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Interaction of saponin 1688 with phase separated lipid bilayers

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ABSTRACT

Saponins are a diverse family of naturally occurring plant triterpene or steroid glycosides that have a wide range of biological activities. They have been shown to permeabilize membranes and in some cases membrane disruption has been hypothesized to involve saponin/cholesterol complexes. We have examined the interaction of steroidal saponin 1688-1 with lipid membranes that contain cholesterol and have a mixture of liquid-ordered (L_o) and liquid-disordered (L_d) phases as a model for lipid rafts in cellular membranes. A combination of atomic force microscopy (AFM) and fluorescence was used to probe the effect of saponin on the bilayer. The results demonstrate that saponin forms defects in the membrane and also leads to formation of small aggregates on the membrane surface. Although most of the membrane damage occurs in the liquid-disordered phase, fluorescence results demonstrate that saponin localizes in both ordered and disordered membrane phases, with a modest preference for the disordered regions. Similar effects are observed for both direct incorporation of saponin in the lipid mixture used to make vesicles/bilayers and for incubation of saponin with preformed bilayers. The results suggest that the initial sites of interaction are at the interface between the domains and surrounding disordered phase. The preference for saponin localization in the disordered phase may reflect the ease of penetration of saponin into a less ordered membrane, rather than the actual cholesterol concentration in the membrane. Dye leakage assays indicate that a high concentration of saponin is required for membrane permeabilization consistent with the supported lipid bilayer experiments.

1. Introduction

Saponins are a diverse family of naturally occurring plant triterpene or steroid glycosides [1–3]. They occur in a wide range of plants and exhibit diverse types of biological activity. Many of the properties of saponins can be traced to their amphiphilic structure with a hydrophobic steroid or triterpene core and with one (monodesmosidic) or two (bidesmosidic) linear or branched saccharide chains attached to the steroid or triterpene scaffold. A number of saponins have been shown to permeabilize membranes [4–8], with evidence from electron microscopy for formation of distinct membrane pores [9]. Some of these effects appear to require cholesterol, leading to the hypothesis that saponin/cholesterol complexes may be responsible for membrane disruption [10]. However there are other findings that suggest that the presence of cholesterol is not needed for membrane permeabilization particularly for bidesmosidic saponins [11,12]. Saponin and cholesterol interactions are affected by the configuration and the type of sugar moiety in the glycone moiety of saponin. More hydrophobic saponins are found to have higher lytic activity; for example, *Quillaja saponaria Molina* extract QS 21 (with a long acyl chain) is more lytic compared to QS 7 (lacking the acyl chain) from the same tree [13].

In addition to their membrane permeabilizing activity, saponins have been shown to induce apoptosis, inhibit the growth of cancer cells, and stimulate the immune system, leading to significant interest in their use as vaccine adjuvants [11,12,14–16]. Lytic activity is a desirable property for the potential therapeutic use of saponins as anti-cancer or anti-microbial agents. However, the lytic activity of the saponins usually results in acute toxicity or lower therapeutic indexes, although no clear correlation between cytotoxicity and hemolytic activity has been established [3,17]. Many of these studies have been carried out in red blood cells which have a high cholesterol content, up to 30–40 mol%, in the plasma membrane.

Saponins have been hypothesized to interact with cholesterol-enriched membrane raft domains, either disrupting them or causing their coalescence [3,18,19]. Rafts are believed to modulate the activity and location of various membrane receptors, thereby influencing signal transduction pathways [20–22]. Rafts in some cancer cells have high cholesterol content, which could be exploited for anticancer activity, potentially for cholesterol-binding saponins. Several studies have examined the effect of saponins on the lateral organization of model membranes as a model for lipid raft formation in cells. In one case a steroidal saponin was observed to colocalize with ganglioside-enriched

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domains, a mimic of cellular rafts [23]. A triterpenoid saponin, α-hederin, was shown to promote phase separation in giant unilamellar vesicles, the formation of worm-like aggregates in DMPC/cholesterol membranes and membrane disruption in supported lipid bilayers [8]. Finally, the saponin-mediated removal of the protein alkaline phosphatase from liposomes occurs only in the presence of cholesterol, indicating cholesterol-dependent disruption of domains [24].

A large number of saponins have been isolated from various sources, but there are very few that exhibit both strong cytotoxic and low hemolytic behavior [3,17]. 1688-1 is a bisdesmosidic triterpenoid saponin (molecular weight of 1688) extracted from *Saponaria vaccaria*, commonly known as cow cockle in Canada [25]. This saponin led to apoptosis in prostrate, breast and colon cancer cell lines with negligible cytotoxicity to normal human cells and non-cancerous cell lines. Furthermore, it required eight times higher concentration to cause hemolytic effects compared to the commercial saponin QS21. Among the various commercial *quillaja saponins* extracted and purified from *Quillaja saponaria*, QS21 is the least hemolytic and is used as an adjuvant in several cancer treatment formulations that have reached clinical testing for use in humans [26].

Understanding the molecular mechanisms leading to the lytic activity of saponins and their effects on cholesterol-enriched membranes could facilitate the development of saponins as high specificity and low toxicity therapeutic agents. We have examined the interaction of saponin 1688-1 with DOPC/sphingomyelin/cholesterol membranes that have a mixture of liquid-ordered (L_o) and liquid-disordered (L_d) phases as a model for lipid rafts in cellular membranes. A combination of atomic force microscopy (AFM) and fluorescence was used to probe the effect of saponin on the bilayer. The results demonstrate that saponin forms defects in the membrane and also leads to formation of small aggregates on the membrane surface, but does not otherwise alter the phase separation behavior of the membrane. Although most of the membrane damage occurs in the liquid-disordered regions of the bilayer, fluorescence results demonstrate that saponin localizes in both ordered and disordered membrane phases, with a modest preference for the disordered regions. Experiments for bilayer membranes that do not contain cholesterol show qualitatively similar effects, suggesting that the membrane activity of saponin 1688-1 does not depend on cholesterol. Both membrane permeabilization as measured using a vesicle dye leakage assay and hemolysis studies indicate that saponin 1688-1 is much less active at disrupting membranes than other saponins.

2. Materials and methods

2.1. Materials

Saponins used in this study were bisdesmosidic quillaic acid type saponins obtained from seed of *Saponaria vaccaria* var. 'Scott' [27]. The extraction and isolation process was as described in Hickie et al. [25]. Mixed saponins used for preparation of fluorescent labeled material were derived from seed saponins after ammoniolysis and preliminary fractionation on an Amberchrom column as described previously [25]. They consisted mainly of saponins 1688-1 and -2, 1556, 1394, 1526, 1422 and 1554 (see supplementary information, Fig. S1). The HPLC and extracted MS profiles are shown in supplementary Figs. S2 and S3. 1688-1 used in this study was obtained in approximately 90% purity via multiple chromatographies on Amberchrom resins. The HPLC profile is shown in supplementary Fig. S3.

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) as either dry powders or chloroform solutions (DOPC) and were used as received. Carboxy-fluorescein (CF) and Texas Red, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt dye (TR-DHPE) were purchased from Invitrogen (Eugene, OR). Deionized water (18.2 MΩ Milli-Q) was used in the preparation of all aqueous solutions.

2.2. Preparation of fluorescent labeled saponin mixture

4-(*N*-1,3-diaminopropyl)-1,8-naphthalimide was prepared in two steps from 4-bromo-1,8-naphthalic anhydride (Sigma Aldrich) by modifying a literature protocol [28]. Step 1: Ammonium hydroxide/ammonium acetate in THF, reflux; Step 2: Product from step 1 plus 1,3-diaminopropane (excess), 80 °C.

300 mg mixed saponins (average MW ~1600, 0.19 mmol) and *N*-hydroxysuccinimide (0.4 mmol, 46 mg) were dissolved in DMF (7 ml). *N*-(3-dimethyaminopropyl)-*N*-ethyl-carbodiimide hydrochloride (0.4 mmol, 99 mg) was added and the reaction was stirred for 1 h. 4-(*N*-1,3-diaminopropyl)-1,8-naphthalimide (MW 269, 0.2 mmol, 54 mg) was added and the reaction mixture was stirred overnight at ambient temperature. The saponins were precipitated by addition of acetone. The isolated precipitate was dissolved in water and applied to a column packed with Amberchrom CG 300. The column was washed with 2 volumes of water and 2 volumes of 50% aqueous methanol. The fluorescent-labeled saponin mixture was eluted with methanol containing 0.1% acetic acid (*v*/v) to afford, after evaporation of solvent, a yellow solid (290 mg). The product was characterized by HPLC-PAD-MS and UV–vis absorption (see supplementary Figs. S2, S3 and S4).

2.3. Bilayer preparation

Small unilamellar vesicles of DOPC/egg sphingomyelin (SM)/cholesterol were prepared as previously described [29,30]. Briefly, chloroform/methanol solutions of lipids (plus TR-DHPE when required) were mixed in the appropriate ratios. The resulting solution was dried under a gentle stream of nitrogen and placed under vacuum overnight to further remove the solvent. The lipid films obtained after drying were hydrated in water and vortexed to obtain multilamellar vesicles. The sample was sonicated in a water bath sonicator (Cole Parmer, Montreal, QC) at ~45 °C to clarity (~30–40 min) to form small unilamellar vesicles with a final lipid concentration of 1.0 mg/ml. Vesicles were used immediately for bilayer preparation.

DOPC/SM/cholesterol bilayers were prepared using a vesicle fusion protocol following a literature procedure [29]. Vesicle solutions containing 25 μg lipids and a final concentration of 10 mM $CaCl_2$ were deposited on freshly cleaved mica substrates (AFM imaging only) or thin mica substrates (20–30 μm thick for correlated AFM and fluorescence imaging) glued (Norland Optical Adhesive 88, Norland Products, Cranbury, NJ) on glass coverslips affixed to a liquid cell. The sample was incubated at 45 °C for 1 h, and slowly cooled to room temperature. The bilayer was washed extensively with water prior to imaging.

DOPC/DPPC and DOPC/DPPC/cholesterol vesicles and supported bilayers were prepared using a similar procedure to that described above, except that bilayers were formed by incubating the samples at 50 °C for 1 h and slowly cooling to room temperature.

2.4. Imaging

Supported bilayers were imaged in water using a PicoSPM atomic force microscope (Molecular Imaging) in MAC-mode using MAC lever type VII (Agilent Technologies, CA). Correlated AFM and fluorescence images were acquired with a JPK NanoWizard II AFM (JPK Instruments, Germany) installed on an Olympus 1X81 inverted microscope equipped with a high resolution CCD camera (CoolSNAP, Photometrics, US) and a 100× oil immersion objective. Fluorescence images were collected using either a GFP (exc 457–487 nm, em 503–537 nm) or a CFP/YFP filter (ex 426–450 nm, em 529–555 nm) for the naphthalimide channel and a Texas Red filter (exc 540–587 nm, em 602–648 nm) for TR-DHPE. The JPK AFM was also used to image all DPPC containing bilayers using PNP-DB (NanoWorld, Switzerland) cantilevers with spring constants of 0.06 N/m to 0.48 N/m.