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Calcium-Regulated *in Vivo* Protein Phosphorylation in *Zea mays* L. Root Tips[1]

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ABSTRACT

Calcium dependent protein phosphorylation was studied in corn (*Zea mays* L.) root tips. Prior to *in vivo* protein phosphorylation experiments, the effect of calcium, ethyleneglycol-bis-(β-aminoethyl ether)-*N*-*N*′-tetraacetic acid (EGTA) and calcium ionophore (A-23187) on phosphorus uptake was studied. Calcium increased phosphorus uptake, whereas EGTA and A-23187 decreased it. Consequently, phosphorus concentration in the media was adjusted so as to attain similar uptake in different treatments. Phosphoproteins were analyzed by two-dimensional gel electrophoresis. Distinct changes in phosphorylation were observed following altered calcium levels. Calcium depletion in root tips with EGTA and A-23187 decreased protein phosphorylation. However, replenishment of calcium following EGTA and ionophore pretreatment enhanced phosphorylation of proteins. Preloading of the root tips with ^{32}P in the presence of EGTA and A-23187 followed by a ten minute calcium treatment, resulted in increased phosphorylation indicating the involvement of calcium, calcium and calmodulin-dependent protein kinases. Calmodulin antagonist W-7 was effective in inhibiting calcium-promoted phosphorylation. These studies suggest a physiological role for calcium-dependent phosphorylation in calcium-mediated processes in plants.

The recognition of calcium as a second messenger has opened new avenues for examining the biochemical mechanism of calcium action. The involvement of calcium in plant development has recently been reviewed by Hepler and Wayne (8) and Poovaiah and Reddy (15). The role of calcium has been implicated in various physiological processes such as hormone action (17, 18, 22), chloroplast movement (24), geotropism (6), and senescence (14). Calcium is known to regulate various biochemical processes after binding to calmodulin, a modulatory protein. The presence of calmodulin and of calcium-calmodulin-activated enzymes has been reported in plants (1, 7, 21).

Protein phosphorylation and dephosphorylation are considered important regulatory mechanisms by which the activity of key enzymes and receptor molecules is altered within cells in response to a wide variety of external stimuli (5). Calcium and calmodulin-mediated protein phosphorylation is one of the major mechanisms for regulating biochemical processes in animals (5). Activation of protein kinases and subsequent promotion of *in vitro* phosphorylation by calcium and calmodulin in plants has been well established (9, 20, 26, 27). In addition, calcium phospholipid-dependent protein kinase (protein kinase C) activity has also been detected in plants (16, 23).

A variety of chemicals such as calcium chelators, calmodulin antagonists and the calcium ionophore (A-23187) have been used to understand the role of calcium and calmodulin (8). Corn roots treated with EGTA solutions failed to respond to gravity, and calcium treatment reversed the effect of EGTA (12). Calcium ionophore (A-23187) has been used to manipulate levels of cytoplasmic calcium. Chloroplast movement in *Mougeotia* (24), cytokinin-induced bud formation in *Funaria* (22), tuberization in potato leaf cuttings (2) and phytochrome mediated germination of *Onoclea* spores (8) can be attained by A-23187 and calcium.

Most of the phosphorylation studies with calcium have been done under *in vitro* conditions (9, 20, 26, 27). However, *in vitro* studies may not reflect the changes occurring in the tissue at the time of stimulus-response. Moreover, the physiological significance of *in vitro* studies is limited. *In vivo* phosphorylation studies could help to understand stimulus-coupled responses in plants. In recent *in vivo* phosphorylation studies, Veluthambi and Poovaiah (28) have reported auxin-dependent changes in the phosphorylation of certain polypeptides. Actively growing corn root tips exhibit high levels of calcium-dependent protein phosphorylation (19), hence they serve as a good plant material to study the effects of calcium on *in vivo* phosphorylation. Briskin and Leonard (4) have reported the existence of a phosphorylated intermediate of H^+,K^+-ATPase in corn roots. Inhibition of proton pumping in corn roots has been shown to be related to increased phosphorylation of membrane proteins (29, 30). Our studies focused on *in vivo* protein phosphorylation in relation to altered calcium levels.

MATERIALS AND METHODS

Chemicals. The chemicals required for electrophoresis were obtained from Bio-Rad Laboratories, Richmond CA. [γ-^{32}P]ATP and carrier-free [^{32}P]orthophosphoric acid (HCl-free) were obtained from New England Nuclear. W-7 was purchased through Seikagaku America Inc., St. Petersburg, FL. Optex-plus intensifying screens were supplied by MCI Optonix Inc., Cedar Knolls, NJ. All other chemicals were purchased from Sigma.

Plant Material. Sweet corn (*Zea mays* L. var Patriot) seeds were a gift from Rogers Brothers Seed Co., Idaho Falls, ID. Corn seeds were sown in vermiculite and germinated in the dark for 3.5 d. The germinated seeds were carefully removed and 5 mm root tips were excised using a sharp blade. The root tips were transferred to incubation buffer.

Uptake of Phosphorus by Roots. Calcium is known to influence the uptake of phosphorus; hence, studies were carried out to understand the effect of calcium, EGTA and calcium ionophore (A-23187) on phosphorus uptake by roots. Root segments

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(0.1 g) were incubated for 2 h in buffer A (10 mM sodium citrate, pH 6.3, 1.5% sucrose, and 5 mM $MgCl_2$), or buffer A containing 5 mM EGTA and 1 μM A-23187. Following incubation, the above buffers were replaced in one set with fresh buffer containing different concentrations of phosphorus (1–25 nM KH_2PO_4). The other set of root segments was transferred to buffer A containing 1 mM calcium and different concentrations of phosphorus. Labeled [^{32}P]orthophosphoric acid (55,000 cpm) was then added to each uptake assay and the incubation was allowed to continue for 1 h. The uptake was stopped by aspirating the incubation buffer, followed by 3 washes with 5 mM KH_2PO_4. Root segments were transferred to scintillation vials containing 10 ml of scintillation cocktail (Scintiverse II, Fisher Scientific Co.). The tissue was left overnight in scintillation cocktail and radioactivity was determined.

***In Vivo* Protein Phosphorylation.** *Manipulation of Calcium Levels*: All incubations were carried out at room temperature with gentle shaking. About 0.5 g of root tips were used for each assay. For calcium depletion, roots were incubated for 2 h in buffer A containing 5 mM EGTA and 1 μM A-23187. For control experiments, roots were incubated for the same period of time in buffer A. Following preincubation, one set of roots from the control was transferred to fresh buffer A and another set was transferred to buffer A containing 1 mM calcium. Similarly, one set of roots from EGTA and ionophore was transferred to buffer A with EGTA and A-23187 and another set was transferred to buffer A with 1 mM calcium. Phosphorylation was immediately initiated by adding different amounts of ^{32}P (orthophosphoric acid), so as to attain similar uptake of ^{32}P by the roots in all treatments, and allowed to proceed for 1 h at room temperature. Additions of ^{32}P (orthophosphoric acid) were determined from the correlation curves obtained from the uptake studies. Phosphorylation was terminated by removing the incubation buffer and washing the roots three times with 10 ml each of homogenization buffer (50 mM Mes-NaOH [pH 7.0], 10 mM KH_2PO_4, 1 mM EDTA, 10 mM NaF, 0.5 mM PMSF,[2] and 1 mM DTT). Root tips were quickly frozen in liquid N_2 and stored at −20°C. Proteins were isolated as described by Veluthambi and Poovaiah (28) with modifications. Frozen root tips were homogenized in a pestle and mortar with 4 ml of homogenization buffer containing 5 μg/ml RNase I. The homogenate was centrifuged at 27,000*g* for 20 min. The supernatant was made up to 5 ml with homogenization buffer, DNase I (5 μg/ml) was added and left for 20 min at 4°C. An equal volume of 20% TCA was added to the supernatant and proteins were allowed to precipitate for 15 min at 4°C. The protein pellet was obtained by centrifuging at 27,000*g* for 20 min. This pellet was washed twice using ice-cold 80% acetone with 2% sucrose followed by 100% acetone. The samples were freeze-dried and dissolved in 150 μl of IEF sample buffer. Protein was estimated as described by Bensadoun and Weinstein (3).

Effect of Calmodulin Antagonist W-7 on Protein Phosphorylation: Root tips were incubated in buffer A for 2 h. After incubation the buffer was replaced either with buffer A containing 1 mM calcium or calcium and 70 μM W-7. Phosphorylation was initiated by adding 1 mCi of [^{32}P]orthophosphoric acid and the incubation was allowed to continue for 1 h. Phosphorylation was stopped and proteins were isolated as described above.

Preloading of Tissue with [^{32}P]orthophosphoric Acid: Root tips were pretreated for 2 h with buffer A containing 5 mM EGTA and 1 μM A23187. The buffer was replaced with fresh solution, to which [^{32}P]orthophosphoric acid was added and left for 1 h. At the end of the incubation period, the ^{32}P solution was aspirated and the tissue was washed with 10 ml of buffer A containing 2 mM KH_2PO_4 followed by two more washes with buffer A. The tissue was treated for 10 more min in 5 ml buffer A containing either EGTA and ionophore or 1 mM calcium. Phosphorylation was terminated and proteins were isolated as described above.

Effect of Calcium Concentration on Phosphorylation: Root tips were preloaded with ^{32}P and washed as described above. Preloaded tips were treated for 10 min in incubation buffer containing 1 mM EGTA and 1 μM A-23187 to which 0.1, 0.5, and 1 mM calcium chloride was added. Free calcium concentration in the media was calculated as described by Steinhardt *et al.* (25). Phosphorylation was terminated as described earlier.

***In Vitro* Protein Phosphorylation.** *In vitro* phosphorylation was performed and the phosphoproteins were analyzed by following the method described earlier (20). The protein phosphorylation assay mixture contained 180 μg soluble protein fraction, 50 mM Mes-NaOH (pH 7), 5 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF. Calcium was added to a final concentration of 0.25 mM.

Two Dimensional Gel Electrophoresis. Protein pellets were dissolved in 150 μl of IEF sample buffer (9.5 M urea, 0.5% DTT, 2% Triton X-100, 1% Biolyte 3/10, 0.5% Biolyte 5/7, and 0.5% ampholine 2.5/4) and centrifuged at 27,000*g* for 15 min. Phosphoproteins were separated on first dimension using 1.5 mm i.d. tube gels following the method described by O'Farrell (13) with modifications. NP-40 was replaced by Triton X-100 in the gel mixture. A mixture of three ampholites was used in preparing IEF gels. The gels were layered with 20 μl of IEF sample buffer and subjected to a pre-run at 200 V for 15 min followed by 300 and 400 V for 30 min each. About 50,000 cpm of radioactivity or 40 μg of protein was loaded on the gels and IEF was carried out at 400 V constant voltage for 16 h followed by 1 h at 1000 V. SDS-gel electrophoresis was performed as described earlier (11). The dried gels were exposed to Kodak X-Omat AR films for autoradiography in the presence of intensifying screens.

RESULTS

Effect of Calcium on Phosphorus Uptake. Phosphorus uptake studies were performed to determine the amount of ^{32}P to be added for different treatments to attain equal amounts of ^{32}P uptake. Uptake of phosphorus increased linearly with increased concentration of phosphorus in the media (Fig. 1). The presence of calcium significantly enhanced the uptake of phosphorus by the roots. Phosphorus uptake nearly doubled in the presence of calcium. A combination of EGTA and calcium ionophore decreased the uptake of phosphorus. Transferring root tips pretreated with EGTA and A-23187 to calcium significantly enhanced the uptake.

Effect of Calcium on *in Vitro* Protein Phosphorylation. Both qualitative and quantitative changes in phosphorylation of polypeptides were observed in the presence of calcium (Fig. 2). Phosphorylation of polypeptides of M_r = 83,000, 74,000, 60,000, 52,000 35,000, 34,000, and 29,000 was enhanced when calcium was included in the assay mixture. Moreover, phosphoprotein bands corresponding to M_r = 137,000 and 24,000 were observed only in the presence of calcium.

Effect of Calcium on *in Vivo* Protein Phosphorylation. Since *in vitro* studies need not necessarily be a reflection of changes occurring in protein phosphorylation in intact tissue, *in vivo* studies were performed. Attempts were made to alter the calcium status of the tissue using EGTA and A-23187. Different quantities of [^{32}P]orthophosphoric acid were added to attain similar phosphorus uptake under all the treatment conditions. The amount of phosphorus to be added was determined from the correlation curves obtained from uptake studies. Phosphoproteins were analyzed on two-dimensional gel electrophoresis by

[2] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl; IEF, isoelectric focusing.

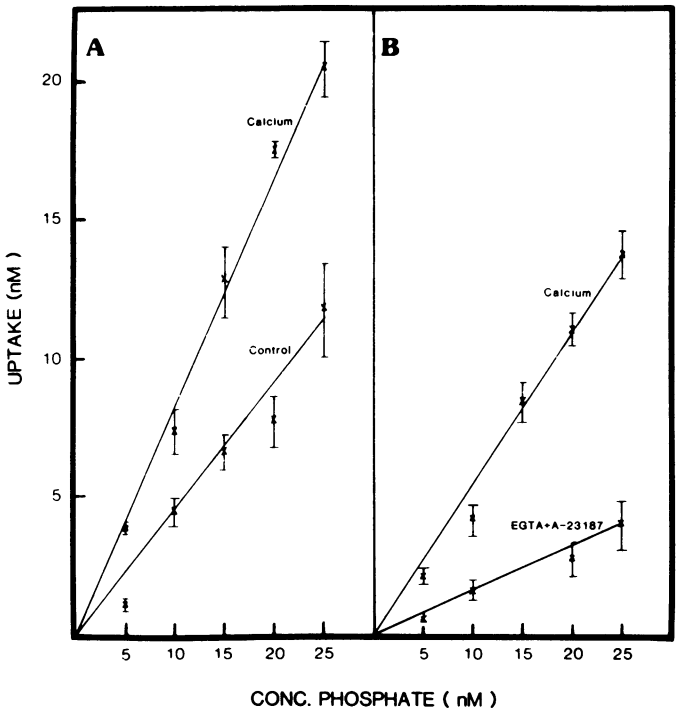

FIG. 1. Effect of calcium, EGTA, and calcium ionophore on phosphorus uptake. Root segments from 3.5-d-old dark grown corn plants were preincubated in buffer A alone (A) or in buffer A containing 5 mM EGTA and 1 μM A-23187 (B) for 2 h. Root tips from A were transferred to buffer containing different concentrations of phosphorus with or without calcium. One set of root tips from B was transferred to the same buffer (EGTA + A-23187) containing different concentrations of phosphorus. Another set of root tips from B was transferred to buffer consisting of calcium and different concentrations of phosphorus. [^{32}P] orthophosphoric acid (55,000 cpm) was added to each treatment as tracer. Phosphorus uptake was stopped and radioactivity in tissue was determined as described in the text. Experiments were performed with three replications. The vertical bars represent standard deviations.

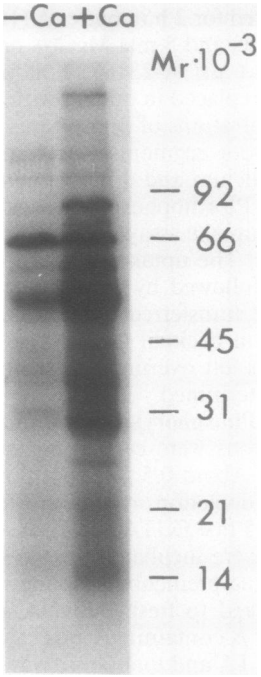

FIG. 2. SDS-PAGE of soluble protein extracted from 3.5-d-old dark grown corn root tips. Phosphorylation was carried out *in vitro* in the presence of 0.2 mM EGTA (minus calcium) or in the presence of 0.25 mM calcium and 0.2 mM EGTA (plus calcium). The mol wt of marker proteins are indicated.

loading an equal amount of radioactivity (Fig. 3). The results indicate the qualitative changes in phosphoprotein patterns. A distinct enhancement in phosphorylation of two polypeptides with M_r = 18,000 was noticed in the presence of calcium. Transferring roots from EGTA and A-23187 treatment to calcium resulted in enhanced phosphorylation of two polypeptides with M_r = 18,000 and a group of polypeptides with M_r = 23,000 to 27,000.

Preloading experiments were performed to further evaluate the role of calcium in protein phosphorylation. A short (10 min) calcium treatment of preloaded root tissue resulted in 33% more incorporation of label into the protein (Table I). The part of the gel showing maximum differences in phosphoprotein pattern is presented (Fig. 4). Calcium distinctly enhanced the phosphorylation of several polypeptides with M_r ranging from 23,000 to 27,000. Preloading studies were also performed to understand the effect of calcium concentrations on phosphorylation in the presence of EGTA and A-23187 (Table II). The preloaded tissue was subjected to a 10 min treatment of 1 mM EGTA and 1 μM A-23187 with or without different concentrations of calcium. One μM free calcium did not result in increased incorporation of label, whereas 10 μM free calcium resulted in significant enhancement of label in the proteins. Higher concentrations of calcium further enhanced phosphorylation.

Calmodulin antagonists are known to inhibit several physiological processes in plants (18). The effect of calmodulin antagonist W-7 on protein phosphorylation was examined (Table III). W-7 treatment was effective in decreasing the label from protein in the presence of calcium.

DISCUSSION

Many key regulatory proteins undergo phosphorylation resulting in small conformational changes in these proteins eventually leading to altered biological properties. Amplification and diversity in some extracellular signal action are achieved by phosphorylation and dephosphorylation of proteins. Recent investigations have shown the regulation of protein kinase activity by calcium in plants (15, 16, 21).

Calcium stimulated the phosphorylation of several proteins under *in vitro* conditions (Fig. 2). These results are in agreement with earlier findings (9, 20, 26, 27). However, *in vitro* results may not necessarily reflect changes occurring under *in vivo* conditions. Furthermore, there is a lack of information on *in vivo* protein phosphorylation regulated by calcium. Hence we focused our studies primarily on *in vivo* changes.

Calcium is known to influence the uptake of anions in plants. Calcium significantly enhanced the uptake of phosphorus as compared to the control (Fig. 1). The treatments (EGTA and A-23187) which decrease calcium concentration in the tissue also resulted in decreased phosphorus uptake. Calcium treatment following EGTA and A-23187 pretreatment resulted in significantly higher uptake of phosphorus. Since the protein phosphorylation experiments were carried out with nanomolar concentrations of labeled phosphorus, we evaluated the effect of calcium on uptake at submicromolar levels of phosphorus. The uptake data was critical in determining the quantity of ^{32}P to be added to achieve similar uptake under different treatments.

Replenishing the roots with calcium following EGTA and calcium ionophore pretreatment resulted in enhanced phosphorylation of several polypeptides (Fig. 3 and 4). Phosphorylation of polypeptides with M_r = 18,000 and a group of polypeptides

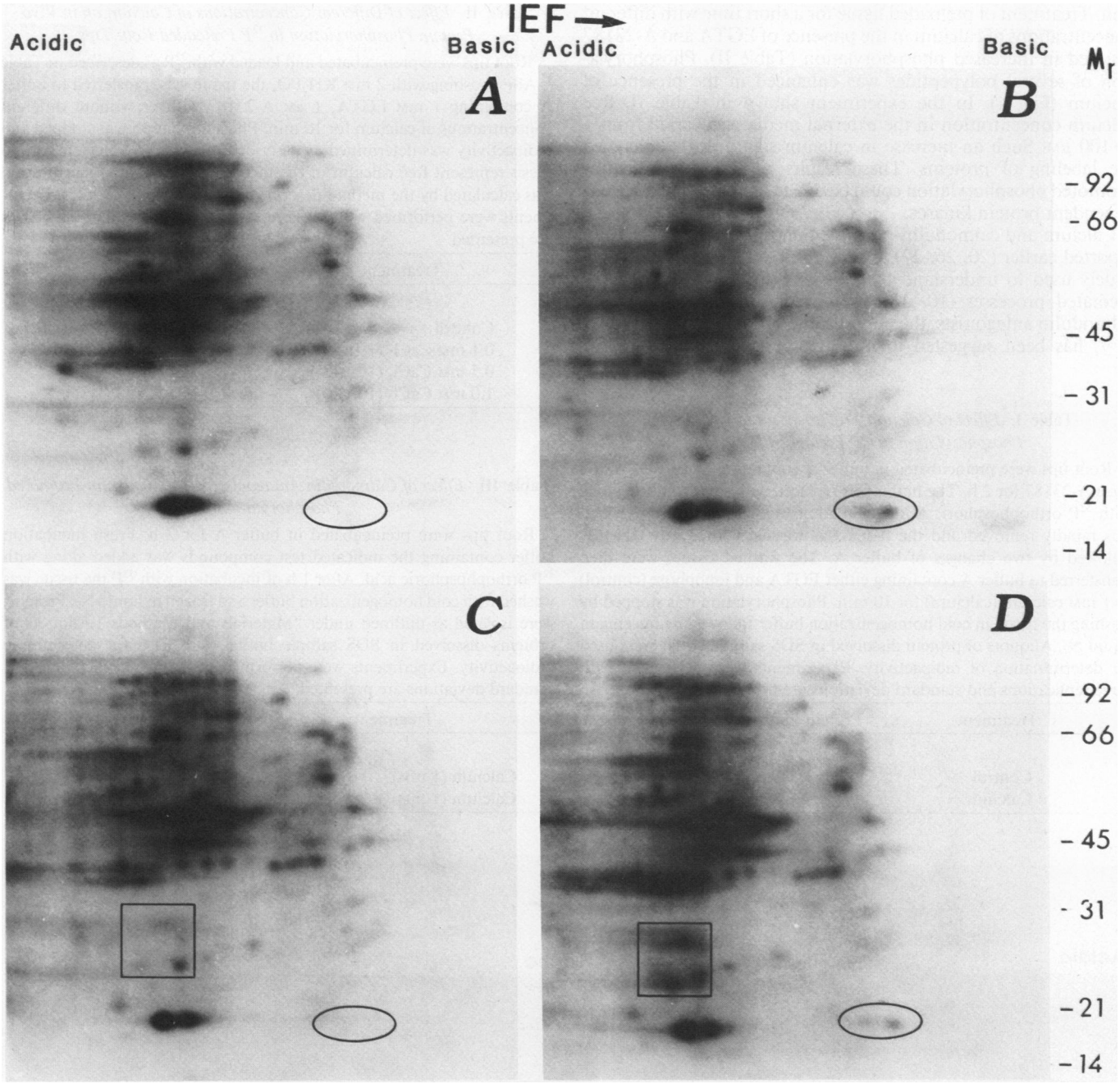

FIG. 3. Two-dimensional gel electrophoresis of *in vivo* phosphorylated polypeptides. Root tips were pretreated with buffer A (A and B) and buffer A containing 5 mM EGTA and 1 μM calcium ionophore (C and D) for 2 h. The buffers were replaced with 5 ml of fresh buffer alone (A) or fresh buffer containing 5 mM EGTA + 1 μM A-23187 (C), or 1 mM calcium chloride (B and D). ^{32}P was added to each of the treatments as described in the text and left for 1 h. Phosphorylation was terminated and proteins were isolated as described in "Materials and Methods." About 50,000 cpm of radioactivity was analyzed on IEF gels (pH 3.5–8.0) for first dimension followed by second dimension on 8 to 16% linear SDS-PAGE. Dried gels were exposed to x-ray films (Kodak X-Omat AR) at −70°C in the presence of intensifying screens.

with M_r = 23,000 to 27,000 appeared to be specific to the increased concentration of calcium. EGTA is known to chelate extracellular calcium (8). This chelation could lead to a decrease in the release of calcium from apoplast to symplast, or the equilibrium in calcium levels between the apoplast and symplast could be altered. Such alterations have been shown to affect the gravitropic response of corn roots (12). A combination of EGTA and calcium ionophore could be effectively used to alter the intracellular concentration of calcium (8). Determination of free calcium concentration in the tissue would be the most ideal way to understand the role of calcium in protein phosphorylation. Due to the technical difficulties associated with the measurements of free calcium in plants, we have been unable to provide the data on the levels of intracellular free calcium under our experimental conditions. However, both EGTA and ionophore have been effectively used to manipulate the levels of intracellular calcium concentration and consequently to alter calcium-mediated physiological processes (2, 8, 12, 22, 24).

Preloading experiments with ^{32}P provided additional evidence for the involvement of calcium in *in vivo* protein phosphoryla-

tion. Treatment of preloaded tissue for a short time with different concentrations of calcium in the presence of EGTA and A-23187 resulted in increased phosphorylation (Table II). Phosphorylation of several polypeptides was enhanced in the presence of calcium (Fig. 4). In the experiment shown in Table II, free calcium concentration in the external media was varied from 1 to 100 μM. Such an increase in calcium significantly enhanced the labeling of proteins. These results suggest that calcium-promoted phosphorylation could be due to activation of calcium-dependent protein kinases.

Calcium and calmodulin-promoted phosphorylation has been reported earlier (20, 26, 27). Calmodulin antagonists have been widely used to understand the role of calmodulin in calcium-mediated processes (10, 18). Among the different groups of calmodulin antagonists, the naphthalene sulfonamide derivative, W-7, has been suggested to be a more specific antagonist of

Table I. *Effect of Calcium Treatment on in Vivo Protein Phosphorylation in ^{32}P Preloaded Root Tips*

Root tips were preincubated in buffer A containing 5 mM EGTA and 1 μM A-23187 for 2 h. The buffer was replaced with fresh solution along with ^{32}P orthophosphoric acid. Incubation was continued for 1 h. ^{32}P was rapidly removed and the tissue was washed with 2 mM KH_2PO_4 followed by two changes of buffer A. The washed tissues were then transferred to buffer A containing either EGTA and ionophore (control) or 1 mM calcium (calcium) for 10 min. Phosphorylation was stopped by washing the tissue in cold homogenization buffer followed by freezing in liquid N_2. Aliquots of protein dissolved in SDS sample buffer were used for determination of radioactivity. Experiments were performed with three replications and standard deviations are presented.

Treatment	Protein Phosphorylation
	cpm/μg protein
Control	849 ± 51
Calcium	1121 ± 21

Table II. *Effect of Different Concentrations of Calcium on in Vivo Protein Phosphorylation in ^{32}P Preloaded Root Tips*

Root tips were preincubated and loaded with ^{32}P as described in Table I. After washing with 2 mM KH_2PO_4 the tissue was transferred to buffer A containing 1 mM EGTA, 1 μM A-23187 with or without different concentrations of calcium for 10 min. Phosphorylation was stopped and radioactivity was determined as described in Table I. Values in parenthesis represent free calcium in the media. Free calcium concentration was calculated by the method described by Steinhardt *et al.* (25). Experiments were performed with three replications and standard deviations are presented.

Treatment	Protein Phosphorylation
	cpm/μg protein
Control	470 ± 61
0.1 mM $CaCl_2$ (1.16 μM)	475 ± 29
0.5 mM $CaCl_2$ (10.1 μM)	622 ± 82
1.0 mM $CaCl_2$ (100 μM)	981 ± 123

Table III. *Effect of Calmodulin Antagonist W-7 on Calcium-Promoted Phosphorylation*

Root tips were preincubated in buffer A for 2 h. Fresh incubation buffer containing the indicated test compounds was added along with ^{32}P orthophosphoric acid. After 1 h of incubation with ^{32}P the tissue was washed with cold homogenization buffer and frozen in liquid N_2. Proteins were isolated as outlined under "Materials and Methods." Aliquots of proteins dissolved in SDS sample buffer were used for determining radioactivity. Experiments were performed with three replications and standard deviations are presented.

Treatment	Protein Phosphorylation
	cpm/μg protein
Calcium (1 mM)	1365 ± 10
Calcium (1 mM) + W-7 (75 μM)	1134 ± 50

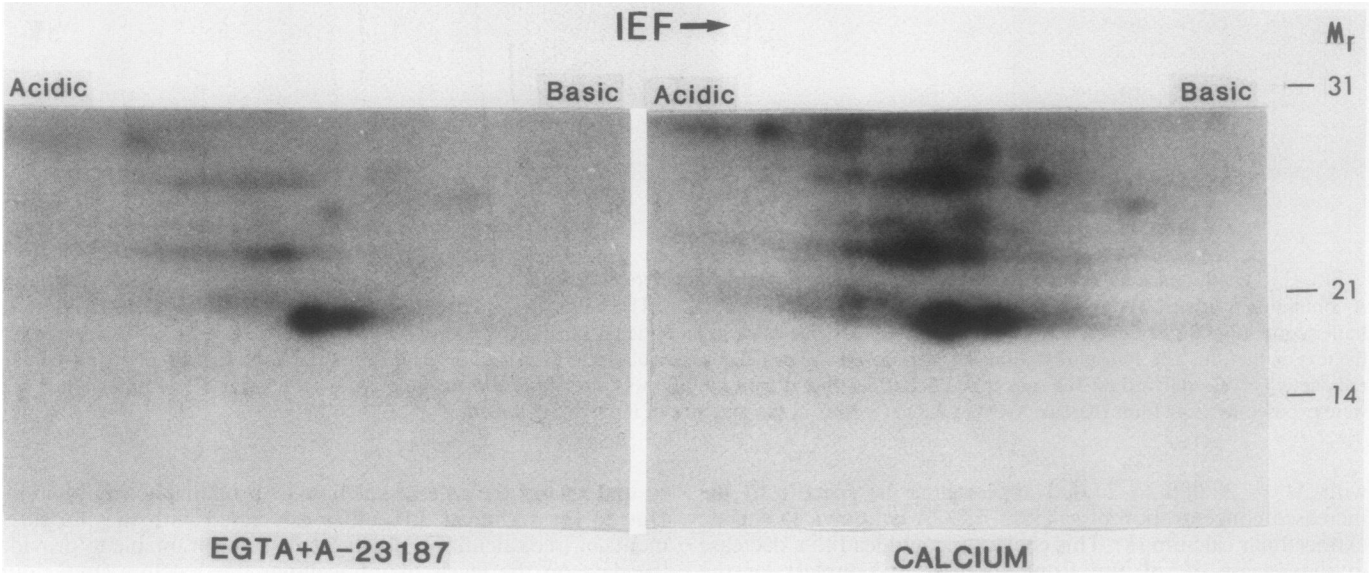

FIG. 4. Two-dimensional gel electrophoresis of *in vivo* phosphorylated proteins. Figures represent the part of the gels obtained from preloading experiment showing distinct differences in labeling. Roots were preloaded with ^{32}P in presence of 5 mM EGTA and 1 μM A-23187 and washed as described in Table I. Preloaded segments were transferred to either 5 mM EGTA and 1 μM A-23187 or to 1 mM calcium for 10 min. Phosphorylation was terminated and proteins were isolated as described in "Material and Methods." About 40 μg of protein was analyzed on two-dimensional electrophoresis.

calmodulin (10). Treating the root tips with W-7 resulted in a substantial decrease in calcium-promoted phosphorylation. This inhibition suggested that calmodulin could be involved in calcium-promoted protein phosphorylation. Since calmodulin antagonists are known to have some nonspecific effects, caution should be exercised in interpreting these findings (18).

Our results indicate an important role for calcium in *in vivo* protein phosphorylation in plants. We further suggest that calcium-dependent protein phosphorylation may play a key role in various physiological processes affected by calcium. The identity and function of calcium-promoted phosphoproteins need to be evaluated to ascertain their physiological relevance.

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