Planta (1991) 184:67–73

Planta

Calcium-dependent phospholipid-binding proteins in plants

Their characterisation and potential for regulating cell growth

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Received 27 September; accepted 12 November 1990

Abstract. There is evidence that Ca^{2+} can regulate vesicle-mediated secretion in plant cells, but the mechanism for this is not known. One possibility is that Ca^{2+}-dependent phospholipid-binding proteins (annexins) couple the Ca^{2+} stimulus to the exocytotic response. Using a protocol developed for the isolation of animal annexins we have identified proteins in maize (*Zea mays* L.) coleoptiles that have similar characteristics to annexins. The predominant polypeptide species run as a doublet of relative molecular mass (M_r) 33 000–35000 on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE); another less-abundant protein of M_r 23000 is also present. In the presence of Ca^{2+} these proteins bind to liposomes composed of acidic phospholipids. Calcium-sensitivity of binding differs for each protein and is also influenced by the pH of the buffer used for the liposome-binding assay. Antiserum raised to the 33 to 35-kDa doublet purified on SDS–PAGE recognises the doublet in crude extracts from maize and proteins of similar M_r in *Tradescantia virginiana* and tobacco (*Nicotiana tabacum* L.). The antiserum also recognises p68 (Annexin VI) from chicken gizzard extracts, indicating homology between animal annexins and the maize proteins. For the maize proteins to be involved in the regulation of exocytosis, binding to phospholipids would be expected to occur at physiological levels of Ca^{2+}. The characteristics of the maize annexin-like proteins are described and attention drawn to the marked effect of pH in lowering the requirement for Ca^{2+} for phospholipid binding.

Key words: Annexin – Calcium – Exocytosis – Phospholipid-binding proteins – *Zea* (annexins)

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Abbreviations: DEAE = diethylaminoethyl; EGTA = ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid; kDa = kilodalton(s); M_r = relative molecular mass; SDS–PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Introduction

Exocytosis in plants is a key process fundamental to the secretion of cell-wall precursors, proteins and plasma membrane. It links the machinery of the cell with its external wall and intercellular milieu. While secretion of proteins is believed to follow the constitutive pathway (Jones and Robinson 1989), there is evidence that Ca^{2+} influences the exocytosis of vesicles that contain cell-wall precursors. Firstly, in sycamore cell suspensions the addition of Ca^{2+} resulted in an increased secretion of polysaccharides into the suspension medium (Morris and Northcote 1977). Secondly, Ca^{2+} has been found to be necessary for *Tradescantia* pollen-tube growth, a process in which vesicle-mediated secretion is known to be essential (Picton and Steer 1985; Steer and Steer 1989 for review). Thirdly, antagonists of Ca^{2+} action block the increase in cell growth that results from auxin treatment; it has been concluded that auxin action is mediated by a change in intracellular Ca^{2+} (Cunninghame and Hall 1986). The concept that Ca^{2+} may act as a secondary messenger to auxin has received some support from the finding that treatment with IAA leads to an increase in cytosolic Ca^{2+} concentration (Felle 1988). A model linking Ca^{2+} as a secondary messenger to auxin, which seeks to explain the regulatory effect of auxin on the secretion of cell-wall precursors from the Golgi apparatus, has been proposed (Brummell and Hall 1987).

We are interested in the mechanism by which a change in the intracellular Ca^{2+} level could control the rate of exocytosis of vesicles containing cell-wall precursors. Although Ca^{2+} per se can promote fusion of artificial liposomes (Wilschut et al. 1980), a concentration of more than 1 mM is required for this effect. The capacity to control exocytosis at more physiological (micromolar) levels of Ca^{2+} has been attributed to some members of a class of Ca^{2+}-dependent phospholipid-binding proteins from animal cells (Drust and Creutz 1988; Ali et al. 1989). In recognition of their high degree of sequence

homology and their putative role as Ca^{2+}-regulated promoters of vesicle fusion these proteins have been given the generic name "annexins" (Geisow and Walker 1986; Crumpton and Dedman 1990 for nomenclature).

We show here that maize coleoptiles contain Ca^{2+}-dependent phospholipid-binding proteins in significant quantities with the major polypeptide species running as a doublet of M_r 33000–35000 on SDS–PAGE. We also show that this doublet and a polypeptide of M_r 23000 bind to liposomes composed of acidic phospholipids in a manner that is dependent on the level of free Ca^{2+} and the pH of the buffer in which the assay is performed. An antiserum raised to the 33 to 35-kDa doublet cross-reacts with the animal annexin p68, and with proteins of $M_r \sim 35000$ from *Tradescantia* and tobacco, which indicates that the proteins are highly conserved between plants and animals. The possible role of these proteins in the regulation of vesicle-mediated secretion in maize coleoptiles and *Tradescantia* pollen tubes is discussed.

Material and methods

Plant materials. Maize (*Zea mays* L. cv. Clipper; J.W. Hull & Co., Rainham, Kent, UK) seed was soaked overnight in running water and then grown for 5 d in moist vermiculite in the dark. The etiolated coleoptiles and enclosed leaf rolls were harvested, frozen in liquid N_2 and stored at $-18°$ C until required. The entire shoot and root tissue of *Tradescantia virginiana* (obtained from Kew Gardens) was harvested, frozen in liquid N_2 and stored at $-18°$ C until required. Tobacco (*Nicotiana tabacum* L. cv. Samsun) cells derived from stem-pith callus (courtesy of Dr S. Shohet, Department of Agricultural Botany, University of Reading, UK) were cultured as a suspension in Murashige and Skoog medium supplemented with IAA and N^6-furfurylaminopurine (kinetin). The cells were harvested by filtration and ground to a fine powder in liquid N_2 using a mortar and pestle.

Isolation of Ca^{2+}-dependent phospholipid-binding proteins. Maize coleoptiles (40 g) were homogenised in 200 ml of 0.15 M NaCl, 10 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid (Hepes), 10 mM EDTA (pH 7.4) in a mortar and pestle, filtered through two layers of muslin, and centrifuged for 30 min at $30000 \cdot g$ (av). The supernatant was decanted and 200 mg bovine brain lipid (B3635; Sigma, Poole, Dorset, UK) added to it, followed by 1 M $CaCl_2$ to give a final concentration of 15 mM. After 30 min on ice the suspension was centrifuged for 30 min at $30000 \cdot g$(av). The pellet was resuspended using a glass-Teflon homogeniser and washed twice in first 100 ml and then 50 ml of 0.15 M NaCl, 10 mM Hepes, 1 mM $CaCl_2$ (pH 7.4). Each wash was followed by centrifugation for 30 min at $30000 \cdot g$(av). The final pellet was resuspended in 20 ml of 0.15 M NaCl, 10 mM Hepes, 15 mM EDTA (pH 7.4) and centrifuged for 1.5 h at $100000 \cdot g$(av). In order to remove nucleic-acid contaminants the supernatant was dialysed for 16 h against 300 vols. of 10 mM potassium phosphate (KPO_4) (pH 6.5) before loading on to a hydroxylapatite (HA–Ultrogel, IBF; Life Science Laboratories, Luton, Beds., UK) column (volume 1.73 ml). The column was washed with 20 ml of 10 mM KPO_4 (pH 6.5) to elute nucleic acids, and the bound protein was then eluted with 500 mM KPO_4 (pH 7.4). For use in lipid-binding assays, the post-HA fraction was dialysed overnight against 500 vols. of 20 mM Hepes (pH 7.4) and concentrated using Centricon-10 microconcentrators (Amicon, Stonehouse, Gloucestershire, UK) according to the manufacturers instructions. All steps were performed at 4° C.

Calcium-dependent phospholipid-binding proteins were also isolated from entire shoots (20 g) and roots (20 g) from *Tradescantia virginiana* and from tobacco cell suspensions (10 g) using the above protocol with appropriate reductions in buffer volumes and omitting the hydroxylapatite purification step. Endonexin II (Annexin V) was isolated from bovine lung as described in Boustead et al. (1988), and chicken-gizzard annexins were also obtained using this method but purification by diethylaminoethyl (DEAE) cellulose chromatography was not performed.

Crude protein samples. Crude protein preparations were obtained from maize and *Tradescantia* shoot and root tissue by removal of an aliquot from the supernatant obtained following the first centrifugation step of the protocol described above. The sample was concentrated by the addition of trichloroacetic acid to a final concentration of 10%.

Protein assay. Protein concentrations were measured using the method of Sedmak and Grossberg (1977) with bovine serum albumin as the standard.

Preparation of antiserum. Antiserum against the 33- to 35-kDa doublet from maize was raised as follows. The protein eluted from membranes and bovine brain lipids with 15 mM EDTA as described above was concentrated by the addition of 1 M $CaCl_2$ to give 16 mM $CaCl_2$, followed by ammonium sulphate to give 80% (w/v) final concentration. The resulting suspension was centrifuged for 30 min at $11000 \cdot g$(av). The pellet was washed with 80% acetone and centrifuged for 10 min at $11500 \cdot g$(max). The dry pellet was taken up in sample buffer and subjected to SDS–PAGE as described below. Using a Coomassie-blue-stained tracking lane the region on gels containing the 33 to 35-kDa doublet were excised from the unstained gels, and electro-eluted at 100 V for 24 h through a non-denaturing (5% acrylamide; Tris-glycine pH 8.3 electrode buffer) tube-gel with dialysis sac attached. The sample was then concentrated on Amicon Centricon-10 microconcentrators and made up to 150 $\mu g \cdot ml^{-1}$ in 10 mM Tris-HCl (pH 7.4). The protein sample was mixed with Freund's adjuvant (complete for the first immunisation and subsequently incomplete) and 50 µg of protein injected into each of three New Zealand white rabbits on three occasions at monthly intervals. The animals were bled 10 d after the final immunisation.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting. Analysis by SDS–PAGE was performed on 12% gels as described by Laemmli (1970), with M_r standards from Sigma (Dalton Mark VII). Gels were stained with Coomassie brilliant blue, or protein separated by SDS–PAGE was electrophoretically transferred to nitrocellulose filters (0.45 µm pore size; Schleicher & Schuell, obtained through Anderman, Kingston-on-Thames, UK) overnight at 30 V in 20% (v/v) methanol-25 mM Tris-192 mM glycine, pH 8.3. The protein trasferred to nitrocellulose membranes was made visible by iodinating with Chloramine T and staining with starch (Kumar et al. 1985). The nitrocellulose was processed for immunoblotting by incubating overnight at 4° C in Tris-buffered saline-Tween (TBS–Tween; 25 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4) containing 3% (w/v) low fat milk. The blot was incubated for 2 h at 20° C in the same buffer containing antiserum at a dilution of 1:1000, washed 3 × 10 min in TBS–Tween and then incubated with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma A6154) at a dilution of 1:200 for 40 min. Following 3 × 10 min washes in TBS–Tween, peroxidase activity was made visible on blots by incubation in 10 ml of 50 mM Tris-HCl (pH 7.4) containing 0.03% (v/v) H_2O_2 and 0.05% (w/v) of 3,3'-diaminobenzidene. Pre-immune serum controls at a dilution of 1:50 were used to detect non-specific cross-reactivity.

Phospholipid binding. Phospholipid vesicles were prepared using L-α-phosphatidylcholine (Sigma P7763), L-α-phosphatidyl-L-serine (Sigma P6641), L-α-phosphatidylethanolamine (Sigma P3511) and L-α-phosphatidylinositol (Sigma P2517), and assays for protein binding were carried out at pH 7.4 (Figs. 4, 5) as described by Boustead et al. (1989). Free-Ca^{2+} levels in the phospholipid-binding assays were set using Ca^{2+}/ethylene glycol-bis(β-aminoethyl-

ether)-N,N,N',N'-tetraacetic acid (EGTA) buffers according to Robertson and Potter (1984). The effect of pH on liposome binding was initially investigated using 20 mM Hepes adjusted to either pH 6.0, 7.0 or 8.0 with 1 N NaOH (Fig. 6); subsequent assays at pH 6.0 were carried out using 20 mM 2-(N-morpholino)ethanesulphonic acid (Mes; Fig. 7).

Results

Purification of maize Ca^{2+}-dependent phospholipid-binding proteins. The initial isolation procedure that utilises the Ca^{2+}-dependent binding of protein to membranes and added phospholipid provides a significant degree of protein purification as relatively few polypeptides are recovered in the supernatant following EDTA elution (Fig. 1, lane B). The most prominent of these is a doublet of M_r 33000–35000. Nucleic acids that co-purify with the polypeptides and interfere with protein estimations can be removed by passage through a hydroxylapatite column with minimal loss of the 33- to 35-kDa doublet (Fig. 1, lanes C, D). The resulting recovery of Ca^{2+}-dependent phospholipid-binding proteins from maize was typically 10 μg protein · g^{-1} fresh weight of tissue.

Immunological reactivity. In order to determine the level of specificity, antiserum raised to the 33 to 35-kDa doublet purified on SDS–PAGE was incubated with blots heavily loaded with crude protein preparations from both maize and *Tradescantia* (Fig. 2). Although the antigen represents only 0.1% of total protein in maize, the antiserum specifically recognises the 33- to 35-kDa doublet (Fig. 2 (ii) lane A). The antiserum also strongly recognises polypeptides of M_r 35000–36000 from *Tradescantia* root and shoot tissues, and there is faint detection of a polypeptide of M_r 68000 (Fig. 2 (ii) lanes B, C). There was no detection of protein on blots loaded with crude protein extracts when incubated with pre-immune serum. The antiserum reacts strongly with maize extracts purified using the protocol for isolating Ca^{2+}-dependent phospholipid-binding proteins, followed by hydroxylapatite chromatography (Fig. 3 (ii) lane A). The antiserum recognition of partially purified Ca^{2+}-dependent phospholipid-binding proteins from *Tradescantia* root tissue and tobacco cell-suspensions (Fig. 3 (ii) lanes B, C) demonstrates that these proteins contain similar epitopes to the 33- to 35-kDa protein from maize. Homology between plant and animal Ca^{2+}-dependent phospholipid-binding proteins is demonstrated by the cross-reactivity of the antiserum with p68 (Annexin VI) present in chicken-gizzard annexin preparations (Fig. 3 (ii) lane D), although bovine endonexin II (Annexin V) is not recognised (Fig. 3 (ii) lane E).

Calcium-dependent phospholipid-binding. In order to investigate the Ca^{2+}-dependent lipid-binding characteris-

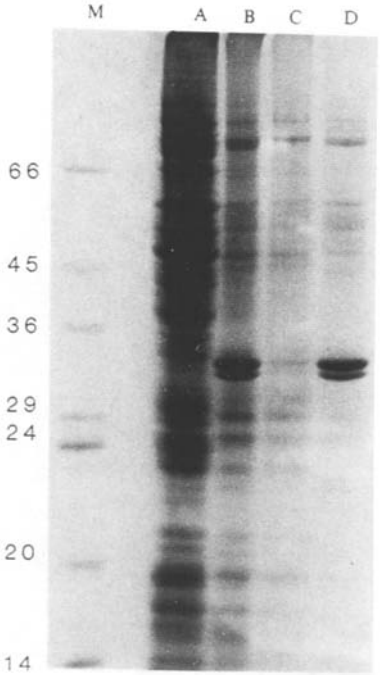

Fig. 1. Purification of Ca^{2+}-dependent phospholipid binding proteins from maize. The SDS-polyacrylamide gels were loaded with: a crude protein extract, equivalent to 0.2 g FW of coleoptile tissue (*A*); proteins isolated using the protocol for Ca^{2+}-dependent phospholipid-binding proteins, equivalent to 2.0 g FW of tissue (*B*); unbound fraction from hydroxylapatite chromatography, equivalent to 2.0 g FW of tissue (*C*) and fraction eluted from hydroxylapatite with 500 mM KPO_4, equivalent to 2.0 g FW (*D*). *M* indicates M_r markers (kDa)

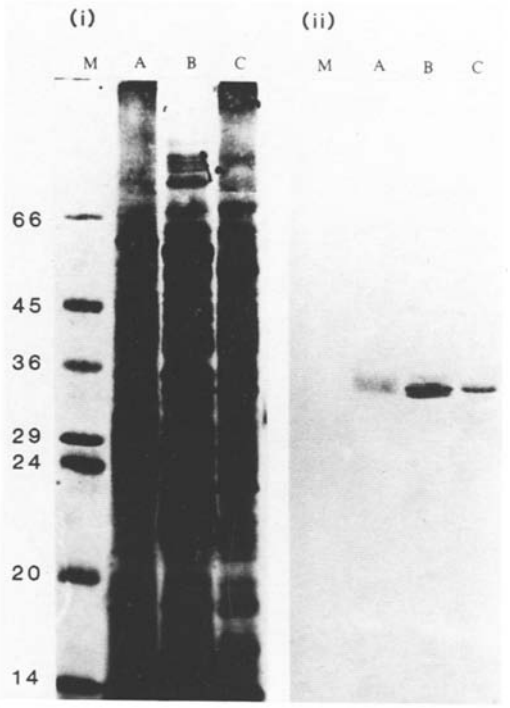

Fig. 2. Cross-reactivity of antiserum raised to the 33- to 35-kDa proteins from maize, with crude protein preparations from plant tissues. (*i*) Protein transferred to nitrocellulose, iodinated with chloramine-T and revealed by starch; and (*ii*) immunoreactive bands visualised with 3,3,-diaminobenzidene. The SDS-polyacrylamide gels were loaded with 20 μg of protein from maize coleoptiles (*A*); 50 μg of protein from *Tradescantia* shoot tissue (*B*) and 50 μg of protein from *Tradescantia* root tissue (*C*). *M* indicates M_r markers (kDa)

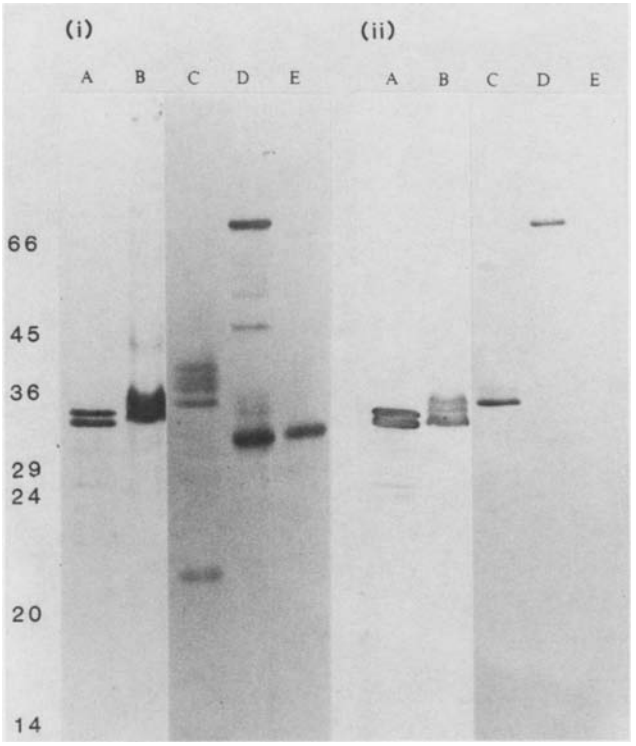

Fig. 3. Cross-reactivity of antiserum raised to the 33- to 35-kDa proteins from maize with animal annexins and Ca^{2+}-dependent phospholipid-binding proteins purified from plants. (*i*) Protein transferred to nitrocellulose, iodinated with chloramine-T and revealed by starch; and (*ii*) immunoreactive bands visualised with 3,3,-diaminobenzidene. The SDS-polyacrylamide gels were loaded with 5 μg of protein from: maize coleoptile extract purified on hydroxylapatite (*A*); *Tradescantia* root tissue (*B*); tobacco cell suspensions (*C*) and purified extracts of chicken-gizzard annexins (*D*) and endonexin II (*E*)

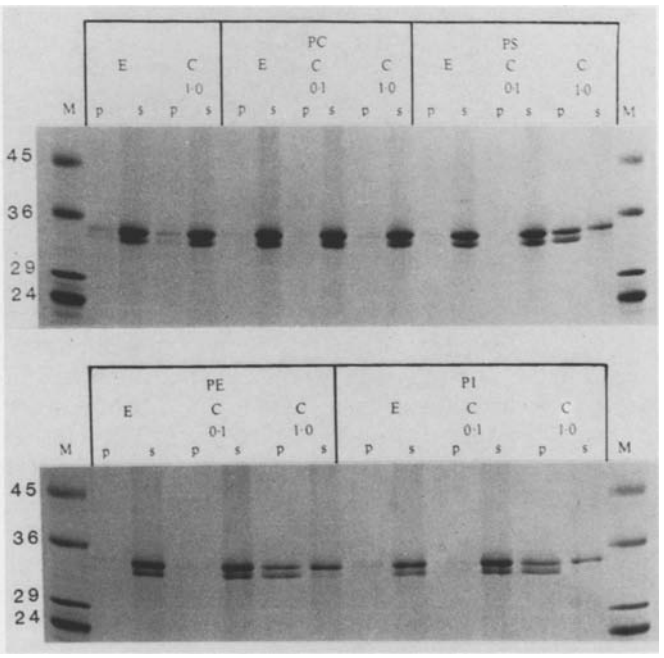

Fig. 4. Calcium-dependent binding of the 33- to 35-kDa proteins from maize to liposomes. The liposomes were prepared from pure phosphatidylcholine (*PC*) or equimolar mixtures of phosphatidylcholine and phosphatidylserine (*PS*) *(upper gel)* and either phosphatidylcholine and phosphatidylethanolamine (*PE*) or phosphatidylcholine and phosphatidylinositol (*PI*) *(lower gel)*. Coomassie-blue-stained 12% SDS–PAGE of equal proportions of supernatants (*s*) and pellets (*p*). Protein (10 μg) was incubated ± liposomes in the presence of 1 mM EGTA (*E*) 0.1 mM free Ca^{2+} or 1 mM free Ca^{2+} (*C*). *M* indicates M_r markers (kDa)

tics of the 33- to 35-kDa doublet from maize the hydroxylapatite-purified protein was incubated at various Ca^{2+} concentrations with liposomes (150 μg per assay) prepared from phosphatidylcholine (PC) or a 1:1 mixture of phosphatidylcholine and phosphatidylserine (PS). Following centrifugation, the proportion of protein bound to the liposome pellet was determined by comparing the pellet and supernatant fractions on SDS–PAGE. There is negligible recovery of protein in pellet fractions in the absence of liposomes or presence of liposomes composed entirely of PC even at 1 mM Ca^{2+} (Fig. 4). However, in the presence of PS-liposomes and Ca^{2+} the protein binds to the liposomes and co-sediments (Fig. 4). The phospholipid specificity for binding was further tested by incubating the maize extract with liposomes composed of a mixture of phosphatidylcholine and either phosphatidylethanolamine (PE) or phosphatidylinositol (PI). Binding of protein to both liposome types is again apparent in the presence of Ca^{2+} (Fig. 4).

These preliminary attempts to determine Ca^{2+}-dependence and phospholipid specificity indicated that more than 100 μM free Ca^{2+} was required for effective phospholipid binding. The critical levels for Ca^{2+}-dependent binding were then investigated using hydroxylapatite-purified proteins (20 μg per assay) and liposomes composed of PS (200 μg phospholipid per assay). These assay conditions revealed that a proportion of the protein binds to liposomes at less than 100 μM free Ca^{2+}; the 33-kDa polypeptide has a requirement for 120 μM free Ca^{2+} for 1/2 maximal binding, while the 35-kDa polypeptide has a requirement for 370 μM free Ca^{2+} for 1/2 maximal binding (Fig. 5). The higher protein loading used for these assays also revealed that a protein of M_r 23000 has the capacity to bind to liposomes in a Ca^{2+}-dependent manner at lower levels of Ca^{2+} (< 100 μM).

To test whether the relatively high Ca^{2+} requirement for binding to liposomes resulted from partial proteolysis and inactivation during extraction and purification, extracts were prepared in the presence of a cocktail of protease inhibitors including phenylmethylsulphonyl fluoride (0.25 mM), leupeptin (2.0 $\mu g \cdot ml^{-1}$), 3,4, dichloroisocoumarin (0.1 mM), aprotinin (1.5 $\mu g \cdot ml^{-1}$) and dithiothreitol (2 mM). Calcium-dependent phospholipid-binding was unaffected by this treatment (results not shown). We consider it unlikely that the 33- to 35-kDa polypeptides appear as a result of proteolytic cleavage of the 68-kDa protein visible in Fig. 1 (lane D) for the following reasons. First, the use of protease inhibitors during extraction and purification of maize extracts did not increase the yield of the 68-kDa protein. Second, although faint detection of a band of similar M_r occurs on Western blots of crude *Tradescantia* extracts (Fig. 2, ii), there is no evidence for Ca^{2+}-dependent

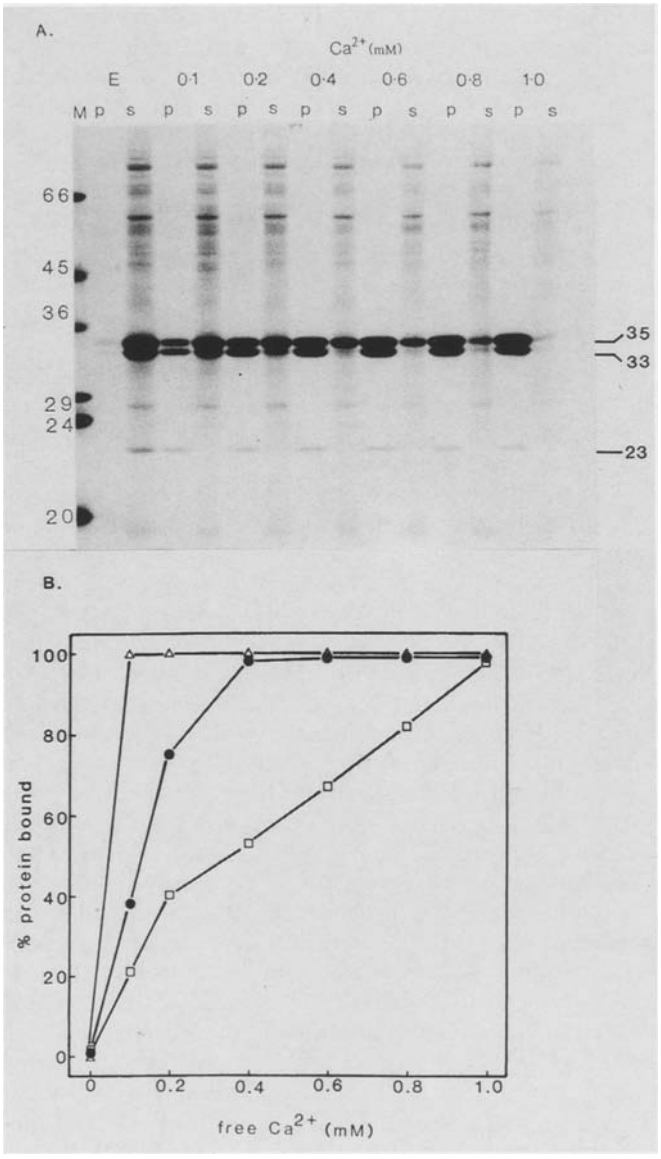

Fig. 5A, B. Calcium-dependent binding of annexin-like proteins from maize to liposomes prepared from an equimolar mixture of phosphatidylcholine and phosphatidylserine. Protein (20 μg) was incubated with liposomes in Ca^{2+}/EGTA buffers to give from 0–1 mM free Ca^{2+}. **A** Coomassie-blue-stained, 12% SDS–PAGE of equal proportions of pellets (*p*) and supernatants (*s*). *E* indicates 1 mM EGTA. *M* indicates M_r markers (kDa). **B** The percentage of protein bound to liposomes as a function of the assay free-Ca^{2+} concentration is shown for the 35-kDa (□—□), 33-kDa (●—●) and 23-kDa (△—△) annexin-like proteins

binding of the 68-kDa protein from maize to phospholipids.

Interaction between Ca^{2+} and pH for phospholipid binding. We also considered the possibility that the capacity of annexin-like proteins to bind to liposomes could be influenced by pH. To test this, the proteins were incubated with liposomes equilibrated with buffers at pH 6.0, 7.0 and 8.0. Total Ca^{2+} was adjusted to give the same free-Ca^{2+} concentration at each pH (Robertson and Potter 1984). At pH 6.0 both the 33- and the 35-kDa

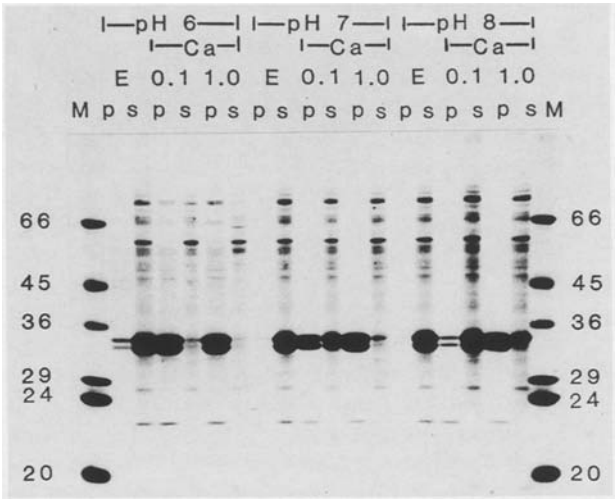

Fig. 6. Interaction between Ca^{2+} and pH for phospholipid binding of annexin-like proteins from maize. The liposomes were prepared from equimolar mixtures of phosphatidylcholine and phosphatidylserine. Coomassie-blue-stained 12% SDS–PAGE of equal proportions of pellets (*p*) and supernatants (*s*). Protein (20 μg) was incubated with liposomes in Ca^{2+}/EGTA buffers at pH 6.0, pH 7.0 and pH 8.0 in the presence of 1 mM EGTA (*E*), 0.1 mM free Ca^{2+} or 1 mM free Ca^{2+} (*Ca*). *M* indicates M_r markers (kDa)

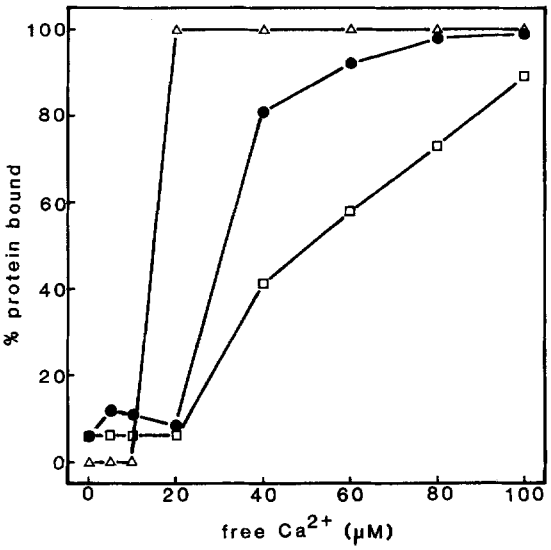

Fig. 7. Calcium-dependent binding of annexin-like proteins from maize to liposomes prepared from an equimolar mixture of phosphatidylcholine (PC) and phosphatidylserine at pH 6.0. Protein (20 μg) was incubated with liposomes in Ca^{2+}/EGTA buffers to give from 0–100 μM free Ca^{2+}. Densitometric scans were made of Coomassie-blue-stained 12% SDS-polyacrylamide gels loaded with equal proportions of pellets and supernatants. The percentage of protein bound to liposomes as a function of assay free-Ca^{2+} concentration is shown for the 35-kDa (□—□), 33-kDa (●—●) and 23-kDa (△—△) annexin-like proteins. Binding to liposomes composed of PC alone at 100 μM free Ca^{2+} was 7% for the 35- and 33-kDa polypeptides, 0% for the 23-kDa polypeptide

proteins bind to liposomes at 100 μM free Ca^{2+}, whereas at higher pH values 1 mM free Ca^{2+} is required for complete binding of both proteins (Fig. 6). The free-Ca^{2+} concentrations for 1/2 maximal binding at pH 6.0 were found to be 30 and 50 μM for the 33- and 35-kDa

polypeptides, respectively (Fig. 7): this represents a four- to eight-fold increase in Ca^{2+}-sensitivity in comparison to liposome binding at pH 7.4 (compare Figs. 7 and 5). In contrast the 23-kDa polypeptide binds to liposomes at < 100 μM free Ca^{2+} at all pH levels (Fig. 6) and at pH 6.0 binding is apparent at a level of 20 μM free Ca^{2+} (Fig. 7).

Discussion

We have identified in maize, Ca^{2+}-dependent phospholipid-binding proteins that have similar characteristics to the extensively studied group of animal proteins known as annexins (Geisow and Walker 1986; Crompton et al. 1988; Burgoyne and Geisow 1989). The most prominent of these proteins run as a doublet (M_r 33000–35000) on SDS–PAGE and in the presence of Ca^{2+} bind to and co-sediment with liposomes composed of acidic phospholipids, but not to liposomes composed entirely of phosphatidylcholine. Antibodies raised to this doublet recognise the animal annexin p68 (annexin VI), indicating homology between plant Ca^{2+}-dependent phospholipid-binding proteins and animal annexins. The same conclusion has been reached for Ca^{2+}-dependent phospholipid-binding proteins of M_r 33000–35000 isolated from tomato cell suspensions: these proteins cross-reacted with antibodies to the animal annexins, calelectrin (annexin V) and p68 (annexin VI) (Boustead et al. 1989), and sequence analysis has identified areas of homology between plant and animal annexins (Smallwood et al. 1990). Use of antiserum to maize annexins has also allowed us to identify proteins of similar M_r that are present in a variety of plant species and tissue types (Figs. 2 and 3).

A Ca^{2+}-dependent phospholipid-binding protein of M_r 23000 is also present in maize (Figs. 5–7). This protein is unusual in that it does not fit into the molecular-size ranges typical of annexins (Geisow et al. 1987), although an annexin-like protein of M_r 22000 has recently been identified in bovine lung extracts (Khanna et al. 1990). The 23-kDa polypeptide in maize has similar characteristics to the 33- to 35-kDa polypeptides, with Ca^{2+}-dependent binding to liposomes composed of acidic phospholipids. However, it is notable that the 23-kDa polypeptide is not recognised by antiserum raised to the 33- to 35-kDa polypeptides and also appears to have a lower requirement for Ca^{2+} for liposome binding. Sequence analysis will enable us to clarify the relationship of the 23-kDa protein to other plant and animal annexins.

The wide distribution of annexin-like proteins at the relatively abundant level of 0.1% total protein indicates an important function in plants. Our hypothesis is that the annexin-like proteins may be involved in the regulation of vesicle-mediated secretion in plant cells. This is based on observations that the animal annexins, synexin (annexin VII) (Creutz et al. 1978; Hong et al. 1981) and calpactin (annexin II) (Ali et al. 1989; Nakata et al. 1990) have the potential to regulate exocytosis by binding to the plasma membrane and vesicle membranes under conditions of elevated Ca^{2+}. It is therefore of great interest that proteins of similar M_r to those studied here (M_r 35000–36000) have been implicated in the Ca^{2+}-dependent fusion of Golgi apparatus- and plasma-membrane-rich fractions from maize (Baydoun and Northcote 1980a, b; 1981). In another experimental system it has been suggested that the elevated concentration of intracellular Ca^{2+} at the tip of *Tradescantia* pollen tubes causes the fusion of vesicles, so providing cell-wall polysaccharides and plasma membrane for continued tip growth (Picton and Steer 1985). Our antiserum to maize annexin-like proteins clearly cross-reacts with *Tradescantia* extracts (Fig. 3). Thus, by immunolocalising at the electron-microscope level we can test the hypothesis that annexin-like proteins are associated with secretory vesicles and the plasmalemma.

For annexin-like proteins in plants to have an equivalent function to animal annexins the plant proteins would be expected to bind to membranes at physiological levels of free Ca^{2+}. There is now considerable evidence that, as in animal cells, resting Ca^{2+} levels lie between 100–400 nM (Miller and Sanders 1987; Nobiling and Reiss 1987; Clarkson et al. 1988; Williams et al. 1990), and that in activated cells Ca^{2+} levels are in the low micromolar range (e.g. Williams et al. 1990; Gehring et al. 1990). Although it is of interest that pH has a marked effect on the Ca^{2+} sensitivity of liposome binding of the plant annexins (Fig. 6), both pCa and pH for effective binding are outside their accepted physiological range. Assessment of the importance of our observation therefore awaits the development of a more realistic in vitro assay (e.g. using native secretory vesicles) for annexin binding. However, we are interested in further characterising this observation because of the reported interaction between oscillations in cytoplasmic pH and free Ca^{2+} that occur in maize coleoptile cells following auxin treatment (Felle 1988). Since auxin treatment causes an increase in the incorporation of cell-wall precursors (Brummell and Maclachlan 1989), and these precursors are secreted via a vesicle-mediated pathway, it is clearly of interest that plant annexins might be subject to dual regulation by changes in intracellular pH and pCa.

This work was funded by the Agricultural and Food Research Council. Our thanks also to Professor P. Lowry and Dr R. Woods, Department of Biochemistry, University of Reading for facilities and advice for antiserum production, and C. Boustead, Department of Biochemistry, University of Leeds for advice on immunoblotting and phospholipid-binding assays.

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