Molecular basis of glycoalkaloid induced membrane disruption

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Abstract

In this study the interaction between the glycoalkaloids α-chaconine, α-solanine and α-tomatine and sterols in model membranes was analysed systematically using techniques like membrane leakage, binding experiments, detergent extraction, electron microscopy, NMR and molecular modelling. The most important properties for sterols to interact with glycoalkaloids turned out to be a planar ring structure and a 3β-OH group, whereas for α-chaconine the 5-6 double bond and the 10-methyl group were also of importance. The importance of sugar–sugar interactions was illustrated by the high synergistic effect between α-chaconine and α-solanine, the leakage enhancing effect of glycolipids, and the almost complete loss of activity after deleting one or more mono-saccharides from the glycoalkaloids. The formed complexes which were resistant against detergent extraction existed of glycoalkaloid/sterol in a 1:1 ratio and formed tubular structures (α-chaconine) with an inner monolayer of phospholipids, whereas with α-tomatine also spherical structures were formed. Based on the results a molecular model for glycoalkaloid induced membrane disruption is presented.

Keywords: Glycoalkaloid; Tubular structure; Sterol; Glycolipid

1. Introduction

Glycoalkaloids are natural toxins occurring in nightshades like potato and tomato. The toxic potential of these compounds is very high and a wide range of organisms has been shown to be susceptible to glycoalkaloids [1,2]. Glycoalkaloids consist of a six-ring steroid structure (aglycon) with a sugar moiety attached to the 3-position of the first ring and a nitrogen atom in the sixth ring end of the molecule. Three of the most well known glycoalkaloids are α-solanine and α-chaconine from potato and α-tomatine from tomato (Fig. 1).

One of the mechanisms whereby these glycoalkaloids cause toxic effects has been partly elucidated in the recent past. Roddick and co-workers showed that glycoalkaloids caused membrane disruption [3,4], leading to release of peroxidase previously enclosed in lipid vesicles. This effect was only induced when sterols were present in the membrane. Membrane disruptive effects were also found when fungal protoplasts [5], rabbit erythrocytes [5], red beet cells [5] and mouse cell lines [6] were exposed to glycoalkaloids. Model membrane systems allow to systematically investigate the molecular mechanism of the glycoalkaloid-membrane interaction and its consequences for membrane organisation. As a first step in this direction we recently used vesicle leakage, monolayer, calorimetric and freeze-fracture techniques to study the membrane interaction of some glycoalkaloids [7]. The results demonstrated that the glycoalkaloids interacted specifically with membrane associated cholesterol resulting in the formation of...
specific complexes which caused disruption of the membrane. This effect was strongly dependent on the type of sugar moiety of the glycoalkaloid and the type of sterol present in the membrane. For instance, α-l-chaconine caused strong lysis of cholesterol containing lipid vesicles whereas α-solanine, which has the same aglycon structure but a different tri-saccharide moiety (Fig. 1), hardly had an effect [7]. In combination, both molecules showed strong synergism illustrating the importance of specific sugar group mediated packing properties in the glycoalkaloid-sterol complexes [5,8]. Typically, the plant sterols β-sitosterol and fucosterol made the bilayer much more susceptible towards the disruptive effects of the glycoalkaloids than cholesterol and ergosterol [7].

In this study we analysed systematically the following aspects of the glycoalkaloid-membrane interaction. Firstly, the structural requirements of the sterol molecule for the membrane disruptive effect of the glycoalkaloids were studied using a series of sterol analogues. Secondly, insight into the importance of sugar residues for the glycoalkaloid-membrane interaction was obtained by specifically deleting mono-saccharides from the glycoalkaloids, by incorporating glycolipids in the membrane and by analysing synergistic interactions between glycoalkaloids. Thirdly, the stoichiometry, the stability and the structure of the glycoalkaloid-sterol complexes were studied using binding experiments, detergent extraction, electron microscopy and nuclear magnetic resonance (NMR) methods. The results obtained illustrate the striking structural specifics of the glycoalkaloid-sterol interaction and lead to a molecular model of the glycoalkaloid-sterol complexes.

2. Materials and methods

2.1. Materials

α-Tomatine, tomatidine, α-solanine, α-chaconine, egg yolk phosphatidylcholine (PC), egg yolk phosphatidic acid (PA), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), fucosterol, β-sitosterol, cholesterol, 5-cholesten-3-one, cholesteryl-β-D-glucuronide, sphingomyelin, di-galactosyldiacylglycerol (DGalDG), monogalactosyldiacylglycerol (MGalDG), lactocerebroside and galactocerebroside were obtained from Sigma (USA). Cholesterol and ergosterol were obtained from Merck (Germany) and ICN (USA), respectively. 1,2-Dilinolenoyl-sn-glycero-3-phosphocholine (DLPC) was obtained from Avanti Polar Lipids (USA). 1,2-Di-n-heptadecanoyl-3-β-D-glucosyl-sn-glycerol (MGluDG) was a gift from D.A. Mannock (Canada). β,β-(without (1-4)-rhamnose)-Chaconine and β,β-(without (1-2)-rhamnose)-chacone were a gift from W. van Gelder (The Netherlands). Epicholesterol was supplied by Mann (USA). 5α-androstan-3β-ol and 5α-pregnan-3β-ol by Steraloids (USA), 5-cholesten-10-nor-3β-ol by Roussel (USA) and coprostanol by Supelco (USA). For chemical structures of the sterols used see Fig. 2. 6-Carboxyfluorescein (CF, Eastman Kodak, USA) was purified by active carbon treatment, recrystallisation from water/ethanol (2:1, v/v) and Sephadex LH20 (Pharmacia, Sweden) column chromatography [9]. The glycoalkaloids were dissolved in dimethylformamide (DMF) up to 20 mM and regularly tested for possible hydrolysis or breakdown by thin layer chromatography (TLC) on silica using methanol/chloroform/1% ammonium hydroxide (in H2O) 50:50:1 (by volume) as eluens. All reagents were analytically pure and solvents used were of analytical grade.

2.2. Vesicle preparation

Large unilamellar vesicles (LUVETS) were prepared from mixtures of PC and other lipids (total lipid 10 μmol) in 1 ml buffer using the extrusion technique (0.4 μm polycarbonate filters, Nuclepore, USA) described by Mayer et al. [10]. Multilamellar vesicles (MLVs) were prepared from PC/PA/sterol mixtures in a 13:1:6 molar ratio by
repeated freeze-thawing of lipid films. Phospholipids were quantified after perchloric acid destruction by the method of Rouser [11] and the cholesterol content was determined by the method of Courchaine et al. [12]. As it was found that extrusion resulted in a loss of 10% of cholesterol from equimolar cholesterol/PC, lipid films with higher initial cholesterol contents were prepared to obtain vesicles with 50% cholesterol. No loss of sterol occurred during extrusion of vesicles containing only 20 mol% sterol. All vesicle experiments were carried out at room temperature.

2.3. CF leakage assay

Leakage of CF from loaded vesicles was determined as described previously [7]. Glycoalkaloids were added to CF loaded vesicles (total lipid 100 nmol) after which leakage of CF was determined on a Perkin Elmer LS50B Luminescence spectrometer.

2.4. α-Tomatine hydrolysis

In order to prepare β1-(without xylose), β2-(without glucose), γ-(without xylose and glucose) and δ-(without xylose and both glucose) tomatine (Fig. 1), 50 mg α-tomatine was dissolved in 100 ml 5% H2SO4 and incubated for 16 h at 60°C. After the solution was cooled down to room temperature, the pH was adjusted to pH 10 with NaOH. Hydrolysis products were extracted three times with 100 ml n-butanol. The pooled butanol fractions were evaporated to dryness on a rota-vapor (Büchi, Switzerland) and dissolved in ethanol. The ethanol solution was injected on a HPLC system (Waters, USA) to separate the different products. HPLC was performed isocratically using a flow rate of 1.0 ml/min and a Nucleosil 5-NH2 column (Macherey, Nagel & Co. (Switzerland)) with a mobile phase consisting of acetonitrile/10 mM potassium dihydrogenphosphate (80:20, v/v). The tomatine derivatives were detected at 200 nm and peaks were collected and evaporated to dryness on a rota-vapor. The identity of the different fractions was determined by 1D and 2D HOHAHA H-NMR [13]. The dry fractions were dissolved in CDCl3/DMSO(D6) in a 1:1 ratio and measured at 300 K and 400 MHz on a Bruker AMX 400 wb I. The 1D spectra of the aglycon parts were compared to the 1D spectra of both the aglycon part of pure α-tomatine and the pure aglycon and were shown to be identical. The composition of the sugar moieties were solved using the 2D HOHAHA NMR method by comparison with the pure α-tomatine sample. Both β-tomatines were not separated on HPLC and used as a mixture. From the NMR spectra it was calculated that the mixture consisted of 70% β1 and 30% β2. The hydrolysis products were dissolved in ethanol and the purity and possible breakdown were tested by TLC using methanol/chloroform/1% ammonium hydroxide (in H2O) 45:55:2 (by volume) as eluting solvent and visualized with iodine staining. They were shown to be pure on TLC.

2.5. Binding experiments

Binding experiments were carried out as described previously [7]. PC LUVETS (total lipid 100 nmol) containing 0 to 50 mol% cholesterol were incubated with glycoalkaloids. For which glycoalkaloids were extracted and subsequently analysed by HPLC as described in the next section.

2.6. Isolation of glycoalkaloid-sterol complexes

In order to isolate the glycoalkaloid-cholesterol complexes, PC LUVETS containing 50% cholesterol were prepared in a 10 mM Hepes buffer (pH 7.4) containing 25 mM NaCl. In a total volume of 3 ml, vesicles (400 nmol lipid) were mixed with different amounts of glycoalkaloids. After an incubation of 5 min at room temperature, 30 μl of a Triton X-100 solution (5%, v/v) was added and the sample was mixed for 1 min. Subsequently, the detergent solubilized samples were centrifuged (15°C, 45 min, 541 000 × g) in a Beckman optima TL table cen-
trifuge (rotor TLA 100.3) resulting in a pellet containing the complex and supernatants which were directly analysed for PC and cholesterol. Glycoalkaloids in the supernatant were extracted three times using n-butanol in a two phase extraction. The pooled n-butanol fractions were dried on a rotary evaporator and subsequently dissolved in 40% methanol (v/v) and prepared for and analysed by HPLC as described by Saito et al. [14] and Keukens et al. [15].

2.7. Freeze-fracture electron microscopy

The isolated complexes were quickly frozen by plunging them in liquid propane and subsequently fractured and etched in a Balzer freeze-etch machine according to standard procedures. Replicas were examined on a Philips EM 400T/ST electron microscope.

2.8. Molecular modelling

The lowest energy of a complex between the aglycon solanidine and cholesterol was calculated using the MAXIMIN 2 minimizer from the Sybyl 6.1 (Tripos, St. Louis, USA) program [16] on a Silicon Graphics Iris workstation. Input parameters were only the crystal structures of cholesterol and demissidine, which lacks the 5-6 double bond compared to solanidine and was adjusted before calculations, obtained from Cambridge Structural Database (Cambridge, UK). Both molecules were aligned in such a way that the length axis of both ring structures were parallel to each other. For all possible orientations the minimum energy was calculated.

2.9. Nuclear magnetic resonance (NMR)

Multilamellar vesicles were prepared from a DOPC/cholesterol mixture (1:2, molar, total lipid 33 μmol) by repeated freeze-thawing of lipid films in a 10 mM Hepes buffer (pH 7.4) containing 25 mM NaCl. Subsequently, the MLVs were diluted in 50 ml buffer and 22 μmol α-chaconine dissolved in 1 ml DMF was added and incubated for 10 min. After centrifugation (2000 × g, 15 min, 15°C) 31P-NMR spectra were recorded from the pellet on a Bruker MSL-300 spectrometer at 121.5 MHz as described by Chupin et al. [17] using an interpulse time of 1.0 s. Prior to Fourier transformation free induction decays where exponentially filtered resulting in a line broadening of 110 Hz. The 0 ppm in the spectrum corresponds to the chemical shift of small unilamellar DOPC vesicles.

3. Results

3.1. Requirements of the sterol structure

In order to determine the specific requirements of the sterol structure for glycoalkaloid induced membrane disruption, vesicles containing the different sterols illustrated in Fig. 2 were tested. Attention was focused on the role of the 3-hydroxy group, the structure of the ring system and the length of the C-17 side chain. These experiments were carried out using MLV because some of the sterols strongly interfered with the extrusion procedure. Phosphatidic acid (PA, 5 mol%) was included to increase the enclosed volume of the vesicles. The kinetics of CF leakage from MLV induced by glycoalkaloids were found to be similar to the kinetics of the leakage observed from LUVETS [7] (not shown). The results revealed that CF leakage decreased when cholesterol in the vesicles was replaced by any of the other sterols (Fig. 3A and B). When cholesterol was replaced by coprostanol, which does not have a planar ring structure, α-chaconine completely lost its ability to disrupt the membrane. Alterations of the β-OH group also
generally resulted in a decreased α-chaconine activity; with the cholestenone and the cholesteryl-glucuronide activity was almost completely lost, whereas with the 3α-OH isomer (epi-cholesterol) some rest activity was observed. A complete loss of α-chaconine induced membrane disruption was also obtained with vesicles containing 10-norcholesterol which lacks the 10-methyl group. Replacing the 5-6 double bond by a single bond thereby leaving the planar ring structure intact (cholestanol) had a less dramatic effect on the α-chaconine activity. Also, shortening the length of the side chain of cholestanol to pregnanol or androstanol resulted in an almost similar decrease of α-chaconine activity compared to cholestanol. α-Tomatine also lost its membrane disruptive activity completely when coprostanol was incorporated in the membrane (Fig. 3B). Changes of the β-OH group caused a high decrease of α-tomatine activity compared to cholesterol; with vesicles containing cholestenone or epicholesterol an almost complete loss of activity was observed whereas a small rest activity was observed with vesicles containing cholestereryl-glucuronide. In contrast with α-chaconine, α-tomatine was able to cause CF leakage from vesicles containing 10-nor cholesterol. Furthermore, the α-tomatine activity was only slightly decreased after addition to cholestanol containing vesicles compared to cholesterol containing vesicles. Decreasing the chain length of cholesterol resulted in a small gradual decrease of α-tomatine induced leakage which was illustrated with pregnanol and androstanol. The differences in effect of α-chaconine compared to α-tomatine show that the latter is less sensitive to structural changes of membrane sterols.

All type of vesicles were also tested for α-solanine induced CF leakage but besides some minor leakage from the cholesterol containing vesicles no leakage occurred.

3.2. Effect of different sugar moieties

To investigate the influence of the size of the sugar moiety on the membrane disruptive effect, α-tomatine (Fig. 1) was hydrolysed in order to obtain β1-(-gal-glu-glu), β2-(-gal-glu-xyl), γ-(-gal-glu) and δ-(-gal) tomatine. The β-tomatines could not be separated with this HPLC method and were used as a mixture. These compounds were tested for their membrane disruptive effect on CF loaded large unilamellar vesicles consisting of PC/cholesterol at equimolar concentration. The striking result was that α-tomatine almost completely lost its ability to disrupt membranes already by removal of one mono-saccharide from the sugar moiety (Fig. 4A). The β-tomatines and the γ-tomatine only induced minor CF leakage whereas δ-tomatine was slightly more effective and approached the value of tomatidine. The limited leakage induced by this aglycon is most likely due to an aspecific effect as it was also observed when cholesterol free vesicles were used (data not shown). Also, the effect of removing either rhamnose moiety of α-chaconine on cholesterol containing vesicles was analysed (Fig. 4B). Both β1- and β2-chaconine lost their ability to disrupt membranes. Thus, the intact tetra- and the tri-saccharide moieties of α-tomatine and α-chaconine, respectively, are of vital importance to those molecules to exert membrane disruption.

3.3. Synergism between α-chaconine and α-solanine

The synergistic effect reported for α-solanine and α-chaconine on cholesterol containing vesicles [5,8] was tested with vesicles containing different sterols to investigate whether this effect is mainly determined by the sugar moiety or that the sterol structure is also of importance. For sensitivity reasons it was decided to measure the synergistic effects at a total glycoalkaloid concentration corresponding to the concentration whereby α-chaconine caused approx. 50% CF release. These half maximal con-
centrations were determined to be 150 and 45 \( \mu \text{M} \) for cholesterol and fucosterol containing vesicles, respectively (Fig. 5A and B), illustrating the preference for the plant sterol. Over this concentration range \( \alpha \)-solanine did not cause a significant CF release. Varying the mol fraction of \( \alpha \)-chaconine and \( \alpha \)-solanine at the half maximal concentration showed for both sterols a striking synergism. Around equimolar \( \alpha \)-chaconine/\( \alpha \)-solanine mixtures, CF leakage from the cholesterol containing vesicles was 1.5 times increased over that induced by \( \alpha \)-chaconine at the half maximal concentration, despite the two-fold lower concentration of \( \alpha \)-chaconine added. At lower concentrations of \( \alpha \)-chaconine the synergism is even more striking. For instance at a 30 \( \mu \text{M} \) \( \alpha \)-chaconine concentration the presence of 120 \( \mu \text{M} \) \( \alpha \)-solanine caused a 10-fold increase in CF leakage. The results obtained with the fucosterol containing vesicles (Fig. 5B) were slightly different from the synergism observed with the vesicles containing cholesterol (Fig. 5B); maximum CF leakage occurred at a \( \alpha \)-chaconine/\( \alpha \)-solanine ratio which was higher than 1:1, whereas the CF leakage was only slightly increased over that at the half maximal concentration of \( \alpha \)-chaconine. Furthermore, the synergism at lower \( \alpha \)-chaconine concentrations is less effective compared to cholesterol. The synergistic effects of both glycoalkaloids on ergosterol and \( \beta \)-sitosterol containing vesicles were nearly identical to the effects on cholesterol or fucosterol containing vesicles, respectively (data not shown). Interestingly, the ratio of the mixtures of \( \alpha \)-solanine and \( \alpha \)-chaconine causing maximum CF release in all type of vesicles, is similar to the ratios found in potato, illustrating the proposed protective properties of those compounds for the plant [18].

These observations demonstrate that the synergistic effect of \( \alpha \)-solanine and \( \alpha \)-chaconine is finely tuned by the chemical structure of the sterol present in the membrane. The specificity of this glycoalkaloid-glycoalkaloid interaction is further demonstrated by the absence of any synergistic effect on the CF release of cholesterol containing vesicles of the combination of \( \alpha \)-tomatine and \( \alpha \)-solanine (data not shown).

3.4. Influence of membrane associated and free sugars

Membrane associated sugars were tested for their influence on glycoalkaloid induced leakage by studying the effect of incorporation of 5% or 10% of the plant galactolipids MGalDG or DGalDG in CF containing vesicles which also consisted of 50% cholesterol and PC. Because these glycolipids are very rich in linolenic acid [19], DLPC was incorporated into the control vesicles to reduce a possible influence of the changed fatty acid composition. The results with the control vesicles containing either 5 or 10% DLPC were almost similar, the average values are presented (Fig. 6). The presence of 5% galactolipids slightly, but significantly increased the CF leakage induced by all glycoalkaloids. With \( \alpha \)-tomatine, the leakage was increased 5–10% in vesicles containing either 5% MGalDG or 5% DGalDG (Fig. 6A). However, when 10% DGalDG was incorporated in the vesicles, the CF leakage was slightly decreased. It was not possible to form stable vesicles consisting of 10% MGalDG, 50% cholesterol and 40% PC, most likely as a result of the phase preference of these lipids. \( \alpha \)-Chaconine caused a higher CF leakage (10–20%) from vesicles containing 5% of the galactolipids, and in contrast to \( \alpha \)-tomatine, increasing the DGalDG concentration to 10 mol% resulted even in a further (30%) increase (Fig. 6B). The low effect of \( \alpha \)-solanine on cholesterol containing vesicles was more than

Fig. 5. Glycoalkaloid induced CF leakage from PC vesicles containing 20 mole % of either (A) cholesterol or (B) fucosterol. CF leakage was measured 5 min after addition of the indicated amounts of either \( \alpha \)-chaconine (C), \( \alpha \)-solanine (O) or mixtures of both (\( \Delta \)). In this latter case the indicated concentration is that of \( \alpha \)-chaconine supplemented with \( \alpha \)-solanine to the half maximal concentration (for further details see text). Each point represents the average of six replicates with a standard deviation of less than 2%.
doubled in vesicles containing 5% MGaIDG or DGaIDG (Fig. 6C) whereas the effect of a-solanine on vesicles containing 10% DGaIDG was only slightly higher compared to the 5% containing vesicles. No glycoalkaloid induced CF leakage from control vesicles containing glycolipids without any sterol was observed. To check whether the observed effects were only restricted to galactose diacylglycerols, experiments were carried out using vesicles consisting of either 5% MGlueDG, which carries a glucose moiety as headgroup, galacto- or lacto-(glucose-galactose) cerebrosides. The control vesicles contained 5% DPPC or sphingomyelin for the MGluDG and the cerebrosides, respectively. The results with these types of vesicles were similar for the three glycoalkaloids compared to those obtained with the vesicles containing the plant glycolipids (results not shown), indicating that the influence of glycolipids is not restricted to the presence of a galactose in the sugar group. It can thus be concluded that in general incorporation of glyco-glycerol and glycosphingolipids slightly but significantly stimulates glycoalkaloid induced CF leakage.

Subsequently, the effects of free saccharides on the glycoalkaloid induced membrane disruption were studied. CF loaded vesicles containing 50% cholesterol were exposed to the glycoalkaloids in the presence of up to 100 mM saccharides both captured in the vesicles and present in the medium. The sugar concentration at the surface of the membrane was calculated to be equal to the sugar concentration present at the surface of the glycolipid containing vesicles. Two monosaccharides (glucose and galactose) and two disaccharides (maltose and sucrose) were tested. No significant differences were observed compared to the control experiment (data not shown).

3.5. Stoichiometry of binding

In order to determine the stoichiometry of the irreversible binding of glycoalkaloids to cholesterol in the vesicle membranes, experiments were carried out using vesicles with different cholesterol contents (total lipid 100 nmol). a-Tomatine did bind very efficiently to vesicles containing 50% cholesterol (Fig. 7A). Addition of up to 50 nmol a-tomatine resulted in complete binding to the vesicles. Addition of higher amounts of o-tomatine did not result in extra binding suggesting a saturation of the cholesterol present in the membrane. Decreasing the cholesterol content of the vesicles resulted in a decreased binding of a-tomatine when low amounts of o-tomatine were added. However, addition of up to 100 nmol a-tomatine eventually resulted in binding of an equimolar amount a-tomatine to the cholesterol present which strongly suggests a binding ratio cholesterol/a-tomatine of 1:1. When the cholesterol content was less than 10%, no significant binding of o-tomatine was observed. The affinity of a-chaconine for the same type of vesicles was slightly decreased compared to a-tomatine (Fig. 7B). However, after addition of high amounts of a-chaconine to vesicles containing 30% cholesterol or more, a 1:1 cholesterol/a-chaconine ratio was observed. Addition of a-chaconine to vesicles containing 20% cholesterol or less only resulted in binding of substoichiometric amounts of this glycoalkaloid. a-Solanine did not show binding when similar amounts of this compound were added to the different vesicles. However, addition of a 1:1 mixture of a-solanine and a-chaconine to 50% cholesterol vesicles resulted in a very efficient and equimolar binding of both compounds (data not shown). This confirmed the strong...
synergistic effects of a mixture of both potato glycoalkaloids.

3.6. Composition and structure of the complex

In order to determine the composition of the complexes formed after the interaction of glycoalkaloids with sterol containing membranes, isolation of the complexes via detergent extraction was attempted. Experiments carried out by monitoring the absorbance at 450 nm revealed that the extinction of a Triton X-100 solubilized sample of cholesterol containing vesicles with glycoalkaloids did not decrease to a level which was obtained after solubilization of vesicles only (Fig. 8). Since α-tomatine does not absorb light at 450 nm this indicated that the complexes were not solubilized. Thus, vesicles containing 50% cholesterol were incubated with different amounts of α-tomatine, α-chaconine, α-solanine and an α-chaconine/α-solanine mixture (1:1) whereafter the membranes were solubilized by Triton X-100. After the complexes were pelleted by centrifugation the supernatant was analysed for PC, cholesterol and glycoalkaloids. The results are shown in Table 1.

In the absence of glycoalkaloids all PC and cholesterol

Table 1
Complex formation of glycoalkaloids with PC/cholesterol vesicles

<table>
<thead>
<tr>
<th>Glycoalkaloid (nmol)</th>
<th>Retrieved in supernatant (nmol)</th>
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<tbody>
<tr>
<td></td>
<td>PC</td>
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<tr>
<td>−</td>
<td>200</td>
</tr>
<tr>
<td>α-Tomatine</td>
<td>100</td>
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<td></td>
<td>200</td>
</tr>
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<td></td>
<td>400</td>
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<tr>
<td>α-Chaconine</td>
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<td>200</td>
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<td></td>
<td>400</td>
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<tr>
<td>α-Solanine</td>
<td>100</td>
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<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>400</td>
</tr>
<tr>
<td>Mix (sol/chac) b</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>196</td>
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<td>200</td>
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PC/cholesterol (1:1) vesicles (total lipid 400 nmol) were incubated with different amounts of glycoalkaloids and after 5 min Triton X-100 was added. After centrifugation (15°C, 45 min at 541 000× g) non-bound components were determined. Data represent the average of three replicates with a standard deviation of less than 3%.

b Ratio α-solanine/α-chaconine = 1:1.

c Total amount of α-solanine and α-chaconine.
was solubilized by Triton X-100, in the absence of Triton X-100 all PC and cholesterol was present in the pellet (data not shown). In the presence of equimolar amounts of glycoalkaloid, some cholesterol but all PC were solubilised except for \( \alpha \)-solanine where also all cholesterol and glycoalkaloid were recovered. After increasing the amount of

Fig. 9. Freeze-fracture electron micrograph (100,000 \( \times \)) of Triton X-100 solubilized samples of unilamellar PC/cholesterol (1:1, molar ratio) vesicles after the addition of (A,D) \( \alpha \)-chaconine, (B,E) \( \alpha \)-chaconine/\( \alpha \)-solanine (1:1, molar) or (F) \( \alpha \)-tomatine. The samples were centrifuged (541,000 \( \times \) g, 45', 4°C) and the pellets were frozen in liquid propane, fractured and some were also etched (D, E, F). The bar corresponds to 0.1 \( \mu \)m.
glycoalkaloids only PC and the additional amount of glycoalkaloids above 200 nmol were recovered from the supernatant. Again α-solanine was not able to form a stable complex with cholesterol. However, with a mixture of α-solanine and α-chaconine both molecules are included equally in the complex. Increasing the amount of glycoalkaloids to 400 nmol did not result in any additional binding of these compounds to the complex. This implies that stable and stoichiometric complexes can be formed between glycoalkaloids and sterols which are resistant to detergent extraction and which do not contain firmly bound PC.

Freeze fracture electron microscopy was performed on the pellets obtained after the detergent extraction in order to study the size and structure of the different complexes. Samples were either directly studied after fractioning or first etched. The results showed that a network of tubular structures was formed after incubations of α-chaconine and α-chaconine/α-solanine with cholesterol containing vesicles which was not solubilized by detergent extraction (Fig. 9A–E). The diameter of these tubuli is approx. 20–30 nm. In the freeze fracture sample of α-solanine/α-chaconine twisted tubuli (Fig. 9C) were observed next to the straight tubuli. α-Tomatine formed more irregular structures (Fig. 9F); both tubular and spherical structures occurred which vary in size from 40 nm for the tubular to 100 nm for the spherical ones. This again illustrated the differences in effect of this glycoalkaloid compared to α-chaconine and α-solanine.

3.7. 31P-NMR

To get insight into the macroscopic organization of DOPC in the tubular structures caused by the addition of α-chaconine to cholesterol containing DOPC vesicles an experimental protocol was designed (see Section 2) which can be expected to lead to maximal complex formation. The resulting complex was isolated by centrifugation and the pellet was analysed by 31P-NMR. This technique can discriminate between various macroscopic organizations of phospholipids because the chemical shift anisotropy of the phospholipid phosphorus is averaged differently in various aggregate structures because of different motional possibilities [20]. Strikingly, Fig. 10 shows that both at 25°C and 40°C the 31P-NMR spectra have an originally symmetrical line shape with a peak at 6 ppm and a shoulder at −14 ppm separated by 20 ppm, the residual chemical shift anisotropy. This corresponds to the theoretical spectra of a phospholipid which can undergo fast axial rotation as in a liquid crystalline system with an additional averaging of the chemical shift anisotropy in a direction perpendicular to the long axis of the molecule. This is characteristic for tubular structures [20] and forms a very strong indication that the phospholipids are co-organized with the chaconine-cholesterol complex in tubular structures. The residual intensity at low field of the 6 ppm peak most likely result from some remaining bilayer structures containing DOPC.

4. Discussion

The results obtained together with previous data lead to a model (Fig. 11) for the membrane disruptive activity of α-chaconine which we would first like to present. After membrane insertion of the glycoalkaloids the aglycon part reversibly binds to sterols (step 1,2) in a 1:1 ratio. When these complexes in the membrane reach a certain density, co-operative sugar-sugar interactions between the sugar moieties of the glycoalkaloids initiate (step 3,4) the formation of a stable irreversible matrix of glycoalkaloid/sterol complexes. Because the sterols in the outer leaflet get immobilised, sterols of the inner leaflet will probably flip and replace them. During the formation of the matrix structure, the latter will be budding of the membrane due to the fact that a glycoalkaloid/sterol complex with a relatively large polar headgroup does not have a cylindrical shape (step 5,6). The phospholipids in the inner leaflet of the membrane opposing the matrix get enclosed in the final structure during separation from the membrane and form a monolayer (step 6). Tubular structures are formed due to the three-dimensional structure of the sugar moiety causing faster growth of the matrix in one direction compared to the other.

Both cholesterol and the aglycon have planar apolar ring structures with two methyl groups sticking out on the
same side of the plane (Fig. 12). Using molecular modelling it was calculated that the complex between solanidine and cholesterol with the lowest energy was obtained when both ring structures opposed each other with the methyl groups sticking to the outside (Fig. 12A). Due to the low energy of this complex caused by van der Waals interactions this complex formation is reversible which was illustrated by the lack of α-solanine binding by cholesterol containing vesicles. Considering the proposed model this means that during the formation of a matrix an interaction between the sides with the methyl groups sticking out is necessary. Of all possible orientations the energy of interaction between solanidine and cholesterol with the methyl groups directed towards each other was calculated to be the second lowest energy which implicates that a matrix is formed as shown in Fig. 11B. The effect of α-chaconine on coprostanol containing vesicles showed that indeed the planar ring structure of the sterol is of vital importance to cause membrane disruption. Furthermore, deletion of only one methyl-group also resulted in a complete loss of disruptive activity. Major changes were observed when the 5-6 double bond was replaced by a single bond. This means that the interaction between α-chaconine and the sterol strongly depends on interactions of both the pi-electrons of both molecules. Changing the length of the side chain of cholestanol only caused a small decrease of α-chaconine activity. The loss of activity observed when the β-hydroxy group was altered is most probably caused by a different orientation of these molecules in the membrane [21].

For the α-tomatine-sterol interactions the planar ring structure of the sterol was also of vital importance. However, the minor decrease observed after deletion of the 10-methyl group differs remarkably for the situation found for α-chaconine. Furthermore, in contrast to α-chaconine, the α-tomatine activity was only slightly decreased when the 5-6 double bond of the sterol was replaced by a single bond. This means that the interactions exerted by the pi-electron of the double bond of the sterol are of minor importance to α-tomatine which already lacks the 5-6 double bond compared to α-chaconine. The difference of activity with cholestanol, pregnanol or androstanol containing vesicles when exposed to α-chaconine compared to α-tomatine strongly suggest that the latter is depending
less on the sterol structure to cause membrane disruption. This might be due to the difference in the strength of sugar–sugar interactions between the sugar moieties of both glycoalkaloids.

The presence of glycolipids in the cholesterol containing membrane resulted in an increased membrane disruption. The structure and composition of the sugar groups of the glycolipids was of minor importance whereas the concentration was. The lipid sugar groups most probably exert hydrogen-bondings with sugar moieties of the membrane associated glycoalkaloids thereby prolonging the presence of the glycoalkaloids in the membrane and enhancing the opportunity to form a membrane disruptive matrix. This was illustrated by the observation that glycoalkaloids with the lowest potency to disrupt membranes have the most benefit of the presence of glycolipids in the membrane. α-Tomatine probably has already reached maximal insertion when 10% glycolipid is incorporated which then disturbed the interactions between the sugar moieties of the glycoalkaloid. This glycolipid effect is very interesting for the toxicologcal effects of glycoalkaloids because they are found in many biological membranes [22].

Although hydrogen-bondings between the sugar moieties of glycoalkaloids are important during matrix formation, the key factors for this process to occur are the structure and composition of these moieties. Reduction of the sugar moiety of α-tomatine and α-chaconine by only one monosaccharide remarkably results in an almost complete loss of membrane disruptive activity. Both molecules are originally branched, and removal of one mono-saccharide yields linear oligosaccharides, suggesting that this property is vital for these compounds to cause disruption. However, this property alone is not sufficient for a glycoalkaloid to induce a loss of barrier function as was seen for α-solane. A three-dimensional fit between the sugar moieties appears to be a prerequisite for membrane disruption. These results differ strikingly from reports on digitonin, a well known saponin [23]. Digitonin interacts like glycoalkaloids with membrane sterols thereby causing membrane disruption. However, reduction of the pentasaccharide of digitonin to a di-saccharide reduced leakage from cholesterol containing vesicles only by 50% as compared to the parent molecule. This di-saccharide is identical to that of γ-tomatine, which did not cause any membrane disruption. This must be due to the different aglycon of digitonin which contains extra hydroxyl-groups in the first and fourth ring that probably give rise to a different sterol–aglycon interaction or even a different position of the sterol-aglycon complex in the membrane.

The synergism between α-solane and α-chaconine is a remarkable example of a very effective interaction between two sugar moieties of glycoalkaloids. Although α-solane hardly causes any formation of complexes with cholesterol, it is incorporated in the complex in a 1:1 ratio to α-chaconine. Because the aglycons of both compounds are identical, this means that the ineffective sugar moiety of α-solane becomes very effective when it is combined with the sugar moiety of α-chaconine. This interaction is only altered in a subtle way when the sterol structure is changed as was illustrated with the plant sterols.

The binding studies as well as the determination of the composition of the isolated complexes clearly demonstrate the formation of a glycoalkaloid/sterol matrix in a 1:1 ratio. The matrix formation depends on the cholesterol concentration and turned out to be a co-operative interaction between glycoalkaloid/cholesterol complexes. A higher cholesterol concentration facilitates a higher membrane binding of the glycoalkaloids despite the much closer packing of the membrane lipids. An increase of the glycoalkaloid concentration eventually results in a bound-glycoalkaloid/cholesterol ratio of 1:1 when the cholesterol content of the membrane is above a certain threshold which is higher for α-chaconine compared to α-tomatine. Below this cholesterol threshold concentration the low density of reversible glycoalkaloid-sterol complexes prevents the co-operative formation of an irreversible matrix.

The isolated complexes, which turned out to be tubular structures for α-chaconine (or α-chaconine/α-solane), consist of glycoalkaloids and cholesterol. Similar tubular structures were observed without detergent extraction as reported earlier [7]. Surprisingly and interestingly, these complexes were fully resistant against detergent extraction. Because eventually all cholesterol is bound by glycoalkaloids we favour the possibility that cholesterol flips from the interior leaflet of the bilayer to compensate for the lack of free cholesterol in the outer leaflet. Alternatively, cholesterol of the inner layer might become accessible for glycoalkaloids during destabilisation and reorganisation of the membrane caused by matrix formation. The PC which forms the inner monolayer of the tubular structures, as can be concluded from the NMR experiment, is replaced by Triton X-100. The structure of the final complexes will be determined by the three-dimensional structures of the single glycoalkaloid/sterol complex; when they have a shape like a piece of a pie (α-chaconine/cholesterol) they will arrange as tubuli, whereas more inverted cone shaped complexes (α-tomatine) will form spherical structures. The twisted structures observed in the mixed sample illustrated that the α-chaconine/α-solane complex formation is not uniform. This could be caused by the irregular composition of the matrix containing different parts with various α-chaconine/α-solane ratios. α-Tomatine also turns out to be able to form different structures which vary from a spherical to a tubular shape. This might be due to different arrangements of the single α-tomatine/sterol units in the matrix. This again illustrates that this molecule acts different compared to α-chaconine/(α-solane) although they share many common features.

This model for sterol-mediated glycoalkaloid induced membrane disruption presents a molecular basis for the toxicological effects related to the consumption of high amounts of these compounds. However, more understand-
ing about the relationship between the membrane disruptive effect and the reported symptoms will require future research on the interaction of glycoalkaloids with biomembranes.

References