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Protein phosphorylation in intact cultured sycamore (*Acer pseudoplatanus*) cells and its response to fusicoccin

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Fusicoccin (FC), a natural diterpene glucoside able to stimulate electrogenic H^+ extrusion in higher plants, has been shown to stimulate the phosphorylation of a polypeptide of molecular mass approx. 33 kDa in intact cultured cells of sycamore (*Acer pseudoplatanus*). The effect is specific, rapid and insensitive to cycloheximide. The presence of the 33 kDa polypeptide and the stimulation by FC have been observed in SDS-containing cell homogenates and in the microsomal and soluble fractions after cell fractionation.

INTRODUCTION

In mammalian cells, protein phosphorylation is known to mediate a number of intracellular responses to hormonal stimuli (Cohen, 1982; Nishizuka, 1984). Protein kinases, stimulated by Ca^{2+} and calmodulin or dependent on Ca^{2+} and phospholipid, have been recently identified in higher plants (Veluthambi & Poovaiah, 1984; Schäfer *et al.*, 1985), whereas a cyclic AMP-dependent protein kinase has been found in the fungus *Neurospora crassa* (Trevillyan & Pall, 1982). A possible involvement of protein phosphorylation in the action of plant hormones has been scarcely investigated, so that the importance and the generality of this process cannot be assessed.

Among the substances endowed with hormone-like effects, FC is peculiar in that it induces proton extrusion and strong hyperpolarization in higher-plant cells (Marrè, 1979). These responses *in vivo* are rapid and are, in part at least, independent of protein synthesis, thus suggesting a stimulatory effect at the level of the plasmalemma H^+-translocating ATPase [see, for regulation of plasmalemma ATPase, Marrè & Ballarin-Denti (1985)]. This hypothesis is supported by indirect evidence and by some stimulatory effect of FC on membrane-bound ATPase activity (Beffagna *et al.*, 1977; Lurie & Hendrix, 1979), but also by the recent finding that FC is able to stimulate ATP-dependent proton transport in microsomal vesicles (Cleland, 1985; Rasi-Caldogno & Pugliarello, 1985). Since a number of other metabolic responses seem to be consequential on those processes just mentioned, we decided to investigate whether the action of FC involved protein phosphorylation as a possible regulatory mechanism of the ATPase; if so, it would be similar to the phosphorylation-mediated activation of the Na^+/H^+ antiport in mammalian cells (Grinstein *et al.*, 1985).

METHODS

Cell cultures of sycamore (*Acer pseudoplatanus*), derived from a strain kindly supplied by Professor J. Guern (CNRS, Gif-sur-Yvette, France), were grown as described by Leguay & Guern (1975). The experiments were performed with subcultures at exponential phase [grown for 8 days at 25 °C and containing $(400–600) \times 10^3$ cells $\cdot$ ml^{-1}]. Portions of these cultures were filtered on nylon net (25 μm pore diameter) and thoroughly washed on the filter with the incubation buffer (15 mM-Mes/0.4 mM-$CaSO_4$, adjusted to pH 6.5 with KOH). The cells were resuspended in a volume of the same buffer such as to obtain a concentration of 3.3×10^6 cells $\cdot$ ml^{-1}, and the suspension was added with carrier-free [^{32}P]P_i (20 μCi $\cdot$ ml^{-1}). Portions (2.5 ml) of the suspension were pipetted into 25 ml flasks, which were put in a rotary shaker (140 rev./min) at 25 °C. After a 3 h incubation, an ethanolic solution of FC was added. Ethanol was also added to the controls at a final concentration of 0.006%. To stop the incubation, the cells of each flask were rapidly filtered, washed on the filter with 100 ml of distilled water, ground for 60 s (mortar and pestle) in 0.6 ml of the denaturing electrophoretic buffer [10% (w/v) sucrose/4.5% (w/v)SDS/3% (w/v)Tris/1% 2-mercaptoethanol/1 mM-phenylmethanesulphonyl fluoride/0.5% dimethylsulphoxide, adjusted to pH 7 with HCl]. The mortars were washed with 0.2 ml of the same buffer, and the samples, collected in glass tubes, were immediately immersed in a water bath at 90 °C for 10 min. After cooling, the tubes were centrifuged at 10000 *g* for 10 min and the precipitate was discarded. The radioactivity in portions of the supernatants was counted by liquid-scintillation spectrometry.

In the experiments involving cell fractionation, 15 ml samples of the initial suspension were incubated in 200 ml flasks under the above-described conditions. After incubation the cells were washed on the filter with 300 ml of distilled water and ground in 4 ml of the homogenization buffer (0.3 M-sucrose/50 mM-Tris/5 mM-EDTA/2 mM-EGTA/50 mM-KF/5 mM-*p*-nitrophenyl phosphate/0.1 mM-ammonium molybdate/4 mM-2-mercaptoethanol/1 mM-phenylmethane sulphonyl fluoride/0.50% dimethyl sulphoxide, adjusted to pH 8 with HCl). The volume of the homogenate was adjusted to 10 ml with the same buffer. The homogenization and the centrifugal fractionation were performed at 0–4 °C. The 12000–140000 *g* pellets were resuspended in 375 μl of the denaturing

Abbreviation used: FC, fusicoccin.

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Table 1. Influence of FC on acidification of the incubation medium

The cells (approx. 20×10^6) were incubated at 25 °C on a rotary shaker (140 rev./min) in 10 ml of a buffer containing 0.1 mM-Mes/0.4 mM-$CaSO_4$/5 mM-K_2SO_4, adjusted to pH 6.5 with NaOH. FC was added after 45 min of preincubation (0 min). The experiment was repeated for each cell culture used for the phosphorylation experiments, and similar results were obtained.

	pH		
Time(min)...	0	30	100
Control	6.73	6.73	6.80
10 μM-FC	6.73	6.37	5.96

electrophoretic buffer, whereas the 140000 *g* supernatants were supplemented with 4.5% SDS. Both of them were denatured as described above.

The SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970), with 8–18%-(w/v)-acrylamide gradient gels, 1.5 mm thick, and containing 0.5% SDS. The gels were stained for 1 h in methanol/acetic acid/(10:2:1:7, by vol.), formaldehyde containing 0.25% Coomassie Brilliant Blue R-250 and destained in a methanol/acetic acid/water (7:2:11, by vol.). After destaining for 2 h, the gels were transferred to boiling 5% (w/v) trichloroacetic acid for 10 min, left to cool in this solution, and again transferred to the destaining solution, with several changes, until complete destaining. The hot trichloroacetic acid treatment, which hydrolyses acyl phosphates, was necessary to reduce the strong radioactive background and to eliminate spurious bands. The gel portion below the protein front was cut off. The gels, dried under vacuum, were exposed to X-ray film (Kodak X-Omat SO-282), which was developed at −30 °C for about 1 week.

Table 2. Influence of FC on P_i uptake

The cells were incubated for 3 h with [^{32}P]P_i, as described in the Methods section, then 10 μM-FC was added (or not) and the incubation continued for an additional 30 or 100 min. The incubation was stopped and the samples were prepared in the electrophoretic buffer as reported in the Methods section. Countings of radioactivity were performed in triplicate on 50 μl aliquots. The values in parentheses are relative to the control (= 100).

	10^{-3} × Radioactivity (c.p.m.)		
		FC added	
Expt. no.	No FC added (control)	30 min	100 min
1	2800 (100)	2945 (105)	—
2	2945 (100)	3125 (106)	—
3	2640 (100)	—	2955 (112)
4	3045 (100)	2710 (89)	3183 (104)
5	2950 (100)	3350 (113)	3005 (102)
Mean ± S.D....	(100)	(103 ± 10)	(106 ± 5)

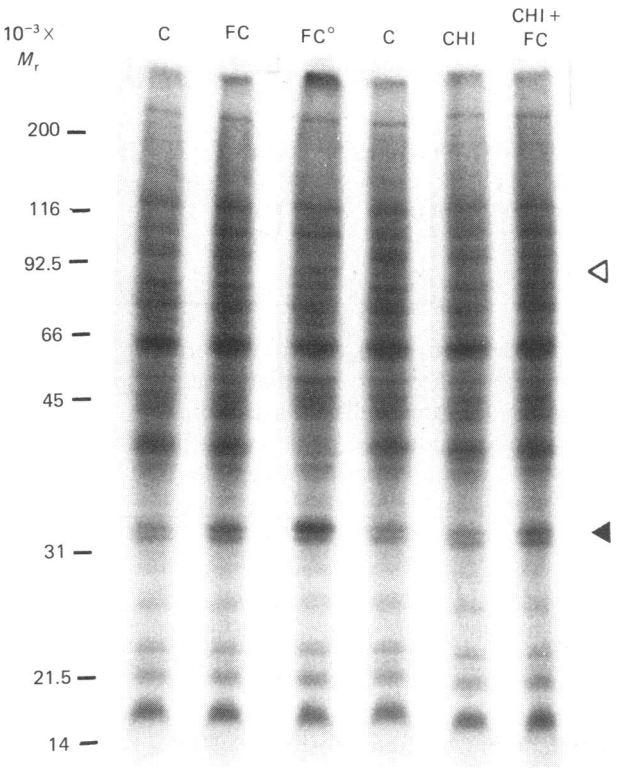

Fig. 1. Effects of FC on protein phosphorylation

The cell incubations and FC additions were performed as reported in Table 2. Cycloheximide was added 20 min before the addition of FC. The cells were homogenized in the SDS-containing buffer and sample volumes with the same radioactivity (1450×10^3 c.p.m. in the exeriment shown) were loaded on the gel. Lane symbols: C, control; FC and FC°, cells incubated with 10 μM-FC for 30 and 100 min respectively; CHI and CHI + FC, cells incubated with cycloheximide (20 $\mu g \cdot ml^{-1}$) and cycloheximide plus FC (30 min) respectively. The triangles indicate the position of the FC-stimulated bands. The molecular masses of these bands, given in the text, represent the mean values ± S.D. for seven experiments. For reference we used the Bio-Rad low- and high-M_r standards. The Figure shows the results of a typical experiment.

RESULTS

As reported (Rollo *et al.*, 1977; Guern *et al.*, 1982), cultured cells of *Acer pseudoplatanus* were sensitive to FC, showing the typical stimulation of medium acidification upon addition of the drug (Table 1). We chose to start investigating protein phosphorylation in intact cells rather than in cell-free systems because cell disruption may alter protein phosphorylation. The use of cultured cells overcomes other limitations encountered in tissues, such as the slow diffusion of FC and the heterogeneity of cells.

Under our experimental conditions (3 h of [^{32}P]P_i loading, followed by 30–100 min incubation with FC), FC did not influence significantly P_i uptake (Table 2). A slight stimulation of P_i uptake by FC had been observed by Lin (1979) and by Ullrich-Eberius *et al.* (1984). Under the present conditions a slight stimulation of P_i uptake by FC may have been abolished by the long period of pre-loading with [^{32}P]P_i.

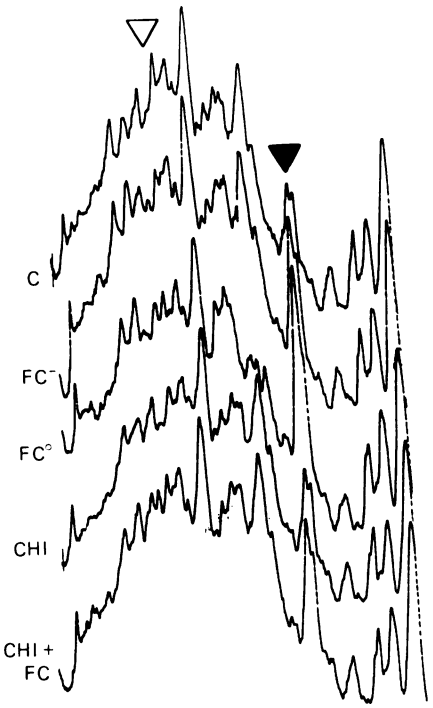

Fig 2. Results of the densitometric scanning of the autoradiograph shown in Fig. 1

Symbols are as in Fig. 1.

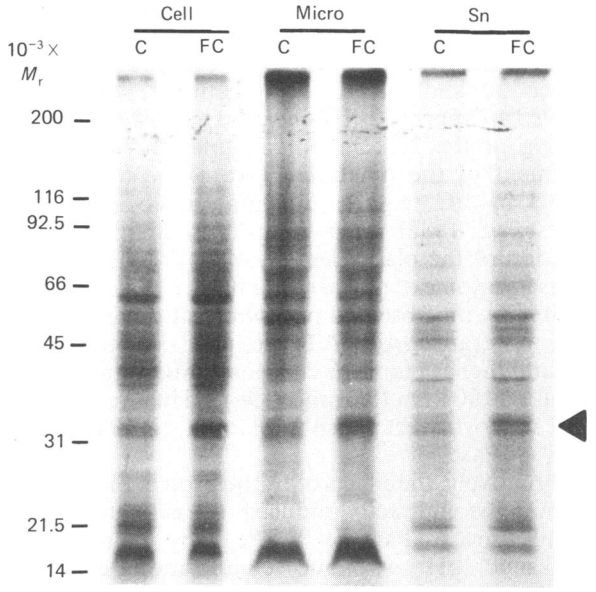

Fig. 3. Effect of FC on phosphorylation of the microsomal and soluble proteins

Cell incubation and FC additions were as reported in Table 2. Symbols: Cell, cell homogenate in SDS-containing buffer; Micro, microsomal fraction; Sn, 140000 *g* supernatant; C, control; FC, 100 min of FC treatment. The samples loaded on to the gel contained the following radioactivities (c.p.m. $\times 10^{-3}$): in the cell homogenate, 2200; in the microsomal fraction, 2400; in the soluble fraction, 1800. The controls and FC samples of each fraction contained the same radioactivity.

The effects of FC added to cell suspensions were first investigated in cell homogenates obtained in the presence of SDS. The SDS-solubilized and -denatured proteins were separated by one-dimensional SDS/polyacrylamide-gel electrophoresis, and their radioactivity was detected by gel autoradiography. The incubation of the cells with FC did not alter the Coomassie Blue-stained protein pattern (result not shown), but stimulated the phosphorylation of a polypeptide band of 33.3 ± 1 kDa (Figs. 1 and 2). An approximate quantification of the FC effects by peak-area calculation and background subtraction gave stimulation values of 60–110% for 30 min of FC treatment (four expts.) and 140–250% for 100 min (three expts.). FC also stimulated the phosphorylation of a feint band of 88 ± 1.4 kDa, which was visible, but not clearly distinguished, by densitometric scanning, owing to the high background in this region. After treatment of cells with FC for 30 min we did not observe any other changes in the phosphorylation pattern. However, we have sometimes noticed a decrease in the amount of label co-migrating with a 40 kDa polypeptide after treatment for 100 min with FC. As shown in Figs. 1 and 2, cycloheximide, added 20 min before FC at a concentration of 20 $\mu g \cdot ml^{-1}$, which blocks protein synthesis in cultured cells (Sung *et al.*, 1981), did not appreciably influence the protein phosphorylation pattern, either in the presence or in the absence of FC. In these experiments the pH of the medium was not changed by FC, owing to the presence of a strong buffer.

We investigated whether the effects of FC treatment *in vivo* were also detectable after cell homogenization in a non-denaturing buffer, followed by centrifugal fractionation. Since FC has been suggested to act at the plasmalemma level, we examined the pattern of phosphorylated proteins in a microsomal fraction (12000–140000 *g* pellet) and in its supernatant. Under our conditions we found the 33.3 kDa phosphorylated polypeptide in both of these fractions, with similar stimulatory effects of FC (Fig. 3).

At present we cannot say whether this distribution may have been influenced by the fractionation procedure or can be taken as an indication of the presence of a membrane-bound and soluble form of the polypeptide.

DISCUSSION

The results show that FC, added to a suspension of cultured plant cells, is able to stimulate the phosphorylation of a polypeptide of 33 kDa. The response is selective and was obtained under conditions in which FC does not show detectable effects on P_i uptake. This result was obtained *in vivo*; therefore it cannot be ascribed to non-specific phosphorylation that may occur upon breakage of cell compartments.

Plant-hormone-influenced protein phosphorylation has been reported by Morrè *et al.* (1984), who observed a 2,4-dichlorophenoxyacetic acid-stimulated phosphorylation of a 45–50 kDa polypeptide in isolated membranes, and by Chapman *et al.* (1975), who described some abscisic acid-induced variations of phosphorylated proteins in nuclei of the duck weed *Lemna*. An influence of plant hormones on protein kinase activity has been reported for naphthalene-1-acetic acid (Van der Linde *et al.*, 1984).

The effect of FC on the phosphorylation of the 33 kDa polypeptide is independent of protein synthesis and might suggest a direct stimulation of a protein kinase. However, between 30 and 100 min of treatment with FC, we observed an approx. 2-fold increase in the FC-stimulated labelling of the 33 kDa polypeptide. By contrast, the attainment of the maximum rate of proton excretion induced by FC requires 3–5 min (Rollo *et al.*, 1977; Guern *et al.*, 1982). Thus this process and, in turn, the activation of the plasmalemma ATPase by FC believed to cause proton extrusion, could hardly be considered as mediated by the observed phosphorylation. The plasmalemma H^+-transporting ATPase, partially purified from fungi and higher plants, is composed of a single catalytic subunit of 100 kDa (Serrano, 1984). The present results do not support direct phosphorylation, stimulated by FC, of this ATPase.

Alternatively, the effects of FC on protein phosphorylation may be indirectly induced by activation of the plasmalemma ATPase by FC, e.g. by changes in the cytoplasmic pH or ionic composition caused by activation of the ATPase. As a third possibility, we cannot exclude at present that the effects of FC on protein excretion and protein phosphorylation are independent.

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