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PHOSPHORYLATION OF COTTONPLANT PROTEINS UNDER THE ACTION OF PROTEIN KINASE INHIBITORS AND ACTIVATORS

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The occurrence of Ca^{2+}-, Ca^{2+}-phospholipid-, Ca^{2+}-calmodulin-, and cAMP-dependent phosphorylation has been shown with the aid of protein kinase activators and inhibitors and by electrophoresis and autoradiography. Specific substrates have been revealed and it has been demonstrated that the pathways of the realization of the action of cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases may intersect.

The phosphorylation of proteins causes a cascade of processes, which is due to a diversity of substrates, not to speak of the diversity of protein kinases and their isomeric forms, the redistribution of the charge of the phosphorylated protein or enzyme, and, of course, a change in its function, and also the subsequent interaction of the modified protein with its environment. The spectrum of processes controlled by this reaction is therefore equally broad. Here must be included the carbohydrate and lipid metabolisms, the biosynthesis of protein, the formation of the cytoskeleton, etc. [1].

In order to approach answers to questions on the role of phosphorylation in the regulation of enzyme activity a detailed investigation of this process is required. The use of effective activators and inhibitors permits the in vitro manipulation of the conditions of the phosphorylation process by isolating a definite protein kinase activity in the multicomponent chain of biochemical reactions proceeding in a plant tissue homogenate. The protein kinase activity exhibited in all fractions of various plant tissues is extremely diverse in relation both to substrate specificity and to the level of regulation of enzyme activity [2-11]. The reason for this is the diversity of pathways by which it realizes the action of signal received, whether this be the action of stress or of phytohormones or othe biologically active substances.

In the present work we have made an attempt to find the specific substrates of cottonplant protein kinases. The activities of the protein kinases were modulated with the aid of known activators and inhibitors. Spectra of the in vitro phosphorylation of the proteins of three-day cottonplant shoots are given in Fig. 1. It must be mentioned that the spectra of in vivo and in vitro phosphorylations may differ substantially with respect to substrate specificity — i.e., with respect to the phosphorylation of proteins [12] — but, in general, we have several proteins characteristic for a given plant in a given experiment that are always phosphorylated under one and the same conditions and serve as a kind of endogenous markers. For the present case such a protein is one with a molecular mass of 30 kDa, which was phosphorylated in all cases, without exception, although the level of phosphorylation was different in each case. This can be explained by the presence of two or more phosphorylated proteins with mobilities close to that of the given band or by the phosphorylation of one and the same protein by different protein kinases, as we can see in Fig. 1.

The protein was phosphorylated on activation by cAMP alone (lane 12) and also by Ca^{2+} ions, Ca^{2+} and phosphatidylserine, Ca^{2+} and phosphatidylcholine, and calmodulin (lanes 4, 5, 6, and 8 and 9), the level of phosphorylation being a maximum on activation by calmodulin. When Ca^{2+} was bound with EDTA the phosphorylation of this protein was slight; i.e., it is a specific substrate of a Ca^{2+}-calmodulin-dependent protein kinase, even though we also observed its phosphorylation to some degree on activation by cAMP, also (lanes 10 and 11). Lane 12 shows the total phosphorylation of this protein by the Ca^{2+}- and cAMP-dependent protein kinases.

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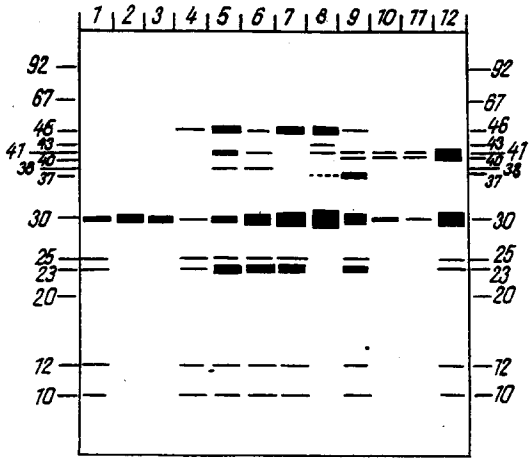

Fig. 1. Spectra of the phosphorylation of the proteins of three-day cottonplant shoots in vitro in the presence of activators and inhibitors. For each lane the activators and inhibitors added to the phosphorylation medium are listed below, while the concentration of each of them and the phosphorylation conditions are described in the Experimental part: 1) control; 2) polymyxin B, nikodipin, trifluoroperazine, leupeptin; 3) polymyxin B, trifluoroperazine, leupeptin; 4) Ca^{2+}; 5) phosphatidylserine, Ca^{2+}; 6) phosphatidylcholine, Ca^{2+}; 7) phosphatidylcholine, Ca^{2+}, nicodipin; 8) calmodulin, Ca^{2+}, polymyxin B, leupeptin, trifluoroperazine; 9) calmodulin, Ca^{2+}; 10) cAMP, polymyxin B, trifluoroperazine, leupeptin, EDTA; 11) cAMP, EDTA; 12) cAMP

If we consider each lane on the electrophoretogram, reflecting the phosphorylation of proteins under the given conditions with the given activator or inhibitor, it can clearly be seen that the protein phosphorylation spectrum has an evident variability. In this connection it must be mentioned that the P_i-proteins with molecular masses of ~25, 23, 12, and 10 kDa behaved similarly and disappeared when inhibitors were added to the phosphorylation medium (lanes 2, 3, 8, 10, 11). This suggests that these proteins are not substrates for the Ca^{2+}-dependent protein kinase, and thata protein kinase the activity of which does not depend on Ca^{2+} is reponsible for their phosphorylation. In the given case, polymyxin B and trifluoroperazine were added to the reaction mixture; i.e., protein kinase C and Ca^{2+}-dependent and Ca^{2+}-calmodulin-dependent protein kinases were inhibited. However, since c-nucleotide-dependent protein kinases remained uninhibited in the reaction medium, the possibility of the phosphorylation of proteins by these protein kinases is not excluded. On the whole, however, some correlation is observed in the spectra of the Pi-proteins for lanes 2, 3, 10, and 11.

In the case of direct activation with the aid of cAMP (lane 12), a Pi-protein with a molecular mass of ~40 kDa appeared, and this was present in traces in lanes 9, 10, and 11, as well, so that it is difficult to consider the corresponding nonphosphorylated protein as an endogenous substrate for the cAMP-dependent protein kinase since, in any case, its phosphorylation nevertheless depends on Ca^{2+}, otherwise we should have detected it also in lanes 2 and 3.

It is known that a special role in the phosphorylation reaction is played by the bivalent ions Ca^{2+} and Mg^{2+}. Thus, Mg^{2+} exerts a concentration influence, changing the whole phosphorylation spectrum [13]. Ca^{2+} likewise inhibits cAMP-dependent protein kinase [14]. In our experiments the concentrations of Mg^{2+} and Ca^{2+} ions were constant (20 and 1 mM, respectively).

In the sample corresponding to lane 4, we added only Ca^{2+} as activator. Here and below (lanes 4-9) we observed the phosphorylation of a protein with a molecular mass of 46 kDa. Thus, this protein may be assigned to t.e specific substrates of Ca^{2+}-dependent protein kinases. The question of to which particular one remains open, in spite of the fact that the level of phosphorylation was everywhere different.

A protein with a molecular mass of 41 kDa was phosphorylated both in samples where activation took place with the aid of cAMP and in samples activated by calmodulin, which clearly shows that the pathways of the action of protein kinases activated by calmodulin and by cAMP are similar. The same phenomenon is observed in the phosphorylation of phospholamban in animal cells [15], where it has been found that amino acid residues phosphorylated under the action of cAMP-dependent protein kinase (serine) and of Ca^{2+}-calmodulin-dependent protein kinase (threonine) are present in close proximity. At the same time, the phosphorylation of one of the residues does not affect the phosphorylation of the other. It is not excluded that in this case we have a similar mechanism.

Particular interest is presented by the appearance of calmodulin-dependent protein kinase activity in samples 8 and 9. It is known that trifluoroperazine, or triftazin, is a selective inhibitor of calmodulin-dependent protein kinase and does not affect the activity of protein kinases activated by Ca^{2+} and phospholipids [16]. If we compare the spectra of the proteins (lanes 8 and 9) we can clearly trace the presence in lane 9 of a specific substrate of calmodulin-dependent protein kinase with a molecular mass of ~37 kDa that is absent from the other samples. Although there are reports [14] in which the possibility of the functioning of calmodulin without calcium ions is shown, the omnipresent calmodulin functions as an intracellular receptor of this ion and participates in the regulation of many physiological-biochemical reactions realized in the presence of Ca^{2+} [17,18].

In our case we have to deal with a specific substrate of calmodulin-dependent protein kinase. The inhibition of calmodulin on the addition of trifluoroperazine, when this ~37 kDa band does not appear, is clearly shown. At the same time, the presence in this sample of inhibitors of protein kinase C and of Ca^{2+}-dependent protein kinases shows the action of cAMP-dependent protein kinases (40 kDa band), which clearly appears in another case when only cAMP was added to the medium (lane 12).

It is particular desirable to mention the difference in the phosphorylation of proteins (lanes 8 and 9) where the reaction took place in the presence of calmodulin and Ca^{2+} (lane 9) and also of polymyxin B, leupeptin, and trifluoroperazine (lane 8). There was practically no phosphorylation of proteins with molecular masses of less than 30 kDa in lane 8, while in lane 9 the whole spectrum of these proteins remained the same as in lanes 5,6, and 7, and the phosphorylation of proteins with a molecular mass of 43 kDa was shown only in lane 8. Here, the phosphorylation of the 37 kDa protein was insignificant in comparison with that in lane 9. Futhermore, the level of phosphorylation of the 30 kDa proteins was a maximum. This can be explained only by the presence of inhibitors.

One would have thought that the addition of phosphatidylcholine to the medium in place of phosphatidylserine would change nothing; however, we see that the levels of phosphorylation of the proteins with molecular masses of 46,41, 38, and 30 kDa did change (lanes 5 and 6).

The facts presented show the particiption of various protein kinases in the system of the regulation of intracellular processes in the cotton plant and permit us to establish the existence of Ca^{2+}-, Ca^{2+}-phospholipid- and Ca^{2+}-calmodulin-dependent, and also cAMP-dependent, protein kinases. Specific susbtrates for these protein kinases have been revealed. It has been demonstrated that the pathways of the realization of the action of cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases may intersect, thereby exerting extremely different influences on identical processes.

EXPERIMENTAL

Homogenization. Three-day shoots of a cotton plant of the variety C-6524 grown at room temperature in the dark were used. Homogenization of the unpigmented part of the shoots was conducted at 4°C in a medium containing; 50 mM Tris-HCl, pH 6.5; 1 mM EDTA; 1 mM PMSF; 5 mM 2-mercaptoethanol; and 1% of Triton X-100 in a porcelain mortar. The homogenate was centrifuged at 10,000 *g* for 20 min, and the supernatant was used for the phosphorylation reaction.

Phosphorylation of the Proteins. In vitro phosphorylation was carried out at room temperature for 5 min. To the reaction mixture were added 20 mM $MgCl_2$, 100 μCi of [γ-^{32}P]ATP, and the appropriate inhibitor or activator. The reaction was stopped by boiling the samples for 2 min on the water bath in the buffer for the samples, containing 2% of sodium dodecyl sulfate, 5% of 2-mercaptoethanol, and 20% of glycerol, after which the samples were used in electrophoretic analysis. The following inhibitors were employed: polymyxin B, a protein kinase C inhibitor, in a concentration of 20 μM; trifluoroperazine (triftazin), an inhibitor of calmodulin-dependent protein kinase, 10 mM; and nikodipin, an inhibitor of the Ca^{2+}-dependent protein kinase, 0.1 μM The activators used were cAMP (20 μM), Ca^{2+} (1 mM), phosphatidylserine (20 gamma per point), phosphatidylcholine (20 gamma per point), and calmodulin (10 gamma per point).

One-dimensional Electrophoresis in Polyacrylamide Gel. The analytical separation of the proteins was conductd in 12% polyacrylamide gel by Laemmli's method [19] at an electric field strength of 12 V/cm for 3 h. After the completion of electrophoresis, the gel plate was soaked in 10% trichlorocetic acid solution for 20 min, and then the gel was stained with Coomassie Blue R-250, washed free from unbound dye, and dried in a special chamber under vacuum. Autoradiographic exposure was made on a RT-1 x-ray plate at 20°C for two days.

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