Digitonin as a Chemical Trigger for the Selective Transformation of Giant Vesicles**

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In memory of Craig West

The remarkable and enigmatic properties of lipid membranes have attracted the interest of many biologically inclined chemists. New challenges are constantly emerging as membrane behavior is defined. Among these is the need to evaluate bilayer interactions with natural and synthetic compounds. Giant vesicles,[1–4] enclosed spherical lipid bilayer assemblies with diameters of 5–200 μm, are ideal vehicles for such studies, as they offer the capabilities for membrane isolation, manipulation, and direct observation under the light microscope.[5] Herein we describe the effects of digitonin on giant vesicles. Digitonin (Figure 1), a member of the saponin family, is a steroid–pentasaccharide conjugate isolated from the european foxglove (*Digitalis purpurea*), which is known to induce lysis in erythrocytes and other cells that contain cholesterol. As will be shown, digitonin acts as a selective chemical "trigger" for membrane layering.[6]

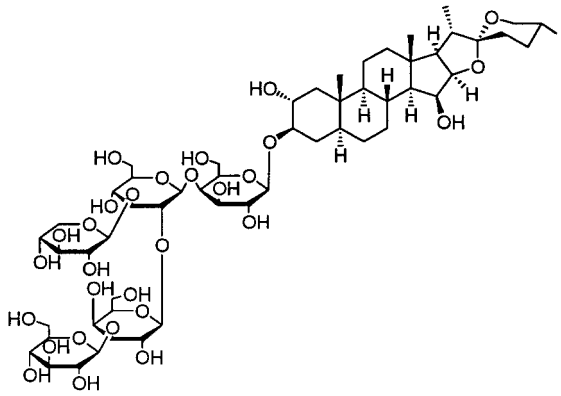

Figure 1. Structure of the steroid–saponin digitonin from *Digitalis purpurea*.

Saponins such as digitonin have wide application as foaming agents, detergents, sweeteners, and adjuvants,[7, 8] but they are perhaps most intriguing because of their ability to enter lipid membranes and form 1:1 complexes with cholesterol.[9–11] Complexation, the exact nature of which is unknown, is believed to be responsible for the lytic activity on cells that contain cholesterol. Although solutions of small vesicles (approximately 100 nm in diameter) have been used previously to study saponin-induced disruption,[12–20] we have taken advantage of the microscopic visibility of giant vesicles to evaluate digitonin's effect upon phospholipid membranes that contain cholesterol.

In the absence of membrane-bound sterols giant vesicles composed of phosphocholine (that is, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)) experienced no observable morphological changes when exposed to over 100 injections (2.7 ± 0.5 pL each) of digitonin solution ($2.5\ mg\,mL^{-1}$). In contrast, inclusion of a sterol such as cholesterol or ergosterol led to reproducible digitonin-induced disruption of the vesicles. For example, a single external 2.7 pL microinjection of a $2.5\ mg\,mL^{-1}$ digitonin solution within 5 μm of a giant vesicle composed of SOPC/50% cholesterol completely destroyed the structure in 1.5 s. Only a mass of precipitate and amorphous lipid material remained.

Intermediate morphologies could be observed by using even lower concentrations of injected digitonin or lower percentages of membrane-bound cholesterol. For example, the exposure of a giant vesicle of POPC/33% cholesterol to 2.7 pL of a $1.25\ mg\,mL^{-1}$ solution of digitonin created transitional "nanocup" structures[21] as seen in Figure 2. The solid complex observed at the lip of the wound likely inhibits reclosure of the sphere. In fact, it required 30 s for the vesicle to erode totally as the circular gap expanded. Interestingly, the intact portion of the vesicle always retained its spherical shape throughout.

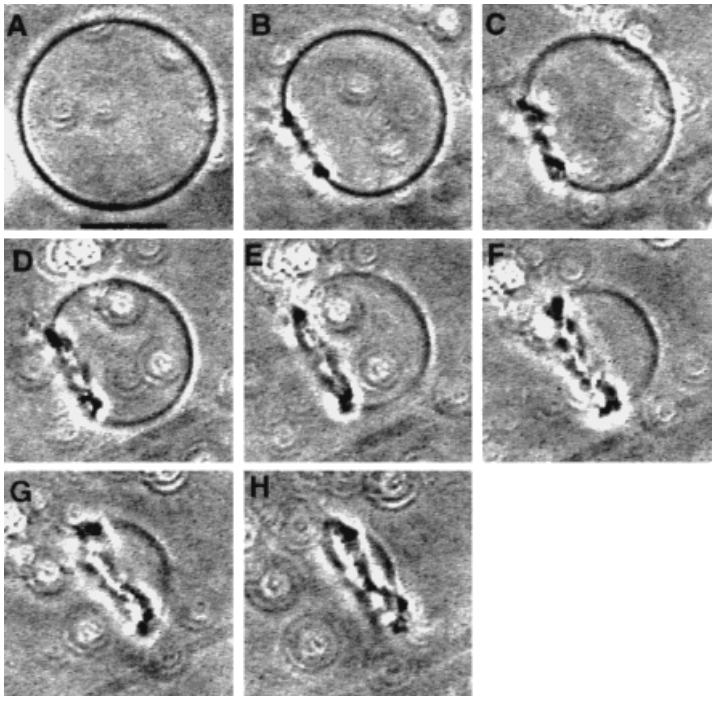

Figure 2. Rupture and gradual disintegration of a 67 μm POPC/33% cholesterol vesicle upon exposure to a single injection of digitonin solution (2.7 pL; $1.25\ mg\,mL^{-1}$). The intermediate "nanocup" morphologies (micrographs D–F) are also shown. Bar = 25 μm; time = 30 s.

In roughly 25% of the experiments, 3–10 μm long fibrous threads were visible at the periphery of the vesicles (Figure 3). Since fibers (believed to consist only of a saponin–sterol complex) have also been detected in submicroscopic vesicles by electron microscopy,[17–19] the behavior of small and giant vesicles overlap here despite their vastly different curvatures. In the giant vesicles' case, however, the fibers are transient and persist for no more than 10 s before they retract onto the

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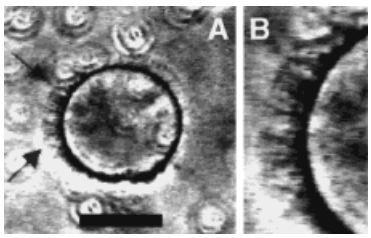

Figure 3. A) Fibrous tubules extend from the surface of a ruptured SOPC/10% cholesterol vesicle exposed to four injections of 2.5 mg mL^{-1} digitonin solution (each 2.7 pL) over a period of 16 s. The tubules retracted back to the surface of the structure 10 s after initial formation. B) Expanded view of the fibrous structures. Bar = 25 μm.

vesicle surface. Visualization of such dynamic processes constitutes a major advantage of light microscopy over electron microscopy.

Figure 4 depicts the molecular ratio of digitonin/cholesterol needed to disrupt SOPC/cholesterol giant vesicles. Data points are based on the minimal amounts of injected digitonin required to induce a rupture event in an isolated giant vesicle. Two conclusions can be deduced from the graph: a) vesicle size is not a critical factor within a 30–70 μm diameter range, with both 10% cholesterol (top plot) and 50% cholesterol (bottom plot); b) cholesterol clearly heightens the sensitivity of SOPC bilayers to digitonin-induced disruption since the digitonin/cholesterol ratio diminishes from about 3 for 10% cholesterol to about 0.15 for 50% cholesterol. Normally, cholesterol is believed to enhance the resistance of membranes to stress-induced ruptures.[1, 23] Formation of an insoluble digitonin/cholesterol complex within the membrane[13, 19, 20] must, therefore, supersede any stabilizing effect of cholesterol.

With the preliminary results in hand we were able to selectively trigger the rupture of giant vesicles within a group

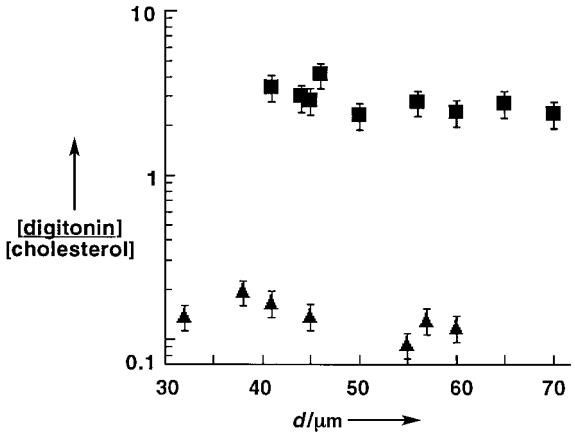

Figure 4. Digitonin/cholesterol molecular ratio for microinjection experiments on SOPC/cholesterol vesicles, based on the minimal amount of injected digitonin solution required to induce rupture of giant vesicles of a given size and composition. (■) SOPC/ 10% cholesterol vesicles; (▲) SOPC/ 50% cholesterol vesicles. Error bars are ±19%, based on the standard deviation of the injection volume. Cholesterol molecules/vesicle values were estimated from area/molecule values determined by Needham and Nunn at 15 °C.[22]

of two or more (the selectivity being based on the cholesterol content of the vesicles). Figure 5 A shows two oppositely charged vesicles that have electrostatically "snapped together" as described previously.[6] The upper cationic vesicle contained POPC/didodecyldimethylammonium bromide/cholesterol in a ratio of 78/2/20 mol%. The lower anionic vesicle contained POPC plus 1-palmitoyl-2-oleoyl-phosphoglycerol (POPG) in a ratio of 98/2 mol% but in the absence of cholesterol. With only 2% cationic and anionic lipid in the respective vesicles, they adhered without change for over 2 h. When the system was exposed to a single injection of a 2.5 mg mL^{-1} solution of digitonin, however, the cationic vesicle with 20 mol% cholesterol burst (Figure 5B) and collapsed upon the stable anionic vesicle (Figure 5C, D). Partial deposition on the anionic vesicle was confirmed by an image profile analysis (Figure 5E, F), which showed a coated membrane in region 2. This selective phenomenon was readily reproducible. When the cholesterol was switched to the anionic vesicle it was now the anionic vesicle that ruptured and collapsed upon its neighbor.

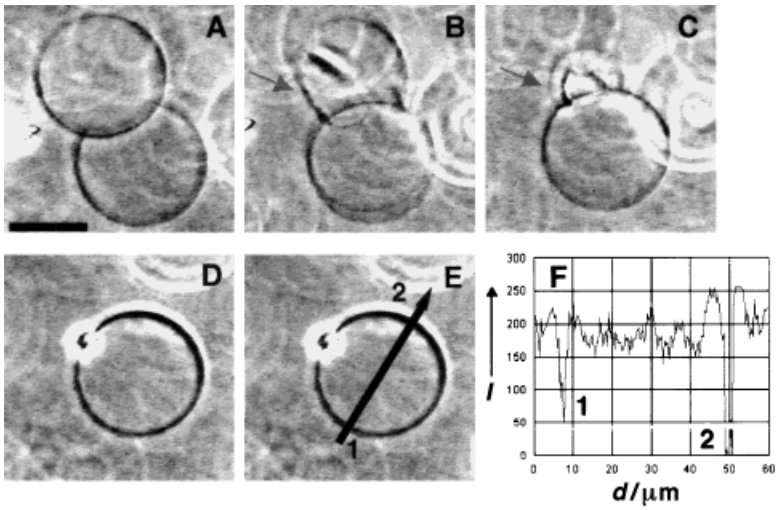

Figure 5. Selective rupture of a 34 μm cholesterol-containing vesicle (POPC/DDAB/cholesterol, 78/2/20 mol%) while adhered to a 40 μm cholesterol-free vesicle (POPC/POPG, 98/2 mol%). Micrographs A–C show the progressive breakdown of the vesicle upon a single 2.7 pL injection of a 2.5 mg mL^{-1} solution of digitonin ($t \sim 2$ s). Micrograph D shows the surviving vesicle, whose partial coating is confirmed with an intensity profile (E,F). The bright spot on the vesicle was precipitate or an anomalous lipid cluster. Bar = 25 μm.

The coating process was also visualized with epi-fluorescence microscopy. Figure 6A shows two adhered vesicles, with the upper one containing both 12 mol% cholesterol and 4-(4-dihexadecylamino)styryl)-*N*-methylpyridinium iodide (DiA; a membrane-soluble fluorescent dye). Since the lower vesicle possessed neither of these components only the upper vesicle with the dye fluoresced (Figure 6B). The upper vesicle burst upon exposure to digitonin and collapsed upon the lower one, to create a partial coating that was observable by fluorescence (Figure 6C).

This work opens new possibilities for selective delivery: A controllable percentile of a disparate giant vesicle population can now be chemically induced to release its contents while the remainder of the population survives intact.[24]

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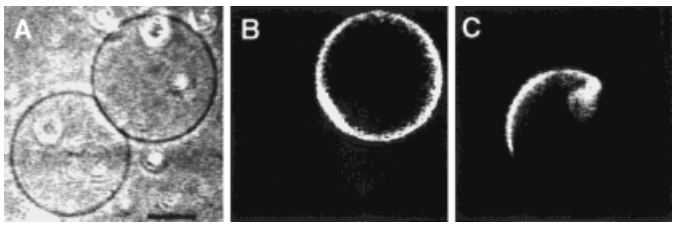

Figure 6. A) Phase-contrast and B) epi-fluorescence visualization of a 70 μm cholesterol-containing, fluorescently-labeled vesicle (POPC/DDAB/DiA/cholesterol, 85/2.5/0.5/12 mol %; upper right hand corner) adhered to a 67 μm POPC/POPG vesicle (98/2 mol %). C) Epi-fluorescence view of a static vesicle with layered lipid from the fluorescently-labeled vesicle, after collapse from exposure to digitonin (2.7 pL; 2.5 mg mL^{-1}). Bar = 25 μm.

Experimental Section

Digitonin (Boehringer Mannheim) was purified (45 %) by the method of Kun et al.[25] (Elemental analysis: calcd for $C_{56}H_{92}O_{29}$: C 54.71, H 7.54; found: C 54.24, H 7.69 %.) Particle sizing (Coulter N4 Plus) of dispersions of purified digitonin in Milli-Q water contained 3 – 8 nm structures at concentrations of 0.5 – 2.5 mg mL^{-1}, which indicated the presence of micellar or premicellar aggregates. DiA was purchased from Molecular Probes (USA). Vesicles were formed by the electroformation method developed by Angelova et al.[26] The typical procedure, which varied slightly with different compositions, was as follows: Droplets (1 μL) of lipid or lipid mixture solutions (0.25 – 1.0 mg mL^{-1} in diethyl ether/methanol (9/1) was placed on two parallel Pt wires (0.5 mm diameter) of the apparatus. After the film had dried under a stream of nitrogen for 45 minutes the apparatus was connected to an alternating current (initial $\nu = 10$ Hz; $V = 0.1$ V) from a function generator (Hewlett Packard, 33120A) coupled to an oscilloscope (Hewlett Packard 54600B), and the chamber filled with Milli-Q water (about 1.5 mL). The voltage (reading on the oscilloscope) was increased to 3 V as the frequency was progressively lowered to 0.5 Hz over a span of 2 – 5 hours. Giant spherical vesicles (presumably unilamellar or of only a few bilayers)[27] with diameters of 10 – 100 μm were formed upon removing the current. All electroformation and subsequent experiments were conducted at 20 °C.

Holding and injection pipettes were prepared from Narishige G-1 capillary tubes using a Sutter P-97 horizontal pipette puller. The holding pipettes were further polished with a Narishige MF-9 microforge. The vesicles were manipulated with Narishige micromanipulation gear connected to a Nikon PLT-188 picoinjector. The vesicles were grasped with holding pipettes and released near the bottom of the chamber to: a) reduce the influence of adventitious air currents that circulated the water and b) rupture the vesicle – wire lipid tether.[27] The tether, which is inherent in vesicles produced by electroformation, allows for water escape, which means that the tethered vesicles are not completely closed spheres. The absence of a tether in vesicles isolated with our method was confirmed by both epi-fluorescence microscopy and the fact that the vesicles did not "rebound" to the wire when released (as connected vesicles do). Microinjections of digitonin solution were performed at a pressure of 3.0 psi, a time of 50 ms, and a balancing pressure (to alleviate capillary action) of 0.042 – 0.055 bar. The injection volume, as determined by optical microscopy evaluation of pipette clearance,[28] averaged 2.7 ± 0.5 pL. External microinjections were performed 5 – 10 μm from the vesicle surfaces.

The samples were examined by phase-contrast microscopy with a Nikon Diaphot TMD microscope in tandem with a Dage-MTI CCD-72 solid-state camera, Panasonic AG-1960 SVHS VCR, Hamamatsu Argus-10 image processor, and a Sony black-and-white monitor. This system was connected to a Micron Millenia 166 PC workstation equipped with Image-Pro Plus image processing software, and images were printed on a Tektronix Phaser 440 color printer. Epi-fluorescence microscopy was achieved with the identical setup except for replacement of the CCD camera with a Dage-MTI VE 1000SIT low-light camera and application of either Nikon V-2A ($\lambda_{excitation} = 380 – 425$ nm; $\lambda_{barrier} = 450$ nm) or B-2A ($\lambda_{excitation} = 450 – 490$ nm; $\lambda_{barrier} = 520$ nm) filter combinations.

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