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Phospholipase A_2 affects the activity of fusicoccin receptors

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Biochemical properties of fusicoccin receptors are strongly influenced by the phospholipid environment. In this report we have studied the effect of different exogenous phospholipases on fusicoccin binding ability of both plasma membrane and solubilised receptors. Among the phospholipases tested only phospholipase A_2 showed an inhibitory effect on fusicoccin binding. In particular, the influence of this enzyme on the time course and reversibility of the fusicoccin binding reaction was studied. The inhibitory effect of phospholipase A_2 was the consequence of fatty acid release. The usual fatty acids of plasma membrane phospholipids were active in inhibiting the interaction of fusicoccin with its receptors. It is concluded that a phospholipid associated to the fusicoccin receptor might play a significant role in the modulation of binding.

Fusicoccin; Membrane receptor; *Zea mays* L.

1. INTRODUCTION

Fusicoccin (FC), the main phytotoxin of *Fusicoccum amygdali* Del. [1], mimics the action of an endogenous ligand [2,3], capable of activating plasmalemma H^+-ATPase [4–8]. The first event of this activation is the interaction of the ligand with high affinity receptors localized at the plasma membrane of plant cells [9–11]. They have been extensively characterized and purified by a number of groups [12,13] . In particular, Aducci et al. [14] noticed a marked influence of phospholipids on the binding properties of FC receptors when some biochemical properties of microsome-bound, solubilised and reconstituted receptors both from a monocot and a dicot plant, were compared. The results suggested a role of phospholipids in modulating the time course, the reversibility and the pH dependence of FC binding.

In the last years studies on the metabolism of phospholipids in plants have been stimulated by the results obtained in animal cells, particularly by the role evidenced for some of them as source of second messengers. The occurrence of phospholipases in plants has been reported, but their involvement in physiological processes is poorly defined. Phosphoinositide responses have been observed after exposure to a number of signal molecules such as auxins [15–17], fusicoccin [18], cytokinins [19], and to light [20]. It was also observed that exogenous PLA_2 can activate the plasmalemma H^+-ATPase in oat roots [21] and that its activity can be stimulated by auxin in zucchini [22].

To better evaluate the influence of phospholipids on FC binding sites we have investigated the effect of these enzymes on the interaction between FC and its receptors.

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Abbreviations: FC, fusicoccin; [^{3}H]FC, tritiated dihydro-FC; PL, phospholipase; DAG, diacylglycerol; HPHT, hydroxylapatite; PC, phosphatidylcholine.

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

FC was prepared according to Ballio et al. [23]. Tritiated dihydro-FC ([^{3}H]FC) was obtained as described by Ballio et al. [24]; its specific activity was 20.5 Ci/mmol.

Fatty acids (99% pure by gas chromatography), phospholipids, choline, phosphorylcholine, glycerophosphorylcholine, diacylglycerol, PLA2 from bee venom, PLC from *Bacillus cereus* and PLD type III from peanuts, were from Sigma.

The activity of commercial enzymes was monitored by TLC [25] using [^{14}C]phospatidylcholine-dipalmitoyl (Amersham; specific activity 58 mCi/mmol) as substrate. Radioactivity was measured in a LKB Wallac 1211 scintillation counter using Beckman Ultima Gold scintillation cocktail. HPLC columns were from Bio-Rad.

2.2. *Plant material*

Maize seeds (*Zea mays* L.) var. Paolo, from Italian Dekalb, were surface-sterilised with 3% calcium hypochlorite, extensively rinsed in running tap water and grown hydroponically at 27°C for six days in the dark.

2.3. *Preparation of membrane-bound and solubilised FC receptors*

Plasmalemma-enriched microsomal fractions were prepared as previously described [24]; solubilised FC receptors were prepared according to Aducci et al. [14]. Briefly, microsomal pellets were resuspended in 0.1 volume (based on tissue fresh weight) of water and the suspension was added to 20 times its volume of cold acetone at −15 °C, with stirring. The precipitate was washed with acetone and dried. This

material (30 mg) corresponding to 30 g fresh weight of tissue, was suspended in 1 ml of 10 mM Tris-MES buffer pH 6.5 and shortly sonicated to provide the solubilised preparation of FC receptors.

2.4. *Binding Assay*

[^{3}H]FC binding to plasma membranes, crude and partially purified solubilised FC receptors was carried out according to a previously described procedure [14].

2.5. *Effect of phospholipases and their metabolites on FC binding activity*

The effect of phospholipases on FC binding was determined both on membrane-bound and solubilised FC receptors. 0.5 mg of proteins were incubated for 60 min at 27°C with 1 nM [^{3}H]FC and with 10 U of one of the following commercial enzymes: PLA2, PLC (both dissolved in 10mM Tris-MES buffer pH 6.5, 1 mM $CaCl_2$), PLD (in 10 mM citrate buffer pH 5.5, 1 mM $CaCl_2$). The final volume was adjusted to 200 μl before enzyme incubation.

The effect of metabolites was measured by suspending 30 mM fatty acids and DAG in 50 mM Tris-MES pH 6.5, sonicating the suspensions and adding them to samples containing FC receptors. Their final concentration in FC binding tests was 60 μM for DAG and 100 μM for fatty acids; all other metabolites were dissolved in the same Tris-MES buffer and diluted to a final concentration of 100 μM.

6 ml of crude solubilised fraction (30 mg of total proteins) were fractionated by HPLC according to Aducci et al. [26]. The active fractions from the HPHT column (the first purification step), as well as those from the anion-exchange column (the second purification step) were pooled, concentrated and the partially purified FC receptors (0.1 mg of protein per sample) incubated with phospholipases or the previously mentioned metabolites under the above described conditions.

3. RESULTS AND DISCUSSION

It is well established that the lipid environment strongly influences some biochemical properties of FC receptors [14].

As a further extension of previous work, the effect of phospholipases A_2, C, D on FC binding in membrane-bound and solubilised receptors was investigated. Among the three phospholipases tested, only PLA_2 displays an interesting activity, producing a 40% inhibition of FC binding both with membrane-bound and solubilised receptors. The same effect is exhibited towards receptors partially purified with a procedure recently worked out in our laboratory [26]. Preparations purified by the first step of the procedure, consisting of adsorption chromatography on Bio-Gel HPHT, are again 40% inhibited; the inhibition of FC binding drops to 20% with preparations further purified by anion-exchange on a DEAE column.

As expected for an enzymatic reaction the effect is dependent on time and enzyme concentration. Fig. 1 shows that after 5 min of incubation the association of [^{3}H]FC to the plasma membrane receptors is nearly 40% inhibited relative to the control without added PLA_2. The overall shape of the two curves, drawn for samples respectively with and without added PLA_2, are similar, but at saturation the bound radioactivity is 40% lower in the sample containing PLA_2.

The dependence of binding activity on PLA_2 concentration is reported in Fig. 2. The inhibition increases sharply up to 10 U of enzyme and is hardly influenced by more than 20 U of enzyme. It is lost on PLA_2 inactivation (data not shown).

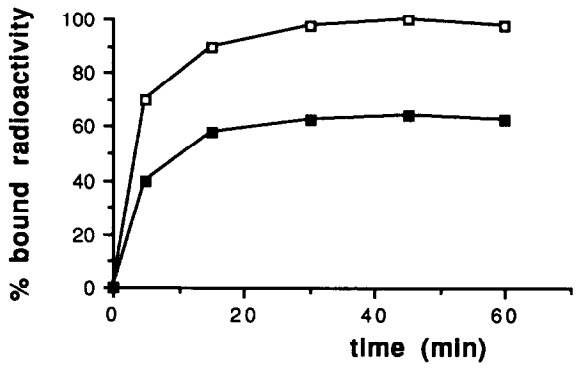

Fig. 1. Time course of FC binding in plasma membrane fractions of maize. Each sample, containing 0.5 mg of proteins, is incubated with 1 nM [^{3}H]FC at 27°C in the presence of 20 U PLA_2 (■) for 60 min in 10 mM Tris-MES buffer pH 6.5, 1 mM $CaCl_2$. Non-specific binding is evaluated by addition of 10 μM unlabelled FC. Control samples do not contain the enzyme (□). The reaction is stopped by centrifuging the samples at 30,000×*g* at 4 °C for 20 min. 100% of bound radioactivity, corresponding to 0.2 pmol [^{3}H]FC, is the maximum of the specifically bound radioactivity. Samples were run in triplicate; variations were less than 10%.

The effect of PLA_2 on FC binding might in fact arise indirectly from the enzymatic release of inhibitor metabolites from endogenous phospholipids. Thus, some of their component fatty acids, lyso-PC and all metabolites derivable from PC by hydrolysis with PLC and PLD were tested for their ability to influence FC binding activity. Results are shown in Table I. All metabolites formed from PC by cleavage with PLC and PLD are ineffective; among the products arising from PC by PLA_2-catalysed hydrolysis, lyso-PC is inactive, but some fatty acids inhibit FC binding. Thus PLA_2, the

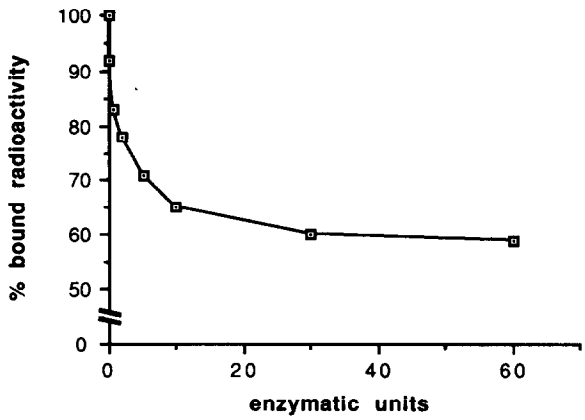

Fig. 2. Dependence of FC binding activity on PLA_2 concentration. Binding conditions and representation of data are the same reported in Fig. 1.

Table I

Effect of different compounds on [^{3}H]FC binding to microsomal and to soluble preparations

Compound	Concentration (mM)	FC binding (% of control)	
		Membranes	Solubilised fractions
Myristic acid (14:0)	0.6	80	80
	0.1	95	100
Myristoleic acid (14:1)	0.6	35	25
	0.1	60	75
Palmitic acid (16:0)	0.6	90	90
	0.1	100	100
Palmitoleic acid (16:1)	0.6	45	45
	0.1	85	80
Stearic acid (18:0)	0.6	75	95
	0.1	95	100
Oleic acid (18:1)	0.6	15	30
	0.1	20	70
	0.06	50	n.d.[a]
Linoleic acid (18:2)	0.6	25	30
	0.1	40	70
Phosphorylcholine	1	100	100
Choline	1	100	100
Glycerophosphorylcholine	1	100	100
Phosphatidic acid	1	100	100
Diacylglycerol	0.6	100	100
Lysophosphatidylcholine	1	100	100

[a]n.d. = not determined.

only PL affecting the interaction of FC with its receptor, promotes the formation of inhibitors of this interaction and might therefore act indirectly through their formation. A comparison of the effects of homologous saturated and unsaturated fatty acids shows that the inhibitory effect is remarkable only when double bonds are present in the hydrocarbon chain. All metabolites inactive on FC binding to membrane-bound receptors are also inactive on binding to solubilised preparations; the latter have a reduced sensitivity to some unsaturated fatty acids in comparison to the binding to microsomal fractions.

Another peculiar aspect of the PLA_2 effect on the binding activity is its ability to dissociate the FC-receptor complex. In fact, 40% of the radioactivity associated to membrane-bound receptors is released on addition of 20 U of PLA_2 at a time when the binding reaction has reached saturation (Fig. 3). A similar result is obtained with 0.6 mM oleic acid, while 1 mM lyso-PC is ineffective. These results are of interest on considering that, at difference with the system FC-spinach receptors, the FC-maize receptor complex is quite resistant to a chase with an excess of FC [14], a behaviour confirmed in the present paper (Fig.3).

As already mentioned, the action of PLA_2 might be ascribed to the fatty acids released from endogenous phospholipids. In fact, they are the only metabolites, among those tested, able to impair FC binding.

The possibility that the inhibition of binding in presence of fatty acids is instead a loss by dissolution of microsomal proteins must be ruled out. In fact, the same inhibition is observed when the binding tests are carried out by a rapid filtration assay to monitor the radioactivity associated to both solubilised and membrane-bound proteins. Moreover, the effect of fatty acids on binding to plasma membrane receptors is still observed with solubilised and partially purified receptors incubated under identical conditions.

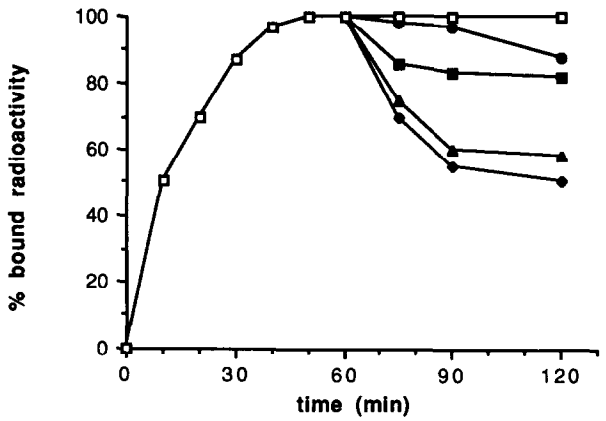

Fig. 3. Effect of FC, LysoPC, PLA_2, oleic acid on the dissociation of the FC-receptor complex. The release of bound [^{3}H]FC from its complex is measured after addition of 0.1 mM lysoPC (●), 1μM FC (■), 20 U of PLA_2 (▲) and 0.6 mM oleic acid (◆) to the saturated receptors. Binding conditions and representation of data are the same as in Fig.1.

The stronger inhibitory activity of cis-unsaturated compared to saturated fatty acids might be explained, at least in part, by their higher solubility. However, extensive sonication or dissolution in propanol of the saturated fatty acids, are unable to increase the inhibition on FC binding up to the level observed with unsaturated fatty acids.

A satisfactory interpretation at the molecular level of the results reported in the present paper would require a structural characterization of the FC receptors which is so far still incomplete. Anyhow some hypotheses can be put forward.

First, the cis-unsaturated fatty acids may affect the receptor activity without a direct interaction with the FC binding site, but rather by combination with an allosteric region.

Second, a role for the metabolites other than fatty acids arising from the enzymatic digestion by PLA_2 cannot be ruled out completely. At difference with the fatty acids, some metabolites might only affect the receptor activity in membranes under physiological conditions and not in a micellar state.

Finally, the PLA_2 effect on FC receptors might also be caused by the hydrolysis of some phospholipids strictly associated to the binding proteins, leading to a change in the microenvironment of the receptors and to a conformational rearrangement of the protein to a

form unable to bind FC. This last hypothesis can also explain why PLA_2 induces a higher release of bound FC from the FC–receptor complex.

Anyhow, the present results, together with previously published data [14], points to the important role of the lipid microenvironment in the regulation of FC receptor activity, perhaps the modification of the structural organization of a lipid–protein complex.

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