Control of Lipid Membrane Stability by Cholesterol Content

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ABSTRACT Cholesterol has a concentration-dependent effect on membrane organization. It is able to control the membrane permeability by inducing conformational ordering of the lipid chains. A systematic investigation of lipid bilayer permeability is described in the present work. It takes advantage of the transmembrane potential difference modulation induced in vesicles when an external electric field is applied. The magnitude of this modulation is under the control of the membrane electrical permeability. When brought to a critical value by the external field, the membrane potential difference induces a new membrane organization. The membrane is then permeable and prone to solubilized membrane protein back-insertion. This is obtained for an external field strength, which depends on membrane native permeability. This approach was used to study the cholesterol effect on phosphatidylcholine bilayers. Studies have been performed with lipids in gel and in fluid states. When cholesterol is present, it does not affect electropermeabilization and electroinsertion in lipids in the fluid state. When lipids are in the gel state, cholesterol has a dose-dependent effect. When present at 6% (mol/mol), cholesterol prevents electropermeabilization and electroinsertion. When cholesterol is present at more than 12%, electropermeabilization and electroinsertion are obtained under milder field conditions. This is tentatively explained by a cholesterol-induced alteration of the hydrophobic barrier of the bilayer core. Our results indicate that lipid membrane permeability is affected by the cholesterol content.

INTRODUCTION

Cholesterol is the major sterol component in most mammalian membranes. It is nonhomogeneously distributed among different organelles. The cholesterol content in the eucaryotic plasma membrane is usually rather high (e.g., larger than 20 wt% in plasma membranes), whereas it is much less in internal membranes. Its level is ~8 wt% in the Golgi apparatus and ~6 wt% in the endoplasmic reticulum, whereas in the mitochondria it is only ~3 wt% (Jamieson and Robinson, 1977).

One of the specific physical features of the cholesterol molecule is the planar steroid ring, a relatively conformationally rigid structure. This governs much of the interactions of cholesterol in a lipid bilayer. The phase of the cholesterol-lipid mixture is a function of the concentration, temperature, nature of the lipid, and pressure. This dependence directly modulates an expansion of the lipid surface produced by cholesterol. As a consequence, binding of membrane-associating species may be influenced (Scarlata, 1997). When cholesterol is present in large amounts, it acts as a permeability barrier for the membrane by introducing conformational ordering of the lipid chains. It increases its mechanical stiffness while keeping the membrane fluid (Bloom et al., 1991). This is in contrast to the case of low cholesterol concentrations, where permeability of bilayers increased dramatically in the phase temperature range (Corvera et al., 1992). When present in small amounts, cholesterol does not prefer one lipid phase to another. This results in a very narrow coexistence region at low cholesterol concentrations (Corvera et al., 1992).

Properties of lecithin-cholesterol model membranes were recently reviewed (Finegold, 1993). It appears that in the case of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) below the gel-to-liquid transition temperature, the molecular organization of the mixture with cholesterol was strongly dependent on the sterol content (Mortensen et al., 1988; Hui and He, 1983). A peculiar system was indeed present at less than 8%. Cholesterol is present as "impurities" in the bottom and/or top of the ripple structure. These "defects" are proposed to induce leaks. It was confirmed by experiments on Na^+ permeability in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) bilayers (Corvera et al., 1992), which showed that 5% cholesterol leads to a significant enhancement of the permeability. The modification in bilayer permeability that occurs according to the cholesterol concentration has a biological consequence (Bretscher and Munro, 1993). It seems possible that the variation in small cholesterol contents provides for an effective control of the membrane stiffness and morphology, thereby controlling the vesicle-budding processes as well as the intermembrane distances in the ER and the Golgi apparatus (Lemmich et al., 1997). A membrane into which proteins are inserted has to be readily deformable and hence be cholesterol-poor (Bretscher and Munro, 1993).

For a few years a physical method called *electropulsation*, using the application of external electric field pulses on cells, has been developed to induce transient membrane potential difference modulation. An external applied electric field pulse (E) induces a potential difference (ΔV) across the membrane capacitor due to charge movement in

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the cytosol and the external medium, giving a charge accumulation at the membrane interfaces. According to Eq. 1 in the Appendix, ΔV is under the control of membrane permeability. One of the most dramatic consequences of electropulsation is a loss of membrane impermeability.

Electropermeabilization of lipid bilayers has been described both in lipid vesicles (Teissié and Tsong, 1981; El Mashak and Tsong, 1985; Needham and Hochmuth, 1989; Raffy and Teissié, 1995) and in planar lipid membranes (Benz and Zimmermann, 1980; Abidor et al., 1979; Weaver et al., 1984; Robello and Gliozzi, 1989). The behavior of lipid vesicles was rather similar to what was observed with cells (Chernomordik et al., 1987), i.e., permeabilization was reversible and a fusogenic state was induced (Teissié et al., 1989; Büschl et al., 1982; Hub et al., 1982; Melikyan and Chernomordik, 1989). Permeabilization was shown to be very transient in pure lipid systems (Benz and Zimmermann, 1981). We recently reported that electropermeabilization of DPPC liposomes, multilamellar vesicles (MLVs), as well as interdigitated-fusion vesicles (IFVs), triggered glycophorin insertion (Raffy and Teissié, 1995, 1997). Therefore the lipid-associated modification supports the spontaneous insertion. The direct consequence of this observation for the processes occurring in biological membranes is limited by the gel state of DPPC in these experiments. In natural membranes, phospholipids are known to be in the liquid state. Moreover, they are mixed with cholesterol, which affects the structural organization of the bilayer (Devaux and Seigneuret, 1985).

Lipid vesicles are membrane models that are useful for showing the involvement of lipid clusters if present in cell membranes. They appear as suitable systems in the present case because it was shown that secretory independent loops were translocated under conditions in which the transmembrane potential difference was not present, a condition that is present in lipid liposomes (Von Heijne, 1994). MLVs were chosen to obtain cell-sized liposomes.

Glycophorin, which is known as being competent for electroinsertion in mammalian cell membranes, was used as a membrane protein. Glycophorin electroinsertion can be considered as a model for posttranslational protein insertion, because the protein is fully made before integration into membranes. As its transmembrane fragment is only 22 amino acids long, it appears to be a reliable model, and the short loop does not seem to require any secretory machinery for translocation or insertion (Von Heijne, 1995). Its primary structure shows a large glycosylated external part, then a stretch of hydrophobic amino acids with a transmembraneous helical conformation, and a short cytoplasmic fragment. Despite its intrinsic character, this protein can be isolated from the membrane without the use of detergent because of its high electrical charge (Springer et al., 1966). For that reason, it is very often used in studies on lipid-protein interactions (De Kruijff et al., 1991).

We studied in this paper the effects of small amounts of cholesterol on lipid bilayer permeability and protein insertion, electropermeabilization, and electroinsertion on multilamellar DPPC lipid bilayers containing different amounts of cholesterol (x_c = 0, 6, 12, and 29 mol%) and on multilamellar egg phosphatidylcholine (egg PC) lipid bilayers containing 0 or 29 mol% cholesterol. The rationale behind this work was to investigate whether cholesterol content can control the membrane permeability of egg PC (fluid phase) and DPPC (gel phase) liposomes and therefore affect electropermeabilization and glycophorin electroinsertion.

MATERIALS AND METHODS

Chemicals

Egg *sn*-3-phosphatidylcholine (egg PC, ref. P7318), 1,2-dipalmitoyl-*sn*-3-phosphatidylcholine (DPPC, ref. P5911), cholesterol (ref. C8667), calcein (ref. C1075), and glycophorin A (ref. G 5017) were purchased from Sigma (St. Louis, MO). Monoclonal antibody antiglycophorin A (ref. 0167) and fluorescein labeled goat polyclonal antibody to mouse IgG (H + L) F(ab′) (ref. 0819) were purchased from Immunotech SA (Marseille, France). All other chemicals and reagents were of analytical grade.

Preparation of lipid vesicles

Multilamellar vesicles (MLVs) were prepared as follows. Ten milligrams of phospholipids mixed with different amounts of cholesterol were dissolved in glass redistilled optical-grade chloroform. They were then evaporated under vacuum to complete dryness. One milliliter of 5 mM HEPES and 25 mM NaCl at pH 7.2 was added. The solution was slowly warmed to 55°C with gentle agitation. The lipid vesicles were then centrifuged four times at 12,000 × *g* in a Hettich mikroliter centrifuge (Germany) for 5 min at 21°C. The pellet was resuspended in a 1 mM HEPES, pH 7.2 buffer. The vesicles obtained had a mean diameter of 5 ± 0.5 μm when observed under a phase-contrast videomicroscope, regardless of the concentration of cholesterol. They were stored at 4°C.

Application of electric pulses to the vesicles

The methods were adapted from those previoulsy described (Teissié et al., 1989). Electropulsation was performed with a CNRS cell electropulsator (Jouan, St. Herblain, France). It delivered square-wave pulses whose parameters (voltage, pulse duration, number of pulses, and duration between pulses) were all independently adjustable. Two flat stainless-steel parallel electrodes, with an anode-cathode distance of 1.5 mm, connected to the voltage generator, gave a uniform electric field for a 50-μl volume. The pulse field intensity kept a constant value during the pulse, regardless of the pulsing buffer composition. The voltage pulse applied to the cell suspension was monitored with an oscilloscope incorporated into the cell pulsator. The field intensities were those directly observed on the oscilloscope. To limit the associated Joule heating, it was not possible to use a pulse intensity higher than 7.5 kV/cm. All experiments were run at 21°C.

Determination of permeabilization

Calcein efflux was used to monitor permeabilization. MLVs were prepared in a 1 mM HEPES, 25 mM NaCl (pH 7.2) buffer to which calcein (2.5 mM) had been added. External dye was washed out while dye molecules remained trapped between the lipid leaflets. Different groups of liposomes were pulsed at each of the different field intensities. Just after application of the pulses, the vesicles were centrifuged for 5 min at 12,000 × *g*, and the vesicle supernatant (50 μl) was diluted in 2 ml HEPES buffer. The fluorescence intensity of this diluted sample of the supernatant was measured on a spectrofluorimeter (JY3; Jobin Yvon, Longjumeau, France) with λ_{exc} = 496 nm and λ_{em} = 520 nm. Permeabilization was quantified through fluorescence intensity.

Supernatant volume and vesicle density were carefully controlled to minimize the possible variations in the measurements of the leakage.

Determination of electroinsertion

Glycophorin A is soluble in aqueous solvent and may take the form of micelles after its extraction from red blood cells by a detergent-free method (Springer et al., 1966). Five microliters of glycophorin A, at a 2 mg/ml concentration in 1 mM HEPES, was added to 50 μl of MLVs (10 mg/ml). The methods were adapted from those previously described in the case of red blood cells or CHO cells (Mouneimne et al., 1989; El Ouagari et al., 1993). After 15 min of incubation at 37°C, the electrical pulses were applied. Immediately after the pulses, the vesicles were washed three times with HEPES buffer (pH 7.4) by centrifugation at 12,000 $\times$ *g* for 5 min. The final pellets were resuspended in 50 μl of phosphate-buffered saline (PBS) (pH 7.4) and incubated at 4°C with 5 μl of 0.2 mg/ml anti-human glycophorin monoclonal antibody for 30 min. They were then washed three times with PBS (pH 7.4) and incubated with 5 μl of 1 mg/ml fluorescein-labeled goat polyclonal antibody to mouse IgG (H + L) $F(ab')^2$ at 37°C for 30 min more. The MLVs were finally washed three times in PBS (pH 7.4). Control samples in which vesicles have been subjected to all steps except electrical pulses were used as references for each corresponding case. Another control was carried out without fluorescein-labeled goat polyclonal antibody. The enhancement of the fluorescent signal was quantified by spectrofluorimetry (λ_{exc} = 487 nm and λ_{em} = 520 nm). The signal was the same whatever the delay between electropulsation and the fluorescence measurement.

Electroinsertion is detected by comparing the immunofluorescence signals between background, nonspecific signals, and experiments.

Because of the methodology, insertion, when present, was detected only on the outer layer of MLVs. It is not possible to assay the occurrence of insertion on the internal leaflets.

RESULTS

Electropermeabilization of egg lecithin MLVs

At the working temperature (i.e., 21°C) egg PC is in fluid phase, whereas DPPC is in gel phase. Different groups of calcein-loaded egg PC liposomes were electropulsed with different electric field intensities while pulse duration, number, and frequency (five pulses of 7 ms at 1 Hz) were kept constant. No calcein release was detected in the supernatant as long as the field intensity was lower than 1.3 kV/cm. Above this strength, the dye leakage was observed to increase with the field intensity (Fig. 1 *A*). This observation shows that egg PC MLVs are electropermeabilized when pulses higher than a critical intensity are applied. It should

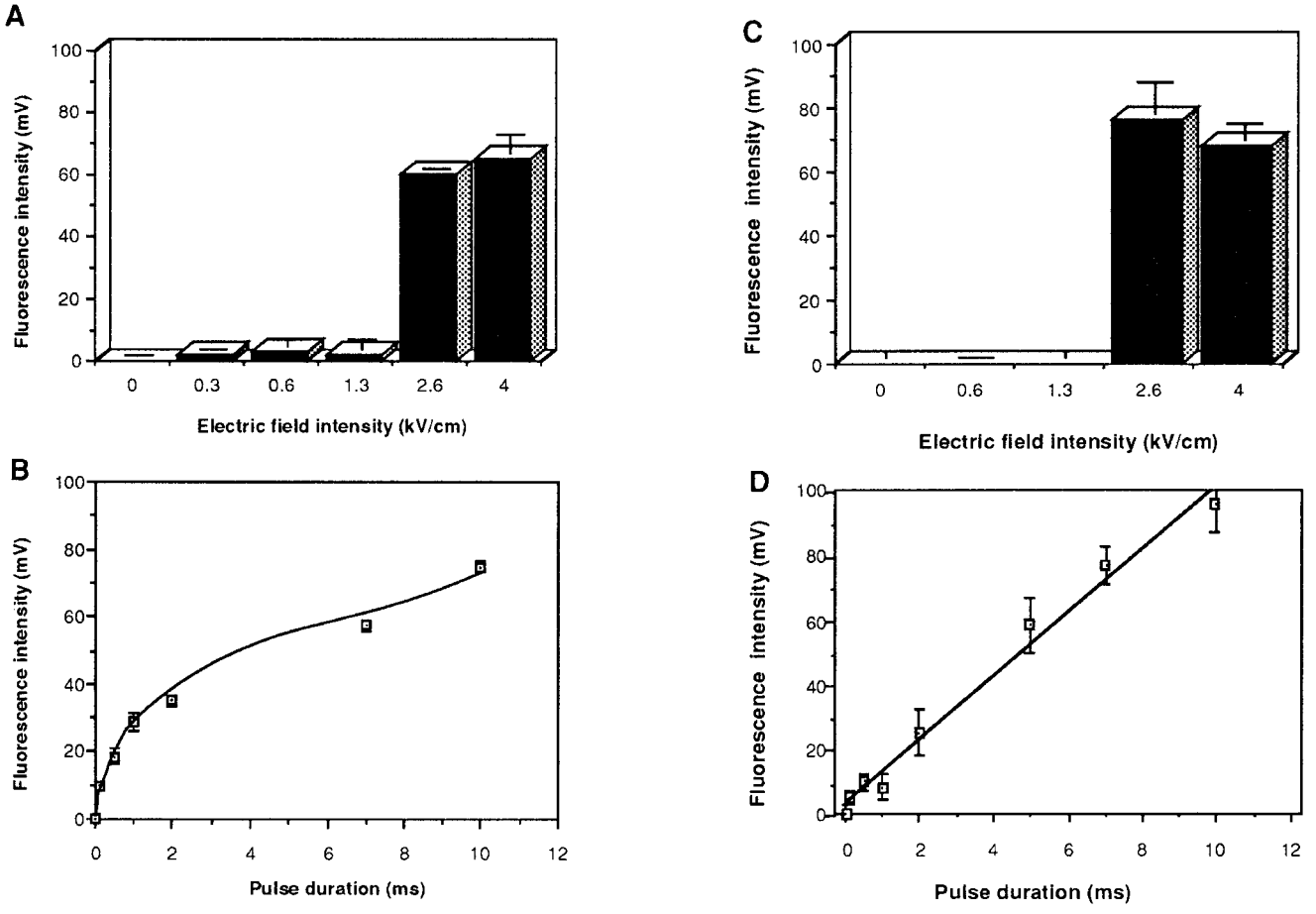

FIGURE 1 Control by electric pulse parameters of egg PC MLV and egg PC-cholesterol MLV electropermeabilization. Liposomes were prepared and loaded with calcein. Permeabilization was assayed by the dye leakage in the external buffer. Five pulses were applied at a frequency of 1 Hz. Egg PC MLVs: (*A*) Effect of the field intensity with a pulse duration of 7 ms. (*B*) Effect of the pulse duration at a field intensity of 2.6 kV/cm. Egg PC-cholesterol MLVs (29% cholesterol): (*C*) Effect of the field intensity with a pulse duration of 7 ms. (*D*) Effect of the pulse duration with a field intensity of 2.6 kV/cm.

be noted that this threshold range is similar to what we had previously observed with DPPC MLVs (Raffy and Teissié, 1995).

When the field strength was held at 2.6 kV/cm (i.e., above the critical permeabilization value), the dye leakage was observed to be dependent on the pulse duration (five successive pulses applied at 1 Hz). The leakage increased strongly with pulse durations up to 1 ms and then more slightly (Fig. 1 *B*). One should notice again that similar behavior was reported in the case of DPPC MLVs (Raffy and Teissié, 1995). This result showed that the fluid state did not prevent membrane permeabilization.

Electropermeabilization of cholesterol-egg PC mixed MLVs

Cholesterol was added to MLVs at a concentration of 29% (mol/mol), a value close to what is found in eukaryotic cell plasma membranes.

The electrical parameters (pulse intensity (*E*) and duration (*T*)) were varied individually to follow their effect on electropermeabilization (calcein leakage) while keeping the pulse number (*N*) and frequency (five pulses at 1 Hz) constant. Dye leakage was detected when the field strength was higher than 1.3 kV/cm, as observed with pure egg lecithin vesicles. The extent of leakage increased with an increase in the field strength before decreasing above that value (Fig. 1 *C*).

When working at a field strength equal to 2.6 kV/cm, higher than the threshold, and at a given number of pulses, calcein leakage was enhanced by longer pulse duration. A linear increase was indeed observed (Fig. 1 *D*).

Electropermeabilization of cholesterol-DPPC mixed MLVs

DPPC MLVs were described as electropermeabilized when pulsed with a field strength higher than 1.3 kV/cm (five pulses lasting 7 ms) (Raffy and Teissié, 1995). Cholesterol DPPC mixed MLVs were prepared with increasing amounts of sterol (6, 12, 29% (mol/mol)). These relative contents of cholesterol were chosen by taking into account the results for DMPC (Mortensen et al., 1988; Hui and He, 1983). Different structural organizations of the lipid bilayer were obtained for these three different percentages when the host lecithin was in the gel state. Observation of the MLVs under a phase-contrast microscope did not show any change in their shape and size from what was detected in pure DPPC systems when the cholesterol content was increased to 29% (data not shown).

When low amounts of cholesterol were present (6%), it was not possible to detect the leakage of calcein, even when the field strength was as high as 6.5 kV/cm, with five pulses of 7 ms applied (Fig. 2). It was not possible to use higher field strengths because of the associated Joule heating.

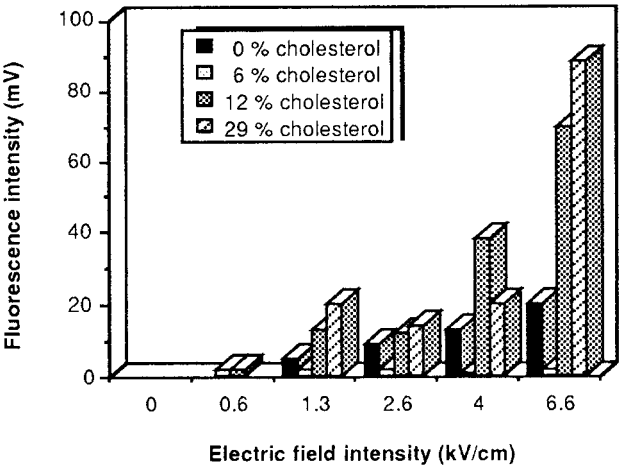

FIGURE 2 Effect of electric field intensity on permeabilization of DPPC/cholesterol liposomes mixtures. Liposomes prepared and loaded with calcein with 0, 6, 12, or 29 mol% cholesterol were pulsed five times at 1 Hz frequency with a 7-ms pulse duration in 1 mM HEPES pulsing buffer. Permeabilization was assayed by the dye leakage in the external buffer. Experiments were carried out four times.

When the liposome cholesterol content was 12%, dye leakage was observed when the field intensity was higher than only 0.6 kV/cm. The same threshold was observed when cholesterol was present at 29%. With 12% cholesterol, the level of leakage rose with increasing electric field intensity, when liposomes were pulsed under similar conditions (same *T* and *N*) (Fig. 2).

Glycophorin A electroinsertion in egg PC MLVs

As shown in Fig. 3, electroinsertion was observed when the field intensity was higher than 1.3 kV/cm. The amount of inserted proteins rose with an increase in the field strength up to a plateau. Nevertheless, a background of immunofluorescence was present as shown in Fig. 4. It was ~40% of

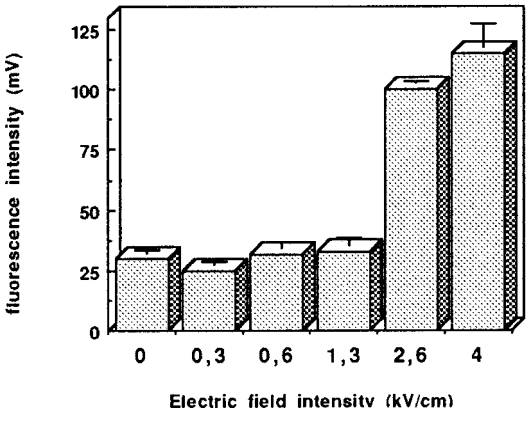

FIGURE 3 Effect of electric field intensity on glycophorin electroinsertion in egg PC liposomes. MLVs were pulsed five times with 7-ms duration and a frequency of 1 Hz. Electroinsertion was assayed by means of immunofluorescence.

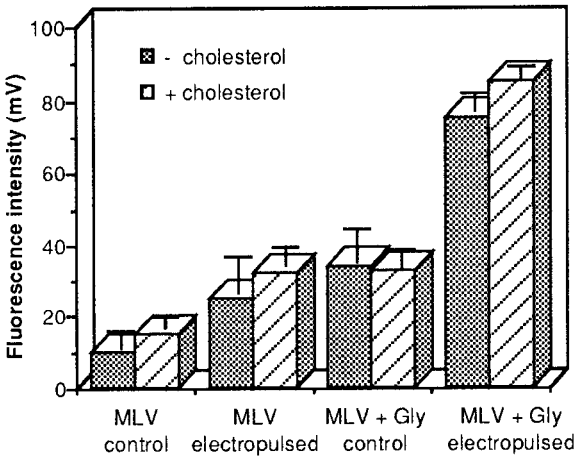

FIGURE 4 Effect of cholesterol on the electroinsertion of glycophorin in egg PC liposomes. Electroinsertion was assayed by means of immunofluorescence. When applied, electropulsation conditions were five pulses of 7 ms at a frequency of 1 Hz and a field strength of 2.6 kV/cm. Cholesterol was present at 29%.

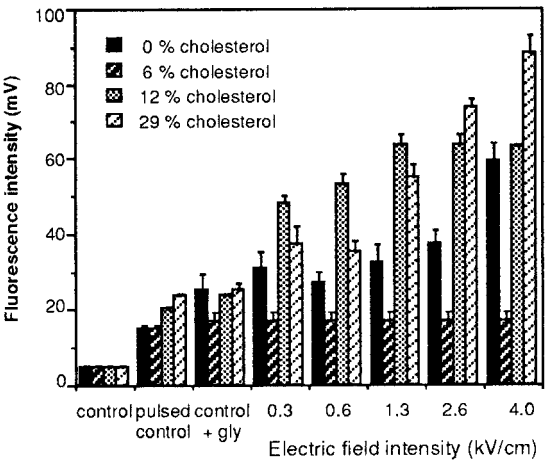

FIGURE 5 Effect of electric field intensity on glycophorin electroinsertion in DPPC-cholesterol mixed MLVs. Liposomes prepared with 0, 6, 12, or 29 mol% cholesterol were pulsed in the presence of the protein five times at 1 Hz frequency with a 7-ms pulse duration in 1 mM HEPES pulsing buffer. *Control*: Unpulsed MLVs without glycophorin. *Pulsed control*: Pulsed MLVs (2.6 kV/cm) without glycophorin. *Control + Gly*: Unpulsed MLVs with glycophorin. Electroinsertion was assayed by means of immunofluorescence.

the signal detected when electroinsertion was present. This has already been reported in the case of electroinsertion in DPPC MLVs (Raffy and Teissié, 1995).

Glycophorin A electroinsertion in egg PC-cholesterol MLVs

Using the same experimental approaches as in the case of egg PC liposomes, electroinsertion was performed in mixed MLVs (29% cholesterol). An immunofluorescence signal was detected above the background, as in the case of egg PC MLVs (Fig. 4). Direct comparison of the observations on egg PC and egg PC-cholesterol MLVs showed that the respective levels of immunofluorescence due to background or to electroinsertion were similar in the two systems.

Glycophorin A electroinsertion in DPPC-cholesterol mixed MLVs

Electroinsertion was assayed in systems where the molar percentage of cholesterol was changed (6, 12, 29%). Experiments on pure DPPC MLVs were repeated (Raffy and Teissié, 1995). Electroinsertion, which is detected by the increase in immunofluorescence intensity above the background level, was detected only when the field intensity was higher than 1.3 kV/cm. The level of electroinsertion was then observed to increase with an increase in field strength (Fig. 5).

When the level of cholesterol was 6% (mol/mol), no increase in immunofluorescence intensity above the background level was observed, regardless of the intensity of the electric field we applied.

When liposomes were formed with 12% cholesterol, electroinsertion was present when electropulsation was operated at very low field strength ($E = 0.3$ kV/cm) (Fig. 5). It rose with an increase in field intensity and reached a plateau value at 1.3 kV/cm. When the level of cholesterol was brought to 29% (mol/mol), electroinsertion was again detected at low field strength (0.6 kV/cm), but in that case, the immunofluorescence intensity rose continuously with an increase in field strength.

A direct comparison of the immunofluorescence of electroinserted glycophorin in the liposomes with different compositions showed that when the same field strength (2.6 kV/cm) was used, the largest signal was detected when 29% cholesterol was present in the DPPC mixed vesicles (Fig. 6). This can be explained either by a higher "affinity" of the electropermeabilized layer for glycophorin or by an increase in the part of the vesicle surface brought to the state competent for insertion.

DISCUSSION

The present investigation shows that as in the case of DPPC large unilamellar vesicles (LUVs) and MLVs (Teissié and Tsong, 1981; Raffy and Teissié, 1995), electropermeabilization can be performed on egg PC liposomes, where the lipids are in the fluid phase. The presence of cholesterol at 29% (mol/mol), a concentration found in mammalian cell plasma membrane, induced minor changes in this process. The same permeabilizing field threshold window of 1.3–2.6 kV/cm was observed. We should mention that these critical intensities were the same for DPPC multilamellar liposomes (MLVs) (Raffy and Teissié, 1995) and for DPPC unilamellar liposomes (IFVs) (Raffy and Teissié, 1997), where the lipids were in the gel phase. Our observation that the critical permeabilizing field intensity window for egg PC was not affected by the cholesterol is then relevant to the conclu-

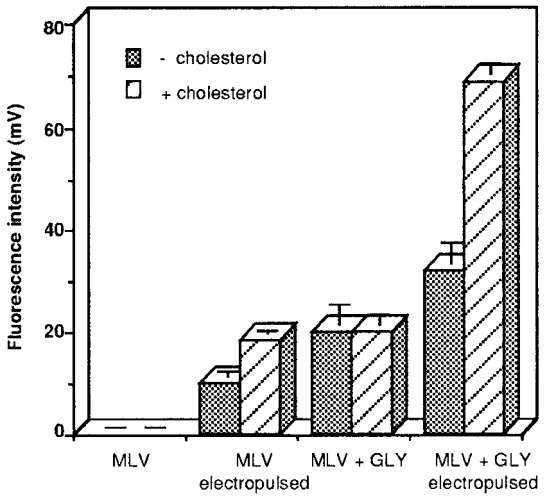

FIGURE 6 Effect of cholesterol on the electroinsertion of glycophorin in DPPC liposomes. Liposomes were prepared with 29 mol% cholesterol. Electroinsertion was assayed by means of immunofluorescence. When applied, electropulsation conditions were five pulses of 7 ms at a frequency of 1 Hz and a field strength of 2.6 kV/cm.

sions on planar membrane electropermeabilization (Genco et al., 1993). It was observed that the critical voltage for electropermeabilization in lipid mixtures was always the same as for the host lipid (egg PC in our case). As far as electroinsertion was concerned, the conclusions of the present work are that it took place not only in the gel phase, as we reported with DPPC (Raffy and Teissié, 1995), but also in the fluid phase.

Glycophorin can be inserted into lipid bilayers containing cholesterol. From the results on egg PC MLVs, it is clear that cholesterol does not affect the insertion process in the permeabilized fluid lipid layer (Figs. 1 and 4). It is important to point out that electroinsertion is obtained only under field conditions leading to electropermeabilization. This is in line with observations with mammalian cells, where electroinsertion was observed in the plasma membrane, which is known to be rich in cholesterol and which is in the fluid phase (El Ouagari et al., 1993, 1994). The structural modifications of lipids associated with electropermeabilization then create a transient membrane state in which a spontaneous insertion of membrane proteins takes place after electric field application (Raffy and Teissié, 1995). The same conclusion was obtained in the case of DPPC vesicles (Raffy and Teissié, 1995). The same critical permeabilizing field intensity was observed with egg PC and DPPC. As the vesicle size was the same, taking into account our observation that the critical permeabilizing transmembrane potential difference is a constant (Teissié and Rols, 1993), membrane permeability is not affected by the lipid phase state or by the presence of a high cholesterol concentration (29 mol%) for egg PC.

Previous studies showed that PC bilayers were electropermeabilizable. In a work with giant unilamellar vesicles made of SOPC (stearoyloleoylphosphatidylcholine), it was observed by an aspiration technique and by making the assumption that the bilayer was perfectly dielectric that the so-called breakdown membrane voltage was very high (more than 1 V) (Needham and Hochmuth, 1989; Zhelev and Needham, 1993). When cholesterol was present, an increase in the "breakdown" membrane voltage of ~66% was observed. Both conclusions for when the cholesterol content was very high (near 50%) are in conflict with the present study and with results on planar lipid membranes obtained with a "current-clamp" method (Robello and Gliozzi, 1989; Genco et al., 1993). It was then observed that the "breakdown" was triggered when the transmembrane voltage reached a value of ~270 mV when the film was made with egg PC at pH 7. When cholesterol was present (29%), no change in the permeabilizing transmembrane voltage was detected. We should mention that in Needham's studies, the relative content of cholesterol was higher than what we were using.

When 6% cholesterol was present in DPPC, mixed vesicles could not be electropermeabilized. The external field could not bring ΔV to its critical value. As the vesicle size was not affected by the presence of cholesterol, this suggests that the g (λ_o, λ_ι, λ_m) parameter (see the Appendix) is strongly reduced in the DPPC MLVs where the cholesterol content was 6%. Several structural analyses have investigated the organization of such systems. Some disorders were observed in the gel phase (Vist and Davis, 1990). By using ^{2}H NMR and differential scanning calorimetry (DSC), it was found that a narrow two-phase region was present between 0 and 6.25% cholesterol, just below the chain-melting transition of the pure phospholipid, i.e., at a higher temperature than the one we were using. X-ray diffraction experiments indicated a dramatic increase in the bilayer repeat spacing when cholesterol was raised to 5 mol% in the gel phase in the case of DMPC (Hui and He, 1983). This was explained as being due to a reduction in the tilt angle. Recent DSC results showed a sharpening of the DPPC phase transition when cholesterol was present at a low percentage (Harris et al., 1995). All of these results gave a description of the DPPC bilayers where cholesterol molecules were shown to exist as transbilayer, tail-to-tail dimers (Harris et al., 1995; Mukherjee and Chattopadhyay, 1996). A direct implication of such a description is that cholesterol would induce defects due to the poor packing. This tends to make the lipid layer leaky, i.e., yields a low value of the g (λ_o, λ_ι, λ_m) parameter in Eq. 1 (see the Appendix). This was indeed predicted by numerical simulations of the thermodynamic properties and passive ionic permeability of lipid-cholesterol bilayers at low cholesterol concentration (Corvera et al., 1992). An increase in permeability was predicted and experimentally observed for Na^+ permeability in the case of DPPC. These descriptions of the DPPC assemblies at low cholesterol content again suggest that the g (λ_o, λ_ι, λ_m) parameter should be lower in these systems than in pure DPPC models. The lack of electroinsertion is then a direct consequence of the fact that electropermeabilization is required for insertion to take place.

A high cholesterol content of mixed DPPC vesicles gives conditions in which electropermeabilization is triggered at a lower field intensity (Fig. 2). Again the correlation between electropermeabilization and electroinsertion is present, as shown by the low critical field for insertion (Fig. 5). Although electropulsation of vesicles without glycophorin induces a fluorescence increase that is different without or with cholesterol, this does not affect the main conclusion that electroinsertion is strongly enhanced when cholesterol is present at a high molar ratio in DPPC. The direct observation of liposomes under the microscope (data not shown) showed that there was no alteration in the size and shape of the mixed MLVs when cholesterol was present at 12% or 29%. As the critical transmembrane potential difference leading to electropermeabilization is obtained for a lower external field value (less than 0.6 kV/cm), our conclusion is then that the $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ parameter in Eq. 1 (see Appendix) has a higher value for a high cholesterol content. Structural organizations of DPPC-cholesterol systems with high sterol contents have been the focus of many studies over the last 30 years (Chapman et al., 1969; Lippert and Peticolas, 1971; Ladbrooke et al., 1968; Darke et al., 1971; Hubbel and McConnell, 1971; Rand and Luzzati, 1968; McMullen and McElhaney, 1995). The major conclusions are 1) a dramatic increase in orientational order of the phospholipid hydrocarbon chains; 2) an associated thickening of the hydrophobic part of the bilayer; 3) a progressive broadening of the thermal transition region, giving an elimination of the phase transition between the Lα liquid-crystalline phase and the Pβ' and Lβ' gel phases; 4) a concomitant suppression of the phase pretransition; 5) a decrease in the chain tilt angle in the gel phase, when present (Vist and Davis, 1990); and 6) a thickening of the hydrophobic part of the bilayer. A direct consequence was the observation that the enhanced ion transbilayer permeability, which is present in the phase transition region, was abolished in mixed systems (Papahadjopoulos et al., 1973). It was then observed that the membrane hydrophobicity was increased in DPPC layers when cholesterol was present. A decrease in membrane defects is associated with a thickening of the membrane (Paula et al., 1996). Formation of effective hydrophobic barriers to permeation of polar species (i.e., a high value for the $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ parameter in Eq. 1) requires cholesterol (Subczynski et al., 1994). These conclusions give experimental support to our observation of a lower value of the critical permeabilizing field intensity. Time-resolved fluorescence anisotropy studies of DPPC layers confirmed these conclusions. Cholesterol increases the membrane order in the core of the lipid layer (Thulborn and Beddard, 1982; Vincent et al., 1982).

From the results shown in Figs. 1, *A* and *C*, and 2 and previous observations on LUVs (Teissié and Tsong, 1981), and by using the evaluation of the permeabilizing membrane potential difference we obtained for many systems (~200–250 mV), in agreement with data on planar films (Genco et al., 1993), the $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ parameter can be obtained for different systems by the use of Eq. 2, developed in the Appendix. It can be estimated that the value of $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ is ~0.5 for DPPC, egg PC, and egg PC-cholesterol assemblies but increases to almost 1 in DPPC-cholesterol models with a high cholesterol content, i.e., λ_m close to 0. At 6% cholesterol, $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ is less than 0.1 for a DPPC-cholesterol bilayer, indicating that λ_m is high.

APPENDIX

Application of an external field to a lipid bilayer or cell membrane induces a modulation of the transmembrane potential difference ΔV, which is associated with the dielectric properties of the membrane. The stationary field effect ΔV obeys the now classical equation (Neumann, 1989)

$$\Delta V = faE \cos\theta g(\lambda_o, \lambda_\iota, \lambda_m) \tag{1}$$

in which f is a shape factor (1.5 in the case of a sphere), a is the radius of the vesicle, E is the intensity of the applied field, and θ is the angle between the normal of the membrane and the direction of the field at the locus of interest. $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ is associated with the electrical conductivity of the membrane (here the lipid bilayer) and could be given by (Neumann, 1989)

$$g(\lambda_o, \lambda_\iota, \lambda_m) = \frac{\lambda_o\lambda_i(2d/a)}{(2\lambda_o + \lambda_i)\lambda_m + (2d/a)(\lambda_o - \lambda_m)(\lambda_i - \lambda_m)} \tag{2}$$

where λ_o is the buffer conductivity, λ_i is the liposome interior conductivity, λ_m is the conductivity of the membrane, and d is the membrane thickness.

According to the liposome preparation, we introduce the approximation that λ_o is close to λ_i and obtain

$$g(\lambda_o, \lambda_\iota, \lambda_m) = \frac{\lambda_o^2(2d/a)}{3\lambda_o\lambda_m + (2d/a)(\lambda_o - \lambda_m)^2} \tag{3}$$

In most cases, the membrane is considered as a pure dielectric, λ_m is set to zero, and $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ is 1.

Electropermeabilization results from the field-induced transmembrane potential difference modulation. Whatever the membrane system (mammalian cell, yeast protoplast, bacterial protoplasts, liposomes), the critical potential difference is always 200–250 mV (Teissié and Tsong, 1981; Teissié and Rols, 1993; Genco et al., 1993; Slawomir et al., 1998). This critical value of ΔV_{perm} is obtained for a critical field strength E_{perm} such as

$$\Delta V_{perm} = fg(\lambda_o, \lambda_\iota, \lambda_m)aE_{perm} \tag{4}$$

When the composition of liposomes is changed, if their shape and size remain unaltered, as ΔV_{perm} is a constant, the observation of a change in E_{perm} is relevant to a change in $g\ (\lambda_o, \lambda_\iota, \lambda_m)$ (i.e., in λ_m).

In our experiments, f is 1.5 (spherical shape), a is 2.5×10^{-6} m (using a value of ΔV_{perm} of 0.25 V), and the numerical application of Eq. 4 gives

$$g(\lambda_o, \lambda_\iota, \lambda_m) = \frac{0.25}{1.5 \times 2.5.10^{-6} \times E_{perm}} = \frac{666}{E_{perm}} \tag{5}$$

$g\ (\lambda_o, \lambda_\iota, \lambda_m)$ values are obtained directly from the experimental determination of E_{perm}, i.e., the threshold for dye leakage or glycophorin electroinsertion (Table 1).

λ_m is acting on the loading time τ_p of the induced potential difference (Neumann, 1989)

$$\tau_p = aC_m \frac{\lambda_i + 2\lambda_o}{2\lambda_i\lambda_o + a\lambda_m/d(\lambda_i + 2\lambda_o)} \tag{6}$$

where C_m is the specific membrane capacitance

TABLE 1 Estimated values of *g* as a function of the nature and the composition of lipid vesicles*

Nature of the lipid bilayer	E_p (kV/cm)	Estimated g
DPPC	1.3	0.5
Egg PC	1.3	0.5
Egg PC-cholesterol	1.3	0.5
DPPC-cholesterol (6 mol%)	>6.5	<0.1
DPPC-cholesterol (12 or 29 mol%)	0.6	1

*See Appendix for details of calculation.

Under our experimental conditions, this can be rewritten as

$$\tau_p = aC_m \frac{3\lambda_o}{2\lambda_o^2 + a\lambda_m/d(3\lambda_o)} \quad (7)$$

As checked by fluorescence spectroscopy (Lojewska et al., 1989), τ_p is affected by λ_m but remains less than 10 μs. As millisecond pulses were used, this does not affect electropermeabilization.

The main consequence of electropermeabilization is to permit a free exchange of small molecules and ions across the plasma membrane. This exchange was shown to obey a free diffusion process across the permeabilized part of the cell surface (Rols and Teissié, 1990). The flow Φ is mathematically described by

$$\Phi(s) = P_s\Delta(S)X(N, T)A\left(1 - \frac{E_{perm}}{E}\right) \quad (\text{if } E > E_{perm}) \quad (8)$$

in which P_s is the permeability coefficient of the hydrophilic solute S (here calcein) across the electropermeabilized membrane, $\Delta(S)$ is the concentration gradient in S, $X(N, T)$ is a function describing the dependence of the membrane reorganization on the pulse number N and duration T, and A is the vesicle surface.

Φ is therefore controlled by E_{perm} at a given field strength (with all other parameters held constant). As E_{perm} is driven by λ_m (Eq. 5), the extend of dye leakage is under the control of g (λ_o, λ_ι, λ_m) (i.e. of λ_m).

Electroinsertion is linearly related to the electropermeabilized fraction of the cell surface (El Ouagari et al., 1993; Raffy and Teissié, 1995, 1997). This fraction is controlled by the ratio E_{perm}/E (Schwister and Deuticke, 1985; Rols and Teissié, 1990).

At a given field strength (with all of the other parameters held constant), the amount of electroinserted proteins is under the control of E_{perm}, i.e., of g (λ_o, λ_ι, λ_m) from Eq. 5 (that is, again of λ_m).

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