Plant Physiol. (1996) 111: 857–865

A Calcium and Free Fatty Acid-Modulated Protein Kinase as Putative Effector of the Fusicoccin 14-3-3 Receptor[1]

Paul C. J. Van der Hoeven[2], Marco Siderius, Henrie A. A. J. Korthout, Artem V. Drabkin, and Albertus H. De Boer*

Faculty of Biology, Department of Molecular and Cellular Biology, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands (P.C.J.V.d.H., H.A.A.J.K., A.H.D.B.); Department of Molecular Cell Biology, BioCentrum, Universiteit van Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands (M.S.); and All-Russian Institute of Agricultural Biotechnology, Timirjazevskajastr. 42, Moscow, Russia (A.V.D.)

A protein kinase that is activated by calcium and *cis*-unsaturated fatty acids has been characterized from oat (*Avena sativa* L.) root plasma membranes. The kinase phosphorylates a synthetic peptide with a motif (-R-T-L-S-) that can be phosphorylated by both protein kinase C (PKC) and calcium-dependent protein kinase (CDPK)-type kinases. Calphostin C and chelerythrine, two PKC inhibitors, completely inhibited the kinase activity with values of inhibitor concentration for 50% inhibition of 0.7 and 30 μM, respectively. At low Ca^{2+} concentrations *cis*-unsaturated fatty acids (linolenic acid, linoleic acid, arachidonic acid, and oleic acid) stimulated the kinase activity almost 10-fold. The two inhibitors of the kinase, calphostin C and chelerythrin, strongly reduced the fusicoccin (FC)-induced H^+ extrusion, and the activators of the kinase, the *cis*-unsaturated fatty acids, prevented [^{3}H]FC binding to the FC 14-3-3 receptor. CDPK antibodies cross-reacted with a 43-kD band in the plasma membrane and in a purified FC receptor fraction. A polypeptide with the same apparent molecular mass was recognized by a synthetic peptide that had a sequence homologous to the annexin-like domain from barley 14-3-3. The possibility of the involvement of a kinase, with properties from both CDPK and PKC, and a phospholipase A_2 in the FC signal transduction pathway is discussed.

Plant cell PMs contain a family of multifunctional H^+-ATPases (Sussman, 1994). These H^+-ATPases provide the driving force for ion and solute uptake, control the cytoplasmic pH, lower the cell-wall pH to enable cell elongation, and control the activity of voltage-sensitive transporters by means of the electrical membrane potential. In view of these numerous functions, it is not surprising that the activity of the pumps is controlled in a number of ways (Palmgren, 1991). Tight control is exerted by the C terminus of the pump, which acts as an autoinhibitor; a series of phosphorylation sites in this domain are potential control points. Also, products of phospholipase A_2, such as lysophosphatidylcholine and free fatty acids, regulate the activity of the pump (Palmgren et al., 1988), but the mode of action is not yet known.

The phytotoxin FC induces a strong proton extrusion in many plant tissues by stimulation of the H^+-ATPase. Hager et al. (1991) demonstrated that the mechanism of FC action on the ATPase is by activation of an inactive pool of ATPases rather than by de novo synthesis of new proton pumps. FC modulates the autoinhibitory C-terminal domain of the H^+-ATPase, which results in an increase of the V_{max} and a decrease of the K_m (Johansson et al., 1993; Rasi-Caldogno et al., 1993; Lanfermeijer and Prins, 1994). How this inhibitory domain is modulated by FC is not known, but it may involve phosphorylation/dephosphorylation. Schaller and Sussman (1988) demonstrated that the ATPase is a substrate for a CDPK in vitro and the phytotoxin syringomycin activates a kinase that is likely to phosphorylate the proton pump (Bidway and Takemoto, 1987).

FC does not seem to act through direct binding to the pump but rather indirectly through association with a binding protein (FCBP) located in the PM. Recently, the FCBP was identified as a member of the so-called 14-3-3 protein family (Korthout and De Boer, 1994; Marra et al., 1994; Oecking et al., 1994). A common theme of 14-3-3 proteins is the regulation of protein kinases through direct association with these kinases (Aitken, 1995). Among these kinases are PKC (Toker et al., 1992), Raf kinase (Freed et al., 1994), and Bcr kinase (Reuther et al., 1994). The phosphorylation of the H^+-ATPases by calcium-dependent kinases and the fact that the FCBP is homologous to 14-3-3 proteins indicate a role for protein kinases in signal transduction from the FCBP to the ATPase.

The plant PM contains so-called CDPKs (Roberts and Harmon, 1992), which are characterized by three major

[1] This work was supported by the Technical Science Foundation. A.H.D.B. was supported by the International Association for the Promotion of Cooperation with Scientists from the New Independent States of the Former Soviet Union (grant no. 94–4358) and the Russian Basic Research Foundation (grant no. 94–04–11217).

[2] Present address: Division of Cellular Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

* Corresponding author; e-mail ahdeboer@bio.vu.nl; fax 31–20–444–7123.

Abbreviations: CDPK, calcium-dependent protein kinase; ECL, enhanced chemoluminescence; FC, fusicoccin; FCBP, FC-binding protein; [^{3}H]FC, tritiated dihydrofusicoccin; IC_{50}, inhibitor concentration for 50% displacement; MJA, methyl jasmonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PLA_2, phospholipase A_2; PM, plasma membrane; PMA, phorbol-12-myristate-13-acetate; PS, phosphatidylserine.

functional domains: (a) a catalytic domain homologous to protein kinases, (b) a C-terminal domain resembling calmodulin, and (c) a junction domain containing an autoinhibitor (Harmon et al., 1994; Harper et al., 1994). The ability of CDPKs to respond to cytosolic calcium changes enables them to function in calcium-regulated signal transduction processes. Nevertheless, a clear role for these kinases in signal transduction pathways still has to be demonstrated. Since calcium is essential for the phosphorylation of the H^+-ATPase (Schaller and Sussman, 1988), CDPKs are likely candidates to mediate the FCBP to ATPase signaling.

In this study we addressed the question of whether membrane-bound kinases play a role in the mechanism of FC signaling. Complex formation of the FC 14-3-3 receptor with kinases was studied as a potential mechanism in signal transduction.

MATERIALS AND METHODS

Two hundred grams of oat (*Avena sativa* L. cv Valiant; H. Blonk C.V., Moerkapelle, The Netherlands) seeds were rinsed in running tap water for 45 min and sown on a stainless steel screen (26 × 45 cm) 1 cm over a 1 mM $CaSO_4$ solution (1.5 L). The solution was renewed daily. The seedlings were sprayed twice a day with the $CaSO_4$ solution. The plants were grown in the dark at 25°C. After 6 d of growth, roots were harvested by cutting them off with a razor blade at 4°C. The roots were weighed and washed with demineralized water. Typically, about 150 g of roots were harvested for every 200 g of seeds.

Treatment of Roots with FC

Prior to the isolation of PM vesicles, the roots were transferred to containers containing aerated $CaSO_4$ solution with or without 10 μM FC and incubated for 30 min at room temperature. PMs that were treated with FC will be referred to as FC-treated vesicles or U_3FC. Untreated PMs will be referred to as PMs or U_3.

Medium Acidification by Coleoptiles

Six-day-old coleoptiles were abraded with silicium carbide and preincubated in 1 mM potassium phosphate buffer, pH 6, 1 mM $CaCl_2$, and 5 mM $MgSO_4$ for 30 min at room temperature. From the 5- to 6-cm-long coleoptiles, two to three sections of 15 mm were cut. Ten of these sections were put in 3 mL of the same buffer in the presence of kinase inhibitors at concentrations indicated and preincubated for 30 min in the light of a luminescent tube at 30°C. This was necessary because the action of calphostin C is light dependent (Bruns et al., 1991). FC was added from a 100 μM stock solution (solvent: water containing 10% methanol) to 1 μM final concentration or solvent as control. The pH of the medium was maintained at 6.0 by titration with 5 mM KOH at 30- to 45-min intervals for 120 min. Chelerythrine was diluted in water from a 1 mM water stock solution. When chelerythrine was used, the medium was adjusted to pH 6.0 with 5 mM KOH before the coleoptile sections were added to the buffer (thereby increasing the K^+ concentration to 1.13 mM). Calphostin C and staurosporine were diluted in water from a 1 mM DMSO solution; DMSO had no effect on acidification.

PM Preparation

All steps were carried out at 0 to 4°C. The roots (100 g) were ground in a Braun blender with 100 mL of buffer I (10 mM Tris-HCl [pH 7.5], 250 mM Suc, and 1 mM Na_2EDTA). The homogenate was filtered through four layers of cheesecloth polyamide sieve gauze (pore size 0.64 μm). The material remaining in the cloth was re-extracted with 100 mL of buffer I. PMSF (0.2 M dissolved in methanol) was added to the filtrate to a 1 mM final concentration. The pooled filtrates were centrifuged for 20 min at 10,000*g* in a fixed angle rotor. The resulting supernatant was centrifuged for 30 min at 100,000*g* in a fixed angle rotor to obtain the microsomal fraction as a pellet; the remaining supernatant is called the cytosolic fraction. This cytosolic fraction was frozen in liquid nitrogen and stored at −20°C. The pellets were resuspended in buffer II (5 mM potassium phosphate [pH 7.8], 250 mM Suc, and 4 mM KCl) in a volume of 5 to 6 mL for every 200 g fresh weight roots. Aliquots of this preparation were frozen in liquid nitrogen and stored at −85°C; the rest was further purified in an aqueous two-phase partitioning system. Briefly, the resuspended microsomal fraction was added to a 30-g two-phase partitioning system (30 g for every 300 g fresh weight roots), resulting in a final concentration of 6.5% (w/w) dextran T500, 6.5% (w/w) PEG (PEG 3350), 250 mM Suc, 5 mM potassium phosphate (pH 7.8), and 4 mM KCl. After the sample was mixed, phases were separated by centrifugation for 10 min at 1,000*g*. The upper phase (U_1) was then mixed with fresh lower phase, the system was centrifuged again, and the U_2 was repartitioned against a fresh lower phase to yield U_3. Likewise, the lower phases (L_1, L_2, and L_3) were partitioned against fresh upper phase added initially to L_1 to yield U'_1, U'_2, and U'_3. U_3 and U'_3 were pooled and diluted 10 times in buffer III (10 mM Tris-HCl [pH 7.5] and 250 mM Suc), pelleted by centrifugation at 100,000*g* for 30 min, resuspended in buffer III to a concentration of about 5 to 8 mg/mL protein, frozen in liquid nitrogen, and stored at −85°C. Typically, 1 g fresh weight roots yielded 30 μg of PM protein. The purity of the PM preparations was evaluated as described by Korthout et al. (1994).

FC-Binding Assay

FC binding was performed in glass tubes in 20 mM Mes-Tris buffer, pH 6.0, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 2.3 mM DTT, 0.2 mM PMSF, 20% (v/v) glycerol, and different concentrations of calphostin C or fatty acids diluted in water from 1 mM DMSO stock solutions. In the case of calphostin C, the PM vesicles were preincubated for 30 min at 30°C under a luminescent tube just before the start of the assay. The binding assay was started with about 2 nM [^{3}H]FC, the final incubation volume was 0.5 mL, and incubation was performed at 30°C in a shaking water bath, 0.005% (w/v) Triton X-100 was added to the binding buffer. The assay contained 1 μg of purified PM protein. The incubation was terminated by adding 3 mL of ice-cold

washing buffer (25 mM Gly KOH, pH 9.5) to the tubes, and rapid filtration was performed through polyethylenimine (1%, w/w) pretreated Whatman GF/B filters, which were washed twice with 5 mL of washing buffer just before filtration. After filtration the filters were washed twice with 4 mL of washing buffer. The whole procedure of filtering and washing took about 30 s. A Brandel (Gaithersburg, MD) filtration apparatus was used, which made it possible to filter 24 samples at the same time. The filters were transferred to 5 mL of Pharmacia OptiPhase HiSafe 3 cocktail. After 3 d of extraction and shaking the samples daily, the radioactivity bound to the filters was measured for 5 min with a LKB Wallac (Turku, Finland) Rackbeta 1219 liquid scintillation counter.

Protein Kinase Assay

Protein kinase activity was assayed using the PepTag nonradioactive PKC assay kit (Promega). This assay uses a fluorescently labeled peptide substrate with the sequence P-L-S-R-T-L-S-V-A-A-K (peptide I). The underlined motif in peptide I can be phosphorylated by both PKC and CDPK. Phosphorylation of peptide I alters the peptide net charge from +1 to −1. The change in the net charge allows the phosphorylated and unphosphorylated forms of the peptide to be separated on agarose gel electrophoresis. The phosphorylated peptide migrates toward the anode and the unphosphorylated species migrates toward the cathode. The kinase activity was assayed in a final volume of 25 μL containing 20 mM Hepes, pH 7.4, 1.3 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 0.005% (w/v) Triton X-100, and 2 μg of peptide I (1.2 nmol). Unless otherwise indicated, the assay contained 0.2 mg/mL lipid or fatty acid. The lipid (5×) stock solution was sonicated prior to the assay for 20 s using a sonicator (Soniprep 150, MSE Scientific Instruments, Manor Royal, Crawley, West Sussex, UK). Rat brain PKC (as a positive control included in the kit) was diluted 10-fold prior to the assay in PKC dilution buffer (1 mM DTT, 20 mM potassium phosphate [pH 7.5], 50% [v/v] glycerol, 20 mM Tris-HCl [pH 7.5]) and 4 μL of this solution (10 ng of PKC) were added to the assay. The PM (5×) sample (5 μL) contained 20 mM Tris-HCl, pH 7.5, 5 μM okadaic acid to inhibit phosphatases, 5 mM PMSF, and 0.25 μg of PM protein. The assay was performed at 30°C, run for 30 min, started with the addition of peptide I, and stopped by heating the samples to 95°C for 10 min. Then, the mixture was cooled to 4°C and 1 μL of 80% glycerol was added to the samples to ensure that it remained in the gel-slot well.

Separation and Quantification of Phosphorylated Peptide I by Electrophoresis

Samples (26 μL) were loaded on a precooled 0.8% (w/v) agarose gel in 50 mM Tris-HCl, pH 8.0 (well size 3 × 1 × 10 mm), and electrophoresis was performed for 25 min at 100 V. The electrophoresis was done at 4°C to minimize diffusion of the peptide in the surrounding agarose. From each sample both the phosphorylated and unphosphorylated bands were cut from the gel, transferred to Eppendorf vials, and centrifuged for 20 s in a microfuge. Then, the volume of the gel pieces was adjusted to 1 mL with gel solubilization solution (Promega, included in the PKC assay kit). The vials were heated for about 10 min to 95°C and vortexed to dissolve the agarose. After the vials were cooled to room temperature, they were centrifuged again for 1 min, and the dissolved agarose was transferred to cuvettes (1 mL). The amount of peptide I present in each vial was quantified by dual-wave spectrophotometry by reading the A_{570} and A_{480} as references on a DW2a UV/Vis spectrophotometer (Aminco, Silver Spring, MD). The spectrophotometer was zeroed with liquefied agarose containing no peptide I.

Calculation of Specific Activity

According to the PepTag assay protocol, only one phosphorylation site exists on each peptide for PKC. Therefore, in the case of PKC the moles of peptide I present in the negatively charged, phosphorylated bands are equivalent to the number of moles of phosphate transferred. For calculation of the specific kinase activity in our experiments, we assumed that the same ratio is valid, because CDPK from different sources phosphorylate several proteins and peptides having a Basic-X-X-Ser motif, where X is any residue. This motif is present in peptide I (R-T-L-S). The specific activity was calculated according to the manufacturer's instructions (Promega), with some modifications.

First, the molar extinction coefficient ϵ was calculated for the system used by measuring the absorbance of 1.2 nmol of nonphosphorylated peptide in dissolved agarose:

$$\epsilon = \frac{A_{570} - A_{480}}{\text{molpeptide} \times \text{L}^{-1}} = 8.33 \times 10^5 \times (A_{570} - A_{480}) \quad (\text{AU} \times \text{mol}^{-1} \times \text{L}). \quad (1)$$

Second, the absorption of phosphorylated peptide I in the negatively charged bands in agarose gel were normalized for losses during gel loading by multiplying this absorption with a normalizing factor $nf = A/B$, where A = absorption of nonphosphorylated peptide I excised from the gel and B = total absorption of a sample as excised from the gel (i.e. phosphorylated plus unphosphorylated band). This yields the corrected absorption of a sample: $A_{570}{}^* = A_{570} \times nf$.

The kinase activity was calculated according to:

$$\text{Kinase activity} = \left(\frac{A_{570}{}^* \times \text{L}}{\text{g protein} \times \text{min} \times \epsilon}\right) \quad (\text{mol Pi} \times \text{g protein}^{-1} \times \text{min}^{-1}). \quad (2)$$

Kinase Activity in the Presence of Fatty Acids, Lipids, Calcium, and Inhibitors

To examine the lipid-dependent kinase activity, lipids and fatty acids were dissolved in chloroform (PS, PC, and PE) or 50 mg/mL DMSO (myristic acid, palmitic acid, stearic acid, arachidonic acid, linolenic acid, linoleic acid,

oleic acid, MJA, and PMA) or water (PA and FC) and dried under nitrogen (in the case of PS, PC, and PE), and the lipids and fatty acids were resuspended in water to a 1 mg/mL 5× stock solution by sonication (20 s without cooling). The standard assay contained 1.3 mM Ca^{2+}. For the calcium dependency experiments, exogenous calcium was added in the form of $CaCl_2$. Calcium was buffered with 1 mM EGTA, and the amount of free calcium was calculated using the dissociation constant for Ca^{2+}-EGTA at pH 7.4. Calphostin C is a potent inhibitor of PKC that acts on its regulatory site, and the inhibition is light dependent (Kobayashi et al., 1989; Bruns et al., 1991). Chelerythrine is a PKC inhibitor that acts on its catalytic site (Herbert et al., 1990). A 1 mM DMSO stock solution of calphostin C or a 20 mM Tris-HCl (pH 7.4) buffered solution of 1 mM chelerythrine was diluted in water or 20 mM Tris-HCl, pH 7.4, respectively, to 10 times the concentration needed in the assay. These solutions (2.5 μL) were added to the standard protein kinase assay buffer together with the U_3 sample and illuminated for 10 min at room temperature to activate the calphostin C. Then the samples were brought to 30°C, and the assay was started with peptide I. The effect of staurosporine was measured in the same way except for the illumination.

Gel Electrophoresis and Western Blotting

Proteins were analyzed by SDS-PAGE using a a 10% acrylamide gel and a 5% stacking gel on a Bio-Rad Protean II xi gel system, according to the method of Laemmli (1970). Proteins resolved by SDS-PAGE were transferred to nitrocellulose filters by electroblotting using a Tris-Gly buffer. Transfer conditions were 30 V and 40 mA overnight at room temperature. The blots were blocked with 4% (w/v) BSA in Tris-buffered saline (TBS-T: 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The blots were probed with the following: (a) Monoclonal anti-CDPK antibodies (1:1000 dilution; 12G8 and 14G5, mixed 1:1 mouse IgG). Then the blots were washed with TBS-T three times. The secondary antibody was a goat anti-mouse-IgG-conjugated horseradish peroxidase (included in Amersham, ECL protein gel blotting detection kit) and was used in 1:5000 dilution. The detection system that was used was an ECL system (Amersham, Buckinghamshire, UK). Chemoluminescence was visualized by short exposure (15–30 s) to blue-light-sensitive autoradiography film (Hyperfilm, enhanced chemiluminescence, Amersham). (b) Anti-14-3-3 antibody (rabbit IgG) raised against the *BMH1* translation product, encoding a 14-3-3 homolog in *Saccharomyces cerevisiae* (1:5000 dilution), and the secondary antibody was included in the ECL kit. (c) PKC antibodies (1:1000 dilution; Boehringer; catalog no. 1428198, mouse IgM, clone 1.9). The secondary antibody was peroxidase-conjugated goat anti-mouse IgM from Sigma (A 8786) used at 1:5000 dilution.

When the same blot was probed with different antibodies, the blot was stripped by submerging in 100 mM mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, and incubated for 30 min at 50°C, then washed thoroughly with TBS-T buffer, blocked, and reprobed again. The sequence of probing was: anti-CDPK, anti-14-3-3, and anti-PKC.

Overlay Assay with the "Annexin-Like" 14-3-3 Peptide

An annexin-like peptide with the sequence biotin-G-D-Y-H-R-Y-L-A-E-F-K-T-G-Q-E (peptide II) was synthesized by Eurosequence bv (Groningen, The Netherlands). The sequence of peptide II was derived from the barley 14-3-3 homolog cloned by Brandt et al. (1992); underlined amino acids were identified in the 31-kD subunit of the oat FCBP (Korthout and De Boer, 1994). The overlay assay was modified after Mochly-Rosen et al. (1991): PM proteins (20 μg) were run on SDS-PAGE and blotted as described above. Next, proteins on the blot were renatured overnight at 4°C with the renaturation buffer: 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaSO_4$, 1 mM DTT, 0.3% Tween 20, and 5% BSA (0.1 mM $ZnSO_4$ was included when renaturation took place in the presence of Zn^{2+} ions). The blot was exposed to the peptide (10^{-5} M) in renaturation buffer for 1 h at room temperature and thereafter washed three times for 10 min each time. Biotinylated peptide II remaining on the blot was visualized using streptavidin conjugated to horseradish peroxidase and the ECL method (see above).

Protein Determination

Protein concentrations were measured using the bicinchoninic acid protein assay reagent (Pierce) with BSA as the standard.

Materials and Chemicals

Calphostin C, chelerythrine, staurosporine, PMA, and okadaic acid were from Biomol Research Laboratories (Plymouth Meeting PA) and were dissolved in DMSO except for chelerythrine. PC, PS, and PE were from Avanti Polar-Lipids (Alabaster AL). All saturated and unsaturated fatty acids and PA were from Sigma. MJA was from Bedouhian Research (Danbury, CT). FC was obtained from Professor Muromtsev (All-Russian Institute of Agricultural Biotechnology, Moscow) and stored in a 1 mM methanolic stock solution at −20°C. The PepTag PKC assay kit was from Promega.

RESULTS

Effect of Kinase Inhibitors on FC-Induced Proton Extrusion

To answer the question of whether a kinase mediates the FC signal, the in vivo effect of several kinase inhibitors was studied on the FC-induced proton extrusion (reflecting increased H^+-ATPase activity). H-7 (concentration 10^{-8}–10^{-4} M) and staurosporine (concentration 10^{-9}–10^{-5} M) both act on the catalytic domain of kinases. H-7 had no effect and staurosporine slightly reduced the effect of FC (data not shown). It is interesting that two typical kinase C inhibitors, calphostin C and chelerythrine (acting on the regulatory and catalytic domain, respectively), effectively reduced the FC-stimulated H^+ extrusion (Fig. 1); IC_{50} values for inhibition were 1 and 10 μM, respectively. Calphos-

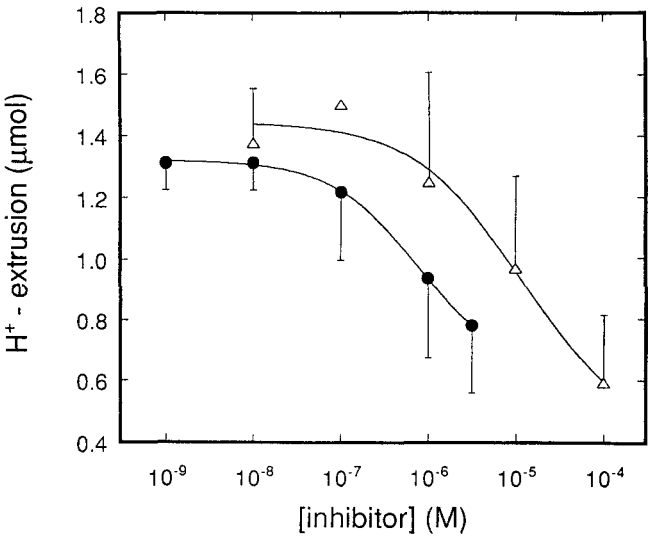

Figure 1. Inhibition of FC-induced proton extrusion by kinase inhibitors. Oat coleoptiles were preincubated for 30 min with calphostin C (●) or chelerythrine (△), and then FC was added (1 µM). In control experiments, the inhibitors had no effect on acidification, which was approximately 0.4 µmol of H^+ per assay. Results are the means ± SD of two experiments.

tin C or chelerythrine alone (controls without FC) had no effect on the proton extrusion. Note that under these control conditions H^+ extrusion was approximately 0.4 µmol of H^+ per assay, irrespective of the amount of inhibitor present.

Effect of Kinase Inhibitors on PM Kinase Activity

Since calphostin C and chelerythrine are known PKC inhibitors, we used a PKC assay kit with a fluorescently labeled peptide as substrate to study kinase activity in the PM. The phosphorylation motif in this peptide (PLSRTLSVAAK; motif is underlined) is identical with the motif in syntide-2 (PLARTLSVAGLPGKK), a peptide preferentially phosphorylated by CDPK (Harmon et al., 1994). Phosphorylation of the peptide by purified oat root PM increased linearly in the range from 0 to 0.5 µg of PM (Fig. 2, inset). Therefore, in all further experiments 0.25 µg of PM protein was used to ensure that the amount of peptide was not limiting. Clearly, the oat root PM contains kinase(s) capable of phosphorylating the synthetic peptide. To investigate whether the kinase(s) responsible for phosphorylation of the peptide is the same as that involved in the FC signaling, we measured the kinase activity in the presence of calphostin C and chelerythrine (Fig. 2). Both inhibitors effectively inhibited the kinase activity with IC_{50} values of 0.7 and 30 µM, respectively. Also, staurosporine (concentration 10^{-8}–10^{-5} M) reduced the kinase activity, but H-7 (concentration 10^{-8}–10^{-4} M) was ineffective (data not shown). The fact that no residual kinase activity was left at higher calphostin C and chelerythrine concentrations (Fig. 2) suggests that the peptide is specifically phosphorylated by one class of kinase(s) with PKC- and CDPK-like properties.

Calcium Dependency of the Kinase Activity

Both CDPK and PKC enzymes are stimulated by cytosolic Ca^{2+}, and also the kinase responsible for the phosphorylation of peptide I was clearly Ca^{2+} dependent; half-maximal stimulation was reached at 10^{-7} M Ca^{2+} (Fig. 3A). To test the idea that FC modulation of the calcium dependency of the kinase might be responsible for the FC modulation of the H^+-ATPase after in vivo treatment of the roots (Lanfermeijer and Prins, 1994) Ca^{2+} dependency of kinase activity of the latter roots was studied as well. However, the calcium dependency of the kinase was unchanged after in vivo FC treatment (Fig. 3A).

If the kinase has an intrinsic calmodulin domain like CDPK-type kinases, one might expect an inhibitory effect of calmodulin antagonists. At 10^{-3} M, the calmodulin antagonist trifluoperazine reduced the kinase activity by 50%, and a series of prenylamine derivatives, synthesized for calmodulin inhibition (Caldirola, 1992), inhibited up to 60% of the kinase activity at 10^{-4} M (data not shown). These assays were done in the presence of high calcium. The concentration of trifluoperazine necessary to inhibit 50% of the kinase activity is higher than reported by others for CDPKs (Abo-El-Saad and Wu, 1995), which may be due to the different substrates used.

Kinase Activity Is Modulated by *cis*-Unsaturated Free Fatty Acids

The PM H^+-ATPase is modulated by free fatty acids in such a way that the pH optimum of the enzyme shifts to more alkaline values, the K_m decreases, and the V_{max} increases (Palmgren et al., 1988). The FC-modulated H^+-

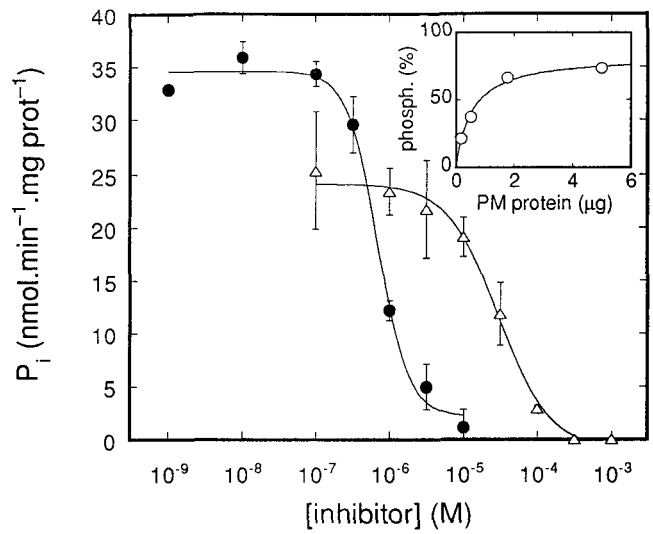

Figure 2. Inhibition of oat root PM protein kinase activity by calphostin C (●) and chelerythrine (△). PM vesicles were preincubated with calphostin C or chelerythrine for 10 min in standard protein kinase assay buffer containing 0.2 mg/mL arachidonic acid as activator. Results are the means ± SD of two experiments. The inset shows the percentage of phosphorylated peptide I (phosph.; total amount 1.2 nmol) in dependence of the amount of PM protein added to the assay. prot, Protein; P_i, Pi.

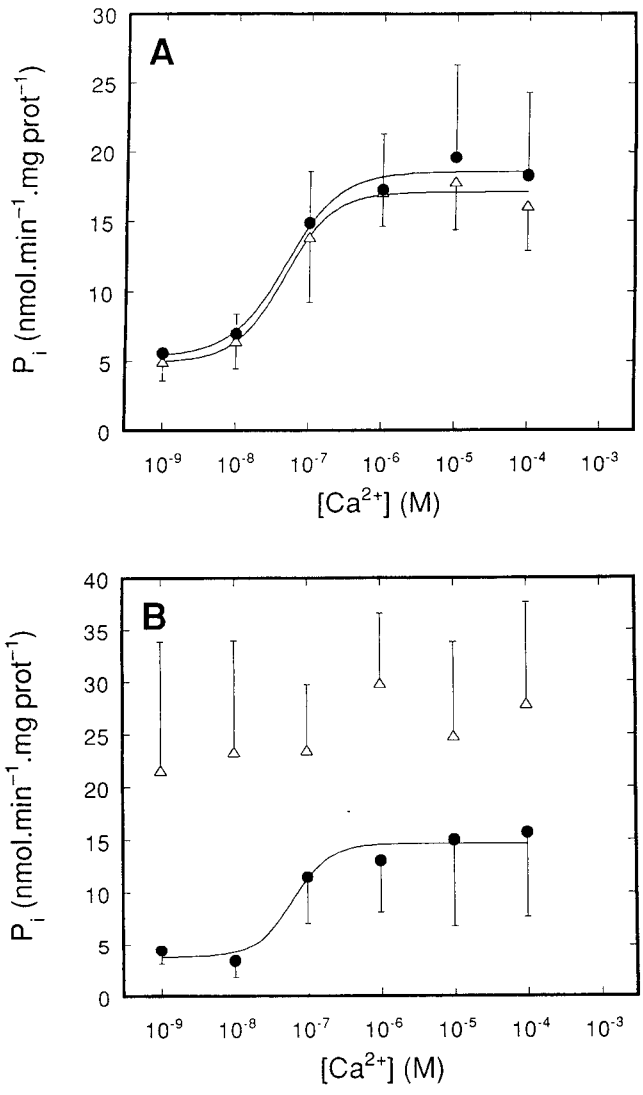

Figure 3. A, Kinase activity in PM vesicles from roots pretreated without FC (△) and with FC (●) in dependence of the free Ca^{2+} concentration in the assay. Results are the means ± SD of three experiments. B, Kinase activity in PM vesicles in dependence of the free Ca^{2+} concentration in the assay. The assay was done in the absence (●) and presence of arachidonic acid (△), 0.2 mg/mL. Results are the means ± SD of three experiments. prot, Protein; P_i, Pi.

ATPase shows similar characteristics (Lanfermeijer and Prins, 1994). Palmgren et al. (1988) and Johansson et al. (1993) hypothesized that these changes in pump kinetics might be the result of (de)phosphorylation. Furthermore, CDPKs have been shown to be stimulated by fatty acids (Klucis and Polya, 1987; Lucantoni and Polya, 1987). Therefore, we tested the effects of free fatty acids and other lipids on the PM kinase activity under conditions of low and high calcium concentrations. Saturated fatty acids (myristic, palmitic, and stearic acids) had no effect, but all of the *cis*-unsaturated fatty acids tested (oleic, linoleic, linolenic, and arachidonic acids) stimulated the kinase activity up to 10-fold in the presence of 10 μM Ca^{2+} (Fig. 4A). PS and PMA, activators of PKC, were not effective; nor were the lipids PA, PE, PC, or MJA, a derivative of linolenic acid (Fig. 4A). Also, the addition of FC to the assay did not significantly change the kinase activity. In the presence of high Ca^{2+} (1.3 mM), the stimulatory effect of the *cis*-unsaturated fatty acids is smaller (linoleic, linolenic, and arachidonic acids) or absent (oleic acid) (Fig. 4B).

Activators of PKC, like diacylglycerol and PMA, dramatically increase the affinity of the kinase for Ca^{2+}, thereby making the kinase Ca^{2+} independent (Nishizuka, 1986). Figure 3B shows that the same may hold true for the kinase that phosphorylates peptide I, since the Ca^{2+} dependency of the kinase is strongly reduced in the presence of arachidonic acid.

Kinase Association with FCBP

CDPK and PKC antibodies showed cross-reactivity with a number of bands in oat root PM on western blot (Fig. 5A). The CDPK antibody cross-reacts with three bands with apparent molecular masses of 67, 54, and 43 kD, and two of

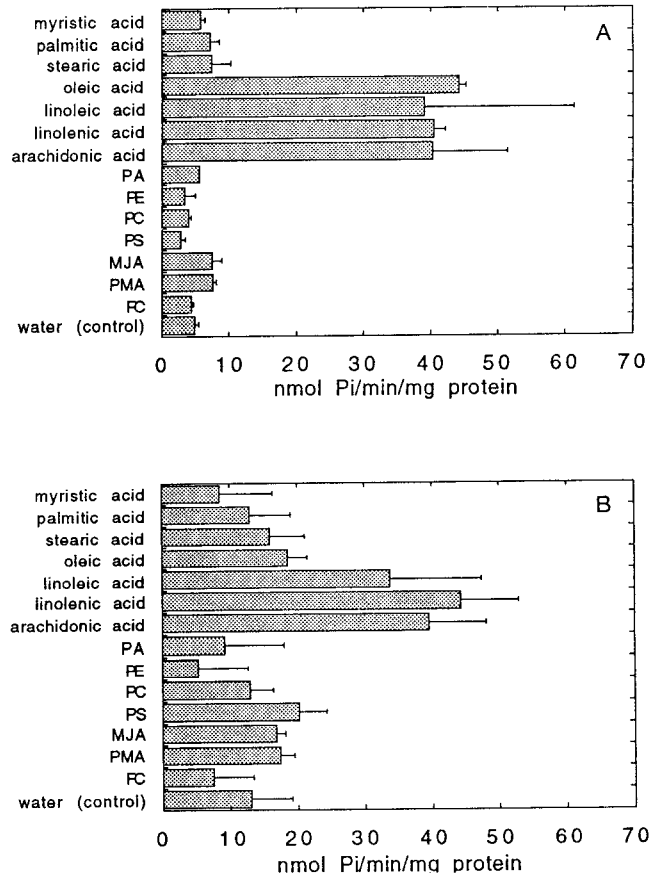

Figure 4. Effects of fatty acids, lipids, and signal transduction reagents on protein kinase activity in oat root PM vesicles. A, Kinase activity measured with 10 μM free Ca^{2+} in the assay medium. B, Kinase activity in the presence of high Ca^{2+} concentration (standard conditions, 1.3 mM Ca^{2+}). The kinase activity was assayed with the addition of 0.2 mg/mL fatty acid or lipid (final concentration). MJA, 400 μM; PMA, 100 μM; FC, 10 μM. The results are the means ± SD of two assays, except for linoleic acid, arachidonic acid, PS, FC, and the water control, which are the means ± SD of three to four assays.

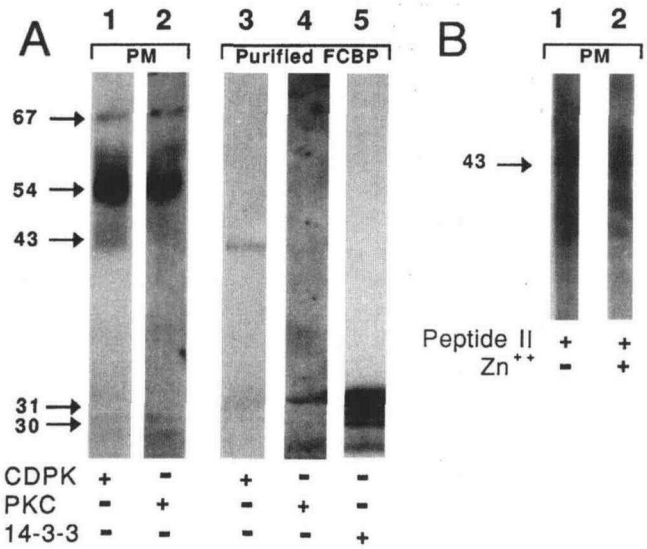

Figure 5. A, Cross-reactivity of proteins in PM vesicles and a purified FCBP fraction with CDPK, PKC, and 14-3-3 antibodies. B, Overlay assay with biotinylated peptide II on western blot with PM vesicles. Renaturation in the blot was done in the absence (−) or presence (+) of 0.1 mM Zn^{2+}. Numbers on the left are molecular masses in kD.

these bands (67 and 54 kD) are recognized by the PKC antibody as well. The 54- and 43-kD bands may be breakdown products, since it is known that CDPKs are readily degraded (Roberts and Harmon, 1992). In a fraction highly enriched in FCBP (Korthout and De Boer, 1994), the 43-kD band cross-reacting with the CDPK antibody was still present (Fig. 5A, lane 3); no cross-reactivity with the PKC antibody was observed (the 31-kD band is due to incomplete stripping of the 14-3-3 antibody; lane 5). This copurification of the 43-kD band and the two FCBP subunits is no proof that the two proteins are physically associated. Therefore, we also tested FCBP fractions purified with the FC-biotin/avidin method (Korthout and De Boer, 1994) for the presence of bands cross-reacting with CDPK antibodies. Also, in this highly pure FCBP fraction, a 43-kD band was visible, although it was very faint (data not shown).

It should be noted that FCBP affinity purified with FC-biotin is activated and the activated state may not be favorable for association of the receptor with other proteins. Therefore, we used a different approach as described by Mochly-Rosen et al. (1991). These authors demonstrated that a synthetic peptide with sequence homology to the so-called annexin-like domain present in 14-3-3 proteins can bind to PKC in an overlay assay. This annexin-like 14-3-3 domain is also conserved in plant 14-3-3 isoforms, and one peptide sequenced from the 31-kD FCBP subunit was in the middle of this motif (Korthout and De Boer, 1994). We synthesized the full-length peptide according to the barley 14-3-3 gene (Brandt et al., 1992) and added a biotin group to the N-terminal end: biotin-G-D-Y-H-R-Y-L-A-E-F-K-T-G-Q-E (peptide II). In an overlay assay, peptide II recognized a 43-kD band on a blot with renatured PM proteins (Fig. 5B). The assay with the peptide was done in the presence and absence of zinc ions, since all kinases known to associate with 14-3-3 proteins contain a zinc finger-like domain (Aitken, 1995). Recognition in the plus-zinc lane was slightly stronger than in the minus-zinc lane.

Effect of *cis*-Unsaturated Free Fatty Acids upon [^{3}H]FC Binding

If the kinase under investigation and the FCBP form one complex under certain conditions, then kinase activation by fatty acids might also affect the binding of [^{3}H]FC. Indeed, the *cis*-unsaturated fatty acids tested (oleic, linoleic, and arachidonic acids) almost fully inhibited the binding with IC_{50} values of approximately 30 μM (Fig. 6). This inhibitory effect is specific for the *cis*-unsaturated fatty acids, since saturated fatty acids have no effect on FC binding (Aducci et al., 1993). It is interesting that when PM vesicles were preincubated for 30 min with calphostin C (in a buffer containing 0.005% Triton X-100) [^{3}H]FC binding was inhibited by 40% at 10^{-5} M calphostin C (Fig. 6, inset). Other kinase inhibitors (staurosporine, H7, and ML-7) tested under identical conditions did not affect the binding of [^{3}H]FC (data not shown).

DISCUSSION

Results reported in this paper provide direct evidence for the involvement of a downstream protein kinase in the FC-signaling pathway. This finding fits very well with the mounting evidence that the direct target enzyme of FC, the H^+-ATPase, is controlled in its activity by phosphorylation/dephosphorylation (Vera-Estrella et al., 1994). More surprising was the observation that, among the kinase inhibitors tested, calphostin C and chelerythrine, known as typical PKC inhibitors (Kobayashi et al., 1989; Herbert et al., 1990), were most effective in blocking the in vivo FC response. Very recently, it was shown that calphostin C does inhibit the phosphorylation of the H^+-ATPase (E.

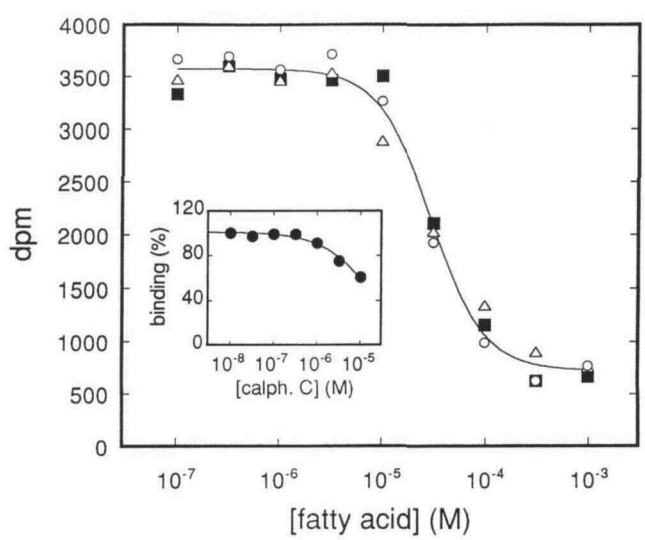

Figure 6. Inhibition of [^{3}H]FC binding to PM vesicles by *cis*-unsaturated fatty acids. ■, Arachidonic acid; △, oleic acid; and ●, linoleic acid. Nonspecific binding was not corrected for. Inset shows the inhibitory effect of calphostin C (calph. C) on [^{3}H]FC binding.

Blumwald, personal communication), thus corroborating our conclusion that a calphostin C-sensitive kinase mediates the signal from the FC receptor to the H^+ pump.

Based on the in vivo effect of the two kinase inhibitors and on the reports that a kinase of the CDPK-type phosphorylates the H^+ pump (Schaller and Sussman, 1988), we choose for the in vitro kinase assay the syntide-2-like peptide I as a substrate. This peptide has a phosphorylation motif that can serve as a substrate for both PKC and CDPK. The specificity of this substrate for the kinase present in oat root PM is apparently very good, as can be judged from the complete inhibition of kinase activity by both calphostin C and chelerythrine (IC_{50} values of 0.6 and 15 μM respectively). It seems very likely that the kinase has some structural similarity to kinase C proteins because the two inhibitors act at different domains of PKC: calphostin C at the regulatory domain and chelerythrine at the catalytic domain. In the regulatory domain, calphostin C inhibits PMA binding to PKC, since it interacts with the Cys-rich zinc-binding domain (Rotenberg et al., 1995), where PMA binds (Gschwendt et al., 1991).

However, a significant difference between the kinase characterized here and most kinase C isoforms is the absence of a stimulatory effect of PS and PMA. On the other hand, the stimulatory effect that *cis*-unsaturated fatty acids have on this kinase (Fig. 4) is shared by one PKC isoform, namely PKC ζ (Nakanishi and Exton, 1992). Stimulation of calcium-dependent kinase activity in plants by *cis*-unsaturated fatty acids has been reported (Klucis and Polya, 1987; Lucantoni and Polya, 1987), but on purified CDPKs, thus far no clear relationship between the nature of the fatty acid and its effect has been established (Roberts and Harmon, 1992). A unique feature of CDPKs is the intrinsic calmodulin domain; the fact that up to 60% inhibition of the kinase activity was inhibited by calmodulin inhibitors (prenylamine derivatives) in the presence of arachidonic acid and calcium (data not shown) indicates the involvement of calmodulin in the regulation of the activity of this kinase. These data suggest that the kinase is a hybrid with features of both kinase C proteins and CDPKs.

These hybrid features are confirmed by the cross-reactivity of CDPK and PKC antibodies with PM proteins: anti-CDPK recognized 67-, 55-, and 43-kD bands, whereas anti-PKC recognized bands with apparent molecular masses of 67 and 55 kD (Fig. 6). CDPKs are known to be readily subject to proteolysis (Roberts and Harmon 1992), and it may well be that the 55- and 43-kD bands are proteolytic products of the 67-kD band. If so, then the 43-kD band does not contain the epitope recognized by the PKC antibody. Based on the fact that the CDPK antibody does recognize an epitope in the catalytic domain of the CDPK protein (Professor A.C. Harmon, personal communication), the 43-kD band should have kinase activity. This was confirmed by gel-filtration experiments showing kinase activity in fractions collected at approximately 40 kD (data not shown).

An emerging theme in the function of 14-3-3 proteins (the class of proteins to which the FC receptor belongs) is the regulation of different kinds of kinases through a direct association (Aitken, 1995). What is the evidence, if any, for a direct association between the kinase characterized in this paper and the FCBP? First, the 43-kD band cross-reacting with the CDPK antibody is still present in a solubilized PM fraction highly enriched in FCBP (Fig. 5A). It is also present, although very faint, in an FCBP fraction purified by means of the ligand FC-biotin (Korthout and De Boer, 1994). Further evidence comes from the use of the annexin-like peptide (Fig. 5B). The recognition by this peptide of a 43-kD band is in line with the observations described above. It is also in line with the results described by Mochly-Rosen et al. (1991), showing that an annexin-like peptide from mammalian 14-3-3 does bind to kinase C isoforms.

All mammalian kinases, known to associate with 14-3-3 (Raf, PKC, and Bcr kinase), contain a so-called zinc-finger domain (Aitken, 1995). This is a domain where a zinc ion is involved in the coordination of the position of Cys and/or His residues. In three CDPK sequences (SK5, Harper et al., 1991; AK1, Harper et al., 1993; ATCDPK2, Urao et al., 1994), we discovered a putative zinc-finger domain, consisting of three His residues and one Cys residue: H-X_3-H-X_{16}-H-X_5-C. Although this motif is unusual, it has been shown that three His residues can bind zinc (Mozier et al., 1991). We are currently investigating whether zinc ions are important for the functioning of the kinase. A first indication may come from the observation that Zn^{2+} strongly inhibits the kinase activity with an IC_{50} of 30 μM (data not shown). A comparable inhibitory effect of Zn^{2+} on the activity of PKC in the presence of high Ca^{2+} has been described by Murakami et al. (1987). Also, the fact that calphostin C interacts with the Cys-rich zinc-binding domain in PKC (Rotenberg et al., 1995) favors the presence of a comparable domain in the kinase described here.

The effect of the *cis*-unsaturated fatty acids on both the enzymatic activity of the kinase and the binding activity of the FCBP is remarkable. It becomes even more intriguing in light of the report by Zupan et al. (1992), who assigned PLA_2 activity to 14-3-3 from sheep platelets. The recombinant and purified 14-3-3 protein did not release fatty acids but formed an acyl enzyme intermediate with arachidonic acid. According to Zupan et al. (1992), a cofactor necessary for the release of the intermediate was lacking in the recombinant sample. If this were true for our 14-3-3 receptor, then the following working model accommodates our observations: (a) under native conditions, the FCBP has or controls PLA_2 activity, and this activity is stimulated by binding of FC; (b) upon binding of FC to the receptor, free fatty acid is released (if the cofactor is present), and the fatty acid binds to the kinase associated with the 14-3-3 protein; and (c) fatty acid induces dissociation of the 14-3-3 receptor and kinase, activates the kinase (Fig. 4), and reduces the receptor affinity for FC.

The fact that we did not observe an effect of FC on kinase activity in vitro could be due to (a) a missing cofactor necessary for the release of fatty acid or (b) the lack of a suitable substrate. The hypothesis that the FC 14-3-3 receptor protein has intrinsic PLA_2 activity or controls the activity of a PLA_2 enzyme is currently under investigation.

ACKNOWLEDGMENTS

We thank Professor A. Harmon and Dr. P. van Heusden for supplying the CDPK and 14-3-3 antibodies, respectively. We are grateful to Dr. K. Krab for his assistance with plotting the graphs.

Received January 2, 1996; accepted April 12, 1996.

LITERATURE CITED

Abo-El-Saad M, Wu R (1995) A rice membrane calcium-dependent protein kinase is induced by gibberellin. Plant Physiol **108:** 787–793

Aducci P, Ballio A, Domini V, Fogliano V, Fullone MR, Marra M (1993) Phospholipase A_2 affects the activity of fusicoccin receptors. FEBS Lett **320:** 173–176

Aitken A (1995) 14-3-3 proteins on the MAP. Trends Biochem Sci **20:** 95–97

Bidway AP, Takemoto JY (1987) Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. Proc Natl Acad Sci USA **84:** 6755–6759

Brandt J, Thordal-Christensen H, Vad K, Gregersen P, Collinge DB (1992) A pathogen induced gene of barley encodes a protein showing high similarity to a protein kinase regulator. Plant J **2:** 815–820

Bruns RF, Miller FD, Merriman RL, Howbert JJ, Heath WF, Kobayashi E, Takahashi I, Tamaoki T, Nakano H (1991) Inhibition of protein kinase C by calphostin C is light-dependent. Biochem Biophys Res Commun **176:** 288–293

Caldirola MP (1992) Diphenylalkylamines: modulators of calcium and calmodulin: synthesis, pharmacology and structure-activity relationships. PhD thesis. Vrije Universiteit, Amsterdam, The Netherlands

Freed E, Symons M, Macdonald SG, McCormick F, Ruggieri R (1994) Binding of 14-3-3 proteins to the protein kinase Raf and effect on its activation. Science **265:** 1713–1719

Gschwendt M, Kittstein W, Marks F (1991) Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role? Trends Biochem Sci **16:** 167–196

Hager A, Debus G, Edel H-G, Stramskey H, Serrano R (1991) Auxin induces exocytosis and the rapid synthesis of a high-turnover pool of plasmamembrane H^+ATPase. Planta **185:** 527–537

Harmon AC, Yoo B-C, McCaffery C (1994) Pseudosubstrate inhibition of CDPK, a protein kinase with a calmodulin-like domain. Biochemistry **33:** 7278–7287

Harper JF, Binder BM, Sussman MR (1993) Calcium and lipid regulation of an *Arabidopsis* protein kinase expressed in *Escherichia coli*. Biochemistry **32:** 3282–3290

Harper JF, Huang J-F, Lloyd SJ (1994) Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin domain. Biochemistry **33:** 7267–7277

Harper JF, Sussman MR, Schaller GE, Putnam-Evans C, Charbonneau H, Harmon AC (1991) A calcium-dependent protein kinase with regulatory domain similar to calmodulin. Science **252:** 951–954

Herbert JM, Augereau JM, Gleye J, Maffrand JP (1990) Chelerytrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun **172:** 993–999

Johansson F, Sommarin M, Larsson C (1993) Fusicoccin activates the plasma membrane H^+-ATPase by a mechanism involving the C-terminal inhibitory domain. Plant Cell **5:** 321–327

Klucis E, Polya GM (1987) Calcium independent activation of two plant leaf calcium-regulated protein kinases by unsaturated fatty acids. Biochem Biophys Res Commun **147:** 1041–1047

Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun **159:** 548–553

Korthout HAAJ, De Boer AH (1994) A fusicoccin binding protein belongs to the family of 14-3-3 brain protein homologs. Plant Cell **6:** 1681–1692

Korthout HAAJ, Van der Hoeven PCJ, Wagner MJ, Van Hunnik E, De Boer AH (1994) Purification of the fusicoccin-binding protein from oat root plasma membrane by affinity chromatography with biotinylated fusicoccin. Plant Physiol **105:** 1281–1288

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lanfermeijer FC, Prins HBA (1994) Modulation of H^+-ATPase activity by fusicoccin in plasma membrane vesicles from oat (*Avena sativa* L.) roots. A comparison of modulation by fusicoccin, trypsin, and lysophosphatidylcholine. Plant Physiol **104:** 1277–1285

Lucantoni A, Polya GM (1987) Activation of wheat embryo calcium regulated protein kinase by unsaturated fatty acids in the presence and absence of calcium. FEBS Lett **221:** 33–36

Marra M, Fullone MR, Fogliano V, Masi S, Mattei M, Pen J, Aducci P (1994) The 30 kDa protein present in purified fusicoccin receptor preparations is a 14-3-3-like protein. Plant Physiol **106:** 1497–1501

Mochly-Rosen D, Khaner H, Lopez J, Smith BL (1991) Intracellular receptors for activated protein kinase C. J Biol Chem **266:** 14866–14868

Mozier NM, Walsh MP, Pearson JD (1991) Characterization of a novel binding site of protein kinase C inhibitor-1. FEBS Lett **279:** 14–18

Murakami K, Whiteley MK, Routtenberg A (1987) Regulation of protein kinase C activity by cooperative interaction of Zn^{2+} and Ca^{2+}. J Biol Chem **262:** 13902–13906

Nakanishi H, Exton JH (1992) Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. J Biol Chem **267:** 16347–16354

Nishizuka Y (1986) Studies and perspectives of protein kinase C. Science **233:** 305–310

Oecking C, Eckerskorn C, Weiler EW (1994) The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. FEBS Lett **352:** 163–166

Palmgren M, Sommarin, M, Ulvkov P, Jorgensen JL (1988) Modulation of plasma membrane H^+-ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A_2. Physiol Plant **74:** 11–19

Palmgren MG (1991) Regulation of plant plasma membrane H^+-ATPase activity. Physiol Plant **83:** 314–323

Rasi-Caldogno F, Pugliarello MC, Olivary C, De Michelis MI (1993) Controlled proteolysis mimics the effect of fusicoccin on the plasma membrane ATPase. Plant Physiol **103:** 391–398

Reuther GW, Fu H, Cripe LD, Collier RJ, Pendergast AM (1994) Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. Science **266:** 129–133

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

Rotenberg SA, Huang MH, Zhu J, Su L, Randidel H (1995) Deletion analysis of protein kinase C inactivation by calphostin C. Mol Carcinogen **12:** 42–49

Schaller GE, Sussman MR (1988) Phosphorylation of the plasma-membrane H^+-ATPase of oat roots by a calcium-stimulated protein kinase. Planta **173:** 509–518

Sussman MR (1994) Molecular analysis of proteins in the plant plasma membrane. Annu Rev Plant Physiol Plant Mol Biol **45:** 211–234

Toker A, Sellers LA, Amess B, Patel Y, Harris A, Aitken A (1992) Multiple isoforms of a protein kinase C inhibitor (KCIP-1/14–3-3) from sheep brain. Eur J Biochem **206:** 453–461

Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K (1994) Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. Mol Gen Genet **244:** 331–340

Vera-Estrella R, Barkla BJ, Higgins VJ, Blumwald E (1994) Plant defense response to fungal pathogens. Activation of host-plasma membrane H^+-ATPase by elicitor-induced enzyme dephosphorylation. Plant Physiol **104:** 209–215

Zupan LA, Steffens DL, Berry CA, Landt M, Gross RW (1992) Cloning and expression of a human 14-3-3 protein mediating phospholipolysis. J Biol Chem **267:** 8707–8710