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Effects of Ca^{++} chelator, Ca^{++} ionophore, and heat shock pretreatment on *in vitro* protein phosphorylation of rice suspension culture cells

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Abstract. We investigated the effects of calcium and heat shock pretreatment on changes of *in vitro* phosphorylation patterns of proteins in extracts of normal and high-salt adapted rice suspension culture cells. Pretreatment of normal culture cells with calcium chelator EGTA [(ethyleneglycol-bis (β -aminoethyl ether)-N,N'-tetraacetic acid)] caused an increase of a 70 kD phosphoprotein and decreases of 12, 25-27, and 40-45 kD phosphoproteins. Pretreatment of the cells with EDTA (ethylenediaminetetraacetate) reversed most of these changes. Pretreatment of the cells with calcium ionophores A23187 and ionomycin also resulted in changes in the accessibility of these proteins to be phosphorylated. In the high-salt adapted cells, the phosphorylation of low molecular weight proteins was increased in the extract of cells pretreated by EDTA followed by heat shock treatment. Heat shock pretreatment of normal culture cells resulted in the prominent presence of three proteins (70-75, 90, and 100 kD). The phosphorylation of 90 kD protein was specifically inhibited by EGTA pretreatment of the cells. In conclusion, pretreatment of cells by calcium chelators and ionophores caused changes in cellular phosphorylation systems which are dependent upon heat shock treatment and salt concentration of the culture media of the cells.

Key words: Ionophore; Chelator; Calcium effect; Heat shock; *Oryza sativa*; Protein phosphorylation.

Introduction

Calcium is important in maintaining membrane integrity and in regulating essential cellular activities. Phosphorylation of proteins is one of the major mechanisms by which calcium exerts its effects on cellular activities. Changes in calcium-regulated physiological processes in plants are often associated with changes in the activities of protein kinases and protein phosphatases which alter the phosphorylation status of intracellular proteins. An increase or a decrease of intracellular calcium level is known to relate closely to these changes (Ranjeva and Boudet, 1987).

Calcium ionophore A23187 has been widely used to manipulate intracellular and extracellular calcium levels in order to study changes in calcium-associated cellular activities. In animal cells, A23187 causes calcium perturbation and stimulates the synthesis of 80 and 100 kD proteins, which are readily phosphorylated (Wu *et al.*, 1981; Resendez *et al.*, 1986). Calcium chelators EGTA and EDTA have also been widely used to manipulate calcium concentrations and to reveal relationships among protein phosphorylation and dephosphorylation, elevated cytosolic calcium concentration, and sequestered calcium levels in cells (Budde and

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Randall, 1990).

Plant cells share many similarities with animal cells in regard to calcium as a signal transduction system, including the role of calcium in protein phosphorylation and dephosphorylation *in vitro* and *in vivo* (Poovaiah and Reddy, 1987; Ranjeva and Boudet, 1987). *In vitro* analyses using cell extracts are particularly useful for examining protein kinases whose activities are dependent on calcium level. *In vivo* analyses can reveal how protein phosphorylation is associated with cellular calcium level. However, the presence of a large central vacuole (which can occupy as much as 90% of the cell volume) and a cell wall often complicate *in vivo* studies. These structures contain large amounts of calcium ions, and their influence on the cytosolic calcium concentration is not known.

Protein kinases, which mediate protein phosphorylation, are universally present and have diverse specificities in plant systems (Blowers and Trewavas, 1989). Most studies on the involvement of calcium in protein kinase activities were performed with *in vitro* systems. Several calcium-dependent protein kinases have been identified, particularly those dependent on calmodulin (Blowers *et al.*, 1985; Polya and Micucci, 1984). *In vitro* assays of [^{32}P] -ATP labelling of proteins reveal that calcium profoundly affects phosphorylation of endogenous proteins in germinating seedlings (Krishnan and Pueppke, 1988).

In rice, phosphorylation of thylakoid proteins is activated by chilling temperatures and divalent ions (Moll *et al.*, 1987). However, there is little information on the effect of calcium ions on protein phosphorylation. This report describes changes in *in vitro* protein phosphorylation of extracts from rice suspension culture cells pre-treated with calcium ionophores and chelators. It demonstrates that such changes are dependent upon heat shock treatment and culture conditions of the cell type.

Materials and Methods

Plant Materials

Rice suspension culture cells were initiated from a callus culture induced from immature embryos of *Oryza sativa* L. cv Tainan 5, and were maintained in Murashige and Skoog (MS) medium supplemented with 5 μM 2,4-D. High-salt adapted rice suspension culture cells were derived from the above suspension culture by recurrent selection of cells tolerant to culture media containing 1% NaCl. Both cell types were kindly provided by Dr. Li-Fei Liu of the Department of Agronomy, National Taiwan University, Taiwan, Republic of China. The high-salt adapted cells were maintained in the MS medium used for the growth of normal suspension culture cells but supplemented with 1% NaCl. To obtain seedlings, seeds of *Oryza sativa* L. Taichung 65 were surface sterilized and germinated in the dark in 0.5-strength Murashige and Skoog (0.5x MS) medium as described previously (Wu, 1987).

Pretreatment of Cells

Rice suspension culture cells were washed three times with 0.5x MS by centrifugation at 500x g for 5 min. The final pellets were resuspended in 0.5x MS at a ratio of 1:1 (v/v) = packed cell: 0.5x MS, and were distributed into 24-well plates at 1 ml per well. The MS medium for cell cultures was adjusted to pH 5.7, and the 0.5x MS medium was adjusted to pH 6.8. Stocks of A23187, EDTA, EGTA and ionomycin were freshly prepared just prior to use at three-fold strengths in 0.5x MS, and were added to each well in a volume of 0.5 ml. The final concentrations of each reagent are indicated in figure legends. After adding the reagents, the suspension culture cells were incubated at 24°C in the dark at 120 rpm for 15 h. For heat shock treatment, the cultures were incubated at 40°C for 2 h following the above incubation period. After incubation, cells were collected by centrifugation at 500 xg for 5 min, washed three times with distilled water, and stored at -70°C.

For seedling pretreatment, four-day old etiolated seedlings were collected under dim light after removing the rice grains and distributed into 24-well plates at 10 seedlings per well. Reagents at the indicated concentration were added to each well in 1.5 ml aliquot, and the seedlings were incubated in the dark for 7 h on a shaker at 120 rpm. The solutions were then removed with a glass pipette connected to a vacuum source. The seedlings were washed 3 times with distilled water before they were collected and stored at -70°C. Samples were stored for no more than one week before they were processed for *in vitro* phosphorylation assays.

In Vitro Protein Phosphorylation Assays

After treatment, suspension culture cells or seedlings were ground into fine powder in liquid nitrogen with a mortar and pestle, and the powder was collected

into centrifuge tubes. After centrifugation at 10,000 xg for 10 min, the supernatants (crude extracts) were quantified for protein concentration by using Bio-Rad's protein assay kit. Aliquots of the crude extract containing 40 μg protein were used for the *in vitro* protein phosphorylation assay after they were adjusted to contain 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1 mM NaCl, 1 mM 2-mercaptoethanol, 0.015% (v/v) Nonidet P-40, and 0.1 mM ATP in a final volume of 20 μl. The reactions were carried out at 24°C for 30 min after adding 1 μCi of [α-^{32}P] -ATP (Amersham, 5000 Ci/mmol) and were terminated by adding an equal volume of 2x electrophoresis sample buffer, which consisted of 0.12 M Tris-HCl, pH 8.0, 10% (v/v) 2-mercaptoethanol, 4% (w/v) sodium lauryl sulfate, 4 mM EDTA, and 30% (w/v) sucrose. The samples were boiled in a waterbath for 3 min and subjected to electrophoresis in 10-18% (w/v) gradient polyacrylamide gels as described previously (Wu and Murry, 1985). Following electrophoresis, the gels were dried and subjected to autoradiography with Kodak x-Omat film at -70°C for the periods of time indicated in the figures. Multiple exposures were made to obtain optimal autoradiographs. Autoradiographs were scanned with an LKB Ultrascan XL densitometer scanner. This densitometer data will mostly be presented qualitatively.

Alkaline Hydrolysis

After electrophoresis, alkaline hydrolysis was carried out by incubating the gels in 1 M KOH at 55°C for 2 h. This was followed by neutralizing the gels with 10% acetic acid, drying, and exposing for autoradiography as described above.

Results

Effect of A23187, EDTA, and EGTA Treatment on in vitro Protein Phosphorylation Patterns of Rice Suspension Culture Cells

When rice suspension culture cells were treated with 2.5 mM EGTA for 15 h, the extracts exhibited an increased phosphorylation of the 36, 70, and 80 kD proteins (Fig. 1, lane g2). When the concentration of EGTA was increased to 5 mM, only an increase in phosphorylation of the 70 kD protein was observed. In addition, the phosphorylation of three groups of proteins with molecular weights of 12, 25-27, and 40-45 kD was greatly reduced.

Pretreatment with 5 mM EDTA produced a pattern largely the reverse of the 5 mM EGTA treatment: Phosphorylation of the 70 kD protein was greatly reduced, whereas phosphorylation of the 12, 25-27, 40-45, and 80 kD proteins was greatly increased.

A23187 or ionomycin treatment was similar to 5 mM EDTA treatment except that phosphorylation of the 70 kD phosphoprotein was not reduced at and above 50 μM of A23187. From densitometer scans of autoradiographs, the integrated areas of the 80 kD pro-

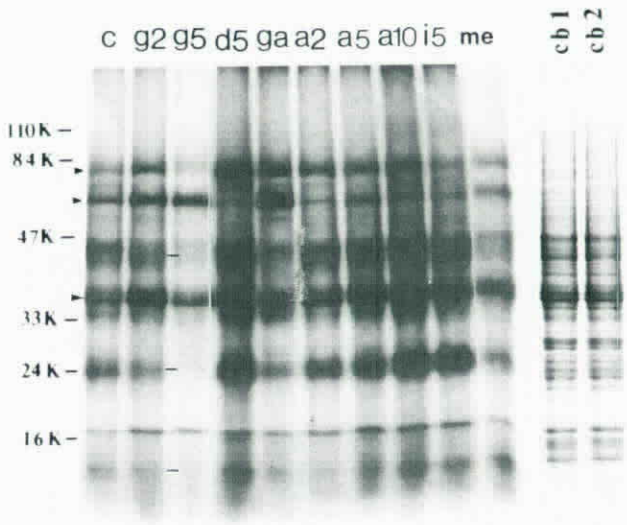

Fig. 1. *In vitro* protein phosphorylation of extracts from rice suspension culture cells pre-treated with A23187, EDTA, EGTA, and 2-mercaptoethanol. The rice suspension culture cells were grown in Murashige and Skoog medium and treated with the reagents at the concentrations indicated in the figure for 15 h at 24°C. The cells were then harvested and their extracts were subjected to *in vitro* phosphorylation assays followed by polyacrylamide gel electrophoresis and autoradiography (exposure time was 15 h) as described in Materials and Methods. c: control; g2 and g5: EGTA at 2.5 and 5 mM, respectively; d5: 5 mM EDTA; ga: 5 mM EGTA for 2 h followed by the addition of 50 μM A23187; a2, a5, and a10: A23187 at 25, 50, and 100 μM, respectively; i5: ionomycin at 50 μm; me: 0.5% of 2-mercaptoethanol. Lanes cb1 and cb2 are coomassie blue stain patterns of lane c and lane g2 respectively. The coomasie blue stain patterns of all other lanes showed no discernible difference from lanes cb1 and cb2 and are not shown here. The 36, 70, and 80 kD phosphoproteins which are increased in 2.5 mM EGTA treated cells are marked with "▶", and the 12, 25-27, and 40-45 kD phosphoproteins which are decreased in 5 mM EGTA treated cells are marked with "–".

tein were 2–3 fold higher in A23187-treated cells than in control cells. However, the increase of this 80 kD phosphoprotein in ionomycin treated cells was not obvious. The reason for this discrepancy is not known.

Co-treatment of the cells with 5 mM EGTA and 50 μM A23187 resulted in a phosphorylation pattern partially resembling that of 2.5 mM EGTA treated cells. However, the increase in phosphorylation of 80 and 70 kD proteins was more notable. Treatment with 0.5% 2-mercaptoethanol did not profoundly affect the protein phosphorylation pattern although it causes some minor changes.

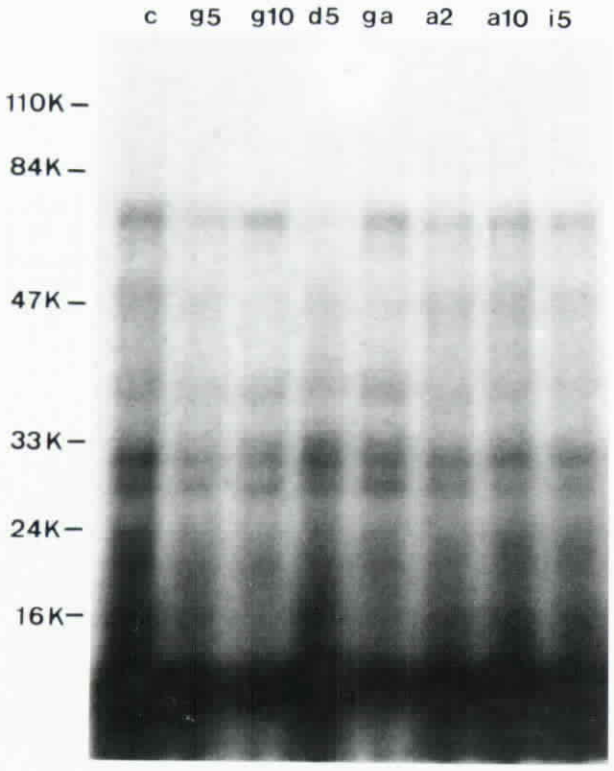

Fig. 2. Effect of A23187, EDTA, and EGTA pretreatment on *in vitro* protein phosphorylation of high-salt adapted rice suspension culture cells. High-salt adapted rice suspension culture cells grown in Murashige and Skoog medium supplemented with 1% NaCl were treated with the reagents at the concentration indicated in the figure for 15 h followed by analyses of *in vitro* protein phosphorylation by polyacrylamide gel electrophoresis and autoradiography (exposure time was 15 h) as described in Materials and Methods. c: control; g5 and g10: EGTA at 5 and 10 mM, respectively; d5: 5 mM EDTA; ga: 5 mM EGTA for 2 h followed by the addition of 50 μM A23187; a2 and a10: A23187 at 25 and 100 μM, respectively; i5: ionomycin at 50 μm.

Coomassie blue stain of the gels showed no discernible difference between protein patterns of treated and untreated cells (Fig. 1, lane cb1 and cb2). This indicated that there were no major changes in the constitutive protein components of the cells.

Effect of A23187, EDTA and EGTA on in vitro Protein Phosphorylation of High-salt Adapted Rice Suspension Culture Cells

The protein phosphorylation patterns from cultures adapted to 1% NaCl differed from those grown in normal medium. The phosphorylated proteins in high-salt adapted cells were enriched in low molecular weight regions (Fig. 2, lane c). Alkaline hydrolysis of phosphorylated proteins indicated that most of the labelled proteins harbored the radioactive phosphate group on serine/threonine residues rather than on tyrosine residue since they could be removed by the 1 M KOH treatment. The major tyrosine-labled protein is a 42 kD protein which was present in both normal and high-salt adapted cells, but its level of phosphorylation was significantly higher in high-salt adapted cells (data not shown). When high-salt adapted cells were subjected to various treatments, no significant changes in the phosphoprotein patterns were observed except that the amount of phosphoproteins in the low molecular weight regions was significantly reduced by EGTA treatment (Fig. 2). The total integrated areas from densitometer scans in regions with molecular weights less than 26 kD were 3–4-fold lower than those of untreated cells.

Effect of Heat Shock on Treatment of A23187, EDTA, and EGTA for Normal Suspension Culture Cells

Heat shock treatment of the cells at 40°C for 2 h resulted in highly elevated levels of phosphorylation of 16, 70–75, 90, and 100 kD proteins (Fig. 3, lane c) compared with those cells grown at 25°C (Fig. 1, lane c). Heat shock treatment combined with 5 mM EGTA treatment specifically inhibited the phosphorylation of a 90 kD protein, and the inhibition was nearly complete when the concentration of EGTA was increased to 10 mM (Fig. 3, lanes g5 and g10). EGTA at 10 mM also slightly reduced the phosphorylation of 70–75 kD protein. EDTA at 5 mM significantly reduced the phosphorylation of all three major phosphoproteins (70–75, 90, and 100 kD). The effect of 5 mM EGTA treatment in the presence of 50 μM A23187 resembled the 5 mM

EDTA treatment. A23187 at 25 μM did not change the protein phosphorylation patterns; but at 50 and 100 μM, it slightly increased the phosphorylation of the 16, 18, 70–75, and 100 kD proteins. The effect of ionomycin resembled that of A23187, except the phosphorylation of the 100 kD phosphoprotein was not observed. Treatment with 2-mercaptoethanol at 40°C stimulated the phosphorylation of several minor proteins (12, 14, 28, and 35 kD) not seen in other treatments.

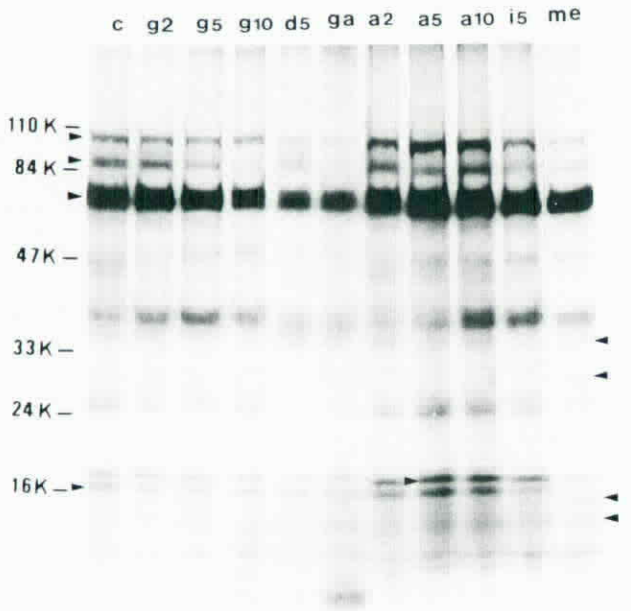

Fig. 3. *In vitro* protein phosphorylation of extracts from cells pre-treated with calcium chelators and calcium ionophores followed by treatment at 40°C. Rice suspension culture cells were pre-treated with the reagents indicated in the figure at 24°C for 15 h followed by heat shock treatment at 40°C for 2 h. The cell extracts were then subjected to *in vitro* protein phosphorylation, gel electrophoresis of the samples, and autoradiography (exposure time was 14 h) as described in Materials and Method. c: control; g2, g5, and a10: EGTA at 2.5, 5, and 10 mM, respectively; d5: 5 mM EDTA; ga: 5 mM EGTA for 2h followed by the addition of 50 μM A23187; a2, a5, and a10: A23187 at 25, 50, and 100 μM, respectively; i5: ionomycin at 50 μm; me: 0.5% of 2-mercaptoethanol. The 16, 70–75, 90, and 100 kD major phophoproteins and an 18 kD protein are indicated by "▶" in lane c and lane a5, respectively. The 12, 14, 28, and 35 kD phosphoproteins which are uniquely present in 0.5% of 2-mercaptoethanol treated cells are indicated by "◀" in lane me.

Effect of Heat Shock Treatment After A23187, EDTA, or EGTA Treatment on the Phosphorylation of Proteins in High-salt Adapted Rice Suspension Culture Cells

Heat shock treatment affected the phosphorylation of various proteins in high-salt adapted cells (Fig. 4) differently from those of normal suspension culture cells (Fig. 2). Most notably, high salt-adapted cells were enriched in the phosphorylation of proteins with low molecular weights and lacked the phosphorylation

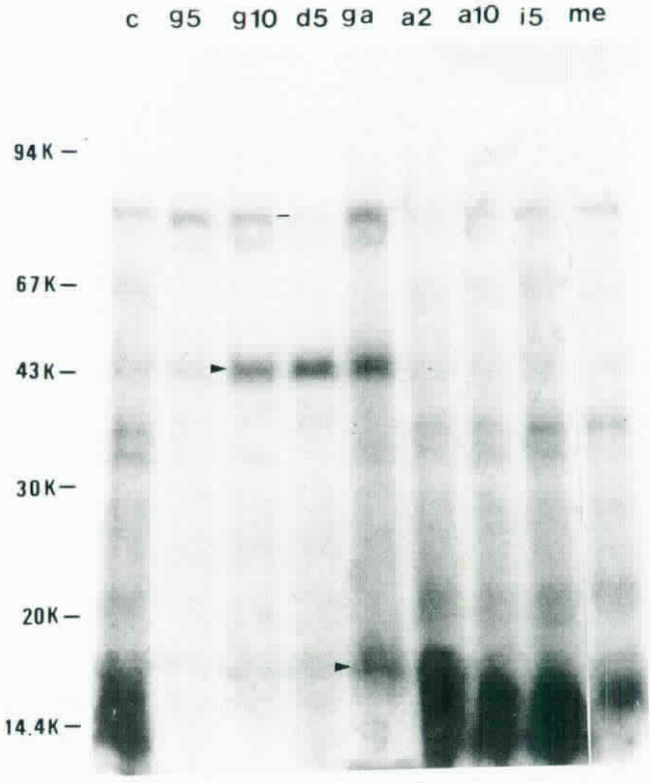

Fig. 4. *In vitro* protein phosphorylation in the extracts from high-salt adapted rice suspension culture cells pre-treated with calcium chelators and calcium ionophores, and then treated with heat shock. High-salt adapted rice suspension culture cells were pre-treated with the reagents indicated in the figure at 24°C for 15 h followed by heat shock treatment at 40°C for 2 h. The cell extracts were then subjected to *in vitro* protein phosphorylation and autoradiography (exposure time 16 h) as described in Materials and Methods. c: control; g5 and g10: EGTA at 5 and 10 mM, respectively; d5: 5 mM EDTA; ga: 5 mM EGTA for 2 h followed by the addition of 50 μM A23187; a2 and a10: A23187 at 25 and 100 μM, respectively; i5: ionomycin at 50 μm; me: 0.5% of 2-mercaptoethanol. The increased amounts of a 45 and an 18 kD phophoproteins in lanes g10 and ga, respectively, are indicated by "▶". The reduced 80 kD phosphoprotein in lane d5 is indicated by "–".

of the 70-75, 90, and 100 kD proteins abundantly present in extracts of cells grown in normal culture medium. Heat shock treatment combined with 10 mM EGTA or 5 mM EDTA treatment almost completely inhibited the phosphorylation of low molecular weight proteins but greatly enhanced the phosphorylation of a 45 kD protein (Fig. 4). Similar to those observed with cells grown in normal medium and treated at room temperature (Fig. 1), treatment with 5 mM EDTA followed by heat shock treatment also greatly reduced the phosphorylation of an 80 kD protein (Fig. 4, lane d5). Co-treatment of the cells with A23187 and EGTA slightly increased the phosphorylation of 18, 45, and 80 kD proteins (Fig. 4, lane ga). In addition, the 33 and 35 kD proteins which were present in control, untreated cells were greatly reduced after treatment with ion chelators EDTA or EGTA. Neither A23187 nor ionomycin treatment caused any conspicuous changes in the patterns of protein phosphorylation.

Discussion

In this report, we have shown that the phosphorylation patterns of proteins *in vitro* of rice suspension culture cells vary considerably among cells grown in normal or high-salt medium and among cells treated with A23187, EDTA, EGTA, or heat shock. Our data suggest that reagents which disturb the delicate calcium balance system in the cell can cause changes in the activity of the protein phosphorylation system. *In vitro* phosphorylation of proteins also varied considerably depending on the concentrations of calcium ions. During the course of this study, we have also attempted *in vivo* [α-^{32}P] -labelling of phosphoproteins under several conditions but found that it required high dosages of radioactive material, prolonged labelling period (over 8 h), and extended exposure time (over 10 days) for autoradiography (unpublished data). These problems compounded to hinder detailed *in vivo* studies of protein phosphorylation changes.

EGTA is a calcium chelator which can remove calcium ions from cell walls as well as from culture media. At near neutral pH, as used in our culture medium, EGTA is charged and is presumed to not readily cross the plasma membrane. Treating the cells with high concentration of EGTA would deplete the availability of external calcium ions and affect the plasma membrane stability and selective ion permeability. Our results indicate that in cells treated with 5 mM EGTA, phosphorylation of a protein (70 kD) was increased and three proteins (12, 25-27, and 40-45 kD) were decreased (Fig. 1). In general, at high concentrations of EGTA, the phosphorylation of proteins were reduced; however, the 70 kD protein was slightly increased. It remains to be determined whether the depleted external calcium level caused by EGTA treatment acts directly as a signal to change the protein phosphorylation system of the cells.

EDTA chelates Mg^{++} and Ca^{++}, and is able to progressively depress mitosis and cause chromosome aberration in plant cells (Halleck *et al.*, 1984). Since the incubation medium (0.5x MS) contains 1.5 mM Ca^{++} and 0.75 mM Mg^{++}, the presence of 5 mM EDTA would deplete these divalent cations. The reversal of changes in protein phosphorylation patterns between EGTA- and EDTA- treated cells is difficult to explain. Since EDTA is thought to readily penetrate into cells (Mehra and Dhiman, 1983), it presumably will not only deplete the extracellular divalent ions but also severely perturb the intracellular ionic balances. Such perturbation might trigger changes in a variety of cellular functions including the protein phosphorylation systems. However, the validity of this explanation needs to be investigated.

The calcium ionophores A23187 and ionomycin can freely cross the cell membrane and alter the intracellular calcium level, leading to changes in gene expression in animal cells (Ranjeva and Boudet, 1987; Wu *et al.*, 1981). Our result shows that treatment with 5mM EGTA in the presence of 50 μM A23187 resulted in a protein phosphorylation pattern partially resembling those of the 2.5 mM EGTA and 50 μM A23187 treatments. This indicates that most changes caused by EGTA treatment remain effective in the presence of A23187, and that A23187 can partially exert its effect in the presence of EGTA. The observation that EDTA and A23187 treated cells share many similarities in protein phophorylation patterns treated at 24°C may be explained by the assumption that both chemicals severely perturbed the intracellular calcium balance.

The phosphorylation of proteins in the extract from high-salt adapted cells differ to those from cells grown in normal culture medium. This indicates that these cells possess a different phosphorylation-dephosphorylation system and that they respond differently from normal cells when treated with high temperature,

ion chelators, and ionophores. Our results indicate that EGTA pretreatment greatly inhibits the phosphorylation of low molecular weight proteins of high salt adapted cells. Such inhibition becomes more severe under heat shock. There is no report that EGTA specifically affects small heat shock proteins(hsps) either *in vivo* or *in vitro*. Although a family of small hsps, some of which can be phosphorylated, is ubiquitous in eukaryotic cells (Lindquist and Craig, 1988), a relationship between EGTA-inhibited protein phosphorylation and the small hsps is not established.

It is difficult to measure the cytosolic free Ca^{++} level in the treated cells because of the heterogeneous nature of the cell aggregates in suspension culture cells. However, it is known that at 1% of NaCl the cytoplasmic calcium concentration can be elevated drastically in maize root protoplasts (Lynch *et al.*, 1990). It is also known that external calcium affects ionic leakage in cells under high NaCl stress (Nakamura *et al.*, 1990). Since our treatments are all related to the membrane permeability of Ca^{++}, it is tempting to suggest that changes in membrane structures in high-salt adapted cells occurred in relation to calcium efflux or influx, and played a role in changing the protein phosphorylation system of the cells.

Plants have a mechanism at transcriptional level that senses and responds to the severity and duration of heat stress by synthesizing heat shock-specific proteins (Kimpeol *et al.*, 1990). Heat shock can also disrupt synthesis of normal proteins (Belanger *et al.*, 1986) and affect the postranslational events, including protein phosphorylation and glycosylation (Lindquist and Craig, 1988). The pronounced presence of three sets of phosphoproteins with molecular weights of 70-75, 90, and 100 kD in extracts of heat shock treated cells suggests that changes in cellular phosphorylation system are involved in the response of cells to heat stress. The high level of these phosphoproteins under heat shock and their low level under normal temperatures is a feature resembling the hsp 70 and hsp 90 protein families in plants. The hsp 90 protein family is phosphorylated *in vivo* and some members of the family are associated with or are the substrates of protein kinases *in vitro*. The hsp 70 protein family binds ATP with high affinity and can be phosphorylated (Lindquist and Craig, 1988). The 80 kD protein induced by calcium ionophore treatment (Wu *et al.*, 1981) is a glucose regulated protein and a member of the hsp 70 family (Resendez *et al.*, 1986). Further investigations are needed to reveal if the phosphoproteins reported here are related to the heat shock proteins.

Our results indicate low levels of phosphorylation of the 70 kD protein in the extracts of A23187 treated cells at 24°C, but dramatically elevated levels when cells were followed by 40°C treatment. Whether or not this 70 kD protein is a cytosolic protein substrate of a protein kinase as reported in rice panicles (Cheng *et al.*, 1991) needs to be investigated. The changes in the phosphorylation of several other proteins observed at 24°C also disappeared at 40°C, indicating that the effect of A23187 is temperature dependent. Results of treatment with EDTA and EGTA are also temperature dependent. Most phosphorylation changes observed in the extract from cells pre-treated with calcium chelators and ionophores at 24°C are not present in the extract of cells following treatment at 40°C. Therefore, it appears that the heat shock effect overrides most of the effects of A23187, EDTA, and EGTA at 24°C. Since heat shock can cause an increase in intracellular free Ca^{++} concentration but not other ions (Vidair and Dewey, 1986), soluble sugars, or amino acids (Lin *et al.*, 1985), it can be assumed that calcium perturbation plays at least a contributory role in heat shock induced changes of cellular activities.

In conclusion, protein phosphorylation may be affected by interactions of heat shock and intracellular calcium perturbation. It may also be affected by the cell type, such as normal *vs* high-salt adapted culture cells and culture cells *vs* seedlings. Whether the changes in *in vitro* protein phosphorylation are calcium -dependent, calmodulin-related, and temperature-sensitive can be investigated by further *in vitro* assays involving calcium and temperature manipulations. The eventual identification and characterization of the endogenous kinases and their substrates would further our understanding on how cells respond to calcium perturbation by modifying their protein phosphorylation systems.

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鈣離子鉗化劑，運送劑與熱處理對細胞體外標定蛋白質磷酸化的影響

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我們以水稻(*Oryza sativa*)液體培養細胞爲材料，經由離體分析法來探討鈣離子與熱處理對正常細胞或耐高鹽細胞之蛋白質磷酸化差異。當細胞以鈣離子鉗合物 EGTA 處理時可增進一個分子量 70 kD 蛋白質的磷酸化，同時使分子量 12, 25-27, 40-45 kD 的另三個蛋白質磷酸化情形降低。此外鈣離子運送劑 A23187 與 ionomycin 的處理亦可造成一些蛋白質磷酸化情形的差異。特別是在 40°C處理條件下，耐高鹽細胞中許多低分子量蛋白質的磷酸化對 EGTA 處理特別敏感。有三個磷酸化蛋白質，分子量分別是 75, 90, 100 kD，在熱處理過的正常培養細胞中一直存在，而另一個 90 kD 的磷酸化蛋白質則只特別出現在 EGTA 處理情況下。歸納上述情形，我們發現鈣離子鉗合物與運送劑所造成的細胞體外標定蛋白質磷酸化現象會受細胞培養溫度及細胞對鹽份適應性的影響。