Exposure of tilapia pituitary cells to saponins: Insight into their mechanism of action

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Abstract

Cell permeation and durable effects of triterpenoidal saponin preparations from soybean (SbS), \textit{Quillaja saponaria} Molina (QsS) and \textit{Gypsophila paniculata} (GypS), were studied. A concentration-dependent change in hemolysis rates was observed when cells were incubated with QsS or GypS, but not with SbS. Dose dependence was also observed for the leakage of lactate dehydrogenase (LDH; MW 142,000) and of Luteinizing Hormone (LH; MW 35,000) from tilapia pituitary dispersed cells. Exposure of pituitary fragments to a combination of GnRH and GypS or QsS, resulted in a significantly high release of LH. GypS were shown to be more potent in inducing hemolysis of human RBC’s and LH release from tilapia pituitary fragments. Interestingly, tilapia pituitary fragments treated with QsS were able to secrete LH in a characteristic manner, in response to a second Gonadotropin Releasing Hormone (GnRH) pulse, while fragments exposed to GypS did not respond to the second hormone pulse. The rapid recovery of pituitary fragments after the removal of QsS, may suggest a rearrangement of membranes rather than pore formation as the mechanism of action of QsS. Understanding the structural features underlying the reversible rearrangement of membranes and the lack of hemolysing activity by specific saponins may lead to the development of novel bioactive drugs.

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1. Introduction

Saponins are widely distributed naturally occurring glycosides, consisting of a non-sugar aglycone coupled to sugar-chain units. \textit{Quillaja saponaria} (Qs) bark and \textit{Gypsophila paniculata} (Gyp; ‘baby’s breath’) are two of the four major commercially available sources of saponins (Fulcheri et al., 1998; Rao and Gurfinkel, 2000; Francis et al., 2002a,b,c). In Qs, saponins may represent 20–25% of the extractable material (Guo and Kenne, 2000). They were recently found to have the potential to improve growth and body-composition parameters in tilapia fry and to stimulate growth and inhibit egg production by female Nile tilapia (Francis et al., 2001a,b; 2002a,b,c). Qs saponins (QsS) have a five-ringed (triterpenoid) quillaic acid (1 in Fig. 1) backbone with small carbohydrate chains consisting of two to five sugar units, attached at the 3 and 28 carbons of the aglycone and are frequently branched (Bomford et al., 1992). Attached to the first fucose sugar unit at the 28 position of the carbohydrate chain is an 18-carbon acyl chain with a small carbohydrate chain, consisting of one or two sugar units at its terminal end.
The five-ringed (triterpenoid) gypsogenic acid (2 in Fig. 1) is the aglycone present in saponins from Gyp, that are of interest to the pharmaceutical industry (Fulcheri et al., 1998). Soybean saponins (SbS) are found in many legumes and are the major dietary source for saponins (Hu et al., 2002). They are triterpenoid saponins of the oleanane-type, i.e., olean-12-ene triterpenes with a C-28 methyl group and a glucuronic acid moiety linked at the C-3. They are divided into two groups, A and B, of which group B (3 in Fig. 1) has been found to contain the primary saponins present in soybeans (Glycine max) (Hu et al., 2002). The genuine group B saponins are conjugated with 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) (Kudou et al., 1994).

It is usually suggested that the biological effects of saponins result from their potent membrane-permeabilizing activities. Common modern uses of saponins in biology include the permeation of cell membranes and active adjuvant components, in their pure form or as part of immuno-stimulating complexes (ISCOMs) (Ronberberg et al., 1995). Using saponins for experimental permeation allows small molecules or macromolecules to cross the cell membrane (Mick et al., 1988; Humbel et al., 1998; Baumann et al., 2000). Although saponins are broadly used as permeabilizing agents, the mechanism by which they allow the entrance of molecules into the cell, and the extent of the reversibility of this process, have not been adequately addressed (Melzig et al., 2001).

Gonadotropin releasing hormone (GnRH) is the most universal and important stimulator of teleost gonadotropins (GtH) such as Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH; reviewed by Yaron et al. (Yaron et al., 2003)). Exposure of pituitary fragments or dispersed cells of tilapia to GnRH results in the dose- and time-dependent release of LH (Levavi-Sivan and Yaron, 1993; Levavi-Sivan et al., 1995).

This study was aimed at exploring the long-lasting effect that triterpenoid saponins may exert on cells. To accomplish this objective, triterpenoid saponin preparations from three different plant sources (Qs, Gyp and Sb) were used, and changes in membrane permeability were measured as LDH and LH release from tilapia pituitary, and hemolysis of human RBC.

2. Materials and methods

2.1. Source of saponins

Preparation and isolation of the SbS (100 gr) were performed as described previously (Hu et al., 2002). Dried, finely ground soybean (Glycine max) powder was extracted with 1 L of 70% aqueous ethanol with stirring for 3 h at room temperature. The extract was condensed to 100 mL with a rotary evaporator (Büchner, Brinkman, R-114, Switzerland) at <30 °C, and loaded on a C-18 extractclean column (High Capacity C18, Alltech, IL) equilibrated with 10:90 (v/v) methanol/water and then fractionated with a linear gradient of aqueous methanol from 30 to 100%. Saponin-rich fractions were visualized on TLC plates (Z. Karem et al., 2005) and pooled. The fractions containing the SbS were collected and evaporated to dryness at <30 °C. The residue was redissolved in water to obtain a crude SbS solution. HPLC showing the characteristic absorbance of the DDMP moiety at 292 and 205 nm (Hu et al., 2002) served to ascertain the presence of the DDMP saponin as the major compound in these preparations (Fig. 2). HPLC was also used to ensure the DDMP stability throughout the experiments.

Quillaja saponaria Molina saponin was obtained from Sigma Chemical (St. Louis, MO). Gypsophila paniculata saponin was obtained from Merck KgaA (Darmstadt, Germany). Commercial powders were dissolved in aqueous methanol (50%) and fractionated on a C-18 extractclean™ column (High Capacity C18, Alltech) with a linear gradient of aqueous methanol from 50 to 100%. Fractions containing saponins were identified using TLC and staining with anisaldehyde reagent (Oleszek, 2002), collected, dried and redissolved as above.

2.2. Fish

The fish used in this study were tilapia hybrids (Oreochromis niloticus x O. aureus). They were collected from the farm of local kibbutzim and then housed at the university’s fish facility, in 500-L tanks under a natural photoperiod and 26±2 °C. For the experiments, the fish (weighing 50–150 g) were sexually mature.
2.3. Primary culture of pituitary cells

The primary culture of tilapia pituitary cells was generally according to Levavi-Sivan and Yaron (Levavi-Sivan and Yaron, 1993). Briefly, pituitaries were collected aseptically from male tilapia into the culture medium (medium 199 containing NaHCO₃ (9 mM), penicillin (100 IU/mL), streptomycin (0.1 mg/mL), nystatin (1.25 IU/mL)) to which 0.3% BSA was added and buffered to pH 7.4 with 10 mM HEPES. The glands were cut and trypsinized in trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA in PBS containing 1% glucose). After counting and determination of viability by trypan blue exclusion, the cells were plated on a 96-well plate (120,000 cells/well/0.2 mL) for LH determinations; or on a 6-well plate (700,000 cells/well/2 mL) for LH or lactate dehydrogenase (LDH) determinations. The cells were cultured for four days at 28°C under an atmosphere of 5% CO₂, and then challenged for 5 h with the saponins dissolved in the culture medium. The medium was collected for later analyses. Experiments were repeated at least three times and a representative experiment is shown.

2.4. Perfusion of tilapia pituitary fragments

The perfusion method was generally according to Levavi-Sivan and Yaron (Levavi-Sivan and Yaron, 1989), and Levavi-Sivan et al. (1995). Briefly, pituitaries were excised from three fish and cut into fragments. The fragments were embedded in Biogel P-2 and perfused at 26 °C±1 °C with Eagle’s basal medium for 14 h before beginning the experiment. The average LH secretion rate during the last 3 h was taken as the baseline. The results of LH release are expressed as mean±SEM of the ratio between the secretion rate after manipulation and the basal secretion rate. Experiments were repeated three times and a representative experiment is shown.

2.5. LH determination

Tilapia LH (taGtH) was determined in the medium by a specific radioimmunoassay according to Levavi-Sivan and Yaron (1989).

2.6. LDH assay

Dispersed tilapia pituitary cells were incubated with saponins for 5 h, as above. At the end of the incubation, the medium was removed and the level of LDH was determined with a commercial kit (Sigma chemical), according to the manufacturer’s protocol. Percentage leakage was calculated on the basis of the LDH value to full lysis, achieved by Triton X-100 (1%).

2.7. Erythrocyte lysis assay

2.7.1. Erythrocytes

Human red blood cells (RBC) were obtained from healthy adult blood-bank donors. Blood was collected with 200 IU/mL sodium heparin, centrifuged for 15 min at 600 g and plasma was separated. Blood cells were washed four times with 0.9% NaCl and finally with PBS. White cells and platelets were carefully aspirated. A series of three independent assays was performed and the mean±SEM is presented.

2.7.2. Lysis assay

Studies were performed by suspending 20 µL of washed RBC in 2 mL isotonic hemolysing buffer (50 mM Tris, 50 mM maleic acid, 100 mM KCl, 0.5 mM EDTA, adjusted with KOH to pH 7.2) containing increasing doses of the various saponins. The suspension was incubated for 30 min on ice. Membranes and intact cells were removed by centrifugation at 18,000 ×g for 30 min, at 4 °C. The absorbance of hemoglobin in the supernatant
was measured at 540 nm. Hemolysis is expressed as percent hemoglobin released in a solution of 1% Triton X-100. Triplicate samples were tested at each saponin concentration.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was employed to compare mean values, followed by Newman–Keuls a posteriori probability test, which simultaneously compares means of all examined groups (GraphPad software). Three independent experiments were carried out, each in triplicate, and the results are presented as mean of three experiments ± SEM.

3. Results

In order to compare the potency of the various saponins erythrocyte lysis was determined. Hemolytic activity was detected at a saponin concentration of 0.5 µg/mL with GypS, and of 10 µg/mL with QsS (Fig. 3). There was a concentration-dependent change in hemolysis rates when cells were incubated with QsS or GypS. No change was noted in the hemolysis rate after exposure of human RBC to either of the tested doses of SbS. The ED50s of QsS and GypS for the hemolysis of RBC were 26.12 ± 0.09 µg/mL and 3.34 ± 0.003 µg/mL, respectively. The highest hemolysis rate recorded in this study was obtained at GypS dose of 10 µg/mL (Fig. 3).

Leakage of LDH, a relatively large protein (MW 142,000), from cultured tilapia pituitary cells was chosen as an index for changes in membrane permeability in these nuclei-containing cells. All saponins used had a significant effect on their permeability, with SbS showing the weakest effect and GypS the strongest (Fig. 4). However, GnRH, which induces the release of LH from pituitary cells, had no effect on their membrane permeability.

Pituitary cells of tilapia were used in order to assess the ability of cells to recover from the damage caused by saponins. All saponins tested in this work stimulate LH release from the pituitary cells of tilapia in a dose-dependent fashion (Fig. 5). However, we found some variation in the potency of the various saponins at the different doses. SbS at the lower doses suppressed GnRH-stimulated LH release, whereas it stimulated LH release only at the highest dose tested (200 µg/mL; Fig. 5A). QsS significantly stimulated LH release at a dose of 100 µg/mL and GypS at a dose of 10 µg/mL (Fig. 5B). The amount of LH discharged into the medium by QsS (100 µg/mL) or GypS (10 µg/mL; Fig. 5C) was in the same order of magnitude as that released in response to the native hormone salmon-GnRH (sGnRH; 10 nM), during the 5 h incubation.

To determine whether saponins can alter the cell’s response to the native hormone, sGnRH and each of the saponins were added simultaneously. The combination of sGnRH and the highest dose of SbS resulted in a significantly higher release of LH (Fig. 5A). However, in the presence of the combination of sGnRH and GypS, an increased response was observed already at 10 and 100 µg/mL of the saponin preparation.

Our next goal was to test whether holes formed in the pituitary cells prevent or alter the response to repetitive pulses of the native decapeptide sGnRH. For this purpose a perfusion system of pituitary fragments of tilapia was used. A high secretion rate of LH from tilapia pituitary fragments was evident after exposure for 1 h to QsS (10 µg/mL; Fig. 6). At the end of the first hour a sGnRH (10 nM) pulse was
given concomitantly with the QsS, but no significant response was recorded. A sharp decrease in LH levels was recorded after withdrawal of QsS from the medium. However, after 4 h of rinsing, a characteristic LH response to sGnRH was recorded from pituitaries exposed to the saponins, similar to that of the control (Fig. 6). Exposure of tilapia pituitary fragments to GypS (10 µg/mL) resulted in a dramatic increase in LH secretion (Fig. 7), that was not changed when the sGnRH pulse was given concomitantly with the saponin. However, in contrast to the characteristic response to a second pulse of sGnRH given after the withdrawal of QsS (Fig. 6; Levavi-Sivan and Yaron, 1989), a second pulse of the hormone given after withdrawal of GypS did not yield any release of LH (Fig. 7). SbS had no

Fig. 5. Luteinizing hormone (LH) release from dispersed tilapia pituitary cells in response to soybean saponins (SbS; A), *Quillaja saponaria* saponins (QsS; B) or *Gypsophila paniculata* saponins (GypS; C). Cells were treated for 5 h with either 10 nM salmon GnRH or with various concentrations of the saponin (1, 10, 100 or 200 µg/mL). LH values are given as the ratio between basal secretion and secretion after stimulation. Each value is the mean±SEM of three different wells from three or four independent experiments. Concentrations that do not share the same letter are significantly different (p<0.05).
significant effect on LH release from pituitary fragments of tilapia in perifusion (data not shown).

4. Discussion

Three saponin preparations, all consisting of a triterpenoidal aglycone, and all shown previously to exert health promoting effects, were studied in the present work. Two of which (QsS and GypS) are broadly used as permeabilizing agents (Mick et al., 1988) in research. All preparations were used after partial purification on a C-18 preparative column, as crude saponin mixtures. In accordance with earlier reports, GypS and QsS showed strong hemolytic activities (Francis et al., 2002a,b,c), while the SbS showed none in the range of concentrations used in this study. ShS have been shown to possess multiple health-promoting properties, such as lowering cholesterol levels by inhibiting its absorption, and being anti-carcinogenic (Rao and Sung, 1995). However, the fact that they do not exert any hemolytic effect may support the suggestion that their mechanism of action does not require pore formation. The potent hemolytic activities of GypS and QsS are usually interpreted as the formation of nonspecific “pores” in membranes, which is widely recognized as the mechanism of action of saponins (Armah et al., 1999). The extent of the permeabilization was shown earlier to differ among various saponins, as a consequence of several factors, such as acylation (Melzig et al., 2001) or the

Fig. 6. Luteinizing hormone (LH) secretion from perifused tilapia pituitary fragments in response to Quillaja saponaria saponins (QsS; 2 h; 10 μg/mL; black arrows) in the presence or absence of salmon GnRH (sGnRH; 5 min; 10 nM; grey arrows), (mean±SEM). Dotted line represents control channels that were exposed only to sGnRH. Other details of the perifusion system can be found in the Materials and methods.

Fig. 7. Luteinizing hormone (LH) secretion from perifused tilapia pituitary fragments in response to Gypsophila paniculata saponins (GypS; 2 h; 10 μg/mL; black arrows; left axis) in the presence or absence of salmon GnRH (sGnRH; 5 min; 10 nM; grey arrows) (mean±SEM). Dotted line represents control channels that were exposed only to sGnRH (right axis). Other details of the perifusion system can be found in the Materials and methods.
degree of glycosilation (Haruna et al., 1995). However, since QsS and GypS are widely used in the pharmaceutical industry and research, the ability of cells to recover calls for a specific attention.

Any application of saponins as therapeutic aids requires an assay system that will enable the study of cellular functions following their application and removal, and the elucidation of the time course of their effect on cells. The induction and the active secretion of LH from tilapia pituitary cells by GnRH, over time, reflect the normal functioning of several cellular components. It was thus chosen here as a candidate model system to study the residual effects following the application and removal of saponins from the medium. First, the general effects of saponins on tilapia pituitary cells were assayed, studying the leakage of LDH from dispersed cells induced by the application of each of the three saponins. Gyps and QsS were very effective causing non-specific leakage of cytoplasmic permeabilization of both cell types tested. However, SbS saponin caused leakage of LDH from pituitary cells, but was unable to cause hemolysis of human RBC, and was ineffective in inducing LH release. This emphasizes the variable effect of different saponin preparations on different cell types.

Exposure of tilapia pituitary fragments to sGnRH results in the release of LH to the medium (Levavi-Sivan and Yaron, 1989; Levavi-Sivan et al., 1995). Exposure of the fragments to QsS or GypS (10 μg/mL) caused a significant increase in LH release; an increase that was not augmented with the addition of sGnRH in the presence of either of the saponin preparations. In the controls, a second exposure to sGnRH after 3 h resulted in a similar increased rate of secretion. However, a differential effect was found in response to a 5-min pulse of sGnRH, 3 h after withdrawal of GypS, while a typical response was evident after the withdrawal of QsS. The hemolysis assay and the LH secretion from tilapia pituitary cells may be interpreted as indicative of pores in the membranes. The LDH assay is usually interpreted as cell viability. Neither cells with pores, nor dead cells, would secrete LH in a normal manner, as early as 3 h after the dramatic release of LH and the withdrawal of QsS. It should be noted that non-membrane-related mechanisms may play a role in the reaction of cells to the application of saponins. However, it may be suggested that conformational changes in membrane proteins, facilitated by QsS, induce various cellular reactions that lead to LH release or LDH leakage, but do not lead to cell death and the effect of the saponin is quickly cured.

In previous experiments it was shown that inclusion of Qs, at low levels (150 mg kg⁻¹) in the diet of common carp resulted in increased growth and metabolic efficiency (Francis et al., 2001a,b, 2002a,b,c). Moreover, dietary Qs was also found to change the sex ratio in favour of males, in tilapia (Francis et al., 2002a,b,c). Also in rat saponins from Petersianthus macrocarpus have been shown to stimulate both FSH and LH release by cultured pituitary cells (Benie et al., 1990).

We investigated the effects of triterpenoid saponins on the release or leakage of proteins from two types of cells. Each group of saponins, all containing structurally related aglycones, exhibited individual modes of action in each assay. It is thus suggested that in the range of assays studied, the side chains, rather than the aglycone structures, are the important module in exerting the specific activity of a saponin. It is generally accepted that saponins exert their effects through their interaction with membrane sterols (El Izzie et al., 1992; Armah et al., 1999; Melzig et al., 2001; Francis et al., 2002a,b,c). It may be suggested that this leads to rearrangement of the membranes, and the observed differences reflect the extent of this rearrangement. In the case of SbS, the overall effect is very limited. In the case of QsS, a considerable effect is observed at small doses, but as the effect is reversible, the extent of rearrangement is probably low. In the case of GypS, the dose–dependence curve is of the same magnitude as that of QsS, but the effect on the cell appears to be irreversible, suggesting a high degree of rearrangement. Understanding the structural features underlying the reversible rearrangement of membranes by specific saponins and the lack of hemolyzing activity of others may lead to the development of novel bioactive drugs. More research is needed in order to determine the effects of various saponins on LH secretion in vivo.

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References


