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Effects of COR6.6 and COR15am Polypeptides Encoded by *COR* (Cold-Regulated) Genes of *Arabidopsis thaliana* on the Freeze-Induced Fusion and Leakage of Liposomes[1]

Matsuo Uemura, Sarah J. Gilmour, Michael F. Thomashow, and Peter L. Steponkus*

Department of Soil, Crop and Atmospheric Sciences, Cornell University, Ithaca, New York 14853 (M.U., P.L.S.); and Department of Crop and Soil Sciences, Michigan State University, East Lansing, Michigan 48824 (S.J.G., M.F.T.)

Several cold-regulated (COR) polypeptides, which have little or no amino acid sequence identity with known proteins, are synthesized during cold acclimation of *Arabidopsis thaliana*. However, the function of the polypeptides has yet to be elucidated. The objective of this study was to determine if COR6.6 and COR15am influence the incidence of either freeze-induced fusion or freeze-induced leakage of small unilamellar vesicles (SUVs) composed of either a single species of phosphatidylcholine (either 1-palmitoyl-2-oleoyl-, dioleoyl-, or dilinoleoylphosphatidylcholine), a mixture of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, and free sterols (1:1:1, mol:mol), or the total lipid extract of the plasma membrane of either nonacclimated or cold-acclimated rye leaves. When the SUVs were suspended in a dilute tris(hydroxymethyl)-aminomethane/2-(*N*-morpholino)ethanesulfonic acid buffer, both COR6.6 and COR15am invariably decreased the incidence of freeze-induced fusion regardless of the lipid composition. However, if the SUVs were suspended in a dilute solution of either sucrose or NaCl, the COR polypeptides had little or no effect on the incidence of freeze-induced fusion. Moreover, the COR polypeptides did not decrease the incidence of freeze-induced leakage—regardless of whether the SUVs were suspended in either the dilute buffer alone or with added sucrose or NaCl. In fact, with SUVs composed of a single species of phosphatidylcholine suspended in the dilute buffer, the COR polypeptides resulted in an anomalous increase in freeze-induced leakage. When considered collectively, these results suggest that neither COR6.6 nor COR15am has a direct cryoprotective effect on SUVs frozen in vitro.

Currently, there is considerable interest in the molecular biology of cold acclimation in plants and the possibility of using genetic engineering techniques for increasing freezing tolerance. Since Guy et al. (1985) first reported altered gene expression during cold acclimation, substantial progress has been made in identifying genes that are regulated during cold acclimation and characterizing the polypeptides that are encoded by these genes (see recent reviews by Guy, 1990; Thomashow, 1993). The expression of "cold-regulated" (*COR*) genes (also referred to as *CAP*, *LTI*, and *KIN* genes, among others) closely parallels the development of freezing tolerance (Guy and Haskell, 1987; Mohapatra et al., 1987a, 1987b; Hajela et al., 1990; Guo et al., 1992), and, in certain cases, the level of expression of *COR* genes correlates with freezing tolerance (Mohapatra et al., 1989). Nevertheless, with few exceptions the function of the polypeptides that are encoded by *COR* genes remains to be elucidated. Because cold acclimation is a complex, developmental phenomenon and freezing injury is a multiform syndrome (Steponkus, 1984; Thomashow, 1990; Steponkus et al., 1993), there are many possible ways, both direct and indirect, in which COR polypeptides may affect freezing tolerance. There is also the possibility that some of the polypeptides synthesized during cold acclimation have no function in freezing tolerance.

It is possible that proteins encoded by *COR* genes function to alter metabolic pathways, the end products of which directly influence freezing tolerance, e.g. alterations in membrane lipid composition. For example, Hughes et al. (1992) have identified a gene in barley that is induced by low temperature, drought, and ABA and that encodes a protein that has a predicted amino acid sequence that has homology with phospholipid transfer proteins in maize. Alternatively, it is possible that alterations in membrane lipid metabolism occur by more subtle regulatory mechanisms that do not require the involvement of *COR* genes. There is also the possibility that COR polypeptides may

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* Corresponding author; e-mail pls4@cornell.edu; fax 1–607–255–2644.

Abbreviations: ANTS, aminonaphthalene-3,6,8-trisulfonic acid; COR polypeptides, polypeptides encoded by cold-regulated genes; DL_2PC, dilinoleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, *N,N′-p*-xylene-bis(pyridinium bromide); FS, free sterol(s); NBD-PE, (7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; PC, phosphatidylcholine; (NA- or ACC-)PM-SUVs, small unilamellar vesicles composed of the total lipid extract of rye plasma membrane isolated from leaves of either nonacclimated or cold-acclimated seedlings, respectively; POPC, 1-palmitoyl-2-oleoylphophatidylcholine; Rh-PE, (lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; SUVs, small unilamellar vesicles; T_m, liquid crystalline-to-gel phase transition temperature.

serve "housekeeping" roles during exposure to the low temperatures (0–5°C) required for cold acclimation. Alternatively, they may function in other metabolic processes that are also stimulated by low temperatures but are not related to cold acclimation. For example, the transcript levels for Phe ammonia lyase and chalcone synthase increase 10-fold in *Arabidopsis thaliana* in response to low temperature (Leyva et al., 1991). Laroche et al. (1992) suggested that amplification and differential methylation of rDNA may be related to vernalization rather than freezing tolerance. Although there are reports that exposure of chilling-sensitive plants to low temperatures alters gene expression (Hahn and Walbot, 1989; Christie et al., 1991; Christie and Walbot, 1991), these responses may be pathological symptoms of stress rather than adaptive responses.

Nevertheless, there is also the possibility that the polypeptides encoded by *COR* genes may have direct cryoprotective functions. Lin and Thomashow (1992a) reported that COR15a exhibits cryoprotection for lactate dehydrogenase, a cold-labile enzyme. Kurkela and Franck (1990) suggested that KIN1, which is a polypeptide encoded by a gene that is expressed during cold acclimation in *A. thaliana*, may function as an antifreeze protein because of some similarities in its amino acid sequence with the antifreeze protein present in winter flounder. Although this suggestion is consistent with recent reports of the occurrence in plants of proteins with antifreeze activity (Griffith et al., 1992; Urrutia et al., 1992; Duman et al., 1993), the significance of the reported sequence similarity is unclear because COR6.6, which was also isolated from *A. thaliana* and is almost identical to KIN1 (Gilmour et al., 1992), does not appear to have antifreeze activity (Thomashow, 1993).

Although the stabilization of cold-labile proteins by COR polypeptides may suggest that they are involved in cold acclimation, freezing injury is primarily a consequence of the freeze-induced destabilization of membranes—not denaturation of proteins (Steponkus, 1984; Steponkus et al., 1993). To date, there are no reports that COR polypeptides function to stabilize membranes during a freeze/thaw cycle. Rather, the most compelling evidence for such a role for COR polypeptides comes from the early studies of Heber and his colleagues (Heber and Kempfle, 1970; Volger and Heber, 1975) and the more recent studies of Hincha and his colleagues (Hincha et al., 1989, 1990; Hincha and Schmitt, 1992). In these studies, the investigators reported that proteins, which are synthesized in spinach and cabbage leaves during cold acclimation but are absent in nonacclimated leaves, minimize freeze-induced damage of thylakoids that are frozen in vitro (Volger and Heber, 1975; Hincha et al., 1990). These proteins, however, have not been purified and nothing is known about the genes that encode them.

The objectives of this study were to determine if two COR polypeptides that are synthesized during cold acclimation of *A. thaliana*, COR6.6 and COR15am, influence the cryostability of liposomes as monitored by freeze-induced fusion of liposomes and freeze-induced leakage of intraliposomal contents. COR6.6, a 6.6-kD polypeptide, is thought to be localized in the cytoplasm (Thomashow, 1993), whereas COR15am, a 9.4-kD polypeptide (the mature polypeptide of COR15a), is localized in the chloroplast (Lin and Thomashow, 1992b). Both COR6.6 and COR15am are hydrophilic, remain soluble after boiling, and are predicted from amino acid sequence analysis to form amphipathic α helices (Lin and Thomashow, 1992b; M.F. Thomashow, unpublished results). The amounts of COR polypeptides increase during cold acclimation in vivo (Lin and Thomashow, 1992b; S.J. Gilmour and M.F. Thomashow, unpublished results). Because the studies reported in this and a companion paper (Webb et al., 1996) required milligram amounts of purified polypeptides, recombinant forms of the polypeptides were synthesized in *Escherichia coli* and purified to near homogeneity as described in the accompanying paper (see Gilmour et al., 1996).

MATERIALS AND METHODS

Lipids

Synthetic phospholipids such as POPC, DOPC, DL_2PC, and DOPE were purchased from Avanti Polar Lipid (Alabaster, AL) and used without further purification. FS, a mixture of β-sitosterol (60%, w/w) and campesterol (40%, w/w), were purchased from Sigma and used without further purification. Total lipids of the plasma membrane were extracted from the plasma membrane fractions isolated from Puma rye leaves of either nonacclimated or 4-week-cold-acclimated seedlings according to the method of Uemura and Steponkus (1994).

Recombinant COR Polypeptides from *Escherichia coli*

Recombinant forms of the COR polypeptides (COR6.6 and COR15am) from *E. coli*, which are described in the companion paper by Gilmour et al. (1996), were used in these studies. Both COR6.6 and COR15am were used at a final concentration of 100 $\mu g\ mL^{-1}$ in all of the studies.

Preparation of SUVs

SUVs were prepared by sonication. Lipids, which were dissolved and mixed in chloroform, were placed in a glass test tube (10 × 100 mm), and the solvent was removed under a stream of nitrogen gas at 40°C. Residual solvent was removed under high vacuum overnight. A solution of the desired composition for each experiment was added over the dried lipid film, and the test tube was filled with nitrogen and capped. The lipids were initially dispersed in the solution by a combination of vortexing and freeze/thawing. Subsequently, the lipid suspension was sonicated at room temperature until the suspension appeared clear (usually after 10–15 min) to obtain SUVs.

Fusion Assay

Freeze-induced fusion of SUVs was determined after a freeze/thaw cycle by quantifying lipid mixing between liposomes using a resonance energy transfer assay according to the method of Struck et al. (1981). This procedure

involves determination of the dilution of nonexchangeable, fluorescent lipid probes NBD-PE and Rh-PE (Avanti Polar Lipid) from labeled liposomes to unlabeled liposomes. Both NBD-PE and Rh-PE were incorporated into one population of liposomes (labeled liposomes) at a concentration of 0.6 mol% of the total lipids. These probe concentrations resulted in a significant quenching of NBD-PE fluorescence by Rh-PE due to an efficient transfer of energy. Liposomes labeled with the fluorescent probes were mixed with the same amount of unlabeled liposomes at a final concentration of 1 μmol lipid mL^{-1}. Solutions used for liposome preparation were either 5 mM Tris/Mes (pH 7.2, 5 mosmol) or 5 mM Tris/Mes + 34 mM NaCl (pH 7.2, 70 mosmol). Either of the COR polypeptides or BSA was added to the liposome suspensions at a concentration of 100 μg mL^{-1} before freezing unless indicated otherwise (i.e. BSA was used at higher concentrations for studies shown in Fig. 3).

Freezing of liposome suspensions was performed by the procedure for freezing of protoplasts described by Uemura and Steponkus (1989). Briefly, an aliquot of the liposome suspension (250 μL, 1 μmol lipid mL^{-1}) in a glass test tube (10 × 100 mm) was placed in an ethanol bath (ULT-80, Neslab, Portsmouth, NH) at −2°C for 10 min, after which ice formation in the suspension was effected by touching the test tube with a spatula precooled in liquid nitrogen. After an additional 30-min incubation at −2°C, the samples were cooled to the specified temperatures at a rate of 0.8°C min^{-1} and kept for 30 min. The samples were then thawed at room temperature and kept on ice until the fluorescence assay was performed.

To determine the extent of fusion of liposomes after a freeze/thaw cycle, fluorescence of NBD-PE from the liposome suspensions before and after addition of Triton X-100 at a final concentration of 1% (w/v) was monitored with an excitation wavelength of 475 nm and an emission wavelength of 530 nm using an SLM500C fluorescence spectrophotometer (SLM Instruments, Urbana, IL). To correct for variation in the amount of NBD-PE in the sample, the ratio of fluorescence intensity (R_{sample}) of the liposomes before addition of Triton X-100 to that after addition of Triton X-100 was used to quantify the extent of lipid mixing after the freeze/thaw cycle as described below. To normalize the extent of lipid mixing of the liposomes, the ratio of fluorescence intensity of NBD-PE of an unfrozen liposome mixture, which contained an equal amount of unlabeled and labeled (0.6 mol% of both NBD-PE and Rh-PE) liposomes, before and after the addition of Triton X-100 (1% [w/v]), was taken as 0% lipid mixing ($R_{0.6\%}$). Because complete mixing of all of the bilayers upon fusion of the liposomes would be expected to result in liposomes containing 0.3 mol%, both of NBD-PE and Rh-PE, the ratio from liposomes containing 0.3 mol% of both NBD-PE and Rh-PE was taken as 100% lipid mixing ($R_{0.3\%}$). Then, lipid mixing (%) of each sample was calculated using the following equation:

$$\text{Lipid Mixing (\%)} = \left(\frac{R_{sample} - R_{0.6\%}}{R_{0.3\%} - R_{0.6\%}}\right) \times 100$$

Leakage Assay

Leakage of intraliposomal contents after a freeze/thaw cycle was monitored by a fluorescence assay using ANTS and DPX, which was based on the method of Düzgünes et al. (1985). SUVs were prepared with a solution of 12.5 mM ANTS (Molecular Probes, Eugene, OR) and 45 mM DPX (Molecular Probes) in 5 mM Tris/Mes (pH 7.2), which yielded a final osmolality of 70 mosmol. With these concentrations of ANTS and DPX encapsulated in the SUVs, the fluorescence of ANTS is effectively quenched by DPX, but dilution of ANTS and DPX caused by leakage into the medium results in increased fluorescence. Prior to the freeze/thaw cycle, unencapsulated ANTS/DPX was removed by gel-filtration column chromatography (Sephadex G-75, 1.5 × 15 cm) with an elution buffer either in the absence of salt (5 mM Tris/Mes, 5 mosmol) or in the presence of salt (5 mM Tris/Mes + 34 mM NaCl, 70 mosmol). Thus, when eluted with a 5 mM Tris/Mes buffer, the liposomes were subjected to hypotonic conditions. In contrast, when eluted with a solution containing 34 mM NaCl and 5 mM Tris/Mes, the liposomes were maintained under isotonic conditions. COR polypeptides or BSA, if used, were added to the liposome suspensions at a concentration of 100 μg mL^{-1} before freezing unless indicated otherwise (i.e. BSA was used at higher concentrations for studies shown in Fig. 3).

After the liposome suspensions were frozen as described above, the fluorescence intensity of ANTS in the liposome suspensions was measured spectrofluorometrically with an excitation wavelength of 360 nm and an emission wavelength of 530 nm before (F_{-Tx}) and after (F_{+Tx}) addition of Triton X-100 (1% [w/v]). The ratio of fluorescence intensity before and after addition of Triton X-100 (F_{-Tx}/F_{+Tx}) was used to express the extent of leakage (%) from liposomes subjected to a freeze/thaw cycle.

RESULTS

Freeze-Induced Fusion and Leakage of SUVs Composed of a Single Species of PC

Effect of COR Polypeptides in the Absence of Added Solutes

The first objective was to determine the effect of COR polypeptides on the occurrence of freeze-induced fusion and leakage in the absence of any additional solutes. For this, SUVs composed of a single species of PC were prepared and suspended in a solution of 5 mM Tris/Mes buffer.

When SUVs composed of either POPC or DOPC were frozen to temperatures over the range of −2 to −30°C, liposome fusion, as measured by lipid mixing, occurred at temperatures below −2°C. However, the temperature at which maximum fusion occurred varied with the lipid composition of the liposomes (Fig. 1). With POPC-SUVs, the incidence of fusion reached a maximum after freezing to −10°C or below (Fig. 1A). With DOPC-SUVs, fusion also occurred after freezing to −10°C, but a substantial increase occurred after freezing to −30°C (Fig. 1B). In contrast,

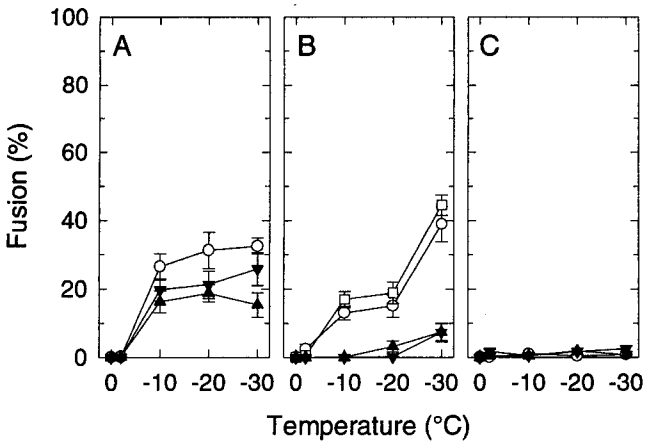

Figure 1. Effect of COR polypeptides on freeze-induced fusion of PC-SUVs frozen in a Tris/Mes buffer in the absence of any additional solutes. A, POPC; B, DOPC; C, DL_2PC. Liposomes were prepared with a 5 mM Tris/Mes buffer and frozen in the same solution. Fusion was determined by measuring lipid mixing between liposomes using a resonance energy transfer assay for the dilution of nonexchangeable, fluorescent lipid probes (NBD-PE and Rh-PE). The concentrations of lipids and polypeptides were 1 μmol mL^{-1} and 100 μg mL^{-1}, respectively. The incidence of fusion (%) was calculated as described in "Materials and Methods." ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are the average and SD of three freezing experiments.

freeze-induced fusion of SUVs composed of DL_2PC was not observed over the range of 0 to −30°C (Fig. 1C).

The studies with DOPC-SUVs revealed that freeze-induced fusion consists of two components: non-T_m-related fusion, which occurred after freezing to temperatures above the T_m (−22°C) of DOPC, and T_m-related fusion, which occurred after freezing to temperatures below the T_m. The extent of T_m-related fusion was somewhat greater than that of non-T_m-related fusion. With POPC-SUVs (T_m = −3°C), these two components were not clearly apparent because the onset of freezing occurred close to the T_m. Thus, freeze-induced fusion of POPC-SUVs reflects both non-T_m-related and T_m-related components. With DL_2PC-SUVs, which were frozen only over the range of −2 to −30°C, T_m-related fusion was not observed because they were not frozen to temperatures below the T_m (−55°C).

Addition of either COR6.6 or COR15am (100 μg mL^{-1}) to the liposome suspensions decreased the incidence of freeze-induced fusion of both POPC- and DOPC-SUVs (Fig. 1). In the presence of the COR polypeptides, fusion of DOPC-SUVs was not observed at −10°C and there was only a low incidence of fusion even after freezing to −30°C (Fig. 1B). In contrast, addition of BSA (100 μg mL^{-1}) to the suspensions of DOPC-SUVs had little effect on the incidence of fusion regardless of the freezing temperature. With POPC-SUVs, the COR polypeptides decreased the incidence of freeze-induced fusion, but the reduction was less than with DOPC-SUVs (Fig. 1A). With both POPC- and DOPC-SUVs, there was little difference between COR6.6 and COR15am. With DL_2PC-SUVs, the effect of COR polypeptides was not apparent because of the low incidence of fusion over the range of −2 to −30°C (Fig. 1C).

When frozen in the absence of COR6.6 or COR15am, there was a substantial increase in leakage of ANTS/DPX after freezing to temperatures below the T_m (Fig. 2). In POPC-SUVs, the extent of leakage reached a maximum after freezing to −10°C or lower (Fig. 2A). In DOPC-SUVs, there was an increase in leakage after freezing to −10°C, but a substantial increase in the extent of leakage did not occur until after freezing to −30°C (Fig. 2B). Although a small increase in the extent of leakage occurred in DL_2PC-SUVs after freezing to −10°C, no further increase in leakage was observed even after freezing to −30°C (Fig. 2C). Therefore, like freeze-induced fusion, a significant increase in leakage occurred after freezing to temperatures below the T_m of the lipids. The occurrence of leakage was not a result of ice formation per se, because freezing to −2°C did not cause a significant increase in leakage, even though more than 99% of the solution was frozen at −2°C.

When either COR6.6 or COR15am (100 μg mL^{-1}) was added to the suspension, leakage from the liposomes unexpectedly reached a maximum after freezing to −10°C or lower, regardless of the lipid composition of the SUVs (Fig. 2). In the presence of COR polypeptides, leakage from DL_2PC-SUVs, which showed no substantial increase in leakage over the range of −10 to −30°C when frozen in the absence of COR polypeptides, increased to the same extent as that from POPC- or DOPC-SUVs. The increase in leakage from DOPC- and DL_2PC-SUVs at −10°C indicated that COR polypeptides increased non-T_m-related leakage, with no significant difference between COR6.6 and COR15am.

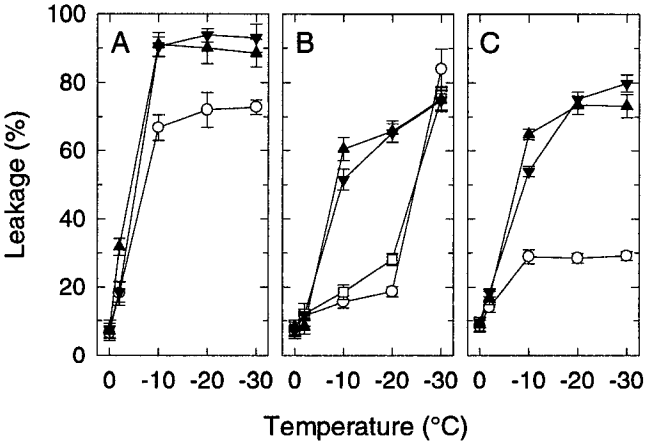

Figure 2. Effect of COR polypeptides on freeze-induced leakage of intraliposomal contents from PC-SUVs frozen in the Tris/Mes buffer in the absence of any additional solutes. A, POPC; B, DOPC; C, DL_2PC. Liposomes containing ANTS/DPX were eluted with a 5 mM Tris/Mes solution during gel filtration, which resulted in hypotonic conditions, and then were frozen in the same solution. Leakage was determined with a fluorescence assay with ANTS and DPX entrapped in the liposomes. The incidence of leakage (%) was determined as described in "Materials and Methods." ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are the average and SD of three freezing experiments.

Addition of BSA (100 μg mL^{-1}) resulted in a small increase in freeze-induced leakage, but the extent was far less than that in the presence of COR polypeptides. The increase in freeze-induced leakage from PC-SUVs in the presence of COR polypeptides was considered to be anomalous because (a) it occurred at −10°C regardless of the lipid composition of liposomes (i.e. irrespective of the T_m); (b) it did not coincide with the large increase in freeze-induced fusion; and (c) it was not consistent with the apparent cryoprotective effect of COR polypeptides on freeze-induced fusion.

Although addition of BSA had little or no effect on either freeze-induced fusion or leakage, the comparison between BSA and COR polypeptides was made using equivalent masses (100 μg mL^{-1}), which results in a large difference in the molar concentration of BSA (1.47 μM) and the COR polypeptides (15.1 μM for COR6.6 and 10.6 μM for COR15am). Therefore, the effect of BSA on freeze-induced fusion and leakage of DOPC-SUVs was also determined at equivalent molar concentrations (10.6 and 15.1 μM). At these concentrations, addition of BSA, like COR polypeptides, resulted in a substantial increase in non-T_m-related leakage over the range of −2 to −25°C (Fig. 3B); but, unlike COR polypeptides, there was no effect on freeze-induced fusion, even when the concentration of BSA was increased to 29.4 μM (Fig. 3A). Thus, the anomalous increase in non-T_m-related leakage was not unique to COR polypeptides; but, the apparent cryoprotective effect on freeze-induced fusion occurred only in the presence of COR polypeptides.

In summary, when frozen in a 5 mM Tris/Mes buffer in the absence of any added solutes, there was an apparent cryoprotective effect of COR polypeptides on freeze-induced fusion of PC-SUVs; but, under these conditions, COR polypeptides increased freeze-induced leakage.

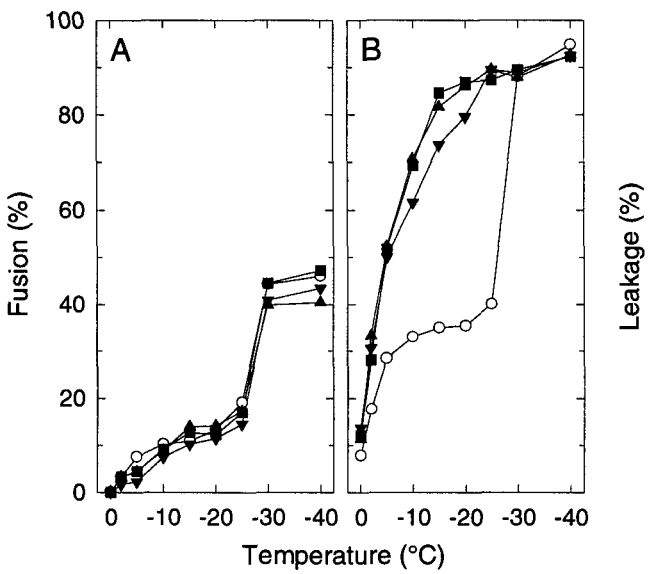

Figure 3. Effect of BSA at higher concentrations on freeze-induced fusion (A) and leakage (B) from DOPC-SUVs frozen in the Tris/Mes buffer in the absence of any additional solutes. Liposomes were prepared as described in Figures 1 and 2. ○, No BSA; ■, 0.721 mg mL^{-1} (10.6 μM) BSA; ▲, 1.027 mg mL^{-1} (15.1 μM) BSA; ▼, 1.999 mg mL^{-1} (29.4 μM) BSA. The results shown are from one freezing experiment.

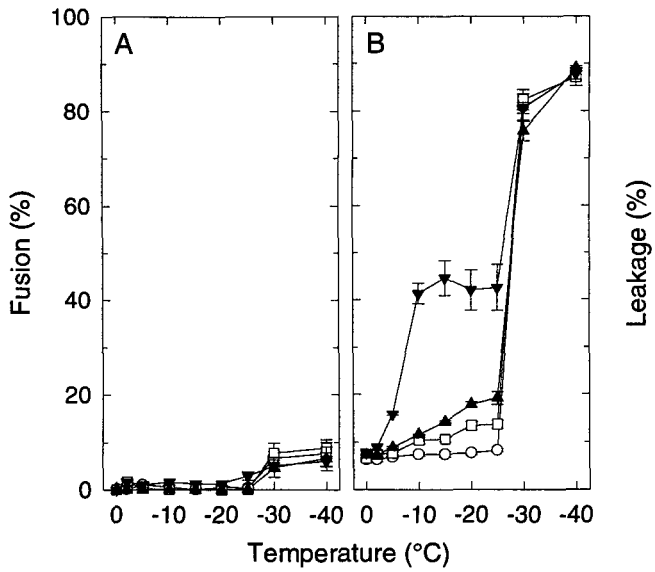

Figure 4. Freeze-induced fusion (A) and leakage (B) from DOPC-SUVs suspended in a solution of 10 mM Suc. Liposomes were prepared as described in Figures 1 and 2, and Suc was then added to the liposome suspensions before freezing. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are the average and SD of two freezing experiments.

Effect of COR Polypeptides in the Presence of Added Solutes

Heber and his colleagues (Heber and Kempfle, 1970; Volger and Heber, 1975) reported that the cryoprotective effect of proteins that are synthesized in cabbage and spinach leaves during cold acclimation is more pronounced when assayed in the presence of low concentrations of Suc (10–30 mM). Therefore, we next determined the effect of COR polypeptides on freeze-induced fusion and leakage of DOPC-SUVs in the presence of various concentrations of Suc (10–50 mM).

Addition of 10 mM Suc precluded non-T_m-related fusion and significantly decreased T_m-related fusion in DOPC-SUVs (Fig. 4A). Fusion did not occur until after freezing to −30°C, and the maximum extent of fusion was <10% (versus 40% in the absence of Suc; Fig. 1B). There was no effect of either COR6.6, COR15am, or BSA (100 μg mL^{-1}) on fusion over the range of −2 to −40°C, but this was because there was little fusion even in the absence of any proteins.

Addition of 10 mM Suc also precluded non-T_m-related leakage from DOPC-SUVs but had no effect on T_m-related leakage (i.e. there was a sharp increase in leakage after freezing at −30°C or below; Fig. 4B). Most important, the anomalous increase in non-T_m-related leakage that oc-

curred when liposomes were frozen in the presence of COR polypeptides, but without any added solutes (Fig. 2), was significantly attenuated when the COR polypeptides were added to liposomes suspended in 10 mM Suc (Fig. 4B). Under these conditions, there was a difference between COR6.6 and COR15am: addition of COR6.6 resulted in only a small (10%) increase in non-T_m-related leakage, whereas addition of COR15am still resulted in a substantial (approximately 30%) increase in non-T_m-related leakage—albeit less than the 50% (Fig. 2B) that occurred in the absence of Suc.

The increase in non-T_m-related leakage from DOPC-SUVs effected by the addition of COR polypeptides was further decreased when the concentration of Suc was increased (Fig. 5). At 20 mM Suc, there was little effect of COR6.6 on non-T_m-related leakage; however, there was still an increase in the presence of COR15am (15%; Fig. 5A). At 50 mM Suc, the increase in non-T_m-related leakage in the presence of COR15am was almost completely precluded (Fig. 5B).

Addition of Suc also affected the extent of T_m-related leakage from DOPC-SUVs depending on the concentration of Suc. At 10 mM Suc the extent of T_m-related leakage (80% leakage at −30°C) was not affected (Fig. 4B), whereas there was a decrease in the T_m-related leakage at 20 mM (60% at −30°C) or 50 mM (50% at −30°C) Suc (Fig. 5).

The increase in non-T_m-related leakage from DOPC-SUVs in the presence of COR polypeptides was also minimized by addition of salts (a mixture of NaCl and $CaCl_2$). When DOPC-SUVs were frozen in a 10 mosmol NaCl: $CaCl_2$ solution (3:2 [mol:mol]), there was a gradual increase in non-T_m-related leakage over the range of −2 to −20°C and a large increase after freezing to −25°C or below (Fig. 6A). Addition of COR6.6 (100 μg mL^{-1}) did not result in an increase in non-T_m-related leakage; COR15am (100 μg mL^{-1}) increased non-T_m-related leakage, but the extent of the increase was less than that in the absence of salts (20 versus 50% in the Tris/Mes buffer alone; see Fig. 2B). Addition of BSA (100 μg mL^{-1}) resulted in an increase in leakage that was intermediate between that effected by COR6.6 and COR15am. When frozen in a 50 mosmol NaCl: $CaCl_2$ (3:2, mol:mol) solution, non-T_m-related leakage was minimized in all of the samples (Fig. 6B). Although the effect of NaCl-$CaCl_2$ was similar to that of Suc in minimizing non-T_m-related leakage and precluding the increase in non-T_m-related leakage in the presence of COR polypeptides, there were some differences between Suc and NaCl-$CaCl_2$: (a) Suc decreased the extent of T_m-related leakage, whereas salts did not; and (b) T_m-related leakage occurred after freezing to −25°C in the presence of salts but occurred at −30°C when frozen in the presence of Suc.

In summary, non-T_m-related leakage from DOPC-SUVs was precluded when the liposomes were frozen in the presence of added solutes, such as Suc or NaCl + $CaCl_2$. Under these conditions, the anomalous increase in non-T_m-related leakage that occurred when the SUVs were frozen in the Tris/Mes buffer plus COR polypeptides was also

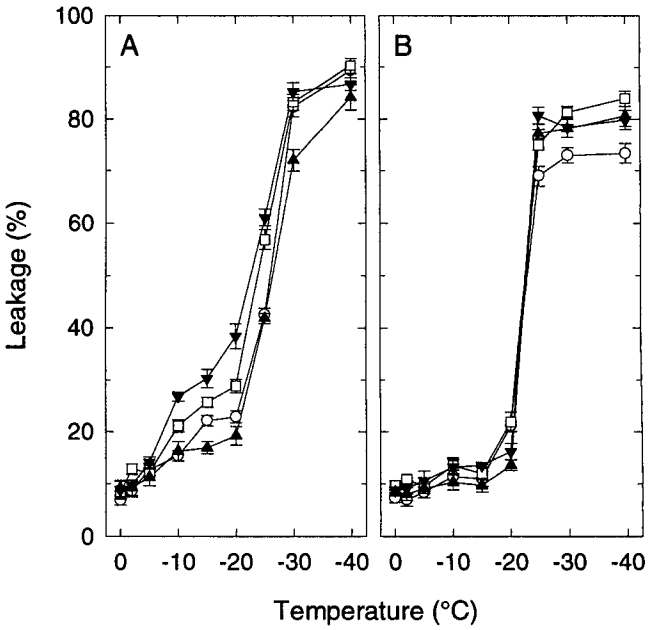

Figure 6. Freeze-induced leakage from DOPC-SUVs suspended in a solution of NaCl + $CaCl_2$ (3:2, mol:mol) with an osmolality of either 10 (A) or 50 mosmol (B). Liposomes were prepared with the same procedure described in Figure 2, and NaCl + $CaCl_2$ was then added to the liposome suspensions before freezing. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are the average and SD of two freezing experiments.

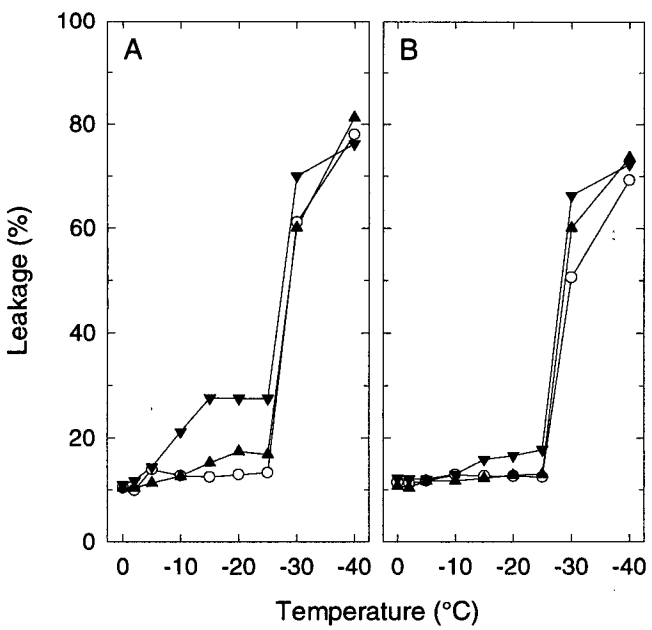

Figure 5. Freeze-induced leakage from DOPC-SUVs suspended in either a 20 (A) or 50 mM (B) Suc solution. Liposomes were prepared with the same procedure described in Figure 2, and Suc was then added to the liposome suspensions before freezing. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am. The results shown are from one freezing experiment.

precluded. Also, freezing the liposomes in the presence of added solutes diminished the incidence of freeze-induced fusion to less than 10%. Under these conditions, neither a positive nor a negative effect of COR polypeptides on freeze-induced fusion could be discerned.

Effect of COR Polypeptides on the Cryostability of Liposomes That Were Purified under Isotonic Conditions

In the studies described above, SUVs that were used in the studies of freeze-induced fusion were prepared by hydrating the lipids with a solution of 5 mM Tris/Mes buffer. Because the lipid probes used for the fluorescence assay of lipid mixing were incorporated into the lipid bilayers, they did not add to the osmolality of either the solution entrapped within the liposome or the suspending medium. In contrast, liposomes that were used in the studies of freeze-induced leakage were prepared by hydrating the lipids in a solution containing 12.5 mM ANTS + 45 mM DPX + 5 mM Tris/Mes buffer, which yielded a final osmolality of 70 mosmol. Prior to the freeze/thaw cycle, unencapsulated ANTS/DPX was removed by gel filtration using a 5 mM Tris/Mes buffer (5 mosmol) as the elution buffer. Under these conditions, the liposomes were subjected to hypotonic conditions. Thus, although the SUVs used for both studies of freeze-induced fusion and freeze-induced leakage were suspended in the same solution (5 mosmol Tris/Mes buffer) during freezing, they differed in that the SUVs used for studies of freeze-induced fusion were suspended and frozen in an isotonic medium, whereas those used for studies of freeze-induced leakage were in a hypotonic medium during purification and were subsequently frozen in a hypotonic medium.

Although there are differing opinions as to whether SUVs are osmotically responsive in a hypotonic medium, there is general agreement that SUVs do not leak when exposed to hypotonic conditions. Johnson and Buttress (1973) first reported that a hypotonic treatment of SUVs composed of a mixture of PC and phosphatidic acid results in little leakage of intraliposomal contents. Sun et al. (1986) also reported that dimyristoylphosphatidylcholine-liposomes (<80 nm diameter) do not expand when subjected to hypotonic solutions. Both reports concluded that SUVs are osmotically insensitive and nondeformable. In contrast, Lerebours et al. (1993) recently reported that dimyristoylphosphatidylcholine-SUVs (20 nm diameter) expand during hypotonic treatment. When liposomes were prepared with a solution containing 3.0 M LiCl and subsequently incubated in 1.5 M LiCl, the mean diameter of the liposomes increased from 17.9 to 24.0 nm. However, under these conditions there was no leakage of intraliposomal contents during swelling of the liposomes. These results suggest that the integrity of the lipid bilayers of SUVs is maintained such that no leakage occurs when the SUVs are expanding in response to an osmotic gradient.

We have confirmed that SUVs do not leak when eluted with a hypotonic buffer during gel filtration. When DOPC-SUVs containing ANTS/DPX were eluted with either a hypotonic solution (5 mM Tris/Mes) or an isotonic solution (5 mM Tris/Mes + 34 mM NaCl) during gel filtration, there was a very little difference in the amount of ANTS/DPX retained in the liposomes after gel filtration (data not shown). Nevertheless, it is possible that the cryostability of SUVs frozen in a hypotonic medium might be different from that of SUVs frozen in an isotonic medium. This was of concern given the desire to compare the incidence of freeze-induced fusion and freeze-induced leakage under similar conditions. But in the studies described above, the SUVs used to assay for freeze-induced fusion were frozen in an isotonic medium, whereas those used in the assays for freeze-induced leakage were frozen in a hypotonic medium.

To determine if a hypotonic treatment during gel filtration affects the cryostability of SUVs during a freeze/thaw cycle, we next measured freeze-induced leakage of ANTS/DPX from DOPC-SUVs that were eluted during gel filtration with a solution containing 5 mM Tris/Mes + 34 mM NaCl, the osmolality of which was equivalent to the ANTS/DPX solution inside the liposomes (70 mosmol). This procedure resulted in the liposomes being suspended in the Tris/Mes + NaCl solution during freezing.

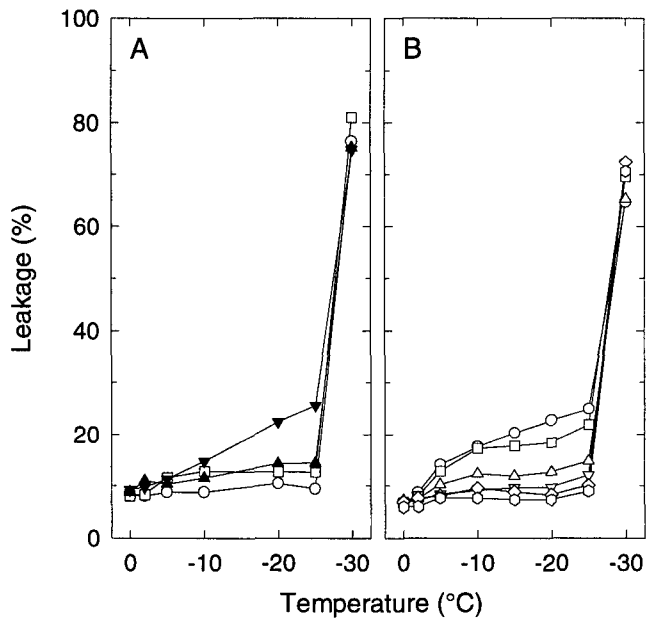

Figure 7. A, Freeze-induced leakage from DOPC-SUVs purified and frozen in the Tris/Mes + NaCl solution (70 mosmol). Liposomes containing ANTS/DPX were purified with a 5 mM Tris/Mes + 34 mM NaCl solution during gel filtration under isotonic conditions and frozen in the same solution. Thus, NaCl and ANTS/DPX were present outside and inside the liposomes, respectively. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are from one freezing experiment. B, Effect of extraliposomal NaCl on freeze-induced leakage from DOPC-SUVs. Liposomes were eluted with a 5 mM Tris/Mes buffer during gel filtration, and NaCl was then added to the liposome suspensions at the desired concentration before freezing. Liposomes were frozen in the absence of NaCl (○) or in the presence of NaCl at a concentration of 10 (□), 30 (△), 50 (▽), 100 (◇), or 200 mosmol (○). The results shown are from one freezing experiment.

Under these conditions, non-T_m-related leakage was precluded; however, there was a large increase in T_m-related leakage after freezing to −30°C (Fig. 7A). Addition of COR15am increased non-T_m-related leakage over the range of −10 to −20°C (<15%), but the extent of the increase was significantly less than that from DOPC-SUVs that were purified under hypotonic conditions and frozen in the absence of added solutes (see Fig. 2B). There was no effect of the addition of either COR6.6 or BSA. In all samples, there was an increase in T_m-related leakage at −30°C, the extent of which was similar in both the presence or absence of the COR polypeptides.

Thus, the incidence of freeze-induced leakage of SUVs that are purified under isotonic conditions and subsequently frozen in an isotonic medium (5 mM Tris/Mes + 34 mM NaCl; Fig. 7A) is substantially different from that of SUVs that are purified under hypotonic conditions and subsequently frozen in a hypotonic medium (5 mM Tris/Mes) without added solutes (Fig. 2B). Specifically, there is a large reduction in the incidence of non-T_m-related leakage from SUVs that are purified and frozen in an isotonic solution containing 70 mosmol NaCl, whether in the presence or absence of the COR polypeptides. These results are similar to those obtained when DOPC-SUVs were purified under hypotonic conditions and frozen in a medium containing Suc or NaCl + $CaCl_2$ (Figs. 4–6). Under these conditions, non-T_m-related leakage was also precluded, including the anomalous increase that occurred when the SUVs were frozen in the presence of COR polypeptides in the Tris/Mes buffer. This suggests that the decrease in non-T_m-related leakage is a result of freezing the SUVs in a medium that contains added solutes rather than whether the SUVs were purified under isotonic or hypotonic conditions.

This possibility was confirmed experimentally. When DOPC-SUVs were prepared with a solution containing ANTS/DPX and eluted during gel filtration with a hypotonic solution (5 mM Tris/Mes), followed by the addition of NaCl to the liposome suspensions (i.e. NaCl was present only outside the liposomes), the extent of non-T_m-related leakage decreased with increasing concentrations of external NaCl (Fig. 7B). At 50 mosmol NaCl or more, non-T_m-related leakage was precluded, which was similar to the results obtained when the SUVs were purified under isotonic conditions by elution with the Tris/Mes + NaCl solution (Fig. 7A). Thus, the decrease in non-T_m-related leakage is a result of the presence of external solutes per se rather than the conditions (isotonic versus hypotonic) used for purification of the SUVs. Furthermore, the anomalous increase in non-T_m-related leakage from SUVs frozen in the presence of COR polypeptides but in the absence of added solutes (Fig. 2B) does not occur when the suspending medium contains a sufficiently high concentration of solutes (50 mM Suc, Fig. 5B; 50 mosmol NaCl + $CaCl_2$, Fig. 6B; or 50 mosmol NaCl, Fig. 7B).

We next determined the incidence of freeze-induced fusion under similar conditions (i.e. freezing in the presence of 70 mosmol NaCl in the suspending medium). For this, DOPC-SUVs labeled with NBD-PE/Rh-PE were prepared with a solution composed of 5 mM Tris/Mes + 34 mM NaCl (70 mosmol). Thus, the liposomes used for assay of freeze-induced fusion and leakage were similar in that both were suspended in a solution containing 5 mM Tris/Mes and 34 mM NaCl (70 mosmol) during freezing. They differed, however, in that the SUVs used in the studies of freeze-induced fusion contained NaCl, whereas those used in the studies of freeze-induced leakage contained ANTS/DPX as the internal osmoticum. Under these conditions, there was no non-T_m-related fusion of DOPC-SUVs frozen in the absence of COR polypeptides (Fig. 8A). In addition, the extent of T_m-related fusion was significantly decreased (15% at −30°C). Addition of either of the COR polypeptides or BSA (100 $\mu g\ mL^{-1}$) did not have a detectable effect on either non-T_m-related or T_m-related fusion.

These results were quite different from those obtained with DOPC-SUVs prepared and frozen in 5 mM Tris/Mes (Fig. 1B). The presence of NaCl both outside and inside the liposomes resulted in a significant decrease in non-T_m-related and T_m-related fusion. However, this comparison did not provide an answer to the question: is the decrease in fusion a result of external NaCl, internal NaCl, or both? The observation that addition of Suc in the suspension of DOPC-SUVs precluded non-T_m-related fusion (Fig. 4A) suggests that the presence of solutes in the suspension

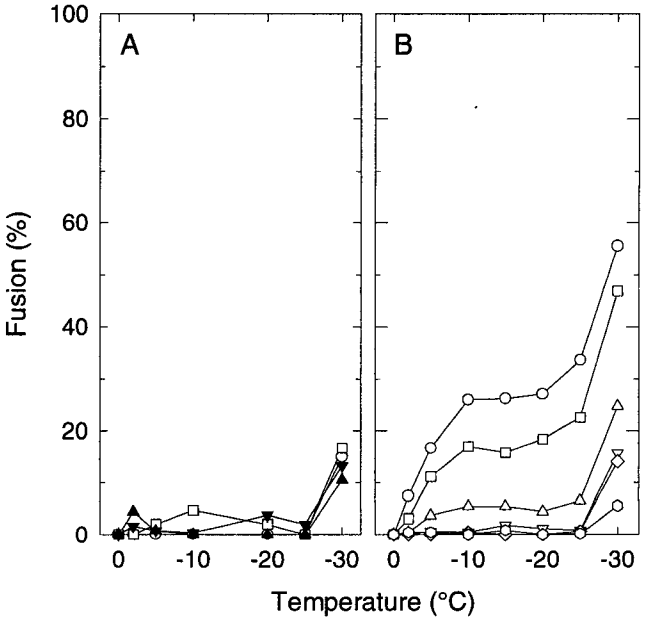

Figure 8. A, Freeze-induced fusion of DOPC-SUVs prepared and frozen in the Tris/Mes + NaCl solution (70 mosmol). NaCl was present both inside and outside the liposomes. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are from one freezing experiment. B, Effect of extraliposomal NaCl on freeze-induced fusion of DOPC-SUVs. Liposomes were prepared with a 5 mM Tris/Mes buffer, and NaCl was then added to the liposome suspensions at the desired concentration before freezing. Liposomes were frozen in the absence of NaCl (○) or in the presence of NaCl at a concentration of 10 (□), 30 (△), 50 (▽), 100 (◇), or 200 mosmol (○). The results shown are from one freezing experiment.

medium is responsible for the large decrease in both non-T_m-related and T_m-related fusion. This possibility was confirmed with DOPC-SUVs that were prepared with a 5 mM Tris/Mes buffer alone and frozen in a solution containing NaCl, i.e. NaCl was present only outside the liposomes. Under these conditions, addition of NaCl (≥50 mosmol) completely precluded non-T_m-related fusion and significantly decreased T_m-related fusion (Fig. 8B), which was very similar to the results when SUVs were prepared and frozen in the Tris/Mes + NaCl solution (i.e. 70 mosmol NaCl was present both inside and outside the liposomes; Fig. 8A). Thus, the decrease in the incidence of both non-T_m-related and T_m-related fusion is a result of the presence of solutes outside the liposomes, and the solutes inside the liposomes have no apparent effect on the cryostability of liposomes.

In summary, when DOPC-SUVs were frozen in a solution containing 70 mosmol NaCl, non-T_m-related leakage was precluded, and both non-T_m-related and T_m-related fusion were significantly decreased. Under these conditions, there was no effect—neither positive nor negative—of COR polypeptides on the cryostability of the liposomes.

Freeze-Induced Fusion and Leakage of SUVs Formed from the Total Lipid Extracts of the Rye Plasma Membrane

In the above studies, liposomes composed of only a single species of PC were used. We next determined freeze-induced fusion and leakage with SUVs formed from a complex mixture of lipids, NA-PM-SUVs and ACC-PM-SUVs.

Effect of COR Polypeptides in the Absence of Added Solutes

When the liposomes were prepared in a 5 mM Tris/Mes buffer and frozen in this solution in the absence of COR polypeptides, there was a pronounced difference in the incidence of freeze-induced fusion of NA-PM-SUVs and ACC-PM-SUVs (Fig. 9). With NA-PM-SUVs, the incidence of fusion increased substantially after freezing to −5°C and reached a maximum at −7.5°C (Fig. 9A). With ACC-PM-SUVs, the incidence of fusion at −5°C was lower than with NA-PM-SUVs, and the maximum incidence of fusion did not occur until after freezing to −20°C or lower (Fig. 9B). The temperatures at which the maximum incidence of freeze-induced fusion of NA-PM-SUVs and ACC-PM-SUVs occurred correspond to the temperature at which 50% electrolyte leakage occurred after a freeze/thaw cycle of nonacclimated (−6°C) and coldacclimated rye leaves (−20°C), respectively (Uemura and Steponkus, 1994).

Addition of either COR6.6 or COR15am (100 $\mu g\ mL^{-1}$) decreased the incidence of fusion of both NA-PM-SUVs (Fig. 9A) and ACC-PM-SUVs (Fig. 9B). However, addition of COR polypeptides did not affect the temperature at which maximum fusion occurred. Instead, addition of either COR6.6 or COR15am resulted in a uniform decrease (approximately 15–20%) in the incidence of freeze-induced fusion—regardless of either the lipid composition of the liposomes (i.e. NA-PM-SUVs or ACC-PM-SUVs) or the temperature at which the liposomes were frozen (i.e. from −5 to −30°C in NA-PM-SUVs or −5 to −40°C in ACC-PM-SUVs).

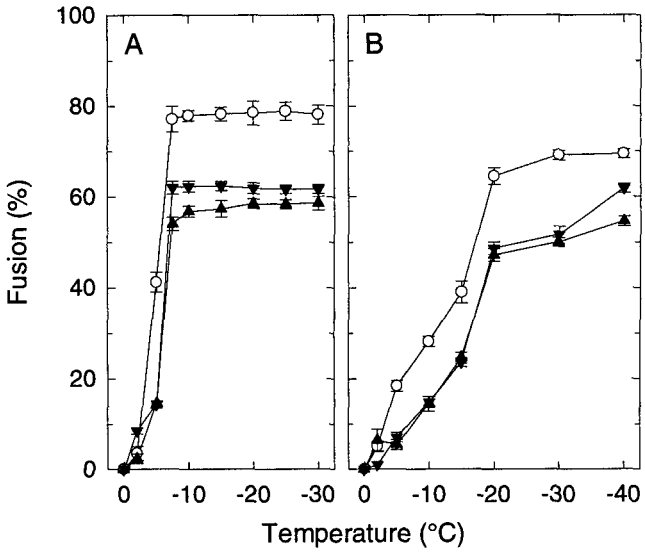

Figure 9. Freeze-induced fusion of SUVs formed from the total lipid extract of the plasma membrane of rye leaves and frozen in the Tris/Mes buffer in the absence of any added solutes. A, NA-PM-SUVs; B, ACC-PM-SUVs. Liposomes were prepared as described in Figure 1. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am. The results shown are the average and SD of three freezing experiments.

There was also a difference between NA-PM-SUVs and ACC-PM-SUVs in the incidence of freeze-induced leakage of ANTS/DPX (Fig. 10). In the absence of COR polypeptides, freeze-induced leakage from NA-PM-SUVs increased after freezing to −5°C and reached a maximum at −10°C (Fig. 10A). With ACC-PM-SUVs, the incidence of leakage increased gradually over the range of −5 to −40°C (Fig. 10B). Neither COR6.6 nor COR15am affected the extent of leakage from either NA-PM-SUVs or ACC-PM-SUVs regardless of the freezing temperature.

It should be noted that the steep increase in freeze-induced fusion and leakage that occurred in NA-PM-SUVs (Figs. 9A and 10A) and to a lesser extent in ACC-PM-SUVs (Figs. 9B and 10B) is unlikely to be a consequence of a liquid crystalline-to-gel phase transition—as observed with SUVs composed of a single species of PC (Figs. 1 and 2). This is because a liquid crystalline-to-gel phase transition in the total lipid extract of the plasma membrane from either nonacclimated or cold-acclimated rye leaves is not detectable by differential scanning calorimetry (Lynch and Steponkus, 1989).

In summary, in the absence of added solutes, COR6.6 and COR15am decreased freeze-induced fusion of PM-SUVs but had no effect on freeze-induced leakage. Thus, the apparent cryoprotective effect of COR polypeptides on freeze-induced fusion of liposomes in the absence of added solutes occurred in both PC-SUVs and PM-SUVs. However, unlike the results with PC-SUVs, the extent of the

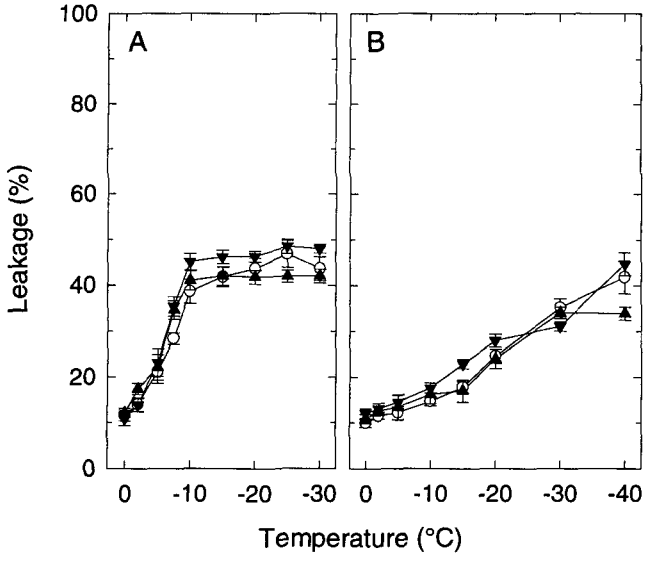

Figure 10. Effect of COR polypeptides on freeze-induced leakage from SUVs formed from the total lipid extract of the plasma membrane of rye leaves and frozen in a Tris/Mes buffer in the absence of any added solutes. A, NA-PM-SUVs; B, ACC-PM-SUVs. Liposomes were prepared as described in Figure 2. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am. The results shown are the average and SD of three freezing experiments.

effect was rather uniform in PM-SUVs regardless of the differences in lipid composition (NA-PM-SUVs versus ACC-PM-SUVs) or freezing temperature (−2 to −30°C or −40°C). Furthermore, the anomalous increase in leakage that occurred in PC-SUVs frozen in the presence of COR polypeptides did not occur in PM-SUVs.

Effect of COR Polypeptides in the Presence of NaCl

Next, freeze-induced fusion and leakage were determined with NA-PM-SUVs and ACC-PM-SUVs frozen in a solution of 5 mM Tris/Mes and 34 mM NaCl (70 mosmol). That is, the SUVs were prepared and frozen in the Tris/Mes + NaCl solution for the fusion assay or purified and frozen in the Tris/Mes + NaCl solution for the leakage assay (i.e. purified under isotonic conditions in the presence of NaCl). Under these conditions, the incidence of freeze-induced fusion in both NA-PM-SUVs (Fig. 11A) and ACC-PM-SUVs (Fig. 11B) was similar to that obtained when the liposomes were prepared and frozen in the Tris/Mes buffer (Fig. 9) in that the increase in fusion occurred over the same temperature range, the maximum extent of fusion was similar, and the differential response of NA-PM-SUVs and ACC-PM-SUVs to freezing was still observed. However, when suspended in the Tris/Mes + NaCl solution, addition of either COR6.6 or COR15am had no effect on the incidence of freeze-induced fusion, which was different from the results obtained when the liposomes were prepared and frozen in the Tris/Mes buffer (i.e. COR polypeptides decreased the incidence of freeze-induced fusion). This is because the incidence of fusion of both NA-PM-SUVs and ACC-PM-SUVs frozen in the presence of COR polypeptides was greater (approximately 80% at −30°C) when the SUVs were prepared and frozen in the Tris/Mes + NaCl solution than when they were prepared and frozen in the Tris/Mes buffer (approximately 60% at −30°C) (compare Figs. 9 and 11).

When liposomes were purified and frozen in the Tris/Mes + NaCl solution (Fig. 12), the incidence of freeze-induced leakage of NA-PM-SUVs and ACC-PM-SUVs, either in the presence or absence of COR polypeptides, was similar to what occurred when the liposomes were purified and frozen in the Tris/Mes buffer (Fig. 10). The incidence of leakage from NA-PM-SUVs reached a maximum at −10°C (Fig. 12A), and there was only a gradual increase in leakage from ACC-PM-SUVs over the range of −5 to −40°C (Fig. 12B). However, the extent of freeze-induced leakage from both NA-PM-SUVs and ACC-PM-SUVs was somewhat greater at any temperature tested when the SUVs were purified and frozen in the Tris/Mes + NaCl solution than when they were purified and frozen in the Tris/Mes buffer (compare Figs. 10 and 12). Addition of either COR6.6 or COR15am had no effect on the extent of freeze-induced leakage.

Thus, as with PC-SUVs, there was no effect of COR polypeptides on either freeze-induced fusion or leakage of PM-SUVs when the liposomes were frozen in the Tris/Mes + NaCl solution. However, there was a difference between PC-SUVs and PM-SUVs on the effect of NaCl on freeze-induced fusion and leakage. With PC-SUVs, NaCl minimized non-T_m-related fusion and leakage and T_m-related fusion, but with PM-SUVs, there was little effect of NaCl on the incidence of freeze-induced fusion and leakage. Under these conditions, there was no cryoprotective effect of COR polypeptides on the incidence of freeze-induced fusion.

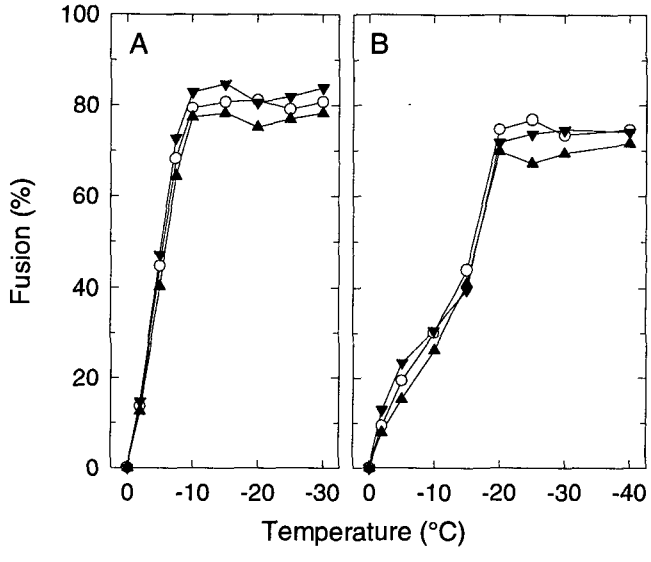

Figure 11. Effect of COR polypeptides on freeze-induced fusion of SUVs formed from the total lipid extracts of the plasma membrane of rye leaves and frozen in the Tris/Mes + NaCl solution. A, NA-PM-SUVs; B, ACC-PM-SUVs. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am. The results shown are from one freezing experiment.

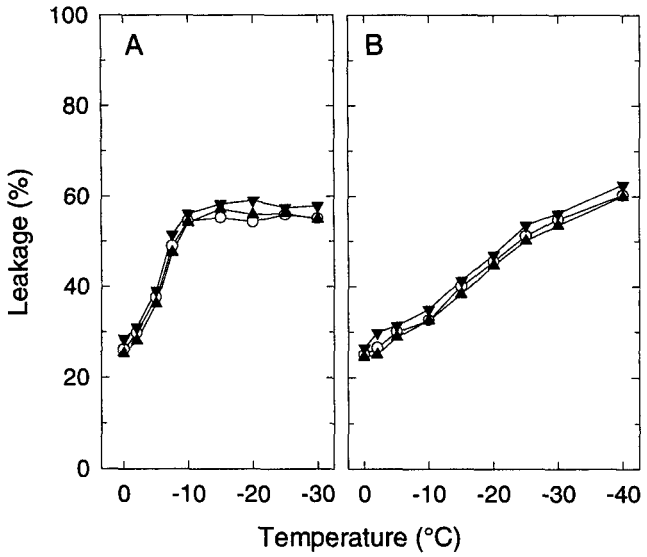

Figure 12. Effect of COR polypeptides on freeze-induced leakage from SUVs formed from the total lipid extract of the plasma membrane of rye leaves and purified and frozen in the Tris/Mes + NaCl solution. A, NA-PM-SUVs; B, ACC-PM-SUVs. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am. The results shown are from one freezing experiment.

Freeze-Induced Fusion and Leakage of SUVs Composed of a Mixture of DOPC-DOPE-FS

We next determined the effect of COR polypeptides on freeze-induced fusion and leakage of SUVs composed of a mixture of DOPC:DOPE:FS (1:1:1, mol:mol). This mixture is more complex than that of PC-SUVs but simpler than that of PM-SUVs.

Effect of COR Polypeptides in the Absence of Added Solutes

When DOPC-DOPE-FS-SUVs were prepared in the Tris/Mes buffer and frozen in this solution in the absence of COR polypeptides, freeze-induced fusion increased after freezing to −2°C and approached a maximum after freezing to −5°C (Fig. 13A). Addition of either COR6.6 or COR15am (100 μg mL^{-1}) substantially decreased the incidence of fusion (<10%) over the range of −2 to −30°C. The extent of the effect (a 45% decrease at −30°C) with DOPC-DOPE-FS-SUVs was greater than that with either PC-SUVs (Fig. 1) or PM-SUVs (Fig. 9). Unlike COR polypeptides, addition of BSA (100 μg mL^{-1}) did not decrease the incidence of freeze-induced fusion.

In the absence of COR polypeptides, there was a gradual increase in freeze-induced leakage from DOPC-DOPE-FS-SUVs (Fig. 13B). Addition of either COR6.6 or COR15am or BSA (all at 100 μg mL^{-1}) had little effect on leakage over the range of −2 to −30°C. Thus, with DOPC-DOPE-FS-SUVs frozen in the absence of COR polypeptides, the increase in the incidence of freeze-induced leakage coincided with the increase in the incidence of freeze-induced fusion. Although COR polypeptides decreased freeze-induced fusion, there was neither a positive nor a negative effect on freeze-induced leakage.

Effect of COR Polypeptides in the Presence of NaCl

When the liposomes were prepared and frozen in the Tris/Mes + NaCl solution and then frozen in the absence of COR polypeptides, the incidence of freeze-induced fusion was decreased substantially (20% when frozen in the presence of NaCl versus 55% when frozen in the absence of NaCl), and the increase in the incidence of freeze-induced fusion occurred only after freezing over the range of −20 to −30°C (Fig. 14A). In suspensions containing COR polypeptides (100 μg mL^{-1}), fusion was detectable only after freezing to −30°C; there was no difference between COR6.6 and COR15am. Addition of BSA (100 μg mL^{-1}) also decreased the incidence of fusion at −20 and −25°C, but not at −30°C.

With liposomes that were purified with the Tris/Mes + NaCl solution during gel filtration and frozen in this solution, the incidence of freeze-induced leakage (Fig. 14B) was somewhat lower than what occurred with liposomes purified and frozen in the Tris/Mes buffer (i.e. without NaCl; Fig. 13B). Addition of COR polypeptides had only a small effect on the incidence of freeze-induced leakage. Addition of BSA (100 μg mL^{-1}), however, did appear to increase the incidence of leakage over that of the control.

Thus, as with PC-SUVs (Fig. 1) and PM-SUVs (Fig. 9) frozen in the Tris/Mes buffer, addition of COR polypeptides resulted in a decrease in the incidence of freeze-induced fusion of DOPC-DOPE-FS-SUVs (Fig. 13A). How-

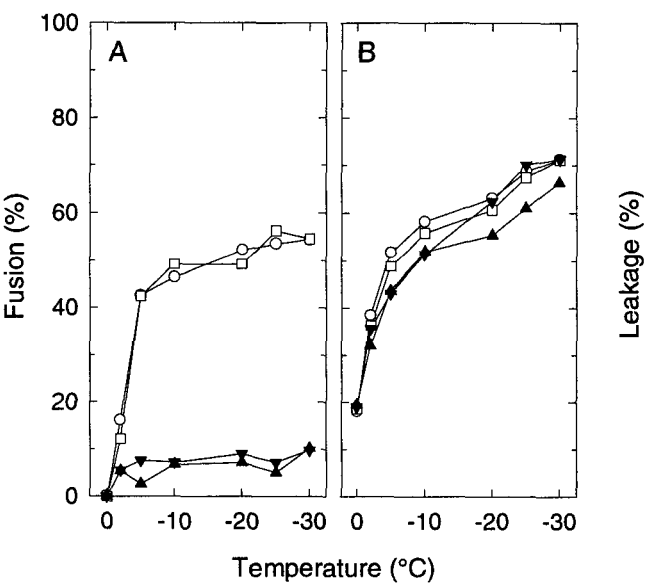

Figure 13. Effect of COR polypeptides on freeze-induced fusion (A) and leakage (B) of DOPC:DOPE:FS-SUVs (1:1:1, mol:mol) frozen in the Tris/Mes buffer in the absence of any added solutes. Liposomes were prepared as described in Figures 1 and 2. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are from one freezing experiment.

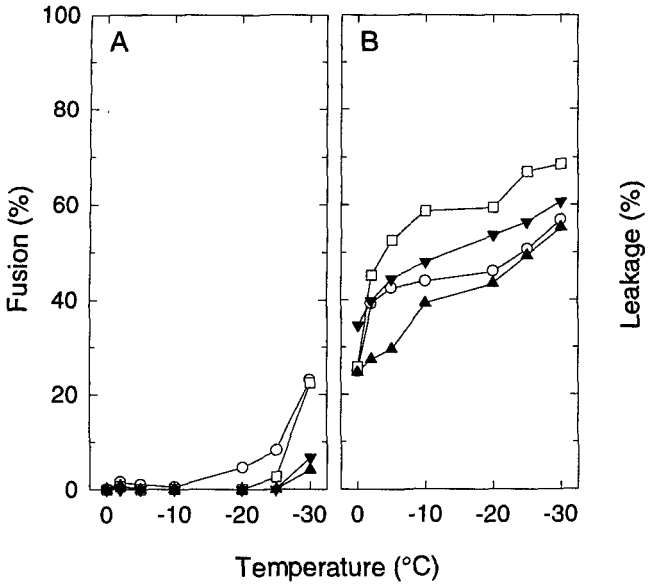

Figure 14. Effect of COR polypeptides on freeze-induced fusion (A) and leakage (B) of DOPC:DOPE:FS-SUVs (1:1:1, mol:mol) frozen in the Tris/Mes + NaCl solution. ○, Liposomes frozen in the absence of polypeptides; ▲, liposomes frozen in the presence of COR6.6; ▼, liposomes frozen in the presence of COR15am; □, liposomes frozen in the presence of BSA. The results shown are from one freezing experiment.

ever, a large diminution of freeze-induced fusion was also effected by freezing in the Tris/Mes + NaCl solution (Fig. 14A); in spite of the very low incidence of freeze-induced fusion, a cryoprotective effect of the COR polypeptides was detected. Addition of COR polypeptides had no effect on the incidence of freeze-induced leakage, regardless of the manner in which the liposomes were prepared.

DISCUSSION

Do COR Polypeptides Have a Cryoprotective Effect on the Cryostability of Liposomes?

The objective of this study was to determine if either COR6.6 or COR15am affects the cryostability of lipid bilayers as measured by their effect on freeze-induced fusion and leakage of liposomes frozen in vitro. Unfortunately, the results of the studies do not yield either a simple or unequivocal answer to this question. Rather, the answer must be qualified depending on the lipid composition of the liposomes and the composition of the solution in which they are frozen.

In considering the question of whether COR polypeptides increase the cryostability of lipid bilayers, the answer is a qualified "yes" if considered from the studies of the effect of COR polypeptides on the incidence of freeze-induced fusion. COR polypeptides increased the cryostability of liposomes as determined by their effect on the incidence of freeze-induced fusion—if the liposomes were prepared and frozen in the Tris/Mes buffer (i.e. in the absence of any added solutes). Under these conditions, addition of COR polypeptides invariably decreased the incidence of freeze-induced fusion regardless of the lipid composition (PC-SUVs, PM-SUVs, or DOPC-DOPE-FS-SUVs; Figs. 1, 9, and 13A). However, the magnitude of the effect varied with the lipid composition of the liposomes: there was a large effect with DOPC-SUVs (addition of COR polypeptides decreased the incidence of freeze-induced fusion from 40% to less than 10% at −30°C; Fig. 1B), but there was less of an effect with POPC-SUVs (a 10% reduction; Fig. 1A); there was a large effect with DOPC-DOPE-FS-SUVs (from 55% to less than 10%; Fig. 13A), such that there was only a very low incidence of freeze-induced fusion over the range of −2 to −30°C, but there was much less of an effect with PM-SUVs (a 15–20% decrease; Fig. 9).

Is the effect of COR6.6 and COR15am on the incidence of freeze-induced fusion unusual? Yes, when compared to BSA, which did not influence the incidence of freeze-induced fusion in any of the liposome systems studied when compared with the COR polypeptides at either equivalent mass concentrations (Fig. 1B) or equimolar concentrations (Fig. 3A). Furthermore, the effect of COR polypeptides on fusion may also be considered unusual when compared to the effect of either aprotinin (6.5 kD) or RNase A (13.7 kD), which, like COR polypeptides, are low-molecular-weight hydrophilic proteins; neither aprotinin nor RNase A affected the incidence of freeze-induced fusion (data not shown).

However, the apparent cryoprotective effect of the COR polypeptides that might be inferred from the studies of their effect on freeze-induced fusion must be carefully considered in relation to two other results. First, under conditions that yielded an apparent cryoprotective effect of the COR polypeptides on freeze-induced fusion (i.e. when frozen in the Tris/Mes buffer in the absence of added solutes), the COR polypeptides had either a negative effect or no effect on the cryostability of the liposomes as measured by the incidence of freeze-induced leakage. With PC-SUVs, freezing in the presence of the COR polypeptides resulted in an increase in leakage at −10°C, regardless of the lipid composition of the liposomes (i.e. irrespective of the T_m; Fig. 2). The large increase in leakage from PC-SUVs occurred in spite of the fact that freeze-induced fusion was largely precluded by addition of the COR polypeptides. We consider this increase in freeze-induced leakage to be anomalous because (a) the large increase in leakage occurred at the same temperature (−10°C) regardless of the lipid composition of the PC-SUVs (i.e. irrespective of the T_m); (b) the large increase in leakage was not coincident with the increase in fusion (as it is when the liposomes are frozen in the absence of COR polypeptides); and (c) the large increase in leakage was not consistent with the apparent cryoprotective effect of COR polypeptides on freeze-induced fusion. With PM-SUVs (Fig. 10) or DOPC-DOPE-FS-SUVs (Fig. 13B), freezing in the presence of COR polypeptides did not have any effect on the incidence of freeze-induced leakage.

Second, a large decrease in the incidence of freeze-induced fusion of either PC-SUVs or DOPC-DOPE-FS-SUVs was also effected by the addition of relatively low concen-

trations of solutes (either Suc or NaCl; Figs. 4A, 8, and 14A). In this respect, diminution of the incidence of freeze-induced fusion is not unique to COR polypeptides. When frozen in the presence of added solutes, freeze-induced fusion of PC-SUVs and DOPC-DOPE-FS-SUVs was greatly decreased, such that any additional effect of the COR polypeptides would be of limited magnitude (e.g. the incidence of freeze-induced fusion of DOPC-DOPE-FS-SUVs that were frozen in the presence of NaCl decreased from approximately 20% to 5% when either COR6.6 or COR15am was present in the suspending medium) and, hence, would be of questionable significance. However, this was not true for PM-SUVs: freezing in the presence of added solutes did not decrease the incidence of freeze-induced fusion, and the incidence of fusion was still relatively high (Fig. 11). Under these conditions, there was no additional effect of COR polypeptides on the incidence of freeze-induced fusion.

Although we considered whether the apparent cryoprotective effect of COR polypeptides was the result of solutes in the COR polypeptide preparation, this is not the case. During purification of COR polypeptides, the COR polypeptide samples were extensively dialyzed against water (Gilmour et al., 1996), and when suspended in 5 mM Tris/Mes buffer at the concentrations used for preparation of the liposomes, the COR polypeptides did not alter the osmolality (5 mosmol) of the solution (data not shown).

Returning to the question of whether COR polypeptides increase the cryostability of liposomes, the answer is an unequivocal "no" if considered from the studies of the effect of COR polypeptides on the incidence of freeze-induced leakage. In no treatment did COR polypeptides decrease the incidence of freeze-induced leakage. If there was an effect of COR polypeptides on freeze-induced leakage, it was invariably a negative effect (i.e. freezing in the presence of COR polypeptides increased the incidence of leakage); however, this was observed only with PC-SUVs (Fig. 2) and not with either PM-SUVs (Fig. 10) or DOPC-DOPE-FS-SUVs (Fig. 13B). However, this does not appear to be a unique effect of COR polypeptides. Although BSA did not result in an increase in freeze-induced leakage when added in mass amounts (100 μg mL^{-1}) that were equivalent to the amounts of COR polypeptides that were used (Fig. 2B), addition of BSA in amounts that were equimolar to the COR polypeptides resulted in an increase in freeze-induced leakage (Fig. 3B). Although this observation tends to diminish the significance of the increased leakage that was elicited by addition of COR polypeptides in some of the treatments, nevertheless, COR polypeptides did not diminish the incidence of freeze-induced leakage in any of the treatments. Also, the negative effect of COR polypeptides on freeze-induced leakage was not observed when the liposomes were frozen in the Tris/Mes buffer in the presence of added solutes. There was, however, a difference in the solute concentration that was required to eliminate the increase in freeze-induced leakage that was effected by addition of the COR polypeptides, with the effective concentration dependent on both the solute and COR polypeptide that was used.

In considering the positive effects of COR polypeptides on freeze-induced fusion (which were observed in only some of the treatments) and the absence of any effect or a negative effect of the COR polypeptides on freeze-induced leakage, it should be noted that freeze-induced fusion requires liposome-liposome interactions, beginning with the close apposition of liposomes. In contrast, freeze-induced leakage does not require liposome-liposome interactions. Therefore, freeze-induced leakage might be considered to reflect the impact of ice formation and the attendant repercussions (increase in osmotic pressure, increase in solute concentration, freeze-induced dehydration) on the stability of the lipid bilayer per se, whereas freeze-induced fusion also depends on the liposomes being in close proximity and on liposome-liposome interactions. Therefore, the lack of a cryoprotective effect of COR polypeptides on freeze-induced leakage suggests that they have little effect on minimizing the deleterious effects of freezing on the lipid bilayer per se, but the apparent cryoprotective effect on the incidence of freeze-induced fusion could be a result of the COR polypeptides minimizing liposome-liposome interactions, which could be effected by either affecting the spatial separation between liposomes or by influencing their behavior when they are in close apposition.

It should be emphasized that, with the exception of the 15% decrease in freeze-induced fusion that was effected by addition of the COR polypeptides to the DOPC-DOPE-FS-SUVs that were suspended in the buffer + NaCl solution, the apparent cryoprotective effect of the COR polypeptides that was demonstrated by a decrease in the incidence of freeze-induced fusion was observed only when the liposomes were frozen in the Tris/Mes buffer without any added solutes. Of the conditions tested, freezing liposome suspensions without any added solutes results in the largest fraction of solution being frozen and, hence, the least spatial separation between liposomes. Thus, it is possible that under these conditions the COR polypeptides act as a steric hindrance to the close apposition of the liposomes. However, if this was only a nonspecific effect, other proteins should also have the same effect; this was not observed. Neither BSA, aprotinin, nor RNase decreased the incidence of freeze-induced fusion of DOPC-SUVs.

Addition of even small amounts of solutes decreases the amount of ice that is formed, and the amount of unfrozen solution remaining is proportionally much greater. Assuming that the liposomes are rejected from the ice crystals and concentrated in the unfrozen portion, it is not unexpected that, if the apparent cryoprotective effect of the COR polypeptides is a consequence of minimizing the close approach of liposomes, this effect would be diminished if the liposomes are frozen in a solution that does not result in as great a concentration of liposomes in the unfrozen portion of the solution (i.e. in the presence of solutes).

Relevance of in Vitro Freezing of Liposomes to Determine if COR Polypeptides Have a Direct Cryoprotective Function during in Vivo Freezing

Although the COR polypeptides had an apparent cryoprotective effect on the cryostability of liposomes during in

vitro freezing under certain conditions (a decrease in the incidence of freeze-induced fusion of PC-SUVs, PM-SUVs, and DOPC-DOPE-FS-SUVs frozen in the Tris/Mes buffer without any added solutes), these results a priori cannot be extrapolated to infer that the COR polypeptides have a direct cryoprotective function during in vivo freezing.

Under conditions of in vitro freezing, the liposomes are directly impacted by the ice matrix; during freezing in vivo, only the outer surface of the plasma membrane is exposed to the ice matrix because ice formation is normally restricted to the extracellular matrix. This is an important consideration if the apparent cryoprotective effect of the COR polypeptides observed in this study was a consequence of an effect of COR polypeptides on ice formation and if freeze-induced fusion of the liposomes was directly dependent on ice-liposome interactions. This possibility is suggested by the fact that during the in vitro freezing conducted in this study, the COR polypeptides were present only in the suspending medium. In studies with large unilamellar vesicles composed of DOPC (M. Uemura and P.L. Steponkus, unpublished results), we have shown that (a) the effect of COR polypeptides added to just the suspending medium on both freeze-induced fusion and leakage is not different from that observed if the COR polypeptides are both inside and outside the liposomes, and (b) the presence of COR polypeptides inside the liposomes (but not outside) yields results that are identical to the control. In other words, COR polypeptides have an effect only when they are in the extraliposomal matrix, and no effect is observed if they are encapsulated only within the liposomes. Therefore, it is possible that the effect of COR polypeptides on the cryostability of liposomes observed under the conditions of in vitro freezing used in this study was a consequence of the effect of COR polypeptides on either ice formation or ice-liposome interactions. However, given that neither COR6.6 nor COR15am is thought to be secreted into the extracellular matrix (Thomashow, 1993), it is unlikely that COR polypeptides interact with ice during freezing in vivo. Therefore, the apparent cryoprotective effect of COR polypeptides on minimizing the incidence of freeze-induced fusion of liposomes frozen in the absence of added solutes should not be extrapolated to conditions that occur during freezing in vivo. This caution also applies to inferences of a cryoprotective effect based on other in vitro freezing studies (Volger and Heber, 1975; Hincha et al., 1990; Lin and Thomashow, 1992a).

Another aspect to be considered is that among the different types of liposomes investigated in the present study, the effect of COR polypeptides on the cryostability of PM-SUVs is most relevant when considering the possible effect of COR polypeptides on the cryostability of biological membranes. COR polypeptides decreased the incidence of freeze-induced fusion of PM-SUVs when frozen in the Tris/Mes buffer in the absence of added solutes; however, there was no effect of COR polypeptides when the liposomes were frozen in the presence of NaCl. Freezing in a solution containing NaCl would be more similar to in vivo freezing than freezing in the Tris/Mes buffer alone. Under these conditions, there was neither a positive nor a negative effect of COR polypeptides on either freeze-induced fusion or leakage of PM-SUVs.

It is possible that different COR polypeptides may influence the cryostability of particular cellular membranes containing specific lipids. For example, since COR15am is located in the chloroplast compartment (Lin and Thomashow, 1992b), it is possible that it may affect the cryostability of the chloroplast envelope or thylakoids, the lipid composition of which is much different from that of the liposomes used in this study. However, in preliminary studies with SUVs composed of a mixture of monogalactosyldiglycerides, digalactosyldiglycerides, and PC (in proportions similar to those found in the chloroplast envelope) and frozen in the Tris/Mes + NaCl solution, there was neither a positive nor a negative effect of either COR15am or COR6.6 on either freeze-induced leakage or fusion (data not shown). In addition, we have determined that there is no effect of COR polypeptides on either light-induced proton uptake or photophosphorylation of isolated spinach thylakoids subjected to in vitro freezing (M. Uemura and P.L. Steponkus, unpublished results).

CONCLUDING REMARKS

In conclusion, although COR6.6 and COR15am invariably decreased the incidence of freeze-induced fusion of liposomes that were suspended in a Tris/Mes buffer without any additional solutes, we do not believe that these results should be taken to conclude that COR6.6 and COR15am have a direct, cryoprotective effect on liposomes frozen in vitro. Rather, we conclude that the decrease in the incidence of freeze-induced fusion effected by the COR polypeptides when the liposomes were frozen in the Tris/Mes buffer alone is atypical and is a consequence of an effect of the COR polypeptides on either ice formation or ice-liposome interactions that occur only during freezing of extremely dilute solutions. The conclusion is consistent with the observations that (a) an anomalous increase in freeze-induced leakage was effected by the COR polypeptides when PC-SUVs were frozen in a medium that did not contain additional solutes, and (b) the COR polypeptides had little or no effect on the incidence of either freeze-induced fusion or leakage when the liposomes were suspended in a buffer containing low concentrations of solutes such as Suc or NaCl. When considered collectively, these results indicate that neither COR6.6 nor COR15am has a direct cryoprotective effect on liposomes frozen in vitro.

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