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Polyamine Stimulation of Protein Phosphorylation in Isolated Pea Nuclei[1]

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ABSTRACT

The phosphorylation of several proteins in isolated nuclei from *Pisum sativum* L. was stimulated by spermine. Although spermine increased the general protein phosphorylation by 10 to 20%, it increased the phosphorylation of a 47 kilodalton polypeptide by 150%. By comparison other polyamines, spermidine, putrescine, and cadavarine had far less effect on the phosphorylation of the 47 kilodalton or any other polypeptide. Sodium fluoride was able to inhibit the phosphorylation of the 47 kilodalton polypeptide in the control, implying the participation of protein phosphatase(s) in the phosphorylation of nuclear proteins. Spermine stimulated the phosphorylation of the 47 kilodalton polypeptide over the controls, even in the presence of NaF. This result indicates that spermine probably activates a nuclear kinase, a conclusion supported also by thiophosphorylation data. The inability of ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid and Compound 48/80, a calmodulin antagonist, to inhibit this spermine stimulated phosphorylation renders improbable any role of calcium and calmodulin in mediating this response.

The role of polyamines in plant growth and development is well documented (11, 25). Polyamines are known to regulate a wide variety of physiological and biochemical processes in the nuclei. Some of these include regulation of cell division (5, 16), and the activities of several enzymes involved in nucleic acid synthesis (14, 27) and repair (23). The activity of at least some of these enzymes is known to be controlled by polyamines via modulation of their phosphorylation state. In slime molds, Kuehn *et al.* (18) showed that polyamines regulate the phosphorylation of a certain nucleolar protein that regulates rRNA synthesis. They later identified this protein as ornithine decarboxylase and showed that polyamines act via regulating the activity of a protein kinase which in turn regulates the phosphorylation state and hence the activity of ornithine decarboxylase (2, 19).

We have already shown that phytochrome stimulates the phosphorylation of certain proteins in isolated nuclei from peas (9). In pea seedlings, phytochrome has also been shown to regulate polyamine biosynthesis by modulating the activity of arginine decarboxylase, one of the first enzymes in polyamine biosynthesis (6). In animal systems, polyamines are known to regulate nuclear protein phosphorylation (4, 15, 18). Given the above results, we decided to test the effect of polyamines on protein phosphorylation in isolated pea nuclei.

In the present paper we show that polyamines, especially spermine, stimulate the phosphorylation of certain nuclear proteins in peas. We also present evidence that this stimulation is probably due to an increased activity of a protein kinase. Since the maximum stimulation of phosphorylation is of a 47 kD polypeptide most of the work in this paper is on the regulation of phosphorylation of the 47 kD polypeptide containing nuclear protein.

MATERIALS AND METHODS

Etiolated, 7-day old pea plumules were used for isolating nuclei. Isolation of the nuclei and the subsequent experiments done with them were all carried out under green safe lights. Isolation of the pea nuclei from *Pisum sativum* L. and the phosphorylation assay were done as described by Datta *et al* (9). The nuclei were preincubated for 15 min with polyamines before the phosphorylation assay was started by the addition of [γ-^{32}P]-ATP or [γ-^{35}S]ATP. The reaction was carried out at room temperature for times indicated in figure legends. The concentration of [γ-^{32}P]ATP used was 10 μM and 10 μCi in a final volume of 100 μl. For thiophosphorylation experiments, [γ-^{35}S]-ATP was used at a final concentration of 50 μM and 2.5 μCi in a 100 μl assay mixture. EGTA was used at pH 7.0 with a final concentration of 200 μM, and the nuclei were preincubated with EGTA for 5 min prior to the assay. The calmodulin antagonist, Compound 48/80, (12) was used at a final concentration of 10 μg/ml. NaF was used at a final concentration of 5 mM and the nuclei were preincubated in it for 30 min prior to the assay. Samples of phosphorylated nuclear proteins were electrophoresed on 8% SDS polyacrylamide gels (20). Autoradiography was done as described earlier (9). When [γ-^{35}S]ATP was used, fluorography was done. For fluorography, gels were soaked in Enlightning (New England Nuclear Research products, NEF-976) for 1 h before drying and exposure to X-Omatic AR (Kodak) films at −70°C.

Autoradiograms/fluorograms were scanned using an IBAS image processor. The relative densities of labeled bands on the autoradiograms were measured by densitometry and normalized to equivalent protein content, as estimated by Coomassie blue staining intensity.

The [γ-^{32}P]ATP and the [γ-^{35}S]ATP were from New England Nuclear. The ultrapure sucrose (Schwarz Mann), glycerol (Fisher), Hepes (Calbiochem), ATP-γ-S[2] (Boehringer Mannheim), polyamines, EGTA, Compound 48/80, Tris-ATP (Sigma), and all other chemicals were of the highest purity available.

RESULTS

Figure 1 shows the effect of various polyamines on nuclear protein phosphorylation. Of the four polyamines tested, spermine stimulated protein phosphorylation the most at the concen-

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[2] Abbreviations: ATP-γ-S. adenosine-5′-*O*(3-thiotriphosphate).

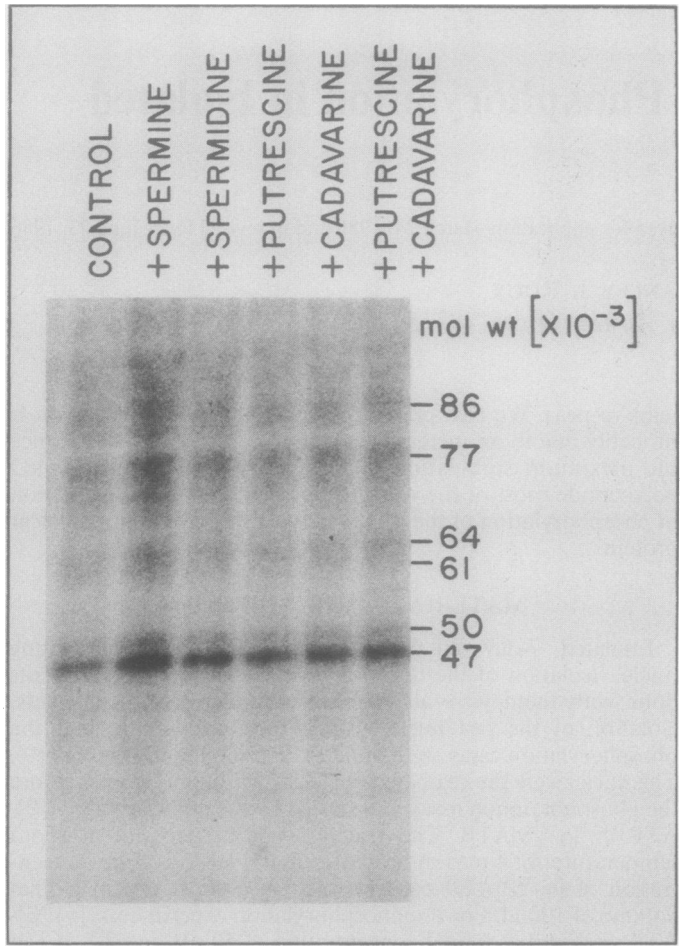

FIG. 1. Effect of various polyamines at 0.33 mM concentration on nuclear protein phosphorylation. The incubation time was 30 s. The concentration of [γ-^{32}P]ATP was 10 μM and 10 μCi.

Table I. *Effect of Varying Concentrations of Spermine on Phosphorylation of the 47 kD Polypeptide*

The assay was carried out for 30 s.

Treatment	Relative Density of the 47 kD Labeled Polypeptide
Dark	100
+ Spermine 0.05 mM	169
+ Spermine 0.10 mM	170
+ Spermine 0.20 mM	168
+ Spermine 0.33 mM	223
+ Spermine 0.66 mM	236

tration used (0.33 mM). Spermine increased overall phosphorylation by about 10 to 20% and it specifically increased the phosphorylation of a 47 kD band by an average of 140%. The other polyamines also increased the phosphorylation of the 47 kD band but only by 20 to 40%. Since the maximum stimulation of phosphorylation was with spermine the rest of the experiments were done with spermine. The effect of spermine was tested at concentrations varying from 0.05 mM to 0.66 mM. Optimal stimulation of phosphorylation of the 47 kD polypeptide was seen at 0.33 mM (Table I).

The time course data for spermine stimulated phosphorylation of the 47 kD polypeptide shows that in the dark phosphorylation increased linearly for 1 min, after which it began to plateau. In the presence of spermine, the rate of phosphorylation was dramatically increased. The highest stimulation occurred at 15 s into the assay, after which the stimulation decreased but remained consistently higher than the dark control (Fig. 2). When [γ-^{35}S]ATP was used, the overall rate of label incorporation was much slower but showed a similar pattern of spermine stimulation as when [γ-^{32}P]ATP was used. In this case phosphorylation in the dark control was linear for 20 min before it began to plateau (Fig. 3). Figure 4 shows the time course of the phosphorylation of the 47 kD polypeptide in the presence of spermine + 5 mM NaF. In the control, without spermine and without NaF, the phosphorylation increased linearly for 1 min. In the presence of NaF, the rate of phosphorylation was the same as in the control for 20 s, after which phosphorylation completely stopped. In the presence of both spermine and NaF, the rate of phosphorylation was greater than the control and there was an increase in the overall phosphorylation of the 47 kD band. Neither EGTA nor Compound 48/80 was able to inhibit the spermine stimulated phosphorylation of the 47 kD polypeptide (Fig. 5).

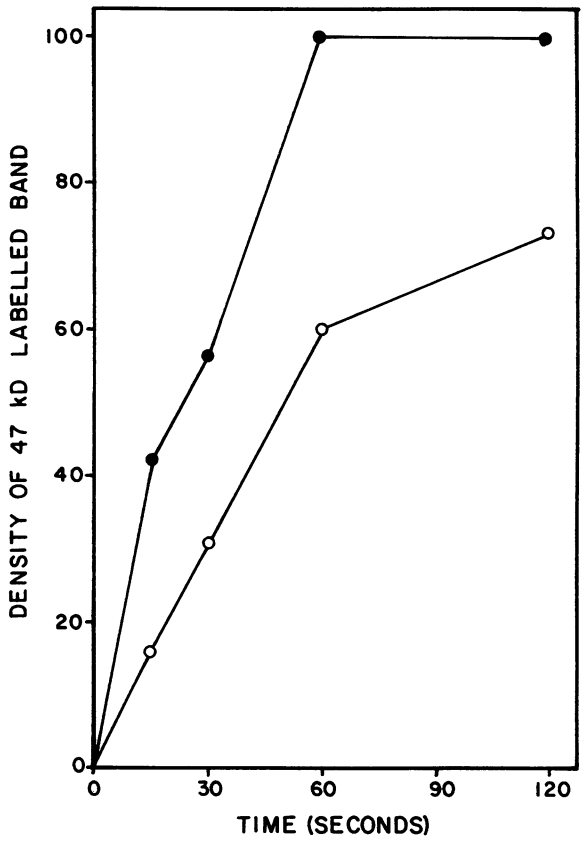

FIG. 2. Time course of the spermine (0.33 mM) stimulated phosphorylation of the 47 kD polypeptide. (○), Control; (●), with 0.33 mM spermine added.

DISCUSSION

Polyamines have a regulatory effect on nuclear protein phosphorylation in chicken embryo (22), rat liver (15), and slime molds (19). In pea nuclei we observed that polyamines, especially spermine, increased protein phosphorylation. In other systems also, spermine has been shown to preferentially affect protein phosphorylation more than the other polyamines (4, 13). The specific increase of phosphorylation of the 47 kD polypeptide would suggest that spermine either increased the activity of one or more protein specific kinase(s), decreased the activity of

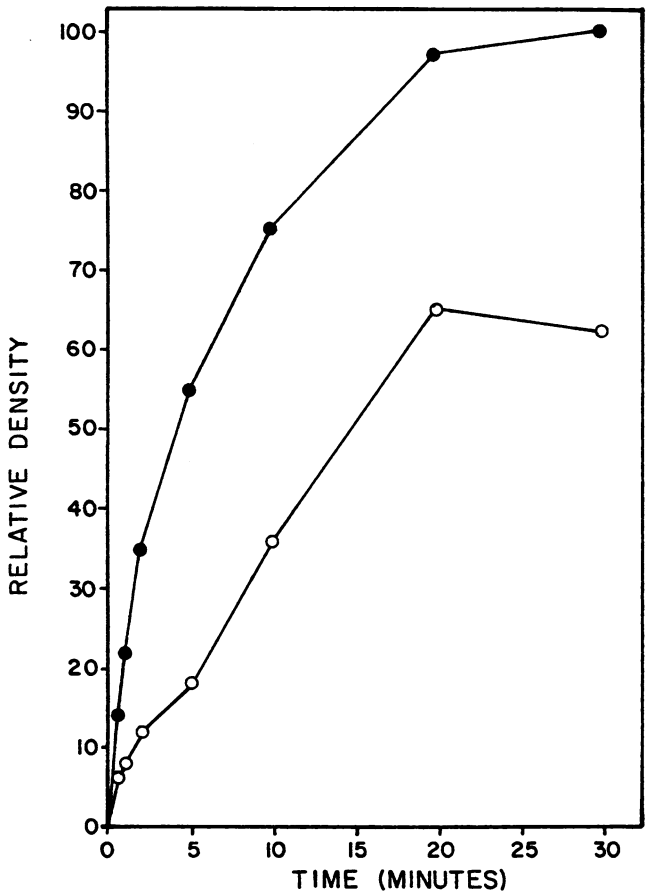

FIG. 3. Effect of spermine on thiophosphorylation. The incubation time was 30 s and the [γ-^{35}S] ATP concentration was 50 μM and 2.5 μCi. (O), Control; (●), with 0.33 mM spermine added.

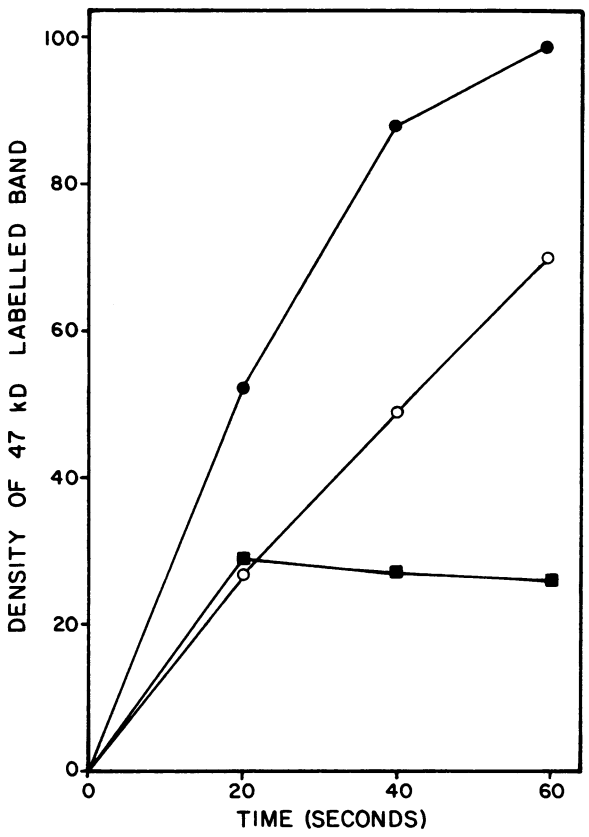

FIG. 4. Time course of the effect of NaF (5 mM) on the spermine (0.33 mM) stimulated phosphorylation of the 47 kD polypeptide. (O), Control; (■), control + NaF; (●), spermine + NaF.

protein specific phosphatase(s), or both.

The experiments done with [γ-^{35}S]ATP showed that spermine definitely increased the activity of one or more kinases. Though [γ-^{35}S]ATP can be used as a substrate by the kinase in lieu of [γ-^{32}P]ATP, phosphatases are unable to recognize the sulfate adduct and remove it (3). Hence, the observed increase in ^{35}S incorporation into the 47 kD nuclear polypeptide could not be due to decreased phosphatase activity, but more likely is due to an increased kinase activity. Polyamines have been shown to regulate the activity of nuclear protein kinases in other systems (8, 15, 22).

The time course data also suggest that spermine stimulates protein kinase(s). In the control, the phosphorylation rate was linear up to 1 min whereas in the spermine treated nuclei, the maximum stimulation of phosphorylation was highest at 15 s and then decreased; the net phosphorylation in spermine-treated sample was always higher than in the control. This would suggest that spermine stimulated and activated protein kinase(s) such that there was not only an increase in the rate of phosphorylation but also some new sites were phosphorylated on the 47 kD polypeptide. An alternative explanation would be that spermine was both stimulating a protein kinase and inhibiting a phosphatase. This was resolved by an experiment with NaF, a protein phosphatase inhibitor (17, 26).

A comparison of the control with the NaF treatment showed that for the first 20 s of the reaction the phosphorylation was the same in the control as it was with NaF. This indicates that the nuclei contain a kinase that will catalyze the incorporation of label onto formerly unphosphorylated sites on the 47 kD polypeptide, *i.e.* no protein phosphatases are required to first remove unlabeled phosphates to allow labeled phosphates to be added on. After 20 s there was no net incorporation of ^{32}P into the 47 kD polypeptide in the presence of NaF, presumably because whatever sites could be phosphorylated under those conditions had been filled, and turnover was not possible in the absence of phosphatase activity. Without NaF present, and with labeled ATP as the main substrate, protein phosphate turnover occurred at a rapid rate and there was a net increase in the incorporation of labeled phosphate for over a min after the addition of [γ-^{32}P]ATP. With the addition of spermine and NaF, label incorporation into the 47 kD polypeptide was higher than in either the untreated control or the sample treated with NaF alone (Fig. 4), indicating that spermine was acting by activating/stimulating a kinase to incorporate label onto sites that are not phosphorylated in the absence of spermine. This could also mean that spermine interacts with the 47 kD polypeptide and changes the configuration such that more sites are available for phosphorylation. This latter possibility has been hypothesized earlier (1).

Polyamines are known to have both inhibitory (21) and stimulatory (7) effects on soybean hypocotyl protein phosphatase and bovine mitochondrial pyruvate dehydrogenase phosphatase, respectively. To the best of our knowledge, there is no report of a protein phosphatase in plant nuclei. Our studies, however, suggest the presence of phosphatase(s) in isolated pea nuclei. Purello *et al.* (24) have, by indirect methods, shown the presence of a phosphatase in the nuclei of rat liver cells. Recently, Friedman (10) has shown that spermine activates a protein phosphatase in isolated nuclei from HeLa cells. Our evidence indicates that, though a role for phosphatases cannot be ruled out, the phosphorylation of a 47 kD polypeptide in pea nuclei involves the stimulation and/or activation of protein kinase(s).

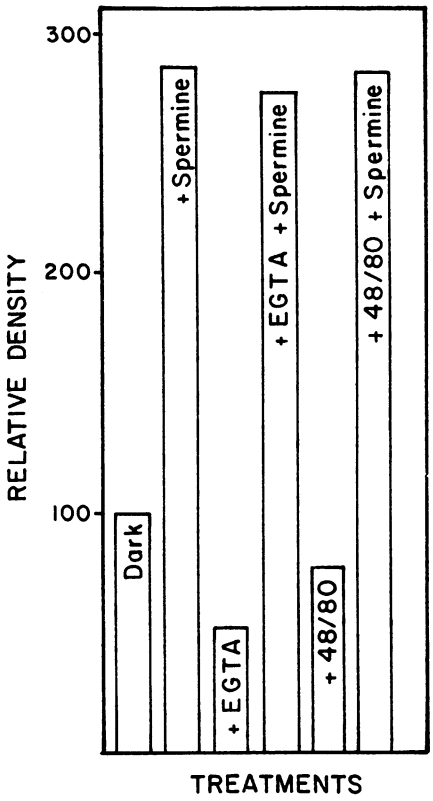

FIG. 5. Effect of EGTA (0.2 mM) and Compound 48/80 (10 μg/ml) on spermine (0.33 mM) stimulated phosphorylation of the 47 kD polypeptide.

The contaminant level of Ca^{2+} in the phosphorylation reaction with no exogenous calcium added was approximately 10 μM. EGTA and Compound 48/80 did not affect the spermine stimulation of the phosphorylation of the 47 kD polypeptide. In an earlier study, we found that phytochrome also stimulated the phosphorylation of a 47 kD polypeptide in isolated pea nuclei, but that this stimulation was Ca^{2+} and calmodulin dependent. If phytochrome and spermine are both regulating the phosphorylation of the same 47 kD nuclear polypeptide, they are doing so by somewhat different mechanisms. Another possibility is that phytochrome could be modulating polyamine levels in a calcium dependent fashion and that this change then regulates protein phosphorylation. Hence, phytochrome might be regulating protein phosphorylation *in vivo* through the mediation of both calcium and polyamines. Studies are presently ongoing to investigate the mechanism by which calcium and polyamines regulate phosphorylation.

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