A family of radish (Raphanus sativus) calmodulin antagonists (RCAs) was purified from seeds by extraction, centrifugation, batch-wise elution from carboxymethyl-cellulose, and high performance liquid chromatography (HPLC) on an SP5PW cation-exchange column. This RCA fraction was further resolved into three calmodulin antagonist polyptides (RCA1, RCA2, and RCA3) by denaturation in the presence of guanidinium HCl and mercaptoethanol and subsequent reverse-phase HPLC on a C8 column eluted with an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The RCA preparation, RCA1, RCA2, RCA3, and other radish seed proteins were phosphorylated by wheat embryo Ca^{2+}-dependent protein kinase (CDPK). The RCA preparation contains other CDPK substrates in addition to RCA1, RCA2, and RCA3. The RCA preparation, RCA1, RCA2, and RCA3 inhibit chicken gizzard calmodulin-dependent myosin light chain kinase assayed with a myosin-light chain-based synthetic peptide substrate (fifty percent inhibitory concentrations of RCA2 and RCA3 are about 7 and 2 μM, respectively). N-terminal sequencing by sequential Edman degradation of RCA1, RCA2, and RCA3 revealed sequences having a high homology with the small subunit of the storage protein napin from Brassica napus and with related proteins. The deduced amino acid sequences of RCA1, RCA2, RCA3, and RCA3′ (a subform of RCA3) have agreement with average molecular masses from electrospray mass spectrometry of 4537, 4543, 4532, and 4560 kD, respectively. The only sites for serine phosphorylation are near or at the C termini and hence adjacent to the sites of protein degradation of RCAl, RCA2, and RCA3 revealed sequences but not synthetic peptides with the Basic-Basic-X-Ser(Thr) amino acid sequence recognized by cyclic AMP-dependent protein kinase (Polya et al., 1989).

Wheat embryo CDPK phosphorylates a number of endogenous protein substrates including histone H1, histone H2B (Polya et al., 1989), an α-amylase inhibitor (Polya and Chandra, 1990), and a wheat putative phospholipid transfer protein (Polya et al., 1992). Wheat CDPK phosphorylates a phospholipid transfer protein from barley (Polya and Chandra, 1990) as well as highly homologous proteins from wheat and pine (Polya et al., 1992). Some plant enzymes are plant CDPK substrates, namely NAD^+-quinone oxidoreductase (Ranjeva and Boudet, 1987), H^+-ATPase (Schaller and Sussman, 1988), and phosphoenolpyruvate carboxylase (Echevarria et al., 1988). The definition of CDPK-mediated signaling pathways in plants requires the resolution of the protein substrates for CDPKs. The present paper describes the purification and amino acid sequencing of three radish (Raphanus sativus) seed calmodulin antagonist proteins that are substrates for plant CDPKs.

MATERIALS AND METHODS

Materials

Radish (Raphanus sativus L.) seeds were obtained from Selected Foods Company, Waterloo, New South Wales, Australia. [γ-32P]ATP (4 Ci/mmol) was obtained from Bresatec, Adelaide, Australia. A synthetic peptide substrate of smooth muscle MLCK (KKRAARATSNNVFA-NH₂) was obtained from Auspep, Melbourne, Australia. Calf thymus histones (type III-S) and ATP were obtained from the Sigma Chemical Co. Affi-Gel blue (100–200 mesh) was obtained from Bio-Rad, DEAE-Sephacel from Pharmacia, and carboxymethyl-cellulose (CM-52) from Whatman. A Protein Pak SP5PW column (75 mm × 7.5 cm) was obtained from Waters, and an Aquapore RP300 (C₈) reverse-phase HPLC guard column (4.6 mm × 30 mm; 7 μm particle size) was obtained from Brownlee Laboratories.

Protein Kinase and Calmodulin Isolation

Wheat embryo CDPK was purified as described previously (Lucantoni and Polya, 1987). MLCK was extensively purified

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Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; MLCK, myosin light chain kinase; PTH, phenylthiohydantoin; RCA, radish calmodulin antagonist.
from chicken gizzard smooth muscle (excised from freshly killed chickens) by a procedure involving muscle extraction and chromatography on Affi-Gel blue and DEAE-Sephacel (Walsh et al., 1983). The active MLCK was dialyzed against 10 mM K phosphate (pH 6) containing 250 mM NaCl and 1 mM DTT and then stored at -70°C. Calmodulin was purified to homogeneity from wheat embryo as previously described (Jinsart et al., 1991).

**Protein Phosphorylation**

Wheat germ CPDPK activity was determined radiochemically by precipitation on phosphocellulose (P-81) disks employing radish proteins or 1.0 mg/mL of calf thymus histones (Sigma type III-S) as substrate as previously described (Lucantoni and Polya, 1987). Phosphoamino acid analysis of phosphorylated polypeptides by high voltage electrophoresis, SDS-PAGE of phosphorylated proteins, and autoradiography were conducted as described previously (Polya et al., 1983). MLCK was routinely assayed in a reaction mixture (final volume 120 μL) containing 7 mM Heps (Na+, pH 7), 1 mM Mg acetate, 0.1 mM CaCl₂, 0.2 mg mL⁻¹ BSA, 0.2% (v/v) Tween 80, 17 μM ATP (γ⁻[32P]ATP, specific activity about 30 Ci mol⁻¹), smooth muscle MLCK peptide substrate (21 μM), 0.2 μM wheat germ calmodulin, and MLCK. Reactions were terminated by spotting onto phosphocellulose (P-81) paper disks, which were then washed successively three times in 500 mL of 75 mM H₃PO₄, dried, and Cerenkov counted. Protein was determined by the Coomassie blue method (Sedmak and Grossberg, 1977) using crystalline BSA as a standard.

**Purification of Radish Seed Proteins**

Unless specified otherwise, all operations were conducted at 0 to 4°C. One hundred grams of radish seeds were homogenized (Omnimixer blender, full power for 1.5 min) in 500 mL of an extraction medium containing 10 mM sodium phosphate (pH 6.0), 0.5 mM PMSF, and 0.25% (v/v) ethanol. The homogenate was filtered through muslin and then centrifuged at 16,000 g for 25 min. The supernatant was added to 25 g of carboxymethyl-cellulose (CM-52). The CM-52 was washed batchwise with 1 L of 10 mM sodium phosphate (pH 6.0) and then basic proteins were eluted in 200 mL of 1 M NaCl in 10 mM sodium phosphate (pH 6.0). This eluate was concentrated to 1 mL by pressure filtration, and applied to a SP5PW cation-exchange column coupled to a Varian HPLC system. The SP5PW column (7.5 mm x 7.5 cm; 10-μm particle size) was eluted over 100 min (flow rate 1 mL min⁻¹) at 25°C with a linear gradient of 0 to 1 M NaCl in 10 mM Na phosphate (pH 6.0) (Fig. 1).

RCA preparation (0.3 mg) was denatured in 3 M guanidinium HCl, 100 mM 2-mercaptoethanol at 100°C for 10 min. Denatured RCA was then subjected to reverse-phase HPLC on a C₈ column (Aquapore RP-300 guard column; 4.6 mm x 30 mm; 7-μm particle size) using an acetonitrile gradient in the presence of aqueous 0.1% (v/v) TFA (a linear gradient increasing from 0-25% acetonitrile in 25 min and then to 50% in a further 100 minutes; flow rate 1 mL min⁻¹).

**Amino Acid Sequencing and Electrospray Ionization MS**

Amino acid sequencing by sequential Edman degradation was conducted with an Applied Biosystems 470A gas-phase peptide sequenator. PTH amino acid analysis employed a Waters HPLC fitted with a Zorbax C₈ column (DuPont, Wilmington, DE), which was eluted with a discontinuous Na acetate buffer (pH 5.0)/acetonitrile gradient (Zimmerman et al., 1977). The RCA polypeptides (50–350 pmol) in 10 μL of methanol:H₂O:acetic acid (49.5:49.5:1) was introduced at a flow rate of 2 μL min⁻¹ into a VG BIO-Q electrospray spectrometer (VG Biotech, Cheshire, UK). The quadrupole spectrometer was scanned from m/z 600 to m/z 1400 (10 s per scan) over 5 min to obtain the final spectra, and the quadrupole was set for unit mass resolution. The mass scale was calibrated by using the multiple charged ions from the separate introduction of myoglobin.

**RESULTS AND DISCUSSION**

**Purification of RCA Polypeptides**

Radish seeds contain a number of basic proteins that can be resolved by HPLC on a SP5PW cation-exchange column and that are phosphorylated by wheat embryo CPDPK (Fig. 1). Basic radish proteins resolved in this fashion include proteins eluted at 0.17, 0.25, 0.35, and 0.82 M NaCl, respectively, from the SP5PW column (Fig. 1).

Because a calmodulin antagonist protein preparation had been previously resolved from radish seeds (Cocucci and Negroni, 1988), we examined the fractions eluting from the SP5PW column for calmodulin antagonist activity. Calmodulin antagonist activity was measured by determining inhibition of chicken gizzard Ca²⁺-calmodulin-dependent MLCK activity, employing as a protein substrate a synthetic peptide substrate (KKRAARATSNVFA-NH₂) of this enzyme (the sequence of this peptide relating to the phosphorylatable sequence of this peptide). A major zone of inhibitory activity corresponds to a protein peak eluting at 0.8 M NaCl concentration (Fig. 1). The yield of this protein from radish seeds by the protocol described in "Materials and Methods" was 26 mg kg⁻¹ fresh weight.

SDS-PAGE of the peak fractions of this RCA fraction revealed two major polypeptide bands migrating between the 14.3- and 3.5-kD standards (estimated molecular mass values of about 7 and 6 kD, respectively) (Fig. 2). This polypeptide pattern is similar to that found for a calmodulin antagonist preparation from radish (Cocucci and Negroni, 1988). In view of this heterogeneity, the RCA preparation was subjected to denaturation by heating in the presence of 3 M guanidinium HCl and 0.1 M 2-mercaptoethanol and subsequent reverse-phase HPLC on a C₈ matrix, as described in "Materials and Methods." This procedure revealed considerable heterogeneity in the RCA preparation, some nine peaks of material absorbing at 230 nm being resolved (Fig. 3A). Calmodulin antagonist activity was associated with three major, closely migrating peaks (RCA1, RCA2, and RCA3).
eluting at about 26% acetonitrile in 0.1% TFA. No other zone of calmodulin antagonist activity was found in the profile. SDS-PAGE of RCA1, RCA2, and RCA3 revealed single bands with mobilities corresponding to molecular mass values of about 6 kD (data not shown). The yields of RCA1, RCA2, and RCA3 from the overall isolation procedure were 0.4, 1.2, and 0.9 mg per 100 g fresh weight, respectively.

RCA1, RCA2, and RCA3 all inhibit Ca\(^{2+}\)-calmodulin-dependent MLCK-catalyzed phosphorylation of a myosin light chain-based synthetic peptide substrate (Fig. 3B). Thirty micrograms per milliliter of RCA2 and 11 µg/mL of RCA3 inhibit MLCK by 55 and 56%, respectively. Thus, with respect to MLCK the concentrations for 50% of inhibition for RCA2 and RCA3 are about 7 and 2 µM, respectively, assuming mol wts for both RCA2 and RCA3 of about 4540 (see below).

Phosphorylation of RCAs

RCA is phosphorylated by wheat embryo CDPK (Fig. 1), as are the RCA polypeptides RCA1, RCA2, and RCA3 (Fig. 3A). Experiments involving SDS-PAGE and autoradiography confirmed that phosphorylated proteins in the RCA preparation comigrate with the RCA polypeptide bands (Fig. 2). Similar experiments confirmed the phosphorylation of RCA1, RCA2, and RCA3 (data not shown). The CDPK rate with the near-saturating concentration of 0.4 mg mL\(^{-1}\) of RCA preparation (Fig. 4) is 8% of that with 1 mg mL\(^{-1}\) of histone III-S. The RCA preparation \(K_m\) is 0.23 ± 0.12 mg mL\(^{-1}\) (as determined from fitting kinetic data to the Michaelis-Menten equation by a least squares curve-fitting program), corresponding to 51 µM if one assumes a mol wt of these proteins of about 4540 (see below). By way of comparison, \(K_m\) values for histone H1 (Polya et al., 1989) and wheat basic protein (Polya et al., 1992) (two of the better endogenous wheat CDPK substrates) are 6 and 2 µM, respectively.

Wheat embryo CDPK phosphorylates a variety of protein and oligopeptide substrates on either Ser or Thr or on both Ser and Thr residues (Polya et al., 1989). The heterogeneous RCA preparation contains polypeptides phosphorylated by wheat embryo CDPK on both Ser (35%) and Thr (65%) residues as determined by the phosphoamino analysis described in "Materials and Methods." However, sequencing of the individual polypeptides RCA1, RCA2, and RCA3 indicates that these are likely to be phosphorylated on Ser residues near the C terminus, as discussed below.

Amino Acid Sequences of RCA1, RCA2, and RCA3

Amino acid sequences of RCA1, RCA2, and RCA3 were determined by sequential Edman degradation (Fig. 5). The three polypeptides have very similar N-terminal sequences that differ in positions 7 (Y, I), 12 (K, R), 29 (K, R), and 32 (R, M). These proteins are highly homologous to the Brassica napin small subunit variants and related proteins (Fig. 5). A minor sequence detected in the sequencing of RCA1 and denoted RCA1' appears to derive from deletion of the first six residues of RCA3 by an Arg-recognizing protease. No new residues (except for a PTH-Pro signal) were observed in cycles 39, 40, 41 (RCA1); 39, 40, and 41 (RCA2); and 39 and 40 (RCA3), consistent with termination of the polypeptide chains at residues 39. Positions 26, 34, 36, and 39 are uncertain in RCA1 and RCA2, and positions 26, 36, 39, and 39 are uncertain in RCA3 (Fig. 5). Residue 26 is likely to be W for
Figure 3. Purification of RCA1, RCA2, and RCA3 by reverse-phase HPLC of the RCA preparation on a C8 column. HPLC was conducted as described in "Materials and Methods." A, Resolution of RCAs 1, 2, and 3 (at 31-35 min) from other proteins. The CDPK activity obtained in the standard assay with 25-μL aliquots of the indicated fraction as protein substrate is indicated by the hatched area. CDPK-catalyzed [32P]phosphoryl incorporation of 1000 cpm in the assay period of 80 min corresponds to a CDPK activity of 1.4 pmol min⁻¹ mL⁻¹. B, Inhibition of MLCK by RCA fractions is indicated by the hatched area.

reasons of homology (Fig. 5), noting that a PTH-W peak is masked by another signal in the analytical system used. Residue 34 is probably S in RCA2 (based on the trace occurrence of a PTH-S signal and of a PTH-dehydroalanine signal deriving from S in the Edman degradation). The remaining assignments as S residues (Fig. 5) are consistent with poor S detectability after 39 Edman cycles, location of S in homologous proteins (Fig. 5), the absence of CDPK-phosphorylatable Ser/Thr residues elsewhere, RCA termination after position 39, and precise molecular mass data from MS, described below.

The average molecular masses of polypeptides in the RCA1, RCA2, and RCA3 preparations were determined by electrospray ionization MS as follows (±sD; average molecular masses of minor components in parentheses): RCA1, 4537.2 ± 0.8 (4613.4 ± 0.8); RCA2, 4542.8 ± 0.4 (4619.2 ± 0.6); RCA3, 4532.1 ± 0.7 (4560.3 ± 0.2). The deduced RCA1, RCA2, and RCA3 amino acid sequences (indicated in square brackets, Fig. 5) give average mol wts (i.e. taking C, O, N, H, and S isotope natural abundances into account) of 4537.1, 4543.1, and 4531.2, respectively, in very close agreement with the mass spectrometric data. The RCA3 preparation contains a minor component (mol wt 4560.3) exactly consistent with the Edman sequencing evidence of K or R at position 12. The average mol wt of RCA3' (the form with R12) is
Calmodulin Antagonist Phosphorylation

Figure 5. N-terminal sequences of RCA1, RCA1', RCA2, RCA3, RCA3', and homologous sequences of related napin small subunits from Sinapis alba, Ricinus communis, Arabidopsis thaliana, Brassica napus, and Bertholletia excelsa. C, S, and T residues are in bold, as are residues where the assignment from Edman sequencing alone is uncertain. X, Nonallocated residues in the Edman sequencing; R, residues are in bold, as are residues where the assignment from Edman sequencing alone is uncertain. N-terminal small residues of processed proteins are underlined. X, Nonallocated residues in the Edman sequencing; the average mol wts of the indicated deduced sequences of RCA1, RCA2, RCA3, and RCA3' are given in square brackets. The references to the numbered napin-related sequences are as follows: 6, Menéndez-Arias et al., 1983; Ericson et al., 1986; Josefsson et al., 1988; 7, Sharief and Li, 1982; lrwin et al., 1990; lrwin and Lord, 1990; 8-11, Krebbers et al., 1988; 12, Crouch et al., 1983; Ericson et al., 1986; Josefsson et al., 1987; 13, Baszczyński and Fallis, 1990; 14, Schofield and Crouch, 1987; 15, Monsalve et al., 1991; 16, Ampe et al., 1986; 17, De Castro et al., 1987.

CDPK-catalyzed phosphorylation occurs on Ser residues deduced to be present in the C-terminal region as discussed above (Fig. 5). Ser residues variously corresponding to S34, S36, or S39 are present in a number of RCA homologs, notably in protein 6 (Fig. 5), which has a very high homology to the RCA proteins (Fig. 5).

Because wheat CDPK is Ser/Thr-specific (Polya et al., 1989) and there are no other Ser/Thr sites, we infer that CDPK-catalyzed phosphorylation occurs on Ser residues deduced to be present in the C-terminal region as discussed above (Fig. 5). Ser residues variously corresponding to S34, S36, or S39 are present in a number of RCA homologs, notably in protein 6 (Fig. 5), which has a very high homology to the RCA proteins (Fig. 5).
Most of the RCA-related polypeptides have G-, P-, and S-rich C-terminal domains (Fig. 5). The P residues in particular could well determine a non-α-helical, open conformation of this segment, making it more accessible to the CDPK in either the precursor protein or as the C-terminal segment of the proteolytically processed proteins studied here. The location of the CDPK phosphorylation site close to the point of precursor protein processing suggests a possible function of signal-regulated phosphorylation in this process.

The RCAs are very basic proteins that inhibit calmodulin function and are similar in these properties and in molecular size to calmodulin antagonist proteins previously partially purified from radish seeds (Cocucci and Negrini, 1988). These calmodulin-inhibiting proteins disappear during germination, and ABA inhibits both germination and the decrease in the levels of the inhibitor proteins (Cocucci and Negrini, 1988). Germination is associated with an increase in calmodulin and a decrease in calmodulin inhibitory activity (Cocucci and Negrini, 1988). The RCA polypeptides resolved and characterized in the present study would have contributed to the set of low mol wt, basic calmodulin inhibitors previously described (Cocucci and Negrini, 1988). The functional consequences of phosphorylation of the RCA inhibitor proteins by signal-regulated CDPK are not known. However, such phosphorylation of these napin homologs could conceivably affect small subunit precursor processing, polypeptide chain folding, interaction with other proteins such as calmodulin, and proteolysis in early germination.

The mechanism of inhibition of Ca\(^{2+}\)-calmodulin-dependent MLCK is likely to be through RCA binding to Ca\(^{2+}\)-calmodulin and prevention of MLCK activation. Thus, a preparation of radish calmodulin antagonist also inhibits Ca\(^{2+}\)-calmodulin-dependent cyclic nucleotide phosphodiesterase (Cocucci and Negrini, 1988). Further, the sequences of the purified RCA proteins (Fig. 5) have the potential to form α-helical structures, as do other calmodulin-binding polypeptides such as melittin (Terwilliger and Eisenberg, 1982; Anderson and Malencik, 1986; Vogel and Jähning, 1986) and polypeptides corresponding to the calmodulin-binding domains of a number of other calmodulin-interacting proteins including MLCK (O’Neil and De Grado, 1990). The RCA sequences 1–14 listed in Figure 5 have homologous regions rich in helix-forming amino acids (corresponding to C\(^{10}\) to Q\(^{33}\) of RCA1) bounded by C-terminal and N-terminal sequences rich in the helix-breaking amino acids Gly and Pro, respectively. Analysis of these potential α-helical domains by helical wheel projections (see Lukas et al., 1986; O’Neil and De Grado, 1990) reveals a surprising confinement of all basic (H, K, and R) residues to one “side” of the α-helices. Likewise, of the potential 24 amino acid α-helical domains of proteins 1–6 and 8–14 (Fig. 5) six to seven basic amino acids in each domain are confined to one sector constituting 200 to 240° of the projection circle and are completely absent from the remaining sector. In the putative helix of protein 7 (Fig. 5) three basic residues are confined to a 120° sector of the projection circle. This arrangement is very similar to the disposition of basic residues in calmodulin-binding polypeptides deriving from the calmodulin-binding domains of smooth muscle MLCK (Lukas et al., 1986; Pearson et al., 1988) and skeletal muscle MLCK (Blumenthal et al., 1985, 1988). Thus, a helical wheel projection applied to the 20-amino acid RS20 calmodulin-binding peptide based on the calmodulin-binding domain of avian MLCK (Lukas et al., 1986) reveals seven basic amino acids confined to a 220° sector of the projection circle. The same analysis applied to the first 17 residues of M13 peptide, a calmodulin-binding peptide corresponding to the calmodulin-binding domain of skeletal muscle MLCK (Blumenthal et al., 1985, 1988) similarly reveals seven basic amino acids confined to a 220° sector of the projection circle.

In contrast with the results obtained with the RCA and MLCK sequences, this analysis applied to the first 21 amino acids of melittin (which have been determined to form an amphiphilic α-helix in membranes [Vogel and Jähning, 1986]) reveals 14 nonpolar residues confined to a 240° sector containing no polar residues and 5 polar residues (including 2 K residues) confined to the remaining 120° sector. In comparison, the potential α-helical domains of the RCAs and related plant proteins (Fig. 5) are much more similar to the amphiphilic α-helices of the RS20 and M13 MLCK-derived calmodulin-binding peptides, in which all of the basic residues (and not simply all of the polar residues as in melittin) are confined to one sector of the helical projection wheel.

The retention of RCA calmodulin antagonist activity after the heat treatment and chromatography in CH\(_3\)CN involved in isolation (Fig. 5B) could suggest nonspecific interaction of denatured basic RCA polypeptides with acidic calmodulin. However, in this connection, the MLCK-derived RS20 peptide that exhibits similarities to the RCA proteins in terms of amphiphilic helix determinants exists as a random coil in aqueous solution but has about 60% α-helix secondary structure in the presence of Ca\(^{2+}\)-calmodulin or in 70% trifluoroethanol (Lukas et al., 1986). The low RCA concentrations required for MLCK inhibition and the conservation in the RCAs and related proteins (Fig. 5) of amphiphilic α-helix-related structural features found in other calmodulin-binding proteins (O’Neil and De Grado, 1990) suggest physiological calmodulin-antagonist functions for these proteins. Such functions could include control of seed germination, as proposed by Cocucci and Negrini (1988).

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