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Cold-Induced Changes in Freezing Tolerance, Protein Phosphorylation, and Gene Expression[1]

Evidence for a Role of Calcium

Antonio F. Monroy, Fathey Sarhan, and Rajinder S. Dhindsa*

Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal, Quebec H3A 1B1, Canada (A.F.M., R.S.D.); and Departmente de Sciences Biologiques, Université du Québec à Montreal, Montreal, Quebec H3C 3P8, Canada (F.S.)

The role of Ca^{2+} in cold-induced changes in protein phosphorylation, gene expression, and development of freezing tolerance has been studied in cell-suspension cultures of a freezing-tolerant cultivar of alfalfa (*Medicago sativa* spp. *falcata* cv Anik). Chemical treatments to block Ca^{2+} channels, antagonize calmodulin action, or inhibit protein kinases markedly inhibited the cellular capacity to develop cold-induced freezing tolerance but had little effect on cell viability. An analysis of phosphoprotein profile by two-dimensional polyacrylamide gel electrophoresis revealed that at low temperature the relative level of phosphorylation of several proteins increased, whereas that of several others decreased. When cold acclimation was carried out in the presence of *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride, an antagonist of calmodulin and Ca^{2+}-dependent protein kinases, or the Ca^{2+} channel blocker La^{3+}, the cold-induced changes in protein phosphorylation were strongly inhibited, cells lost their capacity to develop freezing tolerance, and accumulation of transcripts of cold acclimation-specific genes was substantially reduced. An inhibitor of protein kinases, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, had less pronounced effects on the cold-induced protein phosphorylation and caused only a partial inhibition of the cold-induced development of freezing tolerance and accumulation of the transcripts. The level of phosphorylation of one protein, of about 15 kD, increased more than 10-fold at low temperature and showed a strong positive correlation with cold-induced freezing tolerance and gene expression even when the latter were altered with various chemical treatments. These results suggest that Ca^{2+} and protein phosphorylation, or perhaps a coupling of the two, play an important role during the acquisition of freezing tolerance during cold acclimation.

Freezing tolerance in plants develops in response to, among other factors, declining temperatures before the onset of winter (Levitt, 1980). During this period of cold acclimation (exposure to low but nonfreezing temperatures), novel mRNAs accumulate and new proteins are synthesized (Guy, 1990). There have been several studies, using a variety of plant species, that have demonstrated altered gene expression during cold acclimation (Mohapatra et al., 1988, 1989; Cattivelli and Bartels, 1990; Kurkela and Franck, 1990). Our laboratory has sequenced cDNAs for three cold-induced alfalfa (*Medicago sativa*) genes (Monroy et al., 1993; Wolfraim et al., 1993; G. Dong and R.S. Dhindsa, unpublished data), the expression of which is positively correlated with the capacity of several alfalfa cultivars to develop freezing tolerance (Mohapatra et al., 1989). Although definite clues to the function of cold-induced genes have not emerged from sequence data, we have been interested in studying the mechanisms underlying their induction by low temperature.

For a plant to cold acclimate, it must perceive low temperature signals and transduce them into biochemical responses. Ca^{2+} has been shown to be a modulator of metabolism and development and to serve as a second messenger in the transduction of environmental stimuli in a variety of organisms, including plants (Veigl et al., 1984; Trewavas and Gilroy, 1991). In animal cells, external stimuli cause rapid Ca^{2+} flux, resulting in a transient increase in cytosolic Ca^{2+} concentration. This transient elevation of cytosolic Ca^{2+} is coupled to protein kinases such as protein kinase C (Kikkawa and Nishizuka, 1986) and Ca^{2+}/calmodulin-dependent protein kinases (Hanson and Schulman, 1992) and results in long-lasting effects on gene expression, metabolism, and growth. In plants, external stimuli such as touch, wind, and chilling also evoke transient increases in cytosolic Ca^{2+} (Knight et al., 1991, 1992). The source of cytosolic Ca^{2+} has been shown to depend on the type of stimulus: extracellular (cell wall) Ca^{2+} in response to chilling, and intracellular Ca^{2+} in response to wind (Knight et al., 1992). Although there is no evidence for protein kinase C and Ca^{2+}/calmodulin-dependent protein kinases in plant cells, Ca^{2+} has been shown to regulate protein phosphorylation (Veluthambi and Poovaiah, 1984), and the existence of Ca^{2+}-dependent

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* Corresponding author; fax 1–514–398–5069.

Abbreviations: H7, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride; H8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride; TTC, 2,3,5-triphenyltetrazolium chloride; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.

protein kinases in alfalfa and other plants is well documented (Roberts and Harmon, 1992). The transduction of light (Datta and Cashmore, 1989; Sarokin and Chua, 1992), hormone (Daminov et al., 1992), and pathogen-elicitor (Felix et al., 1991) stimuli has been shown to involve protein phosphorylation and, as in the case of photoregulation, protein phosphorylation has been shown to modulate gene expression (Datta and Cashmore, 1989; Sarokin and Chua, 1992).

Thus, we have reasoned that Ca^{2+} and protein phosphorylation are likely to play important roles in regulating the expression of cold-induced genes and the development of freezing tolerance. To assess the roles of Ca^{2+} and protein phosphorylation, we have determined (a) whether inhibitors of Ca^{2+}/calmodulin action and of protein kinases and Ca^{2+} channel blockers impair the cellular capacity for developing cold-induced freezing tolerance; (b) whether there are cold-induced changes in protein phosphorylation during early stages of cold acclimation and, if so, whether such changes are affected by the inhibitors; and (c) whether the same inhibitors would impair the accumulation of transcripts of previously reported cold acclimation-specific genes (Mohapatra et al., 1989; Monroy et al., 1993; Wolfraim et al., 1993). The results of the present study suggest that Ca^{2+} influx and cold-induced changes in the relative phosphorylation of preexisting proteins regulate the expression of cold acclimation-specific genes and development of freezing tolerance.

MATERIALS AND METHODS

Cold Acclimation of Cell Cultures

Cell-suspension cultures of a freezing-tolerant alfalfa cultivar (*Medicago sativa* spp. *falcata* cv Anik) have been used as an experimental system because compared with intact plants, they are more amenable to uniform and rapid chemical treatment. They have a similar capacity for cold acclimation and they express some of the same cold acclimation-specific genes as the intact seedlings (Monroy et al., 1993; Wolfraim et al., 1993). The cell cultures were grown in a growth chamber under a 12-h photoperiod at 25°C and shaking at 180 rpm in 1-L flasks containing 500 mL of modified B_5h medium (Atanassov and Brown, 1984). One-fifth volume of cell-suspension cultures was routinely subcultured every 3 weeks. To administer cold acclimation, cell cultures were transferred from 25°C to 4°C under otherwise identical conditions. In all experiments, 10-d-old cultures were used because they were at the midpoint of the log-phase of their growth.

In intact seedlings and in cell-suspension cultures, the development of maximum freezing tolerance requires about 2 and 3 weeks of cold acclimation, respectively. However, a period of 8 d of cold acclimation is sufficient to induce a high level of freezing tolerance in seedlings (Mohapatra et al., 1989) and an easily detectable level in cell-suspension cultures (Wolfraim et al., 1993). Thus, to minimize the duration of chemical treatments, cell cultures were cold-acclimated for 8 d and used in experiments where the development of freezing tolerance was examined. The level of freezing tolerance developed at 8 d of cold acclimation is about 62% of that developed by fully cold-acclimated cells. Freezing tolerance was measured as cell survival after being subjected to freezing temperature as determined by the TTC reduction assay (Towill and Mazur, 1974). Briefly, 1 mL of cells, cold-acclimated at 4°C for 8 d, was placed into a 13- × 100-mm culture tube, the medium was removed by aspiration, and the cells were washed twice in 5 mL of water (4°C), resuspended in 2 mL of water at 4°C, and equilibrated at −1.5°C for 15 min. Ice nucleation was then initiated and the cell suspensions were incubated at −1.5°C for 90 min. Control cells (to serve as unfrozen control samples) were removed to 0°C, and the remaining cell-suspension aliquots were further cooled at 3°C/h to −8°C. Cells were thawed at 4°C for 90 min. Cell survival was determined as described (Towill and Mazur, 1974). Values reported here are the means ± SD of three to five independent measurements.

We have used different periods of cold acclimation for studying protein phosphorylation, gene expression, and the development of freezing tolerance. Because this study is a prelude to future, more-detailed studies of low-temperature signal transduction, we wished to study the various aspects as near to the start of cold acclimation as possible, while obtaining unambiguous results. Thus, freezing tolerance was measured after 8 d of cold acclimation because with a shorter period, the differences between nonacclimated and cold-acclimated cells are too small to be unambiguous, particularly in the presence of inhibitors of the processes associated with cold acclimation. The expression of the three cold acclimation-specific genes was determined after 48 h of cold acclimation because this time allowed the transcripts of all three genes to accumulate to easily detectable levels. Protein phosphorylation was examined at 3 h of cold acclimation. Using one-dimensional PAGE, we could detect changes in phosphoprotein profile after 1 h of cold acclimation. However, changes are more readily detectable with two-dimensional PAGE at 3 h of cold acclimation. Based on the results of this study, a few selected changes may now be studied at shorter times of cold acclimation.

Determination of Effects of Chemicals on Cellular Viability

To determine if the chemical treatments affected the general health of cells, the viability of control or treated cells that were not subjected to freezing was determined by TTC reduction assay. Viability under chemical treatments was expressed as percent of viability of cold-acclimated cells. This was done for two reasons. First, because chemicals are used to alter cold acclimation, cells that acclimated to the cold without the chemicals constitute a proper control. Second, TTC reduction is a measure of the activity of the reducing enzymes. During cold acclimation, cellular protein content increases to more than 2 times that of nonacclimated cells (Mohapatra et al., 1987). Also, TTC reduction by cold-acclimated cells measured at room temperature is more than double in cold-acclimated cells than in nonacclimated cells (R. Langis, unpublished data). This would indicate that the cold-acclimated cells are twice as viable as the nonacclimated cells, which is clearly an artifact, due to the increased protein content of the cold-acclimated cells. There is no evidence available in the literature that supports the theory that cold

acclimation changes the viability of plants or cells at room temperature. Thus, the viability of cells cold acclimated in the presence of chemicals has been expressed as a percentage of that of cells cold acclimated in the absence of the chemicals.

Administration of Chemical Treatments

ABA was used at 75 μM. The Ca^{2+} chelator EGTA and the Ca^{2+} channel blockers La^{3+} and verapamil were used at 50 mM, 1 mM, and 0.1 mM, respectively. The compound W7 was used at 100 μM as an antagonist of calmodulin and Ca^{2+}-dependent protein kinases (Hidaka et al., 1981; Roberts and Harmon, 1992) and the compound H7 was used at 100 μM as an inhibitor of protein kinases (Hidaka et al., 1984). W5 and H8 were used at 100 μM as ineffective analogs of W7 and H7, respectively (Hidaka et al., 1981, 1984). To determine whether the cold-induced changes in protein phosphorylation occurred on preexisting or newly synthesized proteins, cycloheximide was used at 150 μM. Experiments were conducted to determine the degree of inhibition of protein synthesis by various concentrations of cycloheximide. The rates of uptake of [^{35}S]Met and of its incorporation into a TCA-precipitable fraction were measured in control and cycloheximide-treated cells following the procedures of Lam et al. (1989). Protein concentration was measured by the dye-binding method of Bradford (1976), and the protein radioactivity was measured by liquid scintillation spectrometry. The results were expressed in cpm μg^{-1} protein h^{-1}. It was found that cycloheximide at 150 μM inhibited the rate of Met incorporation into the TCA-precipitable fraction by more than 90%. This concentration of cycloheximide was used in further experiments.

In experiments to determine the effects of various chemicals on the phosphoprotein profile, the chemicals were added 1 h before the start of in vivo labeling of proteins. To study the effects of these chemicals on the accumulation of cold acclimation-specific mRNAs, the chemicals were added 1 h before the start of cold acclimation. In experiments where the effect of phosphorylation inhibitors on the development of freezing tolerance was determined, the inhibitors were applied 1 h before the start of cold acclimation and again at 4 d of cold acclimation to compensate for their possible metabolism and reduced stability in light during a relatively prolonged period of experimentation.

Analysis of Phosphoprotein Populations

Cells to be labeled in vivo were cold acclimated with or without chemical treatments for 3 h, collected by filtration, and briefly blotted; they were then weighed and divided into 250-mg aliquots. Cells were washed, at 4°C (cold-acclimated cells) or 25°C (nonacclimated cells), three times for 10 min each with 15 mL of 50 mM Na citrate, pH 5.6, 1% Suc (Velutambi and Poovaiah, 1986) to deplete phosphate from the media. [^{32}P]Pi was added to a final radioactivity of 100 to 200 μCi/mL and labeling was carried out for 60 min. ^{32}P-labeled cells were harvested by filtration, rinsed with ^{32}P-free citrate buffer, and ground with pestle and mortar in 2 mL of buffer-equilibrated phenol. Methods of protein extraction with phenol and two-dimensional SDS-PAGE were as described (Monroy et al., 1988). Approximately 4×10^4 cpm of TCA-precipitable radioactivity were loaded on each gel. Labeled proteins were detected by autoradiography. Relative levels of phosphorylation of one particular protein (protein 10), which showed large cold-induced phosphorylation, were compared by scanning the autoradiograms for that protein spot with a model 620 CCD Video Densitometer coupled with the two-dimensional Analyst software program (Bio-Rad Laboratories).

Determination of Cold Acclimation-Specific mRNA Accumulation

Cells, nonacclimated or cold acclimated for 48 h, were harvested as described for protein extraction and homogenized in phenol (4 mL/g fresh weight) at 4°C, and the nucleic acids were extracted in an equal volume of 50 mM Tris, pH 8, 10 mM EDTA, 1% sodium laurylsarcosine, 5% (v/v) 2-mercaptoethanol. The aqueous phase was extracted once with chloroform-phenol and the nucleic acids were precipitated by isopropanol at room temperature. The resulting pellet was dissolved in sterile water, and the RNA was precipitated overnight in 2 M LiCl at 4°C. The RNA was washed once in 2 M LiCl, dissolved in water, precipitated in 1 M ammonium acetate-isopropanol, dried under vacuum, and dissolved in water. Twenty micrograms of each RNA sample were denatured in 95% formamide for 5 min at 90°C and fractionated by electrophoresis in agarose-formaldehyde gels (Sambrook et al., 1989). The RNA was then transferred to a nylon filter by capillary blotting for northern blot hybridization. Filters were incubated with ^{32}P-labeled full-length cDNA inserts from clones pAcs784, pAcs2201, and pAcs2358 corresponding to previously isolated cold acclimation-specific, partial-length cDNA clones (Mohapatra et al., 1989). The cDNA clone p2.1, an unidentified cDNA clone from wheat that is expressed constitutively in alfalfa, was used as a control. Hybridization and filter washes were as described (Sambrook et al., 1989).

In experiments where the effects of H7 and W7 on the stability of cold acclimation-specific transcripts in cold-acclimated cells were examined, these chemicals were added to cold-acclimated cells and, after a further 24-h period at 4°C, the levels of various transcripts were determined by dot-blot hybridization as previously described (Mohapatra et al., 1989). The relative intensities of the hybridization spots on the autoradiogram were compared by scanning with a video densitometer as described for phosphoproteins above.

RESULTS

Ca^{2+} Channel Blockers and Inhibitors of Ca^{2+}-Dependent Proteins and Protein Kinases Inhibit Cold Acclimation

To test for a possible role of Ca^{2+} in cold acclimation, we used the Ca^{2+} chelator EGTA and the potent Ca^{2+} channel blockers La^{3+} and verapamil (Graziana et al., 1988; Hosey and Lazdunski, 1988; Knight et al., 1992). Whereas EGTA was expected to make cell wall Ca^{2+} at least partly unavailable for entering the cytoplasm, La^{3+} and verapamil were expected to block the Ca^{2+} channels at the plasma membrane. Cell cultures were cold acclimated at 4°C with or without the

addition of inhibitors and their freezing tolerance was tested after 8 d. It can be seen from Figure 1 that 8 d of cold acclimation are sufficient to increase freezing tolerance considerably. It can also be seen that, whereas EGTA inhibits the development of freezing tolerance by more than 70%, La^{3+} and verapamil completely abolish the development of freezing tolerance. Thus, it can be concluded that the activity of Ca^{2+} channels is required for the development of cold-induced freezing tolerance. To further test the role of Ca^{2+}, cells were treated with W7, an antagonist of calmodulin and other Ca^{2+}-binding proteins. It can be seen that W7 completely inhibited cold acclimation-induced development of freezing tolerance, whereas W5, which is related to but is known to be severalfold weaker than W7 (Hidaka et al., 1981), had little effect on the development of freezing tolerance.

Two protein kinase inhibitors were then tested to assess the possible role of protein phosphorylation in cold acclimation. When applied in vitro at low concentrations, the compounds H7 and H8 have selectivity for protein kinase C and cAMP-dependent protein kinase, respectively (Hidaka et al., 1984). However, when applied in vivo, their uptake and, therefore, their cellular concentration cannot be manipulated and their effects on a variety of protein kinases are less selective. As seen in Figure 1, H7 inhibited the development of freezing tolerance by nearly 50%, whereas its analog, H8, had little effect.

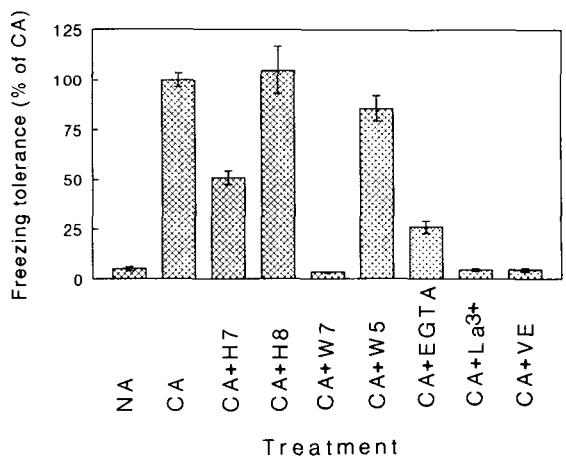

Figure 1. Effects of protein phosphorylation inhibitors and Ca^{2+} channel blockers on cold-induced freezing tolerance in cultured cells of alfalfa. Freezing tolerance of nonacclimated (NA) cells, cold-acclimated (CA) cells, and cells cold acclimated in the presence of 100 μM protein kinase C inhibitor (CA+H7) or 100 μM of its ineffective analog, H8 (CA+H8), 100 μM calmodulin antagonist (CA+W7) or 100 μM of its ineffective analog, W5 (CA+W5), Ca^{2+} channel blockers 1 mM La^{3+} (CA+La^{3+}) or 0.1 mM verapamil (CA+VE), or 50 mM Ca^{2+} chelator EGTA (CA+EGTA) determined as survival at −8°C. The 100% CA value for TTC reduction by cells after being frozen at −8°C was A_{495} of 5.02 ± 0.4/mg dry weight. As noted in "Materials and Methods," these cells are not fully cold acclimated and their freezing tolerance is about 62% of that of fully cold-acclimated cells. Each data point is a mean ± SD of three to five independent measurements.

Table I. *Effects of various treatment chemicals on cell viability*

Cells were treated with these chemicals 1 h before the 8-d cold-acclimation period was started. Chemicals were added again at 4 d of cold acclimation. At the end of 8 d of cold acclimation, cell viability was determined by TTC reduction assay on unfrozen cells as described in "Materials and Methods." CA, Cold acclimation; VE, verapamil. Each value is the mean ± SD derived from three to five independent measurements. The 100% CA control value was A_{495} of 8.13 ± 0.54 units/mg dry weight.

Treatment	Cell Viability
	% of CA control
CA	100
CA + H7	92 ± 12
CA + H8	101 ± 12
CA + W5	95 ± 6
CA + W7	84 ± 10
CA + EGTA	94 ± 5
CA + La^{3+}	102 ± 8
CA + VE	87 ± 6

The viability of cells treated with the above chemicals during 8 d of cold acclimation is shown in Table I. As can be seen, viability is barely affected by any of the treatment chemicals. Even W7-treated cells that completely lose their capacity to cold acclimate show a high level of viability, 84 ± 10% of the control cells. The Ca^{2+} channel blocker La^{3+}, which completely prevents cold acclimation, has no detectable effects on cell viability. Thus, it can be concluded that the decreased freezing tolerance developed in the treated cells is not due to ill effects of these chemicals on the general health of the cells.

Cold-Induced Changes in Phosphoprotein Profile

To examine the role of protein phosphorylation during cold acclimation, we examined cold-induced changes in protein phosphorylation. To eliminate possible artifacts, preliminary experiments were conducted; the results showed that cold acclimation-induced changes in the phosphoprotein profile were not related to growth stages of the culture. To better resolve cold acclimation-induced changes in phosphoprotein profiles, neutral to acidic polypeptides were analyzed by two-dimensional gel electrophoresis. Thus, phosphoprotein profiles have been studied in cells that were nonacclimated, cold acclimated for 3 h, or cold acclimated for 3 h and then deacclimated for 1 h. The results are shown in Figure 2.

It can be seen that many proteins are in the phosphorylated state in nonacclimated cells. Exposure of nonacclimated cells to 4°C for 3 h results in a relative decrease in the level of phosphorylation of several proteins and an increase in that of several others. The majority of these changes are small in magnitude, as can be seen from changes in 10 selected proteins. Thus, proteins 1–4 represent those whose relative phosphorylation decreases, whereas proteins 5–9 represent those whose relative phosphorylation increases. Protein 10 shows a dramatic increase in its phosphorylation level with cold acclimation. On deacclimation, the phosphoprotein profile returns more or less to that of nonacclimated cells.

Because several studies have shown that freezing tolerance

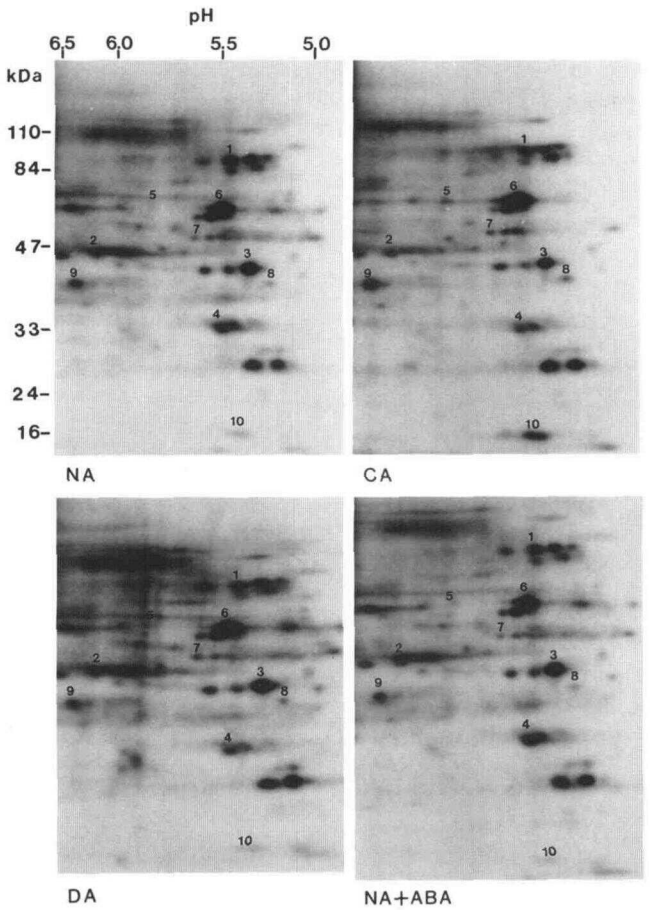

Figure 2. Autoradiographs of in vivo-labeled phosphoproteins from cells nonacclimated (NA), cold acclimated for 3 h (CA), cold acclimated for 3 h and then deacclimated for 1 h (DA), and nonacclimated in the presence of 75 μM ABA (NA+ABA). Numbers 1 to 10 draw attention to the phosphoproteins below them. The relative phosphorylation of proteins 1 to 4 decreases, whereas that of proteins 5 to 10 increases with cold acclimation. Cold-induced phosphorylation of protein 10 is the largest change (see Fig. 4). Experiments were repeated four times and yielded similar results each time.

can be induced by exogenous application of ABA, we tested the effect of this phytohormone on the phosphoprotein profile of nonacclimated cells. As seen in Figure 2 (lower right panel), the phosphoprotein profile of cells treated with ABA was nearly identical to that of nonacclimated cells. Thus, the relative phosphorylation of proteins 5, 7, 8, 9, and 10 increases with cold acclimation but not with ABA. Therefore, it can be concluded that ABA does not reproduce the effects of cold acclimation on protein phosphorylation. This suggests that cold-induced protein phosphorylation is specific to low temperature.

Inhibitors of Cold Acclimation Prevent the Cold-Induced Changes in Phosphoprotein Profile

Because the changes in protein phosphorylation occur early during cold acclimation, it is important to know whether these changes occur on preexisting proteins or on newly synthesized proteins. To answer this question, we examined the effects of cycloheximide on cold-induced changes in protein phosphorylation. As shown in Figure 3 (upper left panel), cold-induced changes in protein phosphorylation also occur when cold acclimation is carried out in the presence of cycloheximide at a concentration that inhibits protein synthesis by more than 90%. Thus, it can be concluded that cold-induced changes in protein phosphorylation occur on preexisting proteins.

In an attempt to assess the relevance of cold-induced protein phosphorylation to cold acclimation, we studied the effects of three different inhibitors of freezing tolerance on phosphoprotein profiles. We chose the protein kinase inhibitor H7, the calmodulin antagonist W7, and the Ca^{2+} channel blocker La^{3+}. The latter was chosen from among other Ca^{2+} channel blockers because of its reported effects on cold shock-induced increase in cytosolic Ca^{2+} (Knight et al., 1992). It can be seen from Figure 3 that the inhibitor of protein kinases,

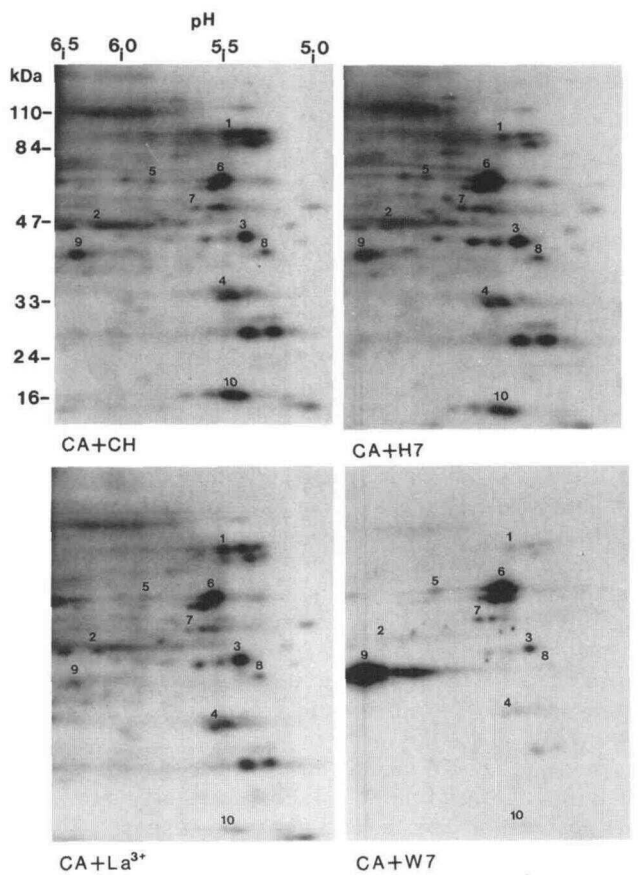

Figure 3. Autoradiographs of in vivo-labeled phosphoproteins from cultured alfalfa cells acclimated at 4°C for 3 h in the presence of 150 μM cycloheximide (CA+CH), 100 μM protein kinase inhibitor H7 (CA+H7), 1 mM Ca^{2+} channel blocker La^{3+} (CA+La^{3+}), or 100 μM calmodulin antagonist W7 (CA+W7). Attention is drawn to proteins 1 to 10, whose relative phosphorylation should be compared with that of proteins shown in Figure 1 (NA and CA). Experiments were repeated four times and yielded similar results each time.

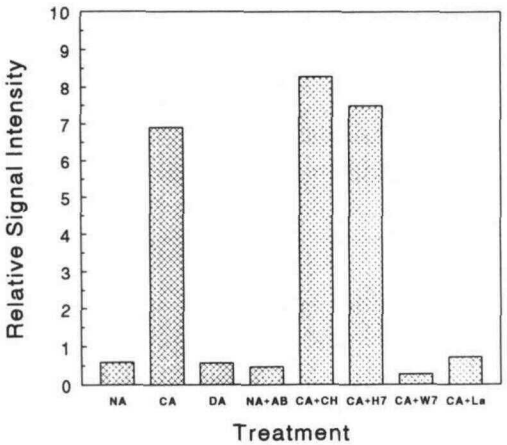

Figure 4. Relative phosphorylation level of protein 10 (Figs. 2 and 3) in nonacclimated (NA) cells, cold-acclimated (CA) cells, deacclimated (DA) cells, or in nonacclimated cells treated with 75 μM ABA (NA+AB), in cells cold acclimated in the presence of 150 μM cycloheximide (CA+CH), 100 μM H7 (CA+H7), 100 μM W7 (CA+W7), or 1 mM La^{3+} (CA+La). Data are derived from scanning of the protein 10 spots on autoradiograms by a model 620 CCD Video Densitometer coupled with the two-dimensional Analyst software (Bio-Rad Laboratories).

H7, has little effect on the cold-induced changes in protein phosphorylation. Cold-induced decrease in protein phosphorylation is not affected by H7. The inhibitory effects of La^{3+} are more severe. The cold-dependent increase in phosphorylation of proteins 5 through 9 is partially reversed. Cold-induced phosphorylation of protein 10 appears to be completely prevented by La^{3+}. Overall, the phosphoprotein profile of cells cold acclimated in the presence of La^{3+} is similar to that of nonacclimated cells. The effects of W7 are more dramatic. The pattern of the phosphoprotein profile is shifted so that the relative phosphorylation of proteins 6 and 9 is increased even more than in cold-acclimated cells. However, the phosphorylation of almost all other proteins, irrespective of their relationship to cold acclimation, is almost completely prevented. Therefore, it can be concluded that La^{3+}, a Ca^{2+} channel blocker, and W7, an antagonist of calmodulin and Ca^{2+}-dependent protein kinases, almost completely prevent the cold-induced phosphorylation of preexisting proteins. The inhibitor of protein kinases, H7, has little effect.

The cold-induced increase in phosphorylation of protein 10 is the largest and shows the strongest association with the development of freezing tolerance. The treatments that inhibit cold acclimation also inhibit cold-induced phosphorylation of this protein. To compare the relative phosphorylation levels of protein 10 under various treatments shown in Figures 2 and 3, the respective protein 10 spots on the autoradiograms were scanned with a video densitometer (see "Materials and Methods") and the results are shown in Figure 4. It can be seen that the relative level of phosphorylation of this protein is barely noticeable in the nonacclimated cells (NA) and increases more than 12-fold with 3 h of cold acclimation. When the cells are subsequently deacclimated for 1 h, phosphorylation declines to nonacclimated levels. Treatment of nonacclimated cells with ABA (NA+AB) does not increase the level of phosphorylation. Cold-induced phosphorylation of protein 10 increases further when cells are treated with cycloheximide. When cold acclimation is carried out in the presence of W7 or La^{3+}, phosphorylation of protein 10 is completely prevented. Treatment with H7, on the other hand, has little effect on the cold-induced phosphorylation of protein 10.

Inhibitors of Cold Acclimation and of Cold-Induced Phosphoprotein Profile Inhibit Accumulation of Cold Acclimation-Specific mRNAs

To determine if the inhibition of the development of freezing tolerance by phosphorylation inhibitors is accompanied by an inhibition of cold acclimation-specific gene expression, the accumulation of transcripts corresponding to the three cold acclimation-specific alfalfa cDNA clones, pAcs784, pAcs2201, and pAcs2358, was examined. An unidentified wheat cDNA clone, p2.1, which was found to be expressed constitutively in alfalfa cells, was used as a control. The accumulation of transcripts with 48 h of cold acclimation in the absence or presence of H7, La^{3+}, or W7 is shown in Figure 5. It can be seen that the level of transcripts corresponding

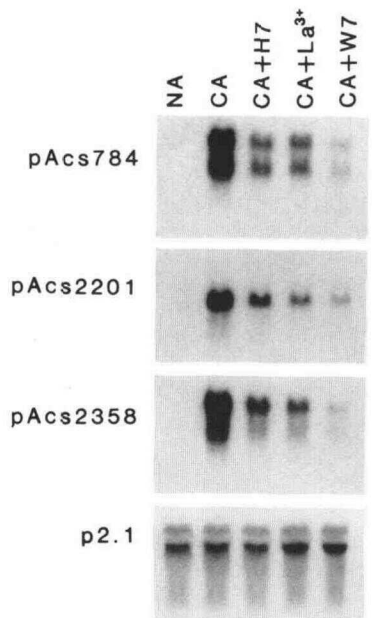

Figure 5. Effects of the protein phosphorylation inhibitors H7 and W7 and the Ca^{2+} channel blocker La^{3+} on cold-induced accumulation of three cold acclimation-specific mRNAs. Total RNA isolated from nonacclimated cells (NA) and cells acclimated to 4°C for 48 h without (CA) or with the addition of 100 μM of the inhibitor of protein kinase C H7 (CA+H7), 1 mM Ca^{2+} channel blocker La^{3+} (CA+La^{3+}), or 100 μM calmodulin antagonist W7 (CA+W7) was subjected to northern hybridization analysis with probes as indicated. pAcs784, pAcs2201, and pAcs2358 are cDNA clones of cold acclimation-specific genes and p2.1 is a cDNA clone for a constitutively expressed gene. Experiments were repeated three times with similar results each time.

to the cold acclimation-specific clones (pAcs784, pAcs2201, and pAcs2358) is undetectable in the nonacclimated cells (NA) but increases sharply with 48 h of cold acclimation (CA). The level of transcripts for the control clone, p2.1, changes little with cold acclimation. Cold acclimation in the presence of H7, La^{3+}, or W7 causes little change in p2.1 transcripts but results in substantial reduction in the levels of transcripts for the three cold acclimation-specific clones. The inhibition is greatest in W7-treated cells and lowest in H7-treated cells. Estimations by semiquantitative densitometry indicated that the inhibition of transcript accumulation is similar to that of freezing tolerance by the respective inhibitors.

The possibility that the inhibitory effects of the above chemicals on transcript accumulation may be due to their effects on the stability of the transcripts was then examined. But the effects of only H7 and W7 were examined because these chemicals, unlike La^{3+}, enter the cells. The effects were studied on the stability of *cas18* transcripts (clone pAcs784 in Fig. 5), which are the most unstable of the three genes examined. Results are presented in Figure 6. It can be seen that cold acclimation for 48 h results in a considerable level of *cas18* transcripts (CA48). During the next 24 h of cold acclimation without any chemical treatment (CA72), transcripts show a further small increase in *cas18* transcripts. When cold acclimation during the last 24 h is carried out in the presence of either H7 (CA+H7) or W7 (CA+W7), the level of transcripts is not different from that in the CA48 cells. However, the level under the chemical treatments is somewhat less than in CA72 cells, suggesting that the treatment with H7 or W7 prevents the increase in the level of transcripts but does not result in their degradation. Thus, it can be concluded that H7 and W7 have no detectable effects on the stability of *cas18* transcripts.

Considered together, these results suggest a coupling of cold-induced protein phosphorylation, expression of cold acclimation-specific genes, and development of freezing tolerance during cold acclimation of alfalfa cells.

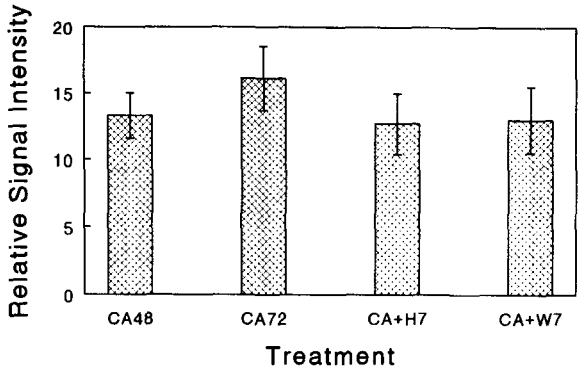

Figure 6. Effects of 100 μM protein kinase C inhibitor H7 or 100 μM calmodulin antagonist W7 on the stability of *cas18* transcripts in cold-acclimated alfalfa cells. Cells cold acclimated for 48 h (CA48) were further cold acclimated for 24 h either without any chemical (CA72) or in the presence of H7 (CA+H7) or W7 (CA+W7). Then, RNA was isolated and the level of *cas18* transcripts was determined by dot-blot hybridization. The relative intensities of the hybridization spots on the autoradiogram were obtained by scanning with a video densitometer (see "Materials and Methods"). Experiments were repeated three times and yielded similar results each time.

DISCUSSION

Cold acclimation, which requires altered gene expression (Guy, 1990), probably involves many biochemical processes that are likely to occur in the following temporal sequence: signal perception and transduction followed by induction of gene expression, which, in turn, is followed by the development of freezing tolerance. An important observation made in the present study is that H7, La^{3+}, and W7 cause a corresponding inhibition of the cold-induced protein phosphorylation, freezing tolerance, and the accumulation of the cold acclimation-specific mRNAs, suggesting that these three cold-induced processes are interlinked during cold acclimation. Furthermore, the data suggest that Ca^{2+}-dependent protein phosphorylation might constitute a signal transduction pathway in triggering cold-dependent gene expression.

The changes in the relative phosphorylation of several proteins occurring relatively early during cold acclimation might comprise at least two classes of events. In one class are those phosphorylation events that are affected by inhibitors and might constitute signal transduction events leading to gene expression. The second class, which is unaffected by inhibitors, could result from low temperature-induced changes in protein kinase activities (such as temperature-dependent conformational changes), which might affect metabolism at low temperatures.

Cycloheximide, applied at concentrations that inhibit protein synthesis by more than 90%, did not inhibit cold-induced changes in protein phosphorylation, leading us to conclude that phosphorylation changes occur on preexisting proteins. This conclusion is also in agreement with our recent observation (Monroy et al., 1993) that the cold-induced accumulation of transcripts of the *cas15* gene (the clone pAcs2201 in Fig. 5) does not require de novo protein synthesis. Because protein phosphorylation is known to regulate the expression of some plant genes (Datta and Cashmore, 1989; Sarokin and Chua, 1992), it is possible that cold-induced phosphorylation observed in the present study might participate in the regulation of cold-induced genes by modulating the interaction of preexisting proteins either directly with DNA or with other proteins that then participate in gene regulation. Regulatory elements will have to be isolated and characterized before these possibilities can be tested.

Cold-induced changes in the relative phosphorylation levels of preexisting proteins were not reproduced by ABA. An analysis of ABA-deficient mutants has shown that ABA, which is known to induce freezing tolerance in several plants (Guy, 1990), may act through signal transduction pathways other than those involved in low-temperature induction of freezing tolerance (Nordin et al., 1991). In alfalfa, the level of endogenous ABA changes little during cold acclimation (Waldman et al., 1975), and its exogenous application has marginal effects on freezing tolerance of alfalfa cell-suspension cultures (Orr et al., 1985; L.A. Wolfraim, R. Langis, and R.S. Dhindsa, unpublished data). Moreover, we have previously reported that, in the case of alfalfa seedlings, ABA is

only about half as effective as low temperature in inducing freezing tolerance (Mohapatra et al., 1988). The results of this study are, therefore, in agreement with the conclusion of Nordin et al. (1991) that low temperature and ABA can induce freezing tolerance through separate signal transduction pathways.

The cold-induced phosphorylation of protein 10 is noteworthy in its magnitude as well as its specificity for cold acclimation. Chemicals, except H7, that inhibit the cold-induced gene expression and freezing tolerance also inhibit the cold-induced phosphorylation of protein 10. Treatment with H7 inhibits cold-induced freezing tolerance by 50% (Fig. 1) but has little effect on the cold-induced phosphorylation of protein 10. It is possible that H7 interferes with the processes of cold acclimation further downstream from the site of involvement of protein 10.

The involvement of Ca^{2+} fluxes in the early events during cold acclimation is highly likely because the Ca^{2+} channel blockers completely prevent the cold-induced development of freezing tolerance. It has been shown (Johannes et al., 1991; Schroeder and Thuleau, 1991; Trewavas and Gilroy, 1991) that plant cell walls and the vacuole act as Ca^{2+} storage sites, and that both plasma membrane and tonoplast possess Ca^{2+} channels. Because EGTA, La^{3+}, and verapamil are unlikely to enter the cell, they probably act by preventing the cell wall Ca^{2+} from entering the cytoplasm, EGTA by chelating it, and the other two by blocking the plasma membrane Ca^{2+} channels. These results also suggest that vacuolar Ca^{2+} plays only a minor role in cold acclimation of alfalfa cells. This suggestion is supported by a recent observation that cold shock-induced increase in cytoplasmic Ca^{2+}, unlike that caused by wind and mechanical pressure, does not appear to come from intracellular stores (Knight et al, 1992). As cytoplasmic Ca^{2+} increases, it probably activates Ca^{2+} pumps on the plasma membrane and tonoplast, which pump it out of the cytoplasm.

The mechanisms underlying the activation of Ca^{2+} channels by cold are not clearly understood at present. Possibly, low temperature decreases the chemical potential of cellular water, thereby altering membrane tension (Zimmerman, 1978). In this respect, even small changes in these parameters may be significant. Although the short-term kinetics of low temperature-induced decrease in cellular water potential have not been examined, evidence for cold-induced decline in cellular water potential has been reported (Kacperska, 1993). Thus, the cold-triggered Ca^{2+} channels may, indeed, be pressure-sensitive channels responding to small changes in the internal cellular hydrostatic pressure rather than to wind pressure from outside. It has been suggested (Braam and Davis, 1990) that physical rubbing, which is known to activate the genes for calmodulin-like proteins (Braam and Davis, 1990) and to enhance cold resistance in several plants (Jaffe and Biro, 1979), results in high cytoplasmic Ca^{2+}. In addition to touch, several stimuli, including cold shock, elicit a transient increase in cytoplasmic Ca^{2+} (Knight et al., 1991, 1992).

Because this Ca^{2+} response occurs in plant cells irrespective of their sensitivity to freezing, the development of freezing tolerance must be determined by a system capable of amplifying the Ca^{2+} signal along a specific pathway. Although the underlying mechanism(s) of how low temperature changes protein phosphorylation patterns remains unclear, our data allow us to propose that Ca^{2+}-binding proteins, including Ca^{2+}-dependent protein kinases, couple the Ca^{2+} response to downstream events, leading to metabolic adaptation to cold and to the development of freezing tolerance. Our proposal, however, should be treated with caution. The effects of W7 on the cold-dependent changes of phosphoprotein profile were more dramatic than those of La^{3+}. Although our use of W5 appears to rule out nonspecific effects of W7 (Gilroy et al., 1987), its inhibition of multifunctional calmodulin (Roberts and Harmon, 1992) implies a complexity of physiological effects that cannot be dissected based on our data. Thus, the participation of calmodulin and other Ca^{2+}-binding proteins, such as Ca^{2+}-dependent protein kinases, as transducers of Ca^{2+} signals during cold acclimation must await further studies.

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