Biochimica et Biophysica Acta 1858 (2016) 289–294

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Phloretin modulates the rate of channel formation by polyenes

Evgeny G. Chulkov *, Olga S. Ostroumova

Institute of Cytology of the Russian Academy of Sciences, Tikhoretsky Ave. 4, St. Petersburg, 194064, Russia

ARTICLE INFO

Article history:
Received 29 June 2015
Received in revised form 5 November 2015
Accepted 1 December 2015
Available online 3 December 2015

Keywords:
Lipid bilayer
Calcein leakage
Polyene
Flavonoid

ABSTRACT

The influence of flavonoids and polyene antibiotics on the permeability of membranes has been investigated through measurements of calcein leakage from large unilamellar vesicles composed of DOPC:cholesterol (67:33 mol%). Phloretin and biochanin A have been shown to induce calcein release from liposomes, but quercetin, daidzein, and catechin have not. Differential scanning calorimetry has indicated a decreasing of melting temperature of DPPC vesicles by 1.5–2 °C in the presence of phloretin and biochanin A. Quercetin, catechin, and daidzein have had almost no effect on the main transition temperature. Phloretin, biochanin A, and quercetin have significantly broadened the main transition peak of DPPC. Phloretin have increased a leakage induced by polyene antibiotics, whereas catechin and daidzein have not. Quercetin has slightly affected it. The effects of tested flavonoids on the polyene-induced calcein leakage and channel forming activity have been similar. The obtained data agree with the previously supposed hypothesis regarding the enhancement of polyene activity by reducing elastic stress near the lipid mouth of the nystatin pore. The inhibition of polyene channel forming activity by biochanin A observed in planar DOPC:cholesterol bilayers may be related to the flavonoid competition with cholesterol in the polyene–sterol channel complexes.

1. Introduction

The calcein release technique is a widely used approach to investigate the membrane permeability of liposomes in the presence of different drugs, nutrients, etc. [1–5]. The changes in the permeability of the membrane of large unilamellar vesicles (LUV) as induced by membrane-active drugs result in calcein flow through the bilayer. Calcein fluoresces very poorly at millimolar concentrations because of strong self-quenching, but its fluorescence increases in diluted solutions. Thus, calcein leakage can be detected in surrounding media, where it is at micromolar concentrations. Various amphiphilic compounds are thought to permeabilize membranes by different mechanisms like membrane thinning and disordering, asymmetric bilayer expansion, toroidal pore formation, and micellization [4,6].

Polyene antifungal antibiotics, such as nystatin and amphotericin B, have been intensively investigated since the 1950s. The mechanism of their action is usually attributed to pore formation in the membranes of target cells [7,8]. Some studies have demonstrated the influence of polyenes on the physical state of the lipid bilayer [9,10]. Added from one side of the membrane, the antibiotics form single-length channels with a lipid mouth of a positive curvature on the opposite side. Polyenes may form single-length channels in the bilayer when they are added from the exterior of a vesicle. The internal radius of a single-length narrow pore (0.36–0.37 nm) allows the transfer through the membrane of small hydrophilic solutes, such as calcein [11].

Flavonoids are a class of plant polyphenols with a wide range of biological activity, such as antioxidant, anti-inflammatory, anticancer, antibacterial, and neuroprotective [12–14]. In addition, some of these compounds may reduce the dipole potential of the membrane [15] and tune the phase segregation scenario of the lipid bilayer [14,16,17]. Due to their structure, more hydrophobic compounds can embed into the boundary between phospholipid polar heads and hydrocarbon core while more hydrophilic ones can interact with membrane surface [18]. Polyene combination with flavonoids provides new opportunities for drug development. We previously showed that some flavonoids affect the steady-state transmembrane current induced by one side addition of nystatin [19]. In particular, phloretin, biochanin A, and quercetin increased the nystatin-induced transmembrane current through DOPC:cholesterol:sphingomyelin bilayers, but catechin and daidzein did not. The effect was attributed to the reduced elastic stress near the lipid mouth by flavonoids that facilitated pore formation. In the present work, we attempt to expand the model of regulation of nystatin activity by flavonoids in the LUV model system.

2. Materials and methods

2.1. Materials

All of the chemicals were reagent grade. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and cholesterol (Chol) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Dimethylsulfoxide (DMSO), phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone), biochanin A

* Corresponding author.
E-mail address: evchulkov@mail.com (E.G. Chulkov).

http://dx.doi.org/10.1016/j.bbamem.2015.12.004
0005-2736/

(5,7-dihydroxy-4′-methoxyisoflavone), quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one), catechin ((+)-cyanidol-3, (2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol), daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 7-hydroxy-3-(4-hydroxy-phenyl) chromone), Triton X-100 (TX-100), sodium chloride (NaCl), sodium hydroxide (NaOH), HEPES, EDTA, Sephadex G-75, amphotericin B, and nystatin A were purchased from Sigma Chemical (St. Louis, MO). The flavonoid and polyene chemicals are presented in Fig. 1.

2.2. Methods

2.2.1. Calcein release

The liposomal membrane permeability was assessed by measuring the fluorescence of the leaked water-soluble dye calcein. LUV were prepared by extrusion using an Avanti Polar Lipid mini-extruder (Pelham, AL). DOPC:Chol (67:33 mol%) stock in chloroform was dried under a gentle stream of nitrogen. Dry lipid film was hydrated by a buffer (35 mM calcein, 10 mM HEPES–NaOH, pH 7.4). The suspension was subjected to five freeze–thaw cycles and then passed through a 100-nm nuclepore polycarbonate membrane 13 times. The calcein that was not entrapped in the vesicles was removed by gel filtration in a Sephadex G-75 column to replace the buffer outside the liposomes with calcein-free solution (150 mM NaCl, 10 mM HEPES–NaOH, 1 mM EDTA, pH 7.4).

To determine the concentration-dependent leakage, the LUV suspension was diluted with calcein-free buffer to obtain a total lipid concentration of 10^{-5} M, which was assessed by a phosphorus assay [20].The typical fluorescence of a 2-ml sample was measured by a Fluorat-02-Panorama spectrofluorometer (wavelength of excitation at 490 nm and emission at 520 nm) before (I_0) and after (I_{max}) the addition of 220 μl of 100 mM TX-100. TX-100 was added to completely disrupt the LUV and to determine the amount of entrapped calcein. Several identical 2-ml samples of LUV suspension were incubated for 1 h at 25 °C in the dark with different concentrations of flavonoid or polyene. Then the fluorescence of the samples (I) was measured. Stock solutions were prepared in ethanol for flavonoids and in DMSO for polyenes and daidzein. The appropriate amount of solvent added alone did not induce a significant release of calcein. The relative intensity of the leaked calcein (Ir) was calculated as $Ir = (I\text{-}I_0)/(I_{max}/0.9\text{-}I_0)\cdot 100\%$. The 0.9 factor corrected for the dilution of the dye by the TX-100 solution. The quenching ratio (Q) was calculated as $Q = I_{max}/I_0$. To investigate the influence of phloretin, biochanin A, quercetin, catechin, and daidzein in the LUV suspension on polyene-induced calcein leakage, the LUV suspension was treated with the flavonoid for 1 h at 25 °C in the dark. Then the appropriate amount of antibiotic was added to the suspension and incubated for 1 h at 25 °C in the dark. Then, the relative intensity of fluorescence was calculated as described above, considering that in this case, the I_0 value corresponded to the control LUV suspension that was incubated with only flavonoid for 2 h. The internal (liposomal) and external (in the bathing solution) concentrations of phloretin, which easily penetrates through lipid bilayer [21], are quickly balanced while internal concentrations of other flavonoids that poorly penetrate through bilayer remain low [22–26]. The reverse order of addition of polyene and flavonoid leads to the less significant effects due to dissipation of calcein gradient by polyene antibiotics.

2.2.2. Planar lipid bilayers

Conductance measurements of lipid membranes modified by polyenes and flavonoids were performed according to protocol described earlier [19]. Briefly DOPC:Chol (67:33 mol%) planar lipid bilayers were formed using a monolayer-opposition technique [27] on an aperture in Teflon film that separated two (*cis*- and *trans*-) compartments filled with 2 M KCl, 5 mM HEPES–KOH, pH 7.4. Ag/AgCl electrodes with agarose/2 M KCl bridges were used to apply the transmembrane voltage (V) and measure the transmembrane current (I). "Positive" voltage caused cationic flux from *cis*- to *trans*-compartment. The current measurements were performed using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. The data were digitized with Digidata 1440A and analyzed using pClamp 10 (Axon Instruments) and Origin 8.0 (OriginLab). The sampling frequency was 5 kHz. The current tracks were processed through an 8-pole Bessel 100-kHz filter. After the membrane formation nystatin or amphotericin B was added to the *cis*-compartment to a final concentration of 20 or 2 μM, respectively. The effects of flavonoids on the polyene channel forming activity were characterized by a ratio (J_∞/J^0_∞), where J^0_∞ and J_∞ are the steady-state polyene-induced transmembrane currents before and after addition of 20 μM flavonoid, respectively. Bilayer normalized conductance (G/G_0) was determined as a ratio of the steady-state membrane conductance at given transmembrane voltage (G) to the approximated zero-voltage membrane conductance (G_0). Flavonoids added alone did not change membrane conductance. The increasing in the number of polyene channels after flavonoid addition can be detected regardless of order of addition of flavonoid and polyene. However, the high variability in the polyene-induced transmembrane current [7,8] strictly limits possible implications of experimental design with reverse order of addition of agents.

2.2.3. Differential scanning calorimetry

Differential scanning calorimetry experiments were performed using a μDSC7 differential scanning microcalorimeter ("Setaram", France). Samples (0.5 μmol DPPC, 800 μl of buffer—5 mM HEPES–KOH, pH 7.4) were formed by electroformation using a commercial Nanion vesicle prep pro (Munich, Germany) as previously described [16] at 50 °C. Liposomal suspension was incubated with flavonoid and heated at a constant rate of 0.2 $K\cdot min^{-1}$. The lipid:flavonoid ratio was

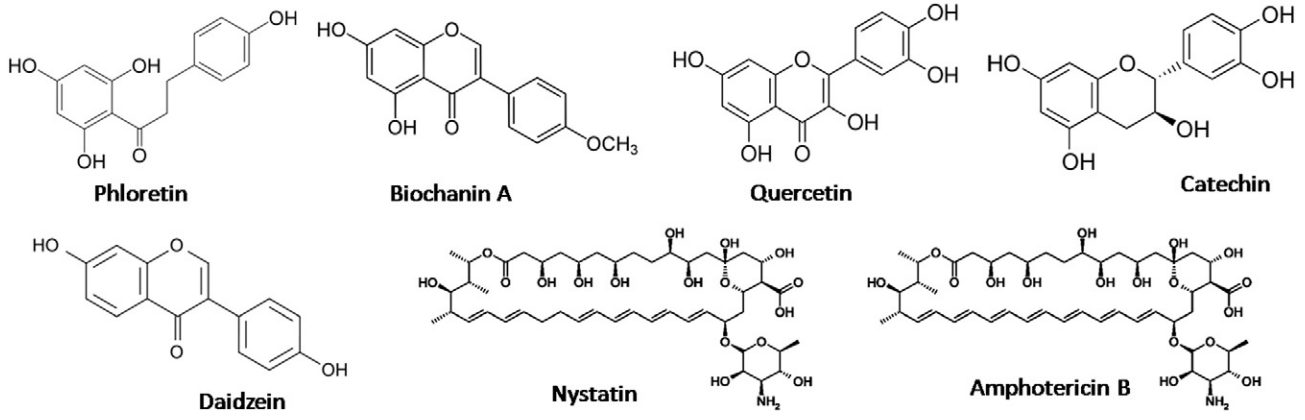

Fig. 1. The chemical structures of flavonoids and polyenes.