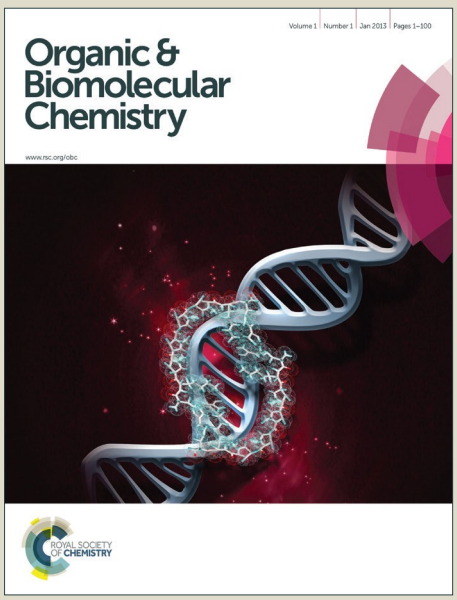
Organic & Biomolecular Chemistry

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Journal Name

ARTICLE

pH-Dependent Membrane Lysis by Melittin-Inspired Designed Peptides

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A. Kashiwada,* M. Mizuno and J. Hashimoto

We developed a membrane-lytic peptide (LP) having 26 amino acid residues composed of a helix-promoting hydrophobic segment (Leu-Ala repetitive sequence) and a cationic segment coming from melittin. In the presence of liposomes, LP interacts with liposomal surfaces to form a hydrophobic helix in the lipid bilayer in a wide pH range. In order to provide LP with a weakly acidic (endosomal) pH-controlled membrane-lytic activity, we have designed an LPE peptide series (a typical peptide, LPE3-1) with a hydrophobic segment in which Leu (L) residues are replaced by acidic Glu (E) ones. To analyze the pH-selective membrane-lytic activity of the designed peptides, both calcein leakage and membrane accessibility assays were performed. In case of membrane disruption induced by the active pore formation, incorporated calcein would leak from the liposomes and simultaneously the aqueous solution in the membrane surrounding would be accessible to the liposome interior at pH 5.0. The assays in the presence of LPE3-1 indicated no significant leakage or accessibility at pH 7.4, but a typical leakage and some accessibility to liposomes were positively observed at pH 5.0. In order to estimate whether the weakly acidic pH-controlled lytic activity is due to a secondary structural change of the hydrophobic segment of LPE3-1 in the liposome membrane, we have measured circular dichroism spectra. In the presence of liposomes, the minimum showing the characteristic helical structure was observed at 222 nm only under a weakly acidic condition. This pH dependence is in good agreement with the results from the leakage and accessibility assays. The pH-dependent membrane disruption properties of the LPE3-1 may open a new avenue to gain an insight into the interaction between peptides and lipids for the development of efficient drug/gene delivery systems.

Introduction

Membrane lytic peptides such as antimicrobial and host-defense peptides, which are innate immune regulators found in the eukaryotic organisms, have capability to disrupt and form pores in lipid bilayer membranes.[1-4] Some of the lytic peptides which have been widely studied forms amphipathic or hydrophobic helices that are essential to their potent hemolytic and antimicrobial activity.[5] These peptides take almost random structures in aqueous environments. On the other hand, they are folded in the helical conformation in the presence of lipid membranes. Due to the formation of the amphipathic or hydrophobic helical surfaces, these peptides are likely to interact with lipid membranes on the basis of these simple physical manners.[6,7] The study of the effective membrane lytic activities of these peptides will help promote a better understanding of the interaction of peptides with lipid membranes and contribute to construction of high-potential peptide mimics. Furthermore, this mechanistic membrane disruption and lytic system controlled by outer stimuli such as pH and metal ions holds great promise for some liposomal drug delivery systems, since acidification of endosomes triggers membrane lysis to release the drugs from liposomes. From the reasons mentioned above, many scientists have been interested to design pH-responsive artificial lytic peptides for enhancing the endosomal escape following internalization of small molecules (drugs or genes)-loaded liposomes via endocytosis.[8,9]

One of the most important pH-dependent lytic peptides is GALA designed by Szoka et al.[10,11] GALA is derived from the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin, which is involved in the fusion of the viral envelope with the endosomal membrane. This peptide is composed of repeating sequences of Glu-Ala-Leu-Ala and has an amphiphilic structure.[10] GALA perturbs biological membranes at acidic pH by forming amphipathic α-helix. At neutral pH, in contrast, the peptide does not interact with the membrane due to electrostatic repulsion of negative charges of deprotonated Glu residues.[12-15]

In addition to GALA, native lytic peptide (magainin 2) whose three Phe residues were substituted by 1-tetrafluorotyrosine as a pH sensing element was designed and synthesized. Incorporation of this modified amino acid into magainin 2 gave the peptide the membrane-lytic activity upon mild acidification.[16]

In this study, we report the design, synthesis, and characterization of artificial lytic peptides inspired by melittin. Melittin is one of the well-known hemolytic peptides derived from venom of the honey

[a] Department of Applied Molecular Chemistry, Graduate School of Industrial Technology, Nihon University, Narashino, Chiba 275-8575, Japan. E-mail: kashiwada.ayumi@nihon-u.ac.jp; FAX: +81-47-474-2579; TEL: +81-47-474-2564

†Electronic Supplementary Information (ESI) available: Identification of designed membrane-lytic peptides (HPLC and mass spectra), further characterization of membrane lytic properties of designed peptides. See DOI: 10.1039/x0xx00000x

bee *Apis melllifera*.[17] Melittin is a 26 amino acid peptide that is composed of a cationic C-terminal segment and a hydrophobic helical segment. In the presence of membranes, the cationic segment strongly interacts with the lipid headgroups at the membrane surface and inserts the hydrophobic helical segment into lipid bilayers to form membrane pores.[18-26] This bi-functional lytic activity of the small-sized peptide has attracted a good deal of attention in constructing novel antibiotics and useful tools for liposomal drug delivery systems.

In preparation for melittin-inspired pH-selective lytic peptides, we first design and then synthesize a typical peptide, LP composed of a Leu-Ala repetitive sequence as a hydrophobic helical segment and a cationic segment from melittin. Since both structural and electrostatic properties of LP are expected to remain unchanged in a wide range of pH, we postulate that LP possesses lytic activity from acidic to neutral condition. Our objective is finally to develop a pH-selective peptides really working in the weakly acidic range and characterize its pH-responsiveness.

In this study, two types of lytic peptides are proposed. One type of the peptides is prepared by the mutation of a hydrophobic helical segment. To be concrete, leucine residues in the hydrophobic segment were replaced with glutamic acid. The substitution of glutamic acid is expected to cause a structural change from a hydrophilic random coil at neutral pH to a hydrophobic helix in an acidic pH range. The other type of the peptide is prepared by replacement of cationic amino acids at C-terminal with histidine. Since the imidazole group of histidine has a p*K*a of around pH 6.0 and thus becomes cationic in a slightly acidic condition, this peptide is expected to exhibit a very efficient membrane lytic activity only in an acidic pH range.

This mutation of cationic segment would have a crucial effect on the peptide-membrane interactions. The knowledge gained in our study will support a new conceptual framework for development of delivery systems for transporting therapeutics through cellular membranes.

Experimental section

Synthesis and purification of the membrane lytic peptides

All polypeptides used in this study were synthesized by the solid-phase synthesis method using Rink amide resin, Fmoc-protected amino acids, HBTU, and HOBt. The deprotection and cleavage were carried out by treatment with 2 ml of a solution containing TFA / triisopropylsilane / water (89 / 10 / 1, v / v) for 2 h. Crude polypeptides were purified by the reversed-phase HPLC on a HITACHI D-7000 system equipped with a Inertsil ODS-3 column (10 μm, 250 mm × 10 mm i.d., GL-science, Japan) and the purity was followed by analytical HPLC (Inertsil ODS-3 column, 5 μm, 250 mm × 4.6 mm i.d., GL-science, Japan). Synthesized peptides were separated by a linear gradient of water / acetonitrile/ 0.1% TFA. All products were identified by a high resolution ESI-TOF MS using an Agilent 6210 ESI-TOF LC-MS spectrometer (Agilent Technologies Inc., Sanat Clara, CA, USA).

Preparation of liposomes

The liposomal vesicles of the following lipid compositions were prepared. Lipid compositions of the typical liposome, the anionic liposome, and cationic liposome were 100% EggPC, EggPC / DOPG = 90 / 10, and EggPC / EthylPC = 90 / 10, respectively. Small unilamellar vesicles (SUVs) were prepared by evaporation of chrloroform solution of lipid mixture in a round bottom flask, followed by hydration in 10-mM buffer solutions of different pH values (pH 5.0: acetic acid/sodium acetate; pH 7.4: Tris-HCl). All buffers contained 100-mM sodium chloride. The suspension was subjected to five freezing-thawing cycles for equilibration. Then, a suspension of heterogeneous size SUVs is passed through 100-nm unipore polycarbonate membranes (Whatman) 10 times on Mini-Extruder Set (Avanti). The final total lipid concentration was 2.0 mM.

Calcein-leakage assay

The lytic peptide-induced membrane disruption was characterized by liposome leakage assays that monitored the increase of fluorescence intensity upon release of the self-quenching concentration of calcein (75 mM) from liposomes to the external medium. Liposome solutions (2.0 mM) was mixed and incubated with peptide samples (peptide / lipid = 1 / 100). The fluorescence of the released calcein was assessed with a spectrofluorometer (HITACHI F2500) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Complete (100%) release was achieved by the addition of Triton X-100 to a final concentration of 0.5%. Spontaneous leakage was negligible at this time scale. The calcein-leakage assays were conducted at 25 °C. The apparent percentage of calcein release was calculated in accordance with the following equation: release % = $100 \times (F - F_0) / (F_t - F_0)$, in which F and F_t represent the fluorescence intensity before and after the addition of Triton X-100, respectively, and F_0 represents the fluorescence of the intact liposomes (before the addition of peptide samples).

Liposome-accessibility assay

SUVs of 100-nm diameter containing 0.5% NBD-labeled phospholipids (NBD-PE) were prepared by the same manner as described above. The fluorescent lipids of the outer leaflet, which were exposed to the bulk medium, were reduced and thereby quenched by sodium dithionite. Thus, only the inner leaflet of the SUVs contributed to the fluorescence emission and the intensity of the emission was measured with fluorescence microscopy. Reduction of the outer leaflet of NBD-PE labeled liposomes was carried out as follows: The NBD-PE labeled liposomal vesicles (2.0 mM) and 10-mM sodium dithionite (in 10-mM Tris-HCl buffer containing 1.0-mM sodium chloride) were incubated at 25 ˚C for 60 min. The complete reduction of NBD in the outer leaflet was confirmed by a decline of fluorescence intensity at an emission wavelength of 530 nm (excitation wavelength: 475 nm). After the complete reduction of the outer leaflet, free sodium dithionite was removed by gel filtration through Sephadex G-25 fine columns. And then the lipid concentration was adjusted to 2.0 mM by the use of Centricon Plus-70 with a membrane NMWL of 3 kDa. In order to assess the accessibility to interior of the liposomes, we measured the quenching of NBD fluorescence in the inner leaflet. Fluorescence of NBD was measured following the addition of fresh

sodium dithionite (100 μM), and then 20 μM of peptide samples was added. The accessibility was quantitated by the decrease in NBD fluorescence in the inner leaflet with membrane permeated sodium dithionite for 40 min.

CD measurements

All CD measurements were performed on a Jasco J-820 spectropolarimeter using a 2-mm path-length cuvette at 25 ˚C (Jasco PTC-348WI peltier thermostat) at a peptide concentration of 20 μM in 10-mM buffer solutions (pH 5.0: acetic acid/sodium acetate; pH 7.4: Tris-HCl). All buffers contained 100 mM sodium chloride. Concentrations of peptides were measured by UV absorbance of the Trp residue at 288 nm. Concentrations of EggPC or liped mixtures were the same as other experiments (2.0 mM) when CD measurements of peptide samples were carried out in the presence of liposomes. Reported data are averaged over 50 measurements obtained from 260 to 200 nm in a 1-nm interval at a rate of 50 nm / min.

Trp fluorescence measurements

Measurements for Trp fluorescence were carried on by using a HITACHI F2500 spectrofluorometer at an excitation wavelength of 288 nm and emission wavelength from 320 to 370 nm. All samples were measured five times at 25 ˚C.

Dynamic light scattering (DLS) measurements

Dynamic light scattering (DLS) of liposome suspensions was studied on an N5 Plus auto correlator (Beckman-Coulter) equipped with a 25-mW He-Ne laser light source (632.8 nm). Single scans with 2-minute averaging time were performed on the sample at an angle of 90.0°. Particle size distributions were calculated from autocorrelation data. All buffer solutions were filtered with a 0.22-μm filter just before liposome preparation. The collection times for the autocorrelation data were 1-4 min.

Results and discussion

Design of a membrane-lytic peptide based on melittin

An amino acid sequence of melittin and a typical membrane-lytic peptide (LP) are shown in Fig. 1. LP has 26 amino acid residues composed of a helix-promoting hydrophobic segment of Leu-Ala repetitive sequences and a cationic Lys-Arg-Lys-Arg segment from melittin. The Leu-Ala repetitive sequence was chosen because of its propensity to take hydrophobic helical conformation and membrane-penetrating property.[27,28] On the other hand, membrane-binding property of the cationic segment of melittin is well known,[20,29,30] and the affinity of cationic Arg or Lys residues for the phospholipid membrane has been demonstrated by NMR technique.[31,32] From these points of view, the cationic C-terminal segment was allowed to have the same sequence as melittin. This simple model peptide is expected to interact with the lipid membranes first, and then penetrate into the membranes and disrupt the membranes, namely LP would offer a bi-functional membrane-lytic system like melittin.

Melittin H_2N-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$

LP H_2N-GWWLALALALALALALASWIKRKRQQ-$CONH_2$

Hydrophobic segment Cationic segment

Fig. 1 Amino acid sequences of melittin and a typical melittin-inspired membrane lytic peptide (LP).

Membrane lytic properties of LP

The membrane lytic activity of LP was monitored by a well-established calcein-leakage assay with small unilamellar EggPC liposomes.[33] The fluorophore calcein entrapped in the liposomes at the self-quenching concentration is released into the surrounding buffer when transmembrane pore formation or membrane disruption occurs. Consequently, quenching of calcein would be relieved and the fluorescence intensity of the solution would increase. A typical calcein release curve on addition of 20 μM LP or melittin to EggPC liposomes (peptide / lipid = 1 / 100) at pH 7.4 and 25 °C is shown in Fig. 2 and the membrane lysis was clearly observed. In addition, LP showed the membrane-lytic activity in the range of pH 4.0 to pH 10.0 (Fig. S4).

In our design of LP, the importance of the hydrophobic helical and the cationic C-terminal segments was specially considered as two functional factors. In order to advance the fundamental understanding of the mechanism of this bi-functional action, we first focused on how membrane-lytic processes affect the electrostatic interactions between the cationic segment of LP and the surface of the liposome. In this characterization we used liposomes with anionic (EggPC / DOPG = 90 / 10) and cationic (EggPC / EthylPC = 90 / 10) lipid compositions. Despite of the favorable electrostatic interaction between LP and anionic liposomes, the lytic efficiency was little improved at pH 7.4 or 5.0

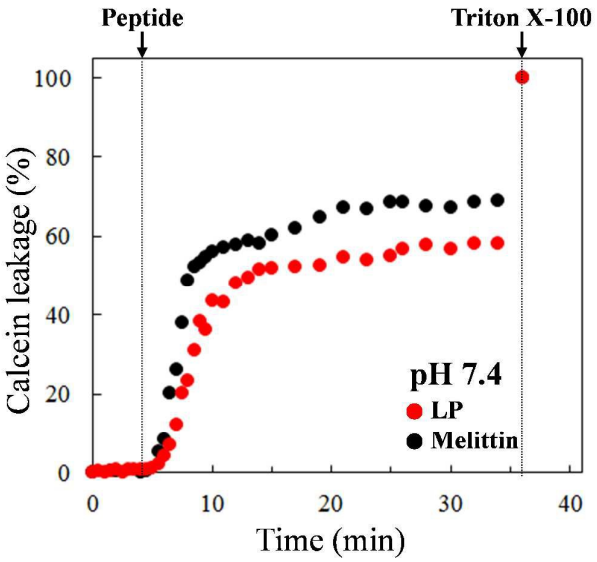


Fig. 2 Membrane lytic activities of melittin and LP at pH 7.4. A typical calcein-leakage assay with melittin and LP is shown. Calcein leakage from EggPC liposomes was measured every 30 or 60 s a following the addition of 20 μM of peptide (melittin or LP) at 25 °C.

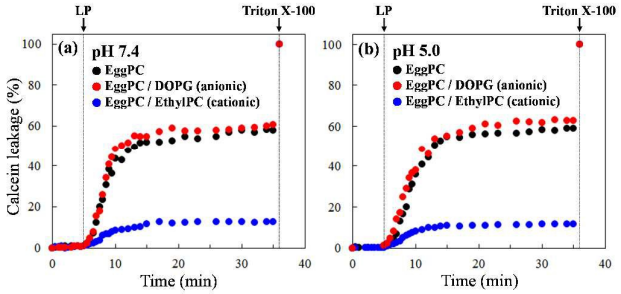


Fig. 3 Membrane lytic activity of LP toward anionic (EggPC / DOPG) and cationic (EggPC / EthylPC) charged liposomes at pH 7.4 (a) and 5.0 (b). Calcein leakage from liposomes was measured every 30 or 60 s a following the addition of 20 μM of LP at 25 °C.

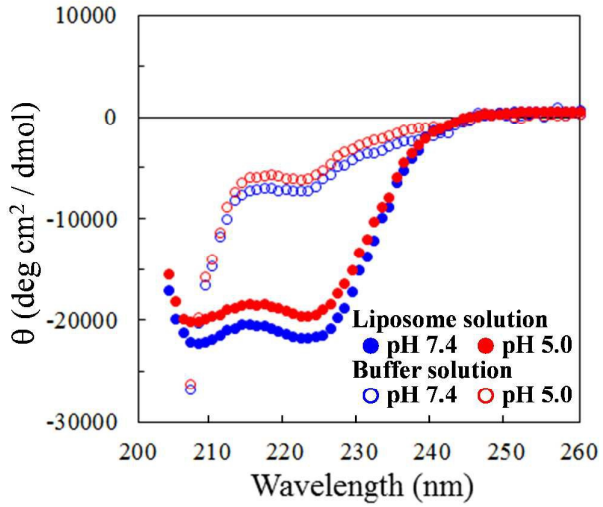


Fig. 4 Circular dichroism spectra of LP in the presence or absence of EggPC liposomes at pH 7.4 and pH 5.0. These spectra were taken for 20 μM LP solutions at 25 °C.

(Fig. 3). This behavior points out the importance of the free energy of membrane transfer as well as electrostatic interactions with lytic activity.[34,35] In contrast, the lytic activity of LP was considerably inhibited for cationic liposomes (Fig. 3). Almost the same results were observed in the case of other EggPC / EthylPC ratios (Fig. S5) and indicate that electrostatic interactions at the surface of the liposomes are necessary for LP to have membrane-lytic activity.

Then we examined the contribution of the hydrophobic helical segment of LP to the membrane-lytic activity. In order to analyze the secondary structure of LP in the presence or absence of EggPC liposomes, circular dichroism (CD) measurements were carried out. In the absence of liposomes, a random coil conformation was confirmed from CD spectra of LP and the pH-dependence on the structure was not observed. On the other hand, LP exhibited a high content of helical parts in the presence of EggPC liposomes (LP / lipid = 1 / 100) at 25 °C (Fig. 4). To further follow the relationship between the conformational change of LP and membrane lysis, we performed CD measurement of LP in the presence of cationic liposomes (Fig. S6). LP exhibited almost random coil, which indicates that no LP penetrate into lipid bilayers of cationic liposomes or form helical conformation with lytic activity. The results from CD measurements agreed with the knowledge from the calcein-leakage assay and indicate that both the hydrophobic helical and the cationic C-terminal segments are closely associated with membrane-lytic activity of LP.

Design of melittin-inspired pH-selective lytic peptides

This study is aimed at designing acidic pH-selective and pH-responsive lytic peptides based on the amino acid sequence of LP and understanding their mechanism of membrane lysis by investigating directly the interaction of these peptides with liposomal vesicles. This acidic pH-selective feature makes the peptides a promising candidate for enhancing molecular transport through lipid membranes in a situation where pH in the membrane-containing environment is reduced. For example, when drug delivery vehicles are internalized via the receptor-mediated endocytosis, acidification of endosomes occurs as they are migrated from the plasma membrane toward lysosomes. This pH reduction would trigger the peptides to activate and enhance endosomal contents to escape into the cytoplasm.

Amino acid sequences of the designed weakly acidic pH-selective lytic peptides are shown in Fig. 5. The first conception of the design of these peptides is the mutation of the hydrophobic helical segment. To be concrete, the leucine residues in hydrophobic segment were replaced with glutamic acid. The substitution of glutamic acid is expected to cause a structural change from a hydrophilic random coil at neutral pH to a hydrophobic helix in the presence of EggPC liposomes under the acidic condition.[28] This structural change would bring about a crucial effect on the membrane-lytic activity. In this study, we synthesized four kinds of designed peptides (LPE series). The peptide named LPE1 has a glutamic acid in the hydrophobic segment. Each of LPE3-1 and 3-2 peptide has three glutamic acids in the hydrophobic segment and LPE5 five ones.

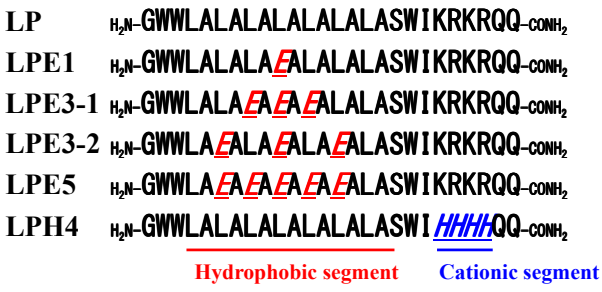


Fig. 5 Amino acid sequences of the weakly acidic pH-selective lytic peptides designed in this study.

As the second conception, we also designed LPH4 to perform the replacement of cationic arginine and lysine residues with histidine. Since histidine is positively charged only in an acidic pH range, the mutation of the cationic segment would have a crucial effect on the peptide-membrane interactions.[36]

Leakage and accessibility assays

To monitor the activity of designed pH-selective lytic peptides by the calcein-leakage assay, the changes in calcein-leakage induced by four kinds of designed peptides were measured (Fig. 6). With the addition of LPE3-1 to EggPC liposomes (LPE3-1 : lipid = 1 : 100) at pH 5.0, a membrane lytic phenomenon began to appear at 15 min and the extent of leakage reached to 55% at 35 min (Fig. 6(c)), indicating that the weakly acidic pH-selective membrane lysis occurred. Moreover, the dependence of the leakage extent on the peptide / lipid ratio was almost consistent with the previous report for GALA.[11,37] Taking the above results into account, we have placed three glutamic acids in the center of the hydrophobic segment in order to prepare another pH-sensitive peptide.

The leakage of calcein from EggPC liposomes with the addition of LPH4 was also examined at pH 7.4 and pH 5.0. As expected, a typical leakage curve was observed upon addition of LPH4 to EggPC liposomes (LPE4 / lipid = 1 / 100) only at pH 5.0. However, LPH4 appeared to be inferior to LPE3-1 in the membrane lytic activity judging from the extent of leakage (Fig. 6(c) (d)). We suppose that the difference in the lytic activity between LPE3-1 and LPE4 is originated from an affinity to lipid surfaces. In other words, the side chains of Arg or Lys interact strongly with the phosphate headgroups.[31,32]

For peptide-triggered membrane lytic mechanisms to be effectively utilized, target specific peptide inserting (pore forming) effect or nonspecific membrane perturbing effect is to be considered. Melittin has been known to insert perpendicularly in the bilayer of the target membrane and form trasmembrane pores.[5,18,38] Synthetic peptide, GALA, has also been reported to form active pores to membrane.[11,37] On the other hand, some peptides exhibit antimicrobial activities by only disturbing the ordered structure of the membranes.[3,39] The calcein-leakage assay provided us the information of lytic activity of the designed peptides, but this assay could only estimate transport of calcein from the inside of the liposomes to the outside. Therefore we could not exclude the possibility of the nonspecific membrane perturbing effect. In order to investigate whether LPE3-1 or LPH4 caused membrane activation by forming active pores in the same manner as melittin, we characterized the membrane accessibility as well as the membrane leakage. To characterize the peptide-induced membrane accessibility we monitored quenching behavior of NBD fluorophore-labeled liposomes by using a membrane-impermeable reductant sodium dithionite. When sodium dithionite was added to the solution of the NBD-labeled liposomes, the fluorescence intensity of NBD reduced to about 45% (or 55% quenching). The value of 45% reflected a percentage of the lipids in the inner-leaflet.[40] Then, further quenching of fluorescence by the reduction of NBD at the inner-leaflet could be found when LPE3-1 was added to the system at pH 5.0 (Fig. 7). In contrast to LPH4, a cationic segment mutated peptide did not show explicit quenching by the accessibility of

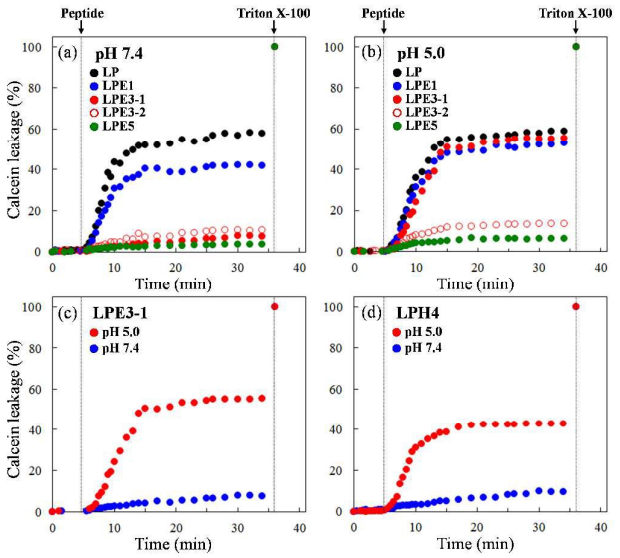


Fig. 6 Membrane lytic activity of the designed peptides mutated at the hydrophobic helical segment of LP (LPE series) toward EggPC liposomes at pH 7.4 (a) and 5.0 (b). Panels (c) and (d) show extent of acidic pH-dependent leakage of LPE3-1 and LPH4, respectively. Calcein leakage from liposomes was measured every 30 or 60 s a following the addition of 20 μM of peptides at 25 °C.

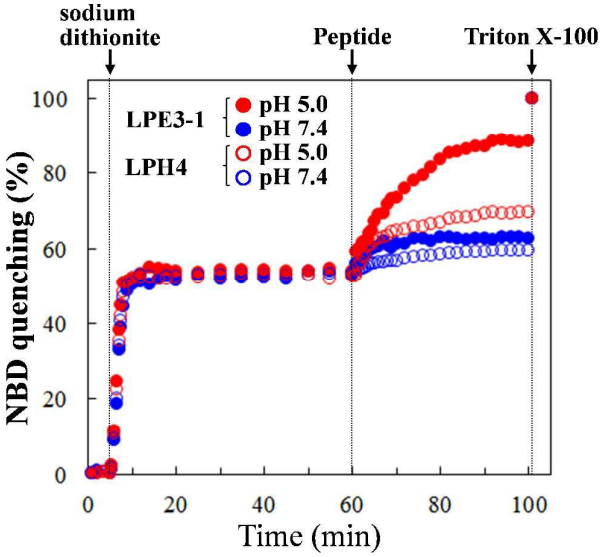


Fig. 7 Membrane lytic activity of LPE3-1 and LPH4 toward EggPC liposomes characterized by the membrane accessibility assay. Membrane accessibility was estimated from quenching of NBD dye-labeled lipids by a membrane-impermeable reductant sodium dithionite. Measurements were carried out by the use of 20 μM of peptides at 25 °C.

sodium dithionite to the inner leaflet at pH 5.0 (Fig. 7). From the results of both membrane leakage and membrane accessibility assays, LPE3-1 appeared to be a more potent membrane active

peptide. Moreover the difference of the membrane lytic activities between LPE3-1 and LPH4 points out the importance of a cationic Lys-Arg-Lys-Arg segment at C-terminal of melittin. Therefore, this cationic segment should be preserved for designing pH-selective LP analogues in this study.

Secondary structure of pH-selective lytic peptide, LPE3-1

For the purpose of investigating the relationship between the pH-dependent structural change of LPE3-1 and lytic activity, circular dichroism (CD) spectra of LPE3-1 were recorded in the presence of EggPC liposome (Fig. 8). From the resultant CD signal, LPE3-1 seemed to be random structure at pH 7.4. Three glutamic acids in the center of the hydrophobic segment of LPE3-1 are dissociated and negatively charged at pH 7.4. Due to the electrostatic repulsion between negatively charged LPE3-1 and surface of EggPC liposomes, LPE3-1 is considered to move into the lipid membranes and disrupt them. On the other hand, LPE3-1 may assume helical structure at pH 5.0. Three glutamic acids of LPE3-1 were protonated and became hydrophobic at pH 5.0. Formation of hydrophobic helix allows LPE3-1 to move into the lipid bilayer of EggPC liposome and thus active pores in the membrane are created.

Characterization of pH-selective membrane-penetrating properties

Membrane-binding and penetrating properties of lytic peptides were also characterized by the fluorescence of the Trp residue. In the aqueous buffer solutions at pH 7.4 and pH 5.0, LPE3-1 showed the fluorescence emission maxima at 352 nm and 353 nm, respectively, typical for Trp in a polar environment (Fig. S7).[41] Similar fluorescence behavior was observed in a buffer solution when the typical lytic peptide, LP, was used as a peptide sample (Fig. S7). Moreover, in the presence of EggPC liposomes, LP showed significant blue shifts in liposome solutions independently of the pH levels. These changes in the characteristics of the Trp fluorescence are in keeping with the results of the insertion of LP into the hydrophobic region of the liposomal bilayers under both physiological and endosomal conditions. On the other hand, LPE3-1 showed a slight blue shift (~3 nm) of the fluorescence emission maximum of Trp at physiological pH or pH 7.4, whereas a remarkable blue shift to 342 nm was observed at endosomal pH or pH 5.0 (Fig. S7). This is good agreement with the pH-responsive calcein-leakage and accessibility activities of LPE3-1 and strongly supports that LPE3-1 causes the membrane activation by forming active pores at endosomal acidic pH.

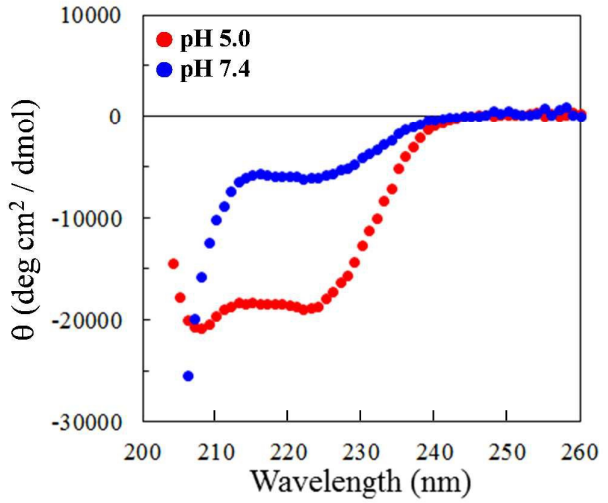


Fig. 8 Circular dichroism spectra of LP in the presence of EggPC liposomes at pH 7.4 and pH 5.0. These spectra were taken for 20 μM LP solutions at 25 °C.

pH-responsive lytic system by the use of LPE3-1

By the use of LPE3-1, the novel membrane lytic system was provided with pH-selectivity. However, "response" to a pH change has not been examined yet. Then, we performed the membrane leakage and membrane accessibility assays when the pH level of the experimental system changed from 7.4 to 5.0. Although addition of LPE3-1 to EggPC liposomes at pH 7.4 brought about no fluorescence changes in calcein leakage or NBD quenching, fluorescence changes indicating membrane lysis was clearly observed by switching of the pH level to 5.0 (Fig. 9 and Fig. S8). From this result, we found that membrane lytic system by the use of novel designed peptide, LPE3-1 possesses "pH-responsiveness" as well as "pH- selectivity". In order to monitor the liposome integrity during the membrane lytic process by LPE3-1, the dynamic light scattering (DLS) measurement was carried out (Fig. S9). When LPE3-1 was added to EggPC liposomes at pH 7.4, no noticeable changes to the liposome

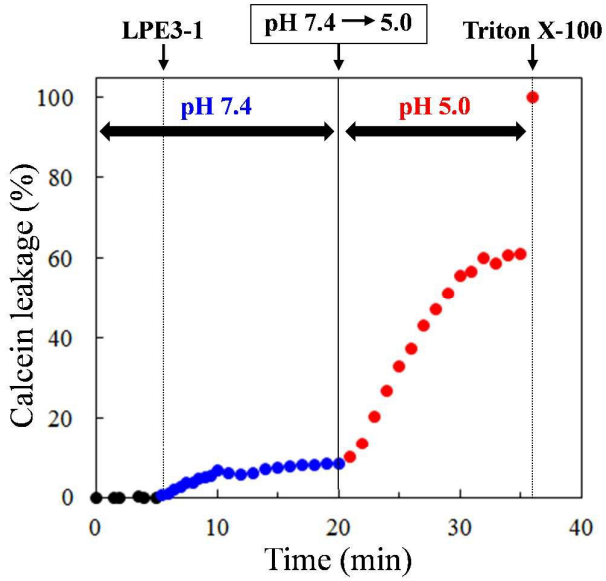


Fig. 9 pH-activated membrane lysis of LPE3-1 characterized by the calcein leakage assay. LPE3-1 was added to calcein-loaded liposome solution at pH 7.4. After 15 min incubation, the pH was lowered to a value of 5.0 by adding hydrochloric acid.

scattering intensity (hydrodynamic diameter) was observed. Moreover after switching the pH to 5.0, the scattering intensity was almost unchanged. Considering the results from the membrane leakage and membrane accessibility assays together, LPE3-1 acts as an endosomal pH-responsive lytic peptide which induces the formation of transmembrane pore without rupturing liposomes. This "pH-responsive" system is attractive to the fields of advanced drug delivery and gene transfer, and also applicable to the generic pharmaceuticals.

Conclusions

In this study we have demonstrated that melittin-inspired lytic peptide (LP) interacting with liposomal surfaces form a hydrophobic helix in the lipid bilayer in a wide pH range. Moreover, we have proposed LP analogues with improved membrane lytic properties at endosomal acidic pH. One of the designed peptide, LPE3-1 which was allowed to alter the helix-promoting hydrophobic segment of LP showed effective interaction to lipid membrane at pH 5.0, which resembled that of melittin or LP by forming active pores. The present approach may open new avenues to gain a deeper understanding of interactions between peptide and cell lipids. Although further studies and precise design are needed to take advantage of the drug release tool for liposomal DDS, our finding has the potential to deliver more drugs or genes into the cytoplasm of target cells via endosome escape process.

Acknowledgements

We are grateful for the financial support from the JSPS KAKENHI Grant Number 24550196. The authors thank Prof. M. Hirata for proofreading of the manuscript.

References

1 M. Zasloff, *Nature*, 2002, **415**, 389–395.
2 K. A. Brogden, *Nat. Rev. Microbiol.*, 2005, **3**, 238–250.
3 R. E. W. Hancock and H. G. Sahl, *Nat. Biotechnol.*, 2006, **24**, 1551–1557.
4 B. Bechinger and K. Lohner, *Biochim. Biophys. Acta*, 2006, **1758**, 1529–1539.
5 B. Bechinger, *J. Membr. Biol.*, 1997, **156**, 197–211.
6 B. B.Finlay and R. E. W. Hancock, Nat. Rev. Microbiol., 2004, **2**, 497–504.
7 K. Matsuzaki, *Biochim. Biophys. Acta, Biomembr.*, 2009, **1788**, 1687–1692.
8 F. Salomone, F. Cardarelli, M. Di Luca, C. Boccardi, R. Nifosi, G. Bardi, L. Di Bari, M. Serresi and F. Beltram, *J. Controlled Release*, 2012, **163**, 293–303.
9 Z. Qian, J. R. LaRochelle, B. Jiang, W. Lian, R. L. Hard, N. G. Selner, R. Luechapanichkul, A. M. Barrios and D. Pei, *Biochemistry*, 2014, **53**, 4034–4046.
10 N. K. Subbarao, R. A. Parente, F. C. Szoka, L. Nadasdi and K. Pongracz, *Biochemistry*, 1987, **26**, 2964–2972.
11 W. Li, F. Nicol and F. C. Szoka, *Adv. Drug Delivery Rev.*, 2004, **56**, 967–985.
12 R. A. Parente, S. Nirs and F. C. Szoka, *J. Biol. Chem.*, 1988, **263**, 4724–4730.
13 F. Nicol, S. Nir and F. C. Szoka, *Biophys. J.*, 1996, **71**, 3288–3301.
14 F. Nicol, S. Nir and F. C. Szoka, *Biophys. J.*, 2000, **78**, 818–829.
15 D. K. Schach, W. Rock, J. Franz, M. Bonn, S. H. Parekh and T. Weidner, *J. Am. Chem. Soc.*, 2015, **137**, 12199–12202.
16 F. Wang, L. Qin, P. Wong and J. Gao, *Org. Lett.*, 2011, **13**, 236–239.
17 M. T. Tosteson and D. C. Tosteson, *Biophys. J.*, 1981, **36**, 109–116.
18 T. C. Terwilliger and D. J. Eisenberg, *Biol. Chem.*, 1982, **257**, 6016–6022.
19 Y. Goto and Y. Hagihara, *Biochemistry*, 1992, **31**, 732–738.
20 A. Okada, K. Wakamatsu, T. Miyazawa and T. Higashijima, *Biochemistry*, 1994, **33**, 9438–9446.
21 T. Benachir and M. Lafleur, *Biochim. Biophys. Acta*, 1995, **1235**, 452–460.
22 C. Subbalakshmi, R. Nagaraj and N. Sitaram, *FEBS Lett.*, 1999, **448**, 62–66.
23 H. Sato and J. B. Felix, *Biochim. Biophys. Acta, Biomembr.*, 2006, **1758**, 1245–1256.
24 A. A. Strömstedt, P. Wessman, L. Ringstad, K. Edwards and M. Malmsten, *J. Colloid Interface Sci.*, 2007, **311**, 59–69.
25 D. Sengupta, H. Leontiadou, A. E. Mark and S. J. Marrink, *Biochim. Biophys. Acta, Biomembr.*, 2008, **1778**, 2308–2317.
26 A. J. Krauson, J. He and W. C. Wimley, *J. Am. Chem. Soc.*, 2012, **134**, 12732–12741.
27 R. N. A. H. Lewis, F. Liu, R. Krivanek, P. Rybar, T. Hianik, C. R. Flach, R. Mendelsohn, Y. Chen, C. T. Mant, R. S. Hodges and R. N. McElhaney, *Biochemistry* 2007, **46**, 1042–1054.
28 A. Kashiwada, I. Yamane, M. Tsuboi, S. Ando and K. Matsuda, *Langmuir*, 2012, **28**, 2299–2305.
29 S. E. Blondelle and R. A. Houghten, *Pept. Res.*, 1991, **4**, 12–18.
30 K. Hall, T. -H. Lee and M. -I. Aguilar, *J. Mol. Recog.*, 2011, **24**, 108–118.
31 M. Tang, A. J. Waring and M. Hong, *J. Am. Chem. Soc.*, 2007, **129**, 11438–11446.
32 Y. Su, T. Doherty, A. J. Waring, P. Ruchala and M. Hong, *Biochemistry* 2009, **48**, 4587–4595.
33 N. Duzgunes, R. M. Straubinger, P. A. Baldwin, D. S. Friend and D. Papahadjopoulos, *Biochemistry*, 1985, **24**, 3091–3098.
34 A. K. Ghosh, R. Rukmini and A. Chattopadhyay, *Biochemistry*, 1997, **36**, 14291–14305.
35 D. Persson, P. E. G. Thorén, P. Lincoln and B. Nordén, *Biochemistry*, 2004, **43**, 11045–11055.
36 Z. Tu, A. Young, C. Murphy and J. F. Liang, *J. Pept. Sci.*, 2009, **15**, 790–795.
37 R. A. Parente, S. Nirs and F. C. Szoka, *Biochemistry*, 1990, **29**, 8720–8728.
38 G. Klocek, T. Schulthess, Y. Shai and J. Seelig, *Biochemistry*, 2009, **48**, 2586–2596.
39 W. C. Wimley and K. Hristova, *J. Membr. Biol.*, 2011, **239**, 27–34.
40 R. A. Moss and S. Bhattacharya, *J. Am. Chem. Soc.*, 1995, **117**, 8688–8689.
41 A. Chattopadhyay and E. London, *Biochemistry*, 1987, **26**, 39–45.

Effective acidic pH-selective liposomal membrane lysis was achieved by a novel designed peptide.