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RICE MEMBRANES CONTAIN A CALCIUM-DEPENDENT PROTEIN KINASE ACTIVITY WITH BIOCHEMICAL FEATURES OF ANIMAL PROTEIN KINASE C

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The presence of calcium-dependent protein kinase activities in rice was investigated. Membrane preparations could phosphorylate the MARCKS peptide, a highly specific substrate for animal protein kinase C (PKC). Phosphorylation, strictly dependent on calcium, was specifically antagonized by a peptide whose amino acid sequence corresponds to the inhibitory, pseudosubstrate domain of mammalian PKC. Similar results have been obtained with rice soluble fractions. Addition of inhibitors of mammalian PKC (staurosporine and calphostin C) also inhibited phosphorylation of specific peptide substrates. Western blot analysis with anti-PKC antibodies identified three major bands (90, 87 and 54 kD) in rice membrane-associated proteins.

Several calcium-dependent protein kinases (CDPKs) have been characterized and partially purified from different organs of many plant species and green algae (1). Three genes encoding for plant CDPKs have been cloned so far (2-4). Sequence data analysis shows for all three proteins the same novel structure in which a calmodulin-like domain is fused with a catalytic domain highly homologous to mammalian Ca^{2+}/calmodulin-dependent protein kinases. On the other hand, antibodies raised against these cloned CDPKs failed to cross-react with other partially purified plant calcium-dependent kinases, suggesting the presence of different forms of these enzymes. Differences have also been observed at the level of substrate specificity in in vitro phosphorylation assays (1).

Some plant CDPK activities have been reported to be stimulated by phospholipids but a clear evidence for the existence of a plant PKC is still lacking. Nevertheless, cross-reaction with antibodies raised against bovine brain PKC has been demonstrated in *Amaranthus tricolor* extracts (5).

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In the present work we investigated, by a direct activity assay, the presence of membrane-associated protein kinases in rice and identified a protein kinase activity with overlapping features to animal PKC.

MATERIALS AND METHODS

Materials: [γ^{32}P]ATP (3000 Ci/mmol) was from Amersham. MARCKS peptide, derived from a sequence of the myristoylated alanine-rich C kinase substrate, was by Tecnogen, Italy. The two peptides with the amino acid sequence of the pseudosubstrate region of PKC, PKC (19-36) and Ser_{25}PKC (19-31) were purchased from GIBCO BRL. Staurosporine was from Sigma and calphostin C from BIOMOL.
Membrane preparations: Rice coleoptiles (*Oryza sativa L.*) were cut from three days-old seedlings and frozen in liquid nitrogen until used. Frozen material (7-8 g) was ground to a fine powder and solubilized in 20 ml homogenization buffer (50 mM Mes-NaOH pH7, 250 mM sucrose, 5mM $MgCl_2$, 0.5 mM DTT, 5mM EDTA, 1mM PMSF). After a first centrifugation at 7,000 x g, supernatant was centrifuged at 130,000 x g for 30 min and the membrane pellet, washed in 10 ml of modified homogenization buffer (0.25 mM EGTA instead of EDTA and pH lowered to 6), was spun again and frozen at -80°C until used.
In vitro kinase assay: Membrane pellets were resuspended in a small volume of 2X kinase assay buffer (100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 µM $CaCl_2$, 200 µM $Na_4P_2O_7$, 2 mM KF, 200 µM PMSF) at a final protein concentration of 0.2 mg/ml. Assays were performed as described by Chakravarthy (6) on 10 µg of membrane proteins, in a final volume of 100 µl, upon addition of the substrate (and inhibitors and cofactors when indicated) and 0.5 µCi of [γ^{32}P]ATP (specific activity 1mCi/mmol). Non specific binding was determined in the absence of added substrates. Calcium dependence was tested in the presence of 0.25 mM EGTA and different concentrations of calcium. Free calcium concentrations were calculated by a computer program based on the method of Perrin and Sayce (7). Assays on the soluble fraction were performed using the supernatant of the membrane fraction, in a buffer containing 1 mM EGTA and 1 mM EDTA; calcium-dependency was assayed by adding millimolar amounts of $CaCl_2$.
Western blot analysis: An antiserum raised against the aminoacid sequence 280 to 292 in the regulatory domain of bovine brain PKC was used. Protein samples (100 µg) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. Filters, saturated with 5% (w/v) non fat dry milk, were incubated overnight at 4°C with the antiserum and signals were detected by chemiluminescence (ECL detection system, Amersham). Autokinase assays were performed according to the method of Ferrel and Martin (8).

RESULTS

In this study we adapted the method described by Chakravarthy et al. (6) by which PKC activity has been measured directly in isolated lymphoma cell membranes, for the detection of protein kinase activities in membranes from rice coleoptiles. In preliminary experiments, crude membrane preparations could successfully phosphorylate, in a dose-dependent manner, two poorly selective substrates as Myelin Basic Protein (MBP) and H1 histone commonly used for

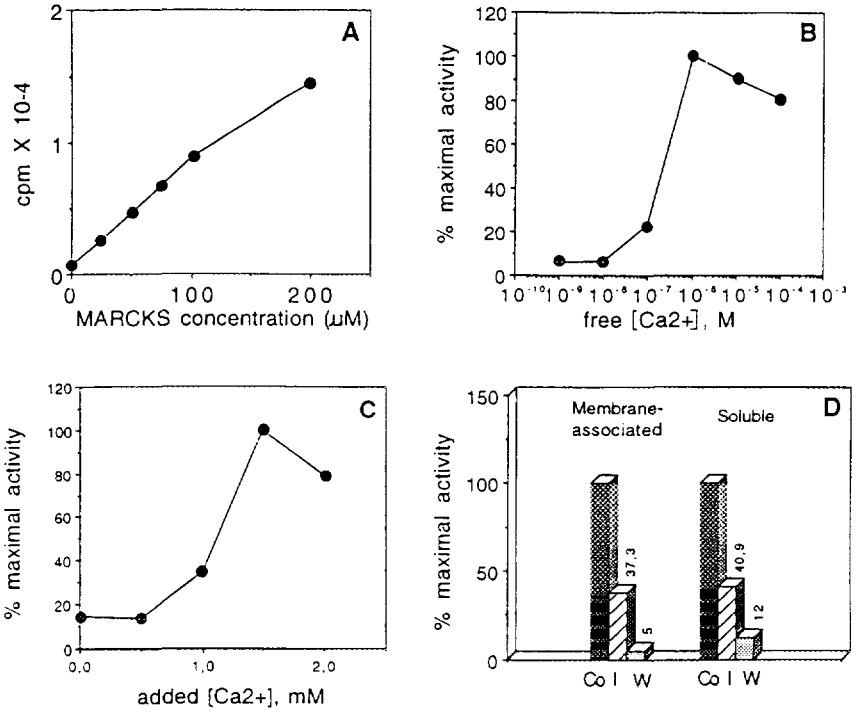

Fig. 1. Characterization of MARCKS peptide phosphorylation by membrane-associated and soluble rice kinase activities.
A) Kinase assays on coleoptile membranes were performed with increasing substrate concentrations and in the presence of 1 μM $CaCl_2$. Radioactivity incorporated into the peptide was counted and cpm values are reported. B) Calcium-dependence of MARCKS phosphorylation in the microsomal fraction was assayed in a Ca^{2+}/EGTA buffer. Free Ca^{2+} concentrations were calculated as described in Materials and Methods. C) Calcium-dependence of MARCKS phosphorylation in the soluble fraction, was measured by adding increasing amounts of calcium. D) The inhibitory effect of the PKC pseudosubstrate peptide PKC(19-36) (180 μM) on the phosphorylation of MARCKS (45 μM) was measured in both the membrane and the cytosolic fractions (I). Peptide phosphorylation in the presence of 0,25 mM EGTA (membranes) or with no added Ca^{2+} (chelators being already present in the buffer of the soluble fraction) was determined (W). Data are reported as percentage values over control samples (Co) with no inhibitors added.

measuring kinase activity (data not shown). Then, we tested the ability of rice membrane preparations to phosphorylate more selective substrates in order to identify specific kinase activities. A peptide (FKKSFKL-NH_2) derived from MARCKS, a physiological substrate of animal PKC, was initially used. As shown in Fig. 1A, coleoptile membrane preparations were able, upon addition of ATP, to phosphorylate the MARCKS peptide in a concentration dependent manner. Peptide concentrations tested (25-200 μM) were in the same range of those reported for similar assays in mammalian cell membranes.

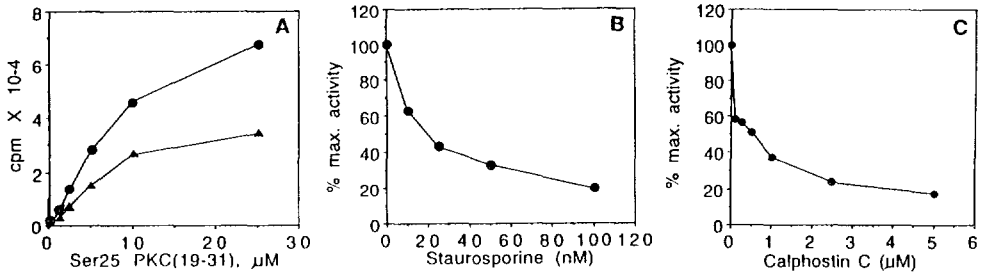

Fig. 2. Phosphorylation of the PKC-pseudosubstrate analog peptide Ser_{25}PKC(19-31) by rice membrane-associated kinase activities.
A) Concentration-dependent phosphorylation obtained with equal amounts of proteins from membrane preparations (●) or soluble extracts (▲), at optimal calcium concentrations. B) Inhibitory effect of staurosporine. Data are reported as percentage of the ^{32}P incorporation value measured without inhibitors. C) Inhibitory effect of calphostin C.

Calcium-dependency of MARCKS peptide phosphorylation was tested in a Ca^{2+}/EGTA buffer (see Material and Methods). Maximal activity was observed in the range of 10^{-6}M (Fig.1B) whereas calcium concentrations up to 10^{-8}M were ineffective in the stimulation of the kinase. Similar values of optimal calcium concentrations have been reported for other plant CDPKs (9,10).

Similarly to animal PKC, several plant CDPKs have been found in both a soluble and a membrane-associated form (9,11,12). Interestingly, MARCKS peptide specific phosphorylation was also obtained when rice soluble extracts were used and phosphorylation was again dependent on Ca^{2+} (Fig. 1C).

The inhibitory peptide PKC(19-36), derived from the pseudosubstrate autoinhibitory sequence of animal PKC and shown to selectively inhibit the enzymatic activity of PKC (13), was also used in our assays. As shown in Fig.1D, phosphorylation of MARCKS substrate by membrane-associated as well as soluble rice CDPK was significantly reduced (60-70%) by the addition of the PKC(19-36) inhibitory peptide.

Phosphorylation of the PKC pseudosubstrate peptide

It has been shown that replacement of Ala_{25} with a Serine residue in the inhibitory PKC(19-36) peptide makes it a good substrate for PKC itself (13). We tested if phosphorylation of an analogous Ser_{25}PKC(19-31) peptide could occur after addition of rice membrane proteins. As shown in Fig.2A, also this PKC substrate was phosphorylated in a concentration dependent manner. Interestingly, maximal ^{32}P incorporation was observed at concentration values about ten fold lower then those reported for MARCKS. Also in this case, phosphorylation was almost completely inhibited by EGTA (data not shown). We also tested the ability of the soluble fraction of rice extracts to phosphorylate the Ser_{25}PKC(19-31) peptide. A calcium-dependent kinase activity on this substrate was observed in the soluble

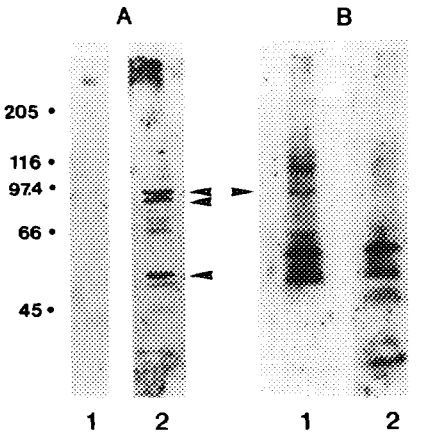

Fig. 3. Immunoblotting and autokinase assay of membrane extracts from rice coleoptiles.
A) Membrane-associated proteins were blotted onto a PVDF membrane and immunostained with non-immune serum (lane 1) or anti-PKC antibodies (lane 2). B) Similar PVDF filters containing identical samples of immobilized membrane proteins were incubated with [γ^{32}P]ATP in the presence of 1 μM $CaCl_2$ (lane 1) or 0.25 mM EGTA (lane 2).

extracts (Fig. 2A). Intriguingly, when we added diacylglycerol and phosphatidylcoline or phosphatidylserine to our soluble extracts we failed to observe a significant increase, over the calcium-stimulatable level, in the amount of ^{32}P incorporation into both the MARCKS and Ser_{25}PKC(19-31) peptides (data not shown).

Use of protein kinase inhibitors

We further extended our analysis by using two other protein kinase inhibitors: staurosporine, known to bind the catalytic domain of PKC, and calphostin C, an inhibitor highly selective for PKC since it interacts with its regulatory, lipid binding domain. As shown in Fig 2B and 2C, both inhibitors showed a dramatic effect in our system. A 10 nM concentration of staurosporine was sufficient to reduce by a 30% ^{32}P incorporation into the Ser_{25}PKC(19-31) peptide, whereas 1μM calphostin C reduced phosphorylation by more than 50%.

Immunological detection of PKC-related proteins

The biochemical similarity between this rice CDPK activity and animal PKC prompted us to look, in membrane extracts from rice coleoptiles, for proteins cross-reactive with anti-PKC antibodies. We used an antibody raised against a conserved sequence present in the regulatory domain of bovine PKC (a gift from Dr. P.J. Parker). With this antibody (antiserum 545) we were able to observe, in Western blot experiments with rice membrane proteins, three major bands of apparent m.w. of 90, 87 and 54 kD, while no signal was observed with pre-immune serum (Fig. 3A). Interestingly, parallel autokinase assays lighted up several discrete

bands, one of which was exactly coincident (90 kD) with the upper band of the doublet recognized by the anti-PKC antibody (Fig. 3B). This band was not evident in slots incubated in the presence of EGTA, suggesting a calcium-dependent ^{32}P incorporation (lane 2, Fig. 3B).

DISCUSSION

Our data show that rice membranes contain a CDPK (or a subfamily of CDPKs) with a substrate and inhibitor specificity overlapping with that of animal PKC. Phosphorylation of any of the substrates used in this study was always calcium-dependent, with its maximum at 1μM Ca^{2+}. This value is consistent with those reported for other plant CDPKs and suggests that this membrane-associated kinase may act as a stimulus-response coupler in calcium regulated processes. The biochemical similarity of this rice CDPK with animal PKC is intriguing. MARCKS peptide (FKKSFKL), previously shown to be selectively phosphorylated by phospholipid-dependent protein kinases present in crude extracts of mammalian cells, is also phosphorylated in a dose dependent manner by membrane extracts prepared from rice. Interestingly, the SFK motif contained in the MARCKS peptide is a consensus sequence also for a plant CDPK purified from alfalfa (14). In addition, the evidence that a peptide corresponding to the pseudosubstrate domain of PKC inhibited the phosphorylation of the MARCKS peptide by rice membrane protein kinase(s), further suggests common features in target sequences recognition between this CDPK and animal PKC. Furthermore, MARCKS and the pseudosubstrate analog Ser_{25}PKC(19-31) peptides were also phosphorylated by rice soluble extracts, suggesting that either two forms of the same CDPK (a membrane-associated and a cytosolic form) exist, or that two very similar enzymes have a different subcellular localization. On the other hand we failed to further stimulate the CDPK activity present in the soluble fraction by addition of phospholipids (phosphatidylserine or phosphatidylcholine) and diacylglycerol to the assay. This could be due to reasons as different as lipid contamination of our extracts, requirement of lipids diverse than those tested or phospholipid-independency of this rice CDPK. In such a case, similarity with PKC may be restricted to the catalytic domain, although the inhibitory effect of calphostin C would remain to be explained.

In any case, as shown in Fig. 3, rice membranes contain proteins cross-reacting with anti-PKC antibodies. At the present, we do not know neither what is the relation among the different predominant bands (90, 87 and 54 kD) we observed, nor if any of these proteins is associated to the kinase activity we have described. However, it is conceivable that the 90 kD product could represent the phosphorylated form of the 87 kD protein, while the 54 kD band could result from proteolytic cleavage. It is also of interest to note that a 90 kD band coincident with that immunologically identified is evident in the autokinase assay only if calcium

is present. This result suggests that only the 90 kD protein is able to autophosphorylate in vitro. Further studies are in progress to better characterize this rice CDPK activity at both biochemical and molecular levels.

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