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INTERACTION OF SAPONIN AND DIGITONIN WITH BLACK LIPID MEMBRANES AND LIPID MONOLAYERS

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The effects of the plant glycosides saponin as well as digitonin on the electrical conductance of black lipid membranes and the effect of these agents on the surface pressure of lipid monofilms was investigated. Both saponin and digitonin induced channel-like fluctuations in planar bilayers made either of diphytanoylphosphatidylcholine (DPhPC) or of DPhPC and cholesterol 2:1 (w/w). In cholesterol-free bilayers the amount needed to induce an increase in conductance was 0.3–1 mg/ml for saponin and about 0.2 mg/ml for digitonin. In contrast, in cholesterol-containing bilayers the concentration needed to induce pores was about 10 μg/ml for both saponin and digitonin. In cholesterol-containing membranes the fluctuating pores induced by saponin were about 3-times more permeable to K^+ than to Cl^- and the macroscopic current showed an ohmic behaviour. Surface pressure experiments demonstrate that both glycosides could penetrate into lipid monofilms of pure DPhPC spread at the air/water interface with an initial surface pressure of 30 mN/m. The increase in surface pressure was considerably enhanced in cholesterol-containing films. It is assumed that the channel-like fluctuations induced by saponin as well as digitonin, in both cholesterol-free and cholesterol-rich bilayers are due to the formation of micellar structures within the lipid lattice. Probably the penetration of the glycosides into the lipid bilayer is considerably enhanced by the presence of cholesterol.

Introduction

Saponin and digitonin are plant glycosides which form water-insoluble complexes with cholesterol [1]. Electron micrographs of mixtures of cholesterol and saponin showed a hexagonal arrangement of holes, where individual holes had a diameter of approximately 8 nm. The hexagonal structure was rarely seen when pure phosphatidylcholine was treated with saponin [2,3]. A similar hexagonal arrangement of pits was observed when cell membranes of Rous sarcoma virus, chicken liver or erythrocyte ghosts were treated with saponin [4]. On the other hand, digitonin-cholesterol complexes showed spherical micelles as well as rigid tubular structures in electron micrographs [5]. Similar particles were observed when membranes of chicken liver or skeletal muscle microsomes were incubated with digitonin [4,5]. The specific interaction of saponin as well as digitonin with cholesterol-rich membranes made these compounds to a versatile tool in cell biology. Electron micrographs revealed that treatment of isolated hepathocytes with digitonin resulted in a loss of the normal staining material present in the cytoplasmic compartment. Consequently a better visualisation of intracellular structures, such as microfilaments, was enabled [6,7]. In addition, it could be demonstrated that both saponin and digitonin could make plasma membranes permeable to various substances, without impairing the function of the intracellular organelles [8,9]. There-

fore saponin and digitonin were used to study intracellular Ca^{2+} transport in sceletal muscle fibres [10], synaptosomes [11], pancreas acinar cells [12–14] and chromaffin cells [15,16]. In order to test the hypothesis that only cholesterol-rich membranes are affected by certain amounts of saponin or digitonin, we performed experiments with artificial planar bilayer membranes. In addition, we intended to get information about the mechanism of the permeability increase of bilayers, caused by these glycosides. Studies with black lipid membranes can reveal, whether an increase in conductance is due, for example, to a carrier mechanism or to pores. Besides the experiments with planar bilayers we investigated the effect of saponin as well as digitonin on the surface pressure of lipid monolayers. These experiments were made in order to elucidate under which conditions the glycosides can penetrate into a monomolecular lipid film. Our experiments with bilayer membranes demonstrate that both saponin and digitonin induce channel-like conductance fluctuations in cholesterol-rich as well as in cholesterol-free membranes. The amount needed to induce such pores is, however, appreciably higher in cholesterol-free than in cholesterol-rich bilayers.

Materials and Methods

Black lipid membranes were formed from a decane solution with the brush technique [17]. Membranes consisted either of a 1.5% solution of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti), or a 2:1 (w/w) mixture of DPhPC and cholesterol (Fluka). Saponin was obtained from Merck and digitonin from Sigma. Saponin was added from a 10 mg/ml stock solution, whereas digitonin was dissolved in ethanol (5 mg/ml). The glycosides were added after membrane formation to one side, which is defined as the *cis* side. The measuring chamber contained 3 ml of electrolyte solution in each compartment. This solution consisted of 150 mM KCl/0.15 mM $CaCl_2$/5 mM Hepes-Tris (pH = 7.4). The measuring chamber and the electrical arrangement were similar to those described previously [18]. Membranes were formed on a hole of 0.4 mm in diameter, if not otherwise stated. The experiments were performed at a temperature of 27 ± 1°C. The sign of the electrical potential is referred to the *cis* side.

Surface pressure measurements of lipid monolayers spread at the air/water interface were performed with a conventional surface balance (Krüss, K10). The surface balance was modified, so that the subphase of the monolayer could be stirred magnetically. Stirring with about 2 cycles per second did not affect the measurements. The experiments were performed with the Wilhelmy plate method. A roughened platin plate, 20 mm long and 10 mm high (Krüss), was used. The experiments were done in a glass vessel with 43 mm diameter, containing 25 ml of solute. The subphase electrolyte was the same as described above for bilayer experiments. The temperature was kept constant at 28 ± 0.5°C. Each measurement was started with pure electrolyte solution. After 5 min small amounts (about 6 μl) of lipid dissolved in hexane was placed onto the surface, so that the surface tension decreased to about 40 mN/m. After 10 min the glycoside was injected from its stock solution into the subphase and the same amount of fluid was withdrawn. After waiting 10 min, the amount of glycoside in the subphase was increased.

Results

Bilayer experiments

In order to investigate the effect of saponin as well as digitonin on the electrical conductance of black lipid membranes, we choose the synthetic lipid diphytanoyl phosphatidylcholine, which is known to form very stable planar bilayers, having a specific resistance in the order of $10^9\ \Omega \cdot cm^2$ [19]. The following experiments were done to elucidate the threshold concentration for saponin, at which a conductance increase appears. At saponin concentrations below 0.3 mg/ml, no effect was observed. In two cases an increase in conductance was recorded with saponin concentrations of 0.3 mg/ml and 0.5 mg/ml, respectively. However, at a concentration of 2 mg/ml, conductance fluctuations occurred in all six experiments, we performed. An example of such current fluctuation is depicted in Fig. 1. When the applied voltage was lower than 50 mV, only weak fluctuations could be observed (not shown). As

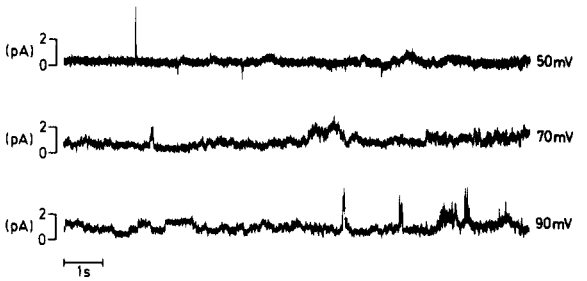

Fig. 1. Current fluctuations of a planar bilayer membrane made of diphytanoylphosphatidylcholine (DPhPC) in *n*-decane. The saponin concentration was 1 mg/ml on the *cis* side.

demonstrated in Fig. 1, at a voltage of 50 mV or more, pronounced current fluctuations appeared, where the number of events as well as their amplitudes increased with increasing voltage. The conductances of the fluctuating events shown in Fig. 1 extend from a few pico-Siemens to about 40 pS. However, in other experiments performed under similar conditions, fluctuations with a conductance up to 800 pS were observed (not shown). Because of the irregularity in amplitude, the saponin-induced fluctuations are quite different from fluctuations caused by antibiotics such as gramicidin [20] or alamethicin [21] or by channel-forming proteins [22]. When the bilayer broke after addition of saponin, a new membrane could be formed, though the glycoside was present in the bath solution. As in the absence of saponin, the bilayer became spontaneously black. In general, channel-like fluctuations occurred already at saponin concentrations of about 0.3 mg/ml, when saponin was present before membrane formation (five observations).

When digitonin was applied to one side of a pure DPhPC bilayer, channel-like fluctuations were observed similar to those recorded in presence of saponin (Fig. 2). The concentration of digitonin,

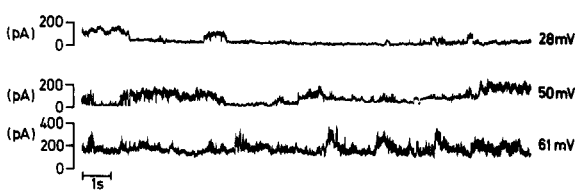

Fig. 2. Current fluctuations recorded in presence of $9.2 \cdot 10^{-5}$ g/ml digitonin on the *cis* side of a planar bilayer consisting of DPhPC.

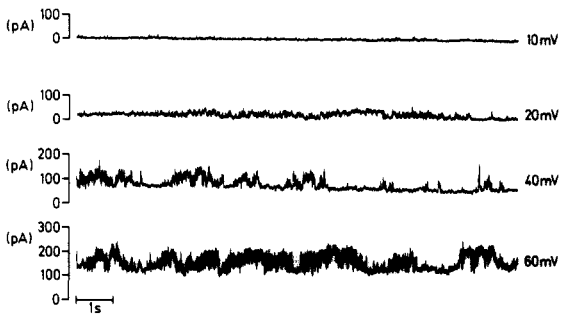

Fig. 3. Current-fluctuation patterns of a bilayer made of a 2:1 (w/w) mixture of DPhPC and cholesterol. $3 \cdot 10^{-5}$ g/ml of saponin was present on the *cis* side.

which was necessary to induce conductance fluctuations, was about 0.2 mg/ml. This concentration is significantly lower than the threshold-concentration of saponin. As Fig. 2 demonstrates, the number of the channel-like fluctuations as well as the baseline current increased with increasing voltage. Both in the presence of saponin as well as digitonin, bilayers were unstable and broke easily. Therefore it was not possible to record current-voltage curves. As in the case of saponin, bilayers became spontaneously black in presence of digitonin. The effect of both saponin and digitonin on bilayers containing cholesterol was investigated in detail only with membranes consisting of a 2:1 (w/w) mixture of DPhPC and cholesterol. The amount of saponin as well as of digitonin needed to increase the permeability was about 10^{-5} g/ml for both glycosides. As demonstrated in Figs. 3 and 4, the fluctuation patterns recorded in presence of saponin as well as

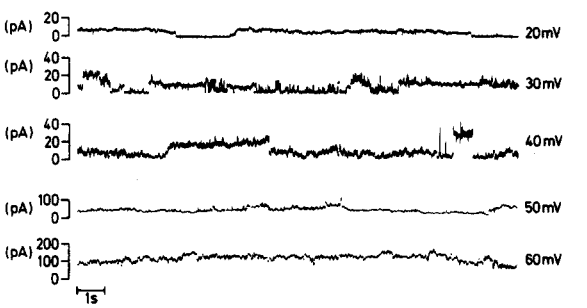

Fig. 4. Current fluctuations recorded in presence of $2 \cdot 10^{-5}$ g/ml digitonin of a bilayer consisting of DPhPC plus cholesterol.

digitonin in cholesterol-rich bilayers, are very similar to those recorded in absence of cholesterol. In addition, Figs. 3 and 4 demonstrate that both with saponin and digitonin the number of channel-like fluctuations as well a the baseline current increase with increasing voltage in cholesterol-containing membranes. Unlike to experiments performed in absence of cholesterol, black lipid membranes could not be formed with a cholesterol-rich lipid solution, when about 10^{-5} g/ml of either saponin or digitonin was present on one side of the bath solution. Under these conditions, a droplet of lipid could be brought across the aperture, but no thinning of the membrane occurred, even not at an applied voltage of 200 mV. With bilayers formed on an aperture of 0.8 mm in diameter, current-voltage curves could be recorded in the presence of saponin. As demonstrated in Fig. 5, the current-voltage behaviour was linear in the indicated voltage range. In the presence of digitonin, membranes were less stable, so that no current-voltage curves could be recorded.

In order to elucidate whether the observed channel-like fluctuations are selective for cations or anions, we performed experiments with different salt concentrations on each side of the membrane. The ion selectivity was only measured in

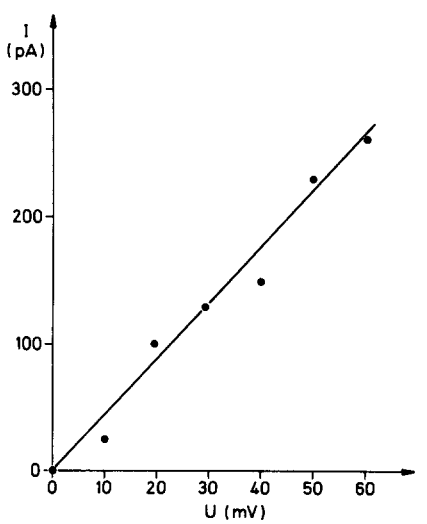

Fig. 5. Current-voltage relationship of a bilayer made of DPhPC plus cholesterol (2:1, w/w) in presence of $2 \cdot 10^{-5}$ g/ml saponin. The aperture of the hole on which the membrane was formed, was 0.8 mm in this case.

Fig. 6. Current-fluctuations of a DPhPC/cholesterol membrane (2:1, w/w) in presence of 10^{-5} g/ml saponin. The KCl concentration was raised by 200 mM on the *cis* side.

presence of saponin and with cholesterol-containing membranes. After membrane formation, the KCl concentration was raised by 200 mM and then saponin was added. The saponin concentration needed to induce channel-like fluctuations in presence of the described salt gradient was about 10^{-5} g/ml, being similar to that used under normal conditions. As shown in Fig. 6, in presence of a salt gradient channel-like fluctuations into the positive directions were recorded at zero applied voltage. These fluctuations increased with positive voltage (upper trace of Fig. 6) and were abolished at about -20 mV (lower trace). These results show that the bilayer is more permeable to K^+ than to Cl^-. Taking -20 mV as the reversal potential, a selectivity of about 3:1 for K^+ with respect to Cl^- was calculated from the Goldman equation:

$$E = \frac{RT}{F} \ln \frac{P_{Na}[Na]_o + P_K[K]_0 + P_{Cl}[Cl]_i}{P_{Na}[Na]_i + P_K[K]_i + P_{Cl}[Cl]_o}$$

where E is the potential, P denote permeability coefficients and the brackets [] denote the activities on the *cis* side (o) and *trans* side (i) of the respective ions.

Monolayer experiments

Experiments with lipid monolayers at the air/water interface were performed under similar conditions as bilayer experiments. The spread monomolecular film consisted either of pure DPhPC or of a 2:1 (w/w) mixture of DPhPC and cholesterol. The initial surface pressure of the lipid monofilm was adjusted to about 30 mN/m. This surface pressure was chosen because it is assumed that it corresponds to the lateral surface pressure of bi-

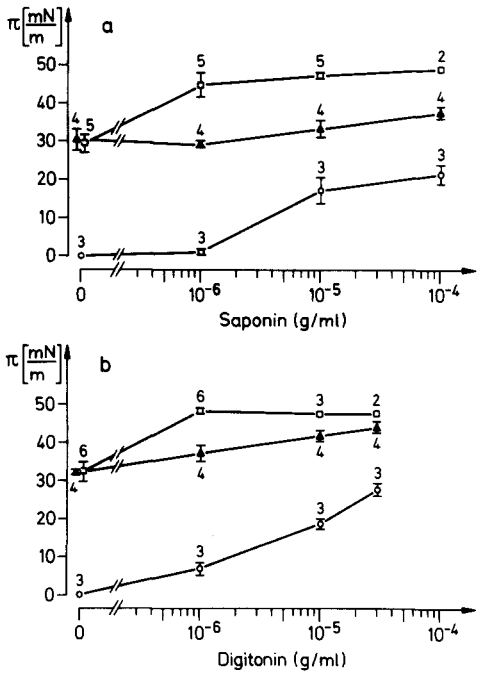

Fig. 7. Dependence of the surface pressure π on the concentration of saponin (a) and digitonin (b) in the subphase medium. Data points with open circles (○) were recorded in absence of a lipid monolayer. Points with triangles (▲) were obtained in presence of a pure DPhPC monolayer and points with squares (□) were recorded in presence of a monolayer consisting of a 2:1 (w/w) mixture of DPhPC and cholesterol. The number of experiments is given for each data point.

layer membranes [23]. The dependence of the surface pressure on the glycoside concentration is depicted in Fig. 7. The points drawn with open circles were obtained in absence of a lipid monofilm. they demonstrate the surface-active properties of both saponin and digitonin, which is expressed in a marked decrease of the surface tension. The surface pressure is defined as $\pi = \nu_0 - \nu_1$ where ν_0 is the surface tension of the pure electrolyte and ν_1 is the surface tension after addition of the surfactant. That means that the surface pressure increases with increasing glycoside concentration. As digitonin was added from an ethanolic stock solution, the effect of ethanol on the surface tension was examined. The maximal ethanol concentration in the subphase was 0.7%, which corresponds to a digitonin concentration of 0.3 mg/ml. The surface tension of a pure electrolyte containing 0.7% ethanol decreased by 3.6 ± 0.4 mN/m ($n = 3$). When ethanol was injected into the subphase of a monofilm consisting of either pure DPhPC or DPhPC plus cholesterol, a final concentration of 0.7% ethanol increased the surface pressure by about 2 mN/m. When saponin was injected into the subphase of a pure DPhPC monofilm, a slight increase in surface pressure was recorded at concentrations of 10^{-5} g/ml and 10^{-4} g/ml (triangles in Fig. 7a). In a mixed phosphatidylcholine/cholesterol film, however, a marked increase in surface pressure was observed at a saponin concentration as low as 10^{-6} g/ml. With further increasing concentration of saponin, the surface pressure increased only slightly, (squares in Fig. 7a). As demonstrated in Fig. 7b, digitonin interacts more strongly both with a pure phosphatidylcholine film and with a cholesterol-rich film than does saponin. In a pure DPhPC film even 10^{-6} g/ml of digitonin increased the surface pressure by about 5 mN/m (triangles in Fig. 7b). In a cholesterol-containing film an increase of the surface pressure by 15 mN/m was recorded with 10^{-6} g/ml digitonin. A further increase of digitonin concentration caused no further increase of the surface pressure (squares in Fig. 7b).

Discussion

The present study demonstrates that both saponin and digitonin interact with model membranes made either of pure phosphatidylcholine or of 2:1 (w/w) mixture of phosphatidylcholine and cholesterol. However, with cholesterol-rich lipids the amount of glycosides needed to induce effects in planar bilayers as well as in monolayers, is much lower than it is the case with cholesterol-free membranes. The observation that also cholesterol-free artificial membranes are affected by digitonin is in agreement with experiments performed by Rosenqvist et al. [24]. The authors observed lysis of liposomes made of pure egg phosphatidylcholine in presence of digitonin. It is evident from our studies that in cholesterol-free as well as in cholesterol-rich model systems the interaction of digitonin is stronger than that of saponin. In bilayers as well as in monolayers made of pure lecithin, digitonin interacts at about 10-fold lower concentration than saponin. Furthermore, cholesterol-rich bilayers break more easily after

addition of digitonin than of saponin, although the concentration of both glycosides needed to induce pores is about the same in both types of bilayers. In cholesterol-rich monolayers the increase in surface-pressure with 10^{-6} g/ml glycoside is more pronounced with digitonin than with saponin. This stronger interaction of digitonin with lipid monolayers is in agreement with previous observations [25]. The increase in surface-pressure of a monomolecular film demonstrates, that both saponin and digitonin can penetrate into a lipid monofilm, having an initial surface pressure as high as 30 mN/m. In cholesterol-rich films, however, the penetration of both glycosides is considerably enhanced. Schulman and Rideal [25] investigated the interaction of saponin as well as of digitonin with pure cholesterol monolayers. These authors observed a dramatic increase in surface pressure, which is in agreement to own observations (unpublished results). In contrast to our results, however, Schulman and Rideal [25] reported that phosphatidylcholine films, compressed to 22 mN/m, could not be penetrated by $1.6 \cdot 10^{-3}$% of saponin. This discrepancy may be due to differences in the subphase medium or to differences in the lipid.

We showed in this study that the increase in electrical conductance caused by saponin as well as by digitonin in planar lipid bilayers is due to fluctuating channels. However, these conductance-fluctuations vary considerably in amplitude. Besides small shortlasting spikes, frequently channels with amplitudes of some 10 pS up to a few nano-Siemens were observed. For example, the channel-like events shown in trace 2 of Fig. 4 are about 40 pS, whereas the fluctuations depicted in the bottom trace of Fig. 3 have a conductance of about 2 nS. If it is assumed that the pores are filled with the same electrolyte solution as the bathing medium, the cross-section of such channels can be estimated. From capacitance measurements performed by Redwood et al. [19], the thickness of a DPhPC bilayer containing *n*-decane is calculated to be about 50 Å. A pore of this length, having a conductance of 2 nS, would have a diameter of 2.9 nm. This is close to a value reported by Seeman et al. [26], who observed 4–5 nm wide pits in electron micrographs of saponin-treated erythrocytes.

At present, the molecular mechanism describing the interaction of the glycosides with artificial bilayers is not clear. A possible mechanism explaining the conductance increase in presence of both saponin and digitonin could be the formation of micellar structures inside the bilayer. The formation of reversed micelles of the glycosides was proposed by Glauert et al. [3]. In this model the more hydrophylic sugar molecules are oriented towards the center of the micelle whereas the hydrophobic parts interact with cholesterol molecules. However, more complex structures such as mixed micelles or block-assembly of micelles, as proposed by Fromherz [27], could be generated. Our observation that channel-like fluctuations of different magnitudes occur, suggests that structures of different sizes are formed. These structures could be due to the formation and decay of different numbers of molecules inside the bilayer. The latter assumption is in agreement with ^{2}H-NMR studies made on multibilayers of egg phosphatidylcholine and cholesterol. Akiyama et al. [28] reported that at low digitonin concentrations no formation of stable digitonin-cholesterol complexes with well defined stoichiometry occurred.

It is interesting to note that both saponin and digitonin interact with monolayers at lower concentrations than it is the case with bilayers. This could mean that a certain number of glycoside molecules must have penetrated into the bilayer before the formation of conducting structures is possible. As demonstrated in Figs. 1–4, the fluctuation patterns recorded with cholesterol-free membranes look similar to the fluctuations recorded with cholesterol-rich bilayers. Unfortunately, a more precise characterization of the pores, such as the evaluation of channel amplitudes or mean open or closed times of the channels is not possible because of the irregular structure of the fluctuations. Therefore the fluctuation patterns can only be compared by inspection. The close similarity between the channel-like structures, recorded with cholesterol-free and with cholesterol-rich membranes suggests, but does not prove, that the micellar structures, supposed to be responsible for the channels, are not directly depended on the presence of cholesterol. However, as demonstrated by the monolayer experiments, the penetration of cholesterol-rich membranes by the glycosides is considerably facilitated.

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