Partial purification of a protein from maize (*Zea mays*) coleoptile membranes binding the Ca^{2+} -channel antagonist verapamil

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A protein that binds the calcium-channel antagonist verapamil has been partially purified from maize (*Zea mays*) coleoptile membranes. The protein was solubilized with the detergent CHAPS {[3-(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate} and purified by a combination of ion-exchange, gel-filtration and hydrophobic-interaction chromatography. This resulted in a 120-fold purification. SDS/polyacrylamide-gel-electrophoretic analysis of the polypeptides from the final purification step indicated that the verapamil-binding protein may have a major component of M_r 169 000. The dissociation constants for specific binding of [^3H]verapamil to crude and CHAPS-solubilized maize coleoptile membrane fractions are 72 nM and 158 nM respectively, with respective binding-site concentrations of 135 pmol/mg of protein and 78 pmol/mg of protein. In both cases the Scatchard plots are linear, indicating a single class of binding sites. [^3H]Verapamil binding to crude maize coleoptile membrane fractions could not be displaced by unlabelled desmethoxyverapamil or by nifedipine, but could be displaced by unlabelled methoxyverapamil.

INTRODUCTION

Ca^{2+} is a ubiquitous regulator of growth and development [1]. It is known that there are similarities between the control systems involving Ca^{2+} in both plants and animals [2–6]. Plant cells regulate their cytosolic Ca^{2+} and maintain it at a submicromolar concentration. Active-transport systems pump Ca^{2+} out of the cell or into organelles. Calmodulin has been isolated from numerous plants and tissues. Changes in cytosolic Ca^{2+} are believed to contribute to the control of diverse phenomena such as tropic bending, stomatal closure and germination.

The entry of Ca^{2+} into plant cells has received little attention. In animal cells (neurons, skeletal muscle and cardiac muscle), voltage-dependent Ca^{2+} channels are an important route for Ca^{2+} entry into excitable cells. They are necessary for smooth-, cardiac- and skeletal-muscle contraction and for secretory processes [7–9]. Ca^{2+} -channel antagonists are a group of drugs (used to treat cardiovascular disorders) of which there are three main structurally unrelated types. These are the dihydropyridines, e.g. nifedipine, the phenylalkylamines, e.g. verapamil, and the benzothiazepines, e.g. diltiazem [10,11]. These compounds bind to specific receptors located in the Ca^{2+} channel. The radioactive forms of the channel antagonists can be used to label specifically the Ca^{2+} -channel proteins, so providing a means of Ca^{2+} -channel detection and identification [12,13].

Experimental evidence suggests that Ca^{2+} channels do exist in plant cells [6,14,15]. Ca^{2+} -channel antagonists have been demonstrated to disrupt a number of plant functions [16–18]. Nifedipine and verapamil have been shown to bind specifically to plant membranes *in vitro* [19–21]. More recently, various phenylalkylamines have been shown to inhibit $^{45}\text{Ca}^{2+}$ influx into carrot (*Daucus*

carota) protoplasts [22]. A direct relationship has been established between membrane binding constants for a range of phenylalkylamines and the ability of the drugs to inhibit Ca^{2+} uptake [22].

Here we present data for [^3H]verapamil binding in maize (*Zea mays*) coleoptile membranes and describe a method for the solubilization and partial purification of the verapamil-binding protein from maize coleoptile membrane preparations.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays* cv. Leader) seeds, from the National Institute of Agricultural Botany, Cambridge, U.K., were grown in moist vermiculite in the dark at 25 °C. After 5 days the coleoptiles, including the leaf rolls, were harvested and collected on ice.

Chemicals

[*N*-methyl- ^3H]Verapamil hydrochloride, with a specific radioactivity of 68.8 Ci/mmol, was obtained from New England Nuclear, Bedford, MA U.S.A. Unlabelled (+/–)-verapamil hydrochloride was obtained from Sigma. All other chemicals were of the highest grade available, obtained from various sources.

Preparation of crude membrane fractions

All preparative procedures were carried out at 4 °C, unless stated otherwise. Freshly harvested tissue was chopped with a razor blade and then homogenized (2 vol. per g fresh weight) in 50 mM-Tris/3 mM-EDTA buffer, adjusted to pH 7.5 with HCl. The homogenate was then filtered through one layer of nylon cloth and centrifuged at 4000 *g* for 20 min. The pellet was discarded

Abbreviations used: PEG, poly(ethylene glycol); PEI, polyethyleneimine; CHAPS, [3-(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; PAGE, polyacrylamide-gel electrophoresis; DTT, dithiothreitol; Con A, concanavalin A; IC_{50} , inhibition constant (concentration at which 50% of the specifically bound radioligand is displaced); B_{max} , maximum concentration of binding sites.

and the supernatant re-centrifuged at 50 000 *g* for 45 min, yielding the crude membrane pellet, which was resuspended in 50 mM-Tris/50 mM-Mops buffer, adjusted to pH 7.5 with HCl, by using a glass/Teflon homogenizer.

Solubilization of the crude membrane fraction

Crude membrane preparations were incubated at a protein/detergent ratio of 1:14 (w/w), in 0.5% (w/v) CHAPS/20% (w/v) sucrose/50 mM-Tris/50 mM-Mops buffer, adjusted to pH 7.5 with HCl, for 30 min on ice and then centrifuged for 1 h at 100 000 *g*. The resulting supernatant contained the solubilized proteins.

Verapamil-binding assay

In the standard assay, [³H]verapamil was added to the crude membrane resuspension, solubilized membrane fraction or column fraction, to give a final concentration of 2.24 nM. Unlabelled verapamil or other compounds were then added as required to a final assay volume of 500 μ l. After an incubation period of 120 min at 20 °C, the reaction was stopped by the addition of 3 ml of ice-cold wash buffer containing 10% (w/v) PEG 8000/50 mM-Tris, adjusted to pH 7.5 with HCl. The mixture was immediately filtered under vacuum through Whatman GF/C filters (pretreated with 0.3% PEI solution for at least 2 h, when solubilized fractions were assayed), using a Millipore multifilter unit. The filters were washed once with 3 ml of ice-cold wash buffer. The radioactivity retained on the filters was determined by liquid-scintillation counting. Non-specific binding was determined by incubating samples with 100 μ M unlabelled verapamil and was subtracted from total binding (obtained from assays containing [³H]verapamil only), to give the specific binding of [³H]verapamil.

Partial purification of the verapamil-binding protein

DEAE-Bio-Gel ion-exchange chromatography. Solubilized membrane fraction from approx. 100 g fresh weight of tissue was loaded on to a column (4.0 cm $\times$ 1.0 cm) of DEAE-Bio-Gel A (Bio-Rad), which had been equilibrated with column buffer containing 50 mM-Tris, 50 mM-Mops, 20% sucrose, 0.1% CHAPS, pH 7.5 (adjusted with HCl). The ratio of sample to matrix was 5:1 (v/v). After loading of the sample, the column was washed with an equal volume of column buffer. Elution with 0.15 M-NaCl in column buffer yielded the verapamil-binding activity in the first 8 ml of the eluate. This was collected, portions were assayed directly for verapamil binding, and the eluate then quick-frozen in liquid N₂ and stored at -80 °C until required for further purification.

Sephacryl S-400 gel filtration. A Sephacryl S-400 column (with a bed volume of 38.8 ml) was equilibrated with column buffer at a flow rate of 0.5 ml/min. The post-DEAE-Bio-Gel fraction (2 ml) was loaded on to the column and eluted with column buffer. Fractions (1 ml each) were collected, and portions were assayed for verapamil-binding activity. Fractions with the highest binding activity were pooled.

Phenyl-Sepharose hydrophobic-interaction chromatography. Pooled fractions from the Sephacryl column were made 1 M with respect to (NH₄)₂SO₄ and 1 mM with respect to CaCl₂. They were loaded on to a column (2.0 cm $\times$ 1.0 cm) of phenyl-Sepharose CL-4B

(Pharmacia) that had been equilibrated with 10 column volumes of buffer containing 50 mM-Tris, 50 mM-Mops, 20% sucrose, 0.1% CHAPS, 1 M-(NH₄)₂SO₄, 0.1 mM-CaCl₂, pH 7.5 (adjusted with HCl) (buffer A). After washing with buffer A, the column was eluted with 18 ml each of the following series of buffers: B, 50 mM-Tris/50 mM-Mops/20% sucrose/0.1% CHAPS/0.1 mM-CaCl₂, pH 7.5 (adjusted with HCl); C, 50 mM-Tris/50 mM-Mops/20% sucrose/0.1% CHAPS, pH 7.5 (adjusted with HCl); D, 0.1% CHAPS/20% sucrose, pH unadjusted. Fractions (2 ml) were collected and 1 ml samples were assayed for verapamil binding.

SDS/PAGE

Samples were denatured in 2.35% (w/v) SDS/10% (v/v) glycerol/62.5 mM-Tris/HCl (pH 6.8)/5% (v/v) β -mercaptoethanol for 10 min at 95 °C and separated by PAGE on 10% (w/v) polyacrylamide gels, using the buffer system of Laemmli [23]. Gels were stained with silver [24]. Molecular-mass markers obtained from Sigma were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (92 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (31 kDa).

Protein determination

This was carried out by a modified Bradford [25] method, with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Kinetics of [³H]verapamil binding to maize coleoptile membranes

Maize coleoptile membranes (crude and CHAPS-solubilized) bind verapamil in a saturable manner, and this binding is reversible. Binding equilibrium is reached after approx. 120 min (Fig. 1). When 100 μ M unlabelled verapamil is added at the 'plateau', dissociation of bound verapamil is initiated and is largely complete after a further 120 min. Similar kinetic data have been found

Fig. 1. Kinetics of [³H]verapamil binding to maize coleoptile membrane fractions

Association was initiated by addition of [³H]verapamil (final concn. 1.12 nM) to the incubation mixture containing membrane protein (0.38 mg/500 μ l assay volume). At the times indicated, 250 μ l samples were withdrawn and assayed as described in the Materials and methods section. Dissociation was initiated by addition of 100 μ M-verapamil.

Fig. 2. Displacement of [^3H]verapamil by unlabelled Ca^{2+} -channel antagonists

Binding of [^3H]verapamil to maize coleoptile membrane fractions was measured under equilibrium conditions, at pH 7.5 and 20 °C, in the presence of either unlabelled verapamil ($\bullet$), methoxyverapamil ($\circ$), desmethoxyverapamil ($\blacktriangle$) or nifedipine ($\triangle$) at the concentrations indicated. Total verapamil binding without addition of unlabelled compounds was 250 fmol, and non-specific binding (determined by the addition of 100 μM unlabelled verapamil) was 4% of the total.

for both zucchini (courgette, *Cucurbita pepo*) membranes and skeletal-muscle microsomes [20,26].

Of the drugs tested for displacement of [^3H]verapamil, only verapamil and methoxyverapamil were able to appreciably displace [^3H]verapamil binding in maize coleoptile membranes, with IC_{50} values of 100 nM and 700 nM respectively (Fig. 2). Desmethoxyverapamil competed poorly, even though it is closely related to verapamil and has an IC_{50} value of about 50 μM . Nifedipine, which is of a different structural class, had little effect, even at 100 μM . These results suggest that there are specific binding sites for the different Ca^{2+} -channel antagonists within a channel.

Figs. 3(a) and 3(b) show Scatchard plots for [^3H]verapamil binding in crude and CHAPS-solubilized membrane fractions. For crude membrane fractions the apparent K_D was 72 nM and B_{max} was 135 pmol/mg of protein. In the CHAPS-solubilized preparation the K_D was 158 nM and the B_{max} 78 pmol/mg of protein. In both cases the plots are linear, suggesting the presence of a single class of binding sites. The similarities in dissociation constants suggest that the solubilized verapamil-binding protein has properties similar to those of the membrane-bound protein.

Scatchard plots were also constructed for [^3H]verapamil binding to crude membrane preparations from maize roots and zucchini hypocotyls, yielding linear plots in both cases (results not shown). Maize roots were found to have a K_D of 233 nM for verapamil binding and a B_{max} of 165 pmol/mg of protein. A K_D

Fig. 3. Scatchard plots of [^3H]verapamil binding in (a) crude and (b) solubilized maize coleoptile membrane fractions

Least-squares regression analysis was used to plot the line of best fit.

of 114 nM and a B_{max} of 96 pmol/mg of protein was obtained for zucchini membrane fractions, which compares favourably with values obtained previously for zucchini, where the K_D was 102 nM and the B_{max} 60 pmol/mg of protein [20]. It is noticeable that the dissociation constants obtained for plant crude membrane fractions indicate a lower affinity than do those obtained for skeletal-muscle microsomes preparations, for which the K_D was 43 nM and the B_{max} 44 pmol/mg of protein [26]. The concentration of verapamil-binding sites also tends to be greater for plant tissues.

Partial purification of the verapamil-binding protein

The verapamil-binding protein was partially purified from maize coleoptile membranes by using anion-exchange and gel-filtration chromatography, followed by phenyl-Sepharose hydrophobic-interaction chromatography. Lectin-affinity chromatography was attempted, but verapamil-binding proteins in solubilized maize coleoptile membrane preparations and DEAE-Bio-Gel fractions did not adhere to Con A-Sepharose or to wheat-germ-lectin columns. The phenyl-Sepharose column was used in order to take advantage of the hydrophobic nature of the Ca^{2+} channel.

After initial studies with a variety of detergents, the verapamil-binding sites were optimally solubilized by 0.5% CHAPS at a protein/detergent ratio of 1:14 (w/w). The inclusion of 20% sucrose during solubilization improved the stability of the solubilized verapamil-binding protein. Under these conditions the recovery of verapamil-binding sites was approx. 50%.

The solubilized receptor was then subjected to DEAE-Bio-Gel anion-exchange chromatography. Verapamil-binding activity was eluted with 0.15 M-NaCl in column buffer. The inclusion of 0.1% CHAPS in the elution buffer was found to be necessary for successful elution of the verapamil-binding protein. Under these conditions,

Fig. 4. Elution profile for the Sephacryl S-400 column

Fractions from the DEAE-Bio-Gel ion-exchange column (2 ml) were loaded on to the column (1.5 cm $\times$ 22 cm) of Sephacryl S-400 and eluted as described in the Materials and methods section. The 1 ml fractions were assayed for verapamil binding. The void volume of the column was determined separately with Blue Dextran 2000.

the degree of purification obtained averaged about 3-fold (varying from 1- to 7-fold, for reasons that are not clear). Recovery of binding activity ranged from 20 to 50 %. Although the purification factor achieved with the DEAE-Bio-Gel column is fairly low, it appeared to be a necessary step in order that the subsequent purification steps should be successful.

Fractions containing verapamil-binding activity eluted from the DEAE-Bio-Gel column were further fractionated on a Sephacryl S-400 column. A typical elution profile is shown in Fig. 4. The recovery of binding activity was more than 100 % of that loaded on to the column, perhaps because of removal of proteins inhibitory to binding. The recovery of binding activity was 74 % of that initially in the solubilized membrane extract. The degree of purification achieved with the gel-filtration step was 76-fold (Table 1). The verapamil-binding protein was eluted in the void volume, suggesting that it chromatographed as a large aggregate with a molecular mass of more than 8000 kDa. The presence of 1 M-NaCl or 1 mM-DTT in the column buffer had no effect on the elution behaviour.

Verapamil-binding fractions from the Sephacryl S-400 column were then applied to a phenyl-Sepharose column. Fig. 5 shows a typical elution profile from the column. The profile indicates that verapamil-binding activity is eluted partly by buffer *B*, and partly by buffer *D*, the latter fraction (eluted with 0.1 % CHAPS/20 % sucrose), having the higher specific activity. The recovery of verapamil-binding activity from the phenyl-Sepharose column (buffer *D* fraction) was 20 %, and overall recovery was less than 4 % of the initial activity in the CHAPS-solubilized membrane extract.

After each purification step, the proteins contained in the different fractions were analysed by SDS/PAGE (Fig. 6). The final purification step gave four stained bands of M_r 169000, 100000, 70000 and 66000 (Fig. 6, lane 5). Silver staining of the gel indicated a band of M_r 169000 which was enriched in the final verapamil-

Fig. 5. Elution profile for the phenyl-Sepharose column

Pooled fractions from the Sephacryl S-400 column were adjusted to 1 M-(NH₄)₂SO₄ and 1 mM-CaCl₂ and loaded on to the phenyl-Sepharose column, which had previously been equilibrated with 10 column volumes of Buffer *A*. Numerous elution protocols were evaluated. The following elution series gave the best recovery of verapamil-binding activity. When sample loading was complete, the column was washed with up to 18 ml of buffer *A*, and then eluted with 18 ml each of buffers *B*, *C* and *D* (arrows). Fractions (2 ml) were collected, and the first four 2 ml fractions of each elution step were assayed for verapamil binding.

binding fraction (Fig. 6, lane 5, see arrow). This band was absent from the protein profiles of inactive fractions (Fig. 6, lane 4). A protein of M_r 100000 was observed throughout the verapamil-binding and non-verapamil-binding fractions from the Sephacryl S-400 column and the breakthrough fractions from the DEAE-Bio-Gel column. A protein of equivalent M_r , 70000, was observed in some of the non-verapamil-binding fractions of the

Table 1. Purification of the verapamil-binding protein

Purification step	Verapamil bound		Protein		Specific activity	Purification (fold)
	(c.p.m.)	(%)	(mg)	(%)		
CHAPS extract	32240	100	25	100	1305	1
DEAE-Bio-Gel	10304	32	4	16	2525	2
Sephacryl S-400	23800	74	0.24	0.108	99166	76
Phenyl-Sepharose	1264	4	0.008	0.032	158000	121

Fig. 6. SDS/PAGE of verapamil-binding fractions from maize coleoptile membranes at different stages of purification

Lane 1, CHAPS-solubilized extract (5 µg of protein); lane 2, pooled DEAE-Bio-Gel fraction (5 µg of protein); lane 3, pooled Sephacryl S-400 fraction (5 µg of protein); lane 4, Sephacryl S-400 fraction with no verapamil-binding activity (5 µg of protein); lane 5, buffer D fraction from the phenyl-Sepharose column (1 µg of protein).

Sephacryl S-400 elution profile (Fig. 6, lane 4). The band at M_r 66000 is a staining artefact frequently observed even in unloaded SDS/PAGE slots and reported by others [27]. On this basis the protein of M_r 169000 is the most likely candidate to contain the verapamil-binding site.

The gel suggests that there has been extensive purification of the verapamil-binding protein. However, the purification factor, 120-fold (Table 1), is higher than might be anticipated from a simple calculation of the abundance of the protein in the initial CHAPS extract. Despite care, we have found the binding-site activity to be unstable during purification. This instability, combined with potential inaccuracies in measuring low levels of protein, probably accounts for this.

To investigate the possible glycoprotein nature of the verapamil-binding protein, gels of the post-DEAE-Bio-Gel verapamil-binding fraction were blotted on to nitrocellulose paper and incubated with peroxidase-linked Con A. The lectin was seen to bind to the band of M_r 169000 and to other proteins present in this DEAE-Bio-Gel purified fraction, suggesting that the M_r -169000 component is a glycoprotein. Binding of the lectin to the later stages of the purification was not obtained, possibly owing to insufficient levels of the purified protein.

The M_r -169000 component of the verapamil-binding protein in maize coleoptile membranes found in the present study is comparable with the major subunit of M_r 142000 found for the dihydropyridine receptor in rabbit skeletal muscle and with the polypeptide of M_r 165000 found for the phenylalkylamine receptor (also in rabbit skeletal muscle) [28–30]. If the Ca^{2+} channel has been conserved during evolution, one would expect to see a similarity in the size of the channel polypeptides in plant and animal tissues. Monoclonal and polyclonal antibodies have been developed for dihydropyridine and phenylalkylamine receptors from skeletal muscle [31,32]. However, these antibodies do not cross-react with plant membranes [22], which suggests some structural differences between the receptors in plants and animals.

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