Receptor for *myo*-inositol trisphosphate from the microsomal fraction of *Vigna radiata*

Susweta BISWAS,*‡ Basudeb DALAL,* Mita SEN* and Birendra B. BISWAS†

*Department of Biochemistry, Bose Institute, Calcutta, India and †Department of Biophysics, Molecular Biology and Genetics, Calcutta University, Calcutta, India

The microsomal fraction from mung-bean (*Vigna radiata*) hypocotyl was found to contain Ins(1,4,5)P_3- and Ins(2,4,5)P_3-binding activity. Preincubation of the microsomal fraction with thiol-containing reagents reduced specific InsP_3 binding. A single class of binding site with a K_d value of 1.5 nM and $B_{max.}$ of 1.1 pmol/mg of protein was detected. Other *myo*-inositol phosphates exhibited little affinity for this protein. The binding protein was purified to homogeneity and the molecular mass of the native form recorded as 400 kDa. However, under denaturing conditions the molecular mass was 110 kDa, suggesting that the protein is a homotetramer. That this protein is associated with Ca^{2+} release was confirmed by including it in proteoliposomes and adding Ins(1,4,5)P_3 or Ins(2,4,5)P_3. The affinity of Ins(1,4,5)P_3 is 3-fold higher than that of Ins(2,4,5)P_3. The binding affinity of InsP_3 is also reflected in the extent of Ca^{2+} released from the microsomal fraction. Heparin inhibits binding of InsP_3 to the protein, the $K_{\frac{1}{2}}$ being 0.26 μM. It is also shown that the protein acts as a receptor for InsP_3 with characteristics of high affinity and low density.

INTRODUCTION

myo-Inositol trisphosphate, particularly Ins(1,4,5)P_3, is the intracellular messenger that mediates the effects of many cell surface receptors on intracellular Ca^{2+} stores [1]. Although specific assays have identified high-affinity Ins(1,4,5)P_3-binding sites in many animal tissues [2,3], these have not been convincingly shown in all cases to be the receptors that mediate Ca^{2+} mobilization, nor is it clear whether the binding sites are different from one tissue to another. The putative specific receptor for Ins(1,4,5)P_3 from cerebellum and other tissues has been characterized and the molecular size reported to be 250–260 kDa [2,4]. Furthermore, the gene for the receptor has been cloned, and at least two distinct genes encoding full-length sequences for Ins(1,4,5)P_3 receptor have been identified [5,6]. All these facts firmly established, at least in animal systems, that the action of Ins(1,4,5)P_3 is mediated via a specific receptor with an expanding family of receptor subtypes [7].

In contrast, a specific receptor for Ins(1,4,5)P_3 has not yet been identified in plant systems. However, there have been several reports on the release of Ca^{2+} by InsP_3 from plant microsomal/vacuolar fractions [8,9]. This has not been correlated with InsP_3 binding in plant systems although it is speculated that intracellular Ca^{2+} mobilization takes place via a receptor. Our interest was to study the role of Ins(1,4,5)P_3 and/or Ins(2,4,5)P_3 in Ca^{2+} mobilization in microsomal or vacuolar fractions from *Vigna radiata*. It has been reported that the phytase product of *myo*-inositol hexakisphosphate isolated as Ins(2,4,5)P_3 can release Ca^{2+} from mung-bean hypocotyl microsomes [10]. The purpose of the present paper is to report on the isolation and purification of the putative receptor from mung-bean microsomal fraction that can bind Ins(1,4,5)P_3 and Ins(2,4,5)P_3 and elicit Ca^{2+} mobilization in plant cells. To our knowledge this is the first report of such a receptor isolated, purified and characterized from a plant source.

MATERIALS AND METHODS

Materials

D-[^{3}H]Ins(1,4,5)P_3 assay kit and D-[^{3}H]Ins(1,4,5)P_3 (1 Ci/mmol) were purchased from Amersham International p.l.c. Quin-2, D-Ins(1,4,5)P_3, L-Ins(1,4,5)P_3, Ins1P, Ins2P, InsP_6, chymotrypsin, trypsin, *p*-chloromercuribenzoylsulphonate (pCMBS), dithiothreitol (DTT), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phospholipase C, phospholipase A_2, heparin, heparin–agarose, diltiazem, verapamil, valinomycin, chlorpromazine and A23187 were from Sigma. Ins(4,5)P_2 and Ins(2,4,5)P_3 were obtained from Boehringer-Mannheim. All other reagents were of analytical grade and obtained indigenously from different agencies.

Preparation of crude membrane fraction containing Ins(1,4,5)P_3-binding activity

Mung-bean (*Vigna radiata* B_1) seeds were allowed to germinate in the dark for 48 h at 37 °C and hypocotyls were homogenized with ice-cold isotonic homogenization buffer containing 0.25 M sucrose, 10 mM 2-mercaptoethanol, 3 mM EDTA, 25 mM Tris/HCl (pH 7.4) and 1 mM phenylmethanesulphonyl fluoride. The homogenate was passed through two layers of cheesecloth and centrifuged at 1000 *g* for 5 min to remove nuclei and debris. The supernatant was centrifuged at 12000 *g* for 40 min. The pellet was monitored for binding of Ins(1,4,5)P_3. All operations were carried out at 4 °C. Protein was measured by the method of Bradford [11] with BSA as standard.

Preparation of microsomal fraction from mung-bean embryos

Microsomes were prepared from Mung-bean hypocotyls of length 10 cm by a previously described procedure [10]. The

Abbreviations used: pCMBS, *p*-chloromercuribenzoylsulphonate; DTT, dithiothreitol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

‡ To whom correspondence should be addressed.

microsomal pellet was resuspended in medium B (0.5 M sucrose, 5 mM Mops/KOH buffer, pH 7.8, 2 mM $MgCl_2$ and 100 mM KCl). Protein was measured as described by Bradford [11].

Measurement of Ca^{2+} release and uptake

For the determination of Ca^{2+} influx and efflux, microsomal suspension (5 mg of protein) was held in a shaking water bath at 25 ± 1 °C and 3 mM NaN_3 (to prevent mitochondrial Ca^{2+} uptake) and 100 μM $CaCl_2$ were added. After incubation for 10 min, 2 mM ATP was added to trigger ATP-dependent uptake of Ca^{2+}. After 30 min, different amounts of Ins(1,4,5)P_3 or Ins(2,4,5)P_3 were added in a total volume of 2 ml. Ca^{2+} release was monitored using 100 μM Quin-2 in an Hitachi F3000 fluorescence spectrometer [excitation at 339 nm (slit width 3 nm) and emission at 492 nm (slit width 5 nm) at 25 °C] [12]. Any change in Ca^{2+} was determined and expressed as nmol of Ca^{2+} released using the equation described in [9]. $^{45}Ca^{2+}$ was also used for flux measurements in the microsomal fraction [10].

Assay of InsP_3 binding

The assay mixture contained 1 mg of protein and 0.09 μCi (10 pmol) of [^{3}H]Ins(1,4,5)P_3 in a total volume of 400 μl made up with 0.1 M Tris/HCl buffer (pH 8.5) containing 4 mM EDTA and 4 mg/ml BSA unless otherwise stated. The mixture was then incubated for 15 min at 4 °C followed by centrifugation at 10000 *g* for 5 min and aspiration of the supernatant. Non-specific binding was determined by including either 10 μM Ins(1,4,5)P_3 or 10 μg/ml heparin in the incubation medium. Both showed identical non-specific binding.

The method of Miyawaki et al. [6] was used to assay the binding of [^{3}H]InsP_3 in the soluble fraction. The gel-eluted protein (5 μg) was incubated with 0.09 μCi (10 pmol) of [^{3}H]Ins(1,4,5)P_3 in a total volume of 400 μl in the buffer described above for 15 min at 4 °C. The sample was then mixed with 10 μl of γ-globulin (50 mg/ml) and 100 μl of a solution containing 30 % (w/v) poly(ethylene glycol) 6000, 1 mM 2-mercaptoethanol and 50 mM Tris/HCl (pH 8.0). After incubation on ice for 5 min, the protein–poly(ethylene glycol) complex was collected by centrifugation at 10000 *g* for 15 min at 4 °C. Supernatant was removed and surfaces of the Eppendorf tube were blotted with tissue paper; the pellet was counted for radioactivity.

Purification of InsP_3 receptor for mung-bean microsomes

Mung-bean microsomal fraction was suspended in 1 % Triton X-100 plus buffer X (50 mM Tris/HCl, pH 8.3, 1 mM EDTA and 1 mM 2-mercaptoethanol), and the protein was solubilized by stirring the suspension for 45 min at 4 °C; it was then centrifuged at 100000 *g* for 2 h at 4 °C. To the supernatant was added NaCl to a final concentration of 0.2 M. The supernatant was then passed through a heparin–agarose column (0.8 cm × 6 cm) pre-equilibrated with buffer X containing 0.2 M NaCl and 0.1 % Triton X-100. The column was washed thoroughly with this buffer and the protein was then eluted with buffer X containing 0.1 % Triton X-100 and 0.5 M NaCl. It was desalted by dialysis, concentrated by lyophilization and then subjected to native PAGE (5 % gel). The gel slice corresponding to the protein band was cut out and electroeluted with buffer X. The binding assay was carried out with [^{3}H]InsP_3. The 400 kDa protein with InsP_3-binding activity was subjected to SDS/PAGE. The gel was sliced (2 mm) and eluted by crushing in buffer X and assayed for InsP_3-binding activity.

Reconstitution of the purified Ins(1,4,5)P_3 receptor into proteoliposomes

After rapid isolation, 100 μg of InsP_3 receptor was reconstituted in 100 μg of PC/PE (at PC/PE ratios of 1:1, 2:1 or 1:2) in buffer (25 mM Tris/HCl, pH 7.2, 1 mM KCl, 10 mM $CaCl_2$ and 1 mM 2-mercaptoethanol) by sonication for 2 min; this was then centrifuged at 100000 *g* for 1 h. The pellet was dispersed gently in the same buffer without Ca^{2+}. These vesicles were used to measure InsP_3-activated Ca^{2+} flux by the method of Samanta et al. [9]. The term flux refers to the mobilization of Ca^{2+} in the presence of InsP_3 rather than to the absolute amount of Ca^{2+} translocated/unit time per membrane area. Similarly prepared liposomes without receptor protein were used as controls.

RESULTS AND DISCUSSION

Effect of channel blockers, ionophores and ATPase inhibitors on microsomal Ca^{2+} mobilization

Verapamil, a well-known Ca^{2+}-channel blocker, had no effect on Ca^{2+} efflux (Table 1), suggesting that the channels are not voltage-dependent. Diltiazem inhibited 30 % Ca^{2+} uptake but did not affect Ca^{2+} mobilization from Ca^{2+}-preloaded microsomes. Valinomycin, a K^+ ionophore, did not affect uptake or release of Ca^{2+}. Vanadate had no effect but chlorpromazine completely blocked both ATP-dependent Ca^{2+} influx and InsP_3-dependent Ca^{2+} efflux, suggesting that the microsomal storage organelles are not associated with endoplasmic reticulum but are possibly of vacuolar origin. In the case of plant systems, the vacuolar origin of Ca^{2+} efflux has previously been stressed [13].

Localization of binding sites for Ins(1,4,5)P_3 and Ins(2,4,5)P_3 in the mung-bean cell fractions

Mung-bean hypocotyls were collected after imbibition of seeds for 48 h and membrane and microsomal fractions were monitored for [^{3}H]Ins(1,4,5)P_3 binding. Both crude membrane and microsomal fractions exhibited binding which could be competed for by Ins(1,4,5)P_3, and the IC_{50} value (0.125 μM and 0.8 μM for microsomal and membrane fractions respectively) showing a 6–7-fold higher affinity in the case of the microsomal fraction.

[^{3}H]Ins(1,4,5)P_3 binding to mung-bean embryo microsomes was determined in the presence of increasing concentrations of

Table 1 Effect of channel blockers, ionophores and ATPase inhibitors on microsomal Ca^{2+} mobilization

Different reagents (10 μM) were added at appropriate times; assay methods were as described in the Materials and methods section: 100% Ca^{2+} uptake = 367 nM; 100% Ca^{2+} release = 109 nM. Ca^{2+} release in the presence of channel blockers was monitored after the uptake of Ca^{2+} by the microsomes in the absence of blockers.

Agent	Ca^{2+} uptake (%)	Inhibition of Ca^{2+} release (%)
Control	100	0
Diltiazem	68	0
Verapamil	100	0
Valinomycin	100	0
Chlorpromazine	0	100
Vanadate	100	0

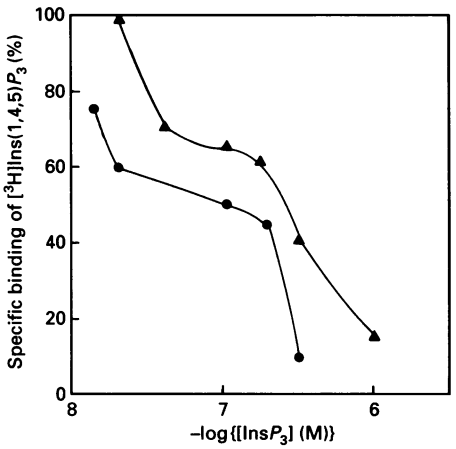


Figure 1 Specific binding of [^{3}H]Ins(1,4,5)P_3 to mung-bean microsomal fraction

The membrane fraction was prepared as described in the Materials and methods section. The 400 μl assay mixture included 0.09 μCi of [^{3}H]Ins(1,4,5)P_3, 1 mg of protein and various concentrations of competing InsP_3. Incubations were performed at 4 °C for 15 min. The concentration of InsP_3 required to displace half of the specific [^{3}H]Ins(1,4,5)P_3 binding was determined. Data are representative of at least two experiments performed in duplicate which varied by less than 10%. ●, Ins(1,4,5)P_3; ▲, Ins(2,4,5)P_3.

Table 2 Displacement of [^{3}H]Ins(1,4,5)P_3 binding by various inositol phosphates

Inositol phosphate	IC_{50} (μM)
Ins(1,4,5)P_3	0.125
Ins(2,4,5)P_3	0.40
InsP_6	> 50
Ins(4,5)P_2	> 10
Ins2P	> 100
Ins1P	> 100

unlabelled Ins(1,4,5)P_3 or Ins(2,4,5)P_3. The IC_{50} for Ins(2,4,5)P_3 was 3-fold greater than that of Ins(1,4,5)P_3, indicating that the affinity of Ins(1,4,5)P_3 for the binding site is 3-fold higher than that of Ins(2,4,5)P_3 (Figure 1).

Specificity of Ins(1,4,5)P_3 binding

The binding sites in the microsomal fraction are specific for InsP_3 because various other inositol phosphates were shown to be ineffective at binding (Table 2). Ins(2,4,5)P_3 was found to bind specifically with one-third the affinity of Ins(1,4,5)P_3. Ins(4,5)P_2 was about 1/25th as potent as Ins(2,4,5)P_3. InsP_6 produced virtually no inhibition of binding at concentrations as high as 50 μM, and Ins1P and Ins2P were similarly inactive. Other phospho compounds did not bind to the putative binding sites at all.

InsP_3 binding as a function of pH

Ins(1,4,5)P_3 and Ins(2,4,5)P_3 binding varied over the pH range 7.0–9.5. Binding was enhanced between pH 7.5 and 8.5, reached a maximum at pH 8.5 and finally declined at pH 9.5.

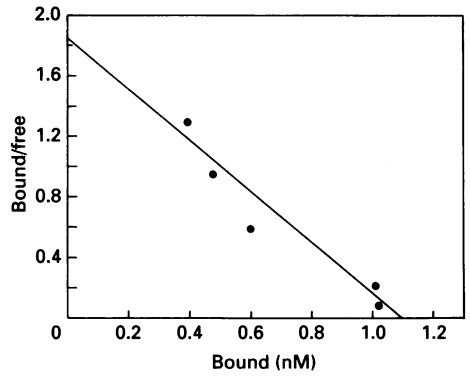


Figure 2 Saturation analysis of [^{3}H]Ins(1,4,5)P_3 binding to microsomal membranes

The assay mixture included 1 mg of protein and 0.09 μCi (10 pmol) of [^{3}H]Ins(1,4,5)P_3 in a total volume of 400 μl as described in the Materials and methods section and was incubated for 15 min at 4 °C followed by centrifugation. Non-specific binding was determined by including 10 μM Ins(1,4,5)P_3 in the incubation medium.

Table 3 Effect of different compounds on InsP_3 binding by microsomal protein

Microsomal membrane (1 mg) was tested with various reagents and enzymes as indicated. After appropriate time intervals, samples were centrifuged at 10000 *g* in an Eppendorf Microfuge for 5 min at 4 °C. The supernatant was aspirated, and the pellet assayed for [^{3}H]Ins(1,4,5)P_3 binding as described in the Materials and methods section. Values are means of three sets of each experiment; 100% binding = 640 c.p.m./mg of protein.

Compound	Concentration	Treatment	[^{3}H]Ins(1,4,5)P_3 binding (% of control)
None	–	–	100
pCMBS	1 mM	–	0
2-Mercaptoethanol	2 mM	–	100
pCMBS	1 mM	Pretreatment with 2-mercaptoethanol	50
DTT	1 mM	–	100
Heat	–	1 h, 60 °C	0
Trypsin	0.1 unit	10 min, 25 °C	0
α-Chymotrypsin	1.0 unit	10 min, 25 °C	0
Phospholipase A_2	1.5 unit	10 min, 25 °C	100
Phospholipase C	1.25 unit	10 min, 25 °C	100
Xylanase	1 unit		100

Saturation of InsP_3 binding

Saturation of binding sites was analysed by using progressively increasing concentrations of [^{3}H]InsP_3. Scatchard analysis indicated a single binding site with a K_d value of 1.5 nM and a $B_{max.}$ of 1.1 pmol/mg of protein (Figure 2), which are very close to those reported for receptors from several animal peripheral tissues.

Effects of enzymes and reagents on InsP_3 binding

Before solubilization of the receptor(s) the effect of various reagents and enzymes on [^{3}H]InsP_3 binding was monitored. InsP_3 binding was completely abolished by α-chymotrypsin and trypsin (Table 3), suggesting that the receptor is protein in nature.

Table 4 Quantification of Ins(1,4,5)P_3 using the InsP_3-binding-protein kit in the crude membrane fraction of developing embryo

The results are means ± S.E.M. (3 determinations).

Period of imbibition (h)	Endogenous InsP_3 (pmol/mg of fresh tissue)
24	0.20 ± 0.02
48	0.26 ± 0.03
72	0.22 ± 0.03

Table 5 Effects of various inositol phosphates on Ca^{2+} mobilization from preloaded microsomes

The effect of Ins(1,4,5)P_3 was taken as 100% and is equivalent to 109 nM Ca^{2+} released/mg of protein.

Ligand	Concentration (μM)	Relative effect (%)
D-Ins(1,4,5)P_3	2	100
D-Ins(2,4,5)P_3	2	33
L-Ins(1,4,5)P_3	2	8
Ins(4,5)P_2	10	0
Ins2*P*	100	0
Ins1*P*	100	0
InsP_6	50	0

Denaturation of the putative receptor by heating the microsomal fraction at 60 °C for 1 h also eliminated binding. Binding was unaffected by bacterial preparations of phospholipase C and A_2 as well as xylanase. Treatment with 1 mM pCMBS totally abolished microsomal [^{3}H]InsP_3 binding, an effect that was partially prevented (50%) by pretreatment with 2 mM 2-mercaptoethanol. DTT and 2-mercaptoethanol did not affect microsomal [^{3}H]InsP_3 binding.

Variation in InsP_3 level during embryo development

A higher amount of InsP_3 was detected in the crude membrane fraction isolated from embryos after 48 h of imbibition than after 24 and 72 h imbibition periods (Table 4). A sensitive assay method based on binding reactions of InsP_3 to bovine adrenal preparations was used to determine the levels of endogenous InsP_3 in embryo at different developmental stages. The amount of InsP_3 found in these tissues ranges from 0.2 to 0.3 pmol/g of fresh tissue (Table 4) which was 4–5-fold lower than that detected in maize roots and coleoptiles [14].

InsP_3-receptor-mediated microsomal Ca^{2+} release

Ins(1,4,5)P_3 and Ins(2,4,5)P_3 both elicited Ca^{2+} release from ATP-dependent azide-insensitive Ca^{2+}-preloaded microsomes. Ca^{2+} was also mobilized by the Ca^{2+} ionophore, A23187. The amount of Ca^{2+} released by 2 μM Ins(1,4,5)P_3 (maximum release) was higher than that induced by 2 μM Ins(2,4,5)P_3 (maximum release) (Table 5). Ins(4,5)P_2, Ins2*P*, Ins1*P* and InsP_6 were all ineffective at releasing Ca^{2+}.

Effect of heparin on Ca^{2+} release

The glycosaminoglycan, heparin, proved to be a potent inhibitor of InsP_3-induced Ca^{2+} efflux from plant vacuolar/microsomal fractions. When applied to mung-bean embryo microsomes at a concentration of 4 μg/ml it completely eliminated InsP_3-mediated Ca^{2+} release (Figure 3). The $K_{\frac{1}{2}}$ for heparin inhibition was 1.3 μg/ml or 0.26 μM.

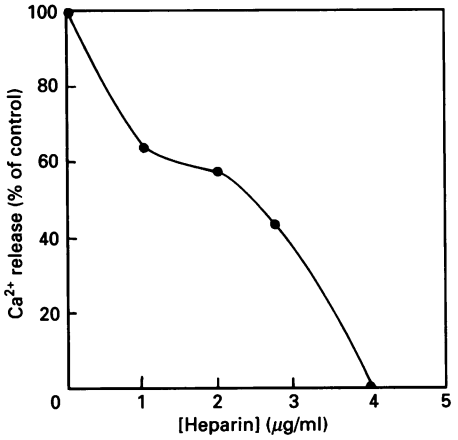


Figure 3 Effect of heparin on Ca^{2+} release from mung-bean embryo microsomes

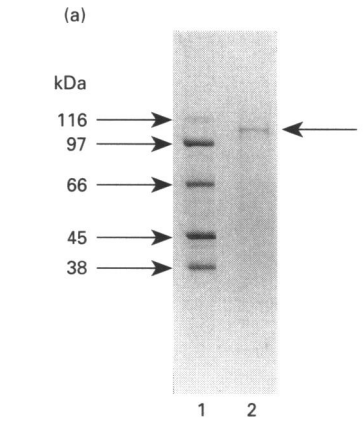
Table 6 Purification of InsP_3 receptor

Native PAGE was carried out as described previously [15]. Protein analysed by SDS/PAGE loses 50% of its activity compared with that analysed by native PAGE.

Fraction	Protein obtained (mg)	Specific binding (c.p.m./mg)	Purification
Crude microsome	10.2	444	1.0
Solubilized	7.5	1988	4.4
Heparin–agarose	1.8	60000	135.0
PAGE-eluted fraction (native)	0.08	448000	1004.5

Purification of [^{3}H]Ins(1,4,5)P_3-binding protein

The activity increased 4-fold after solubilization of the microsomal fraction with 1% Triton X-100, and after heparin–agarose chromatography, the receptor was purified 135-fold over the activity in the crude microsomal fraction (Table 6). There was a 6-fold decrease in protein after heparin–agarose chromatography with an enhancement of about 22-fold in binding activity (assuming 100% recovery), suggesting the removal of component(s) that interfered with InsP_3 binding. In fact, a 30% inhibition of binding was observed when the pellet portion was added to the Triton X-100-solubilized portion (results not given). In this case the apparent $B_{max.}$ for the intact microsomal fraction might be 22-fold higher than the value reported (1.1 pmol/mg of protein). This is still much lower than that reported for crude rat cerebellum extract [2]. A native-PAGE analysis revealed that the fraction obtained from the heparin–agarose column contained a single protein band of molecular mass approx. 400 kDa with binding activity. The purification obtained at this stage was 1004-fold. When this band was electroeluted and subjected to SDS/PAGE, predominantly one protein band (110 kDa) was visible after staining (Figure 4a), and InsP_3-binding activity coincided with this band as assayed by slicing the gel (Figure 4b).



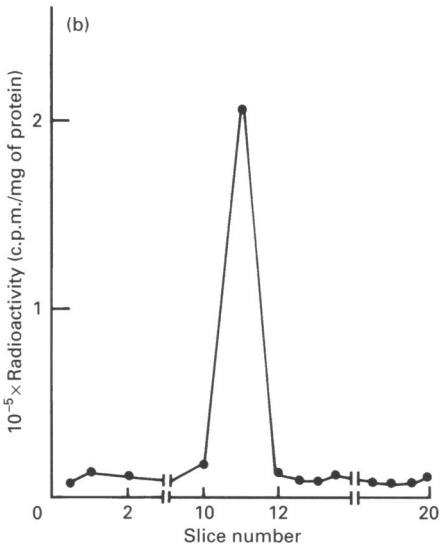


Figure 4 SDS/PAGE of fraction of Ins(1,4,5)P_3-binding protein eluted from heparin–agarose

(**a**) Electrophoresis was carried out by the method of Laemmli [16] on a 7.5% polyacrylamide gel. The gel was stained with Coomassie Blue. Lane 1, marker (25 μg); lane 2, fraction eluted from heparin–agarose (25 μg). (**b**) A replica of the gel was sliced (2 mm) and assayed for binding after elution of the protein.

Native-PAGE (5% gel) and gel-exclusion chromatography (using Sephacryl S-300) showed the molecular mass of the protein to be about 400 kDa, revealing that the Ins(1,4,5)P_3 receptor from mung-bean microsomes is a homotetramer.

Effect of Ins(1,4,5)P_3 on Ca^{2+} mobilization from proteoliposomes

Reconstitution of proteoliposomes using various concentrations of PE and PC revealed that a particular proportion of phospholipid (PE/PC = 1:1) was required for optimal activity of Ins(1,4,5)P_3-receptor-mediated Ca^{2+} mobilization (Table 7). Analysis of the phospholipids (results not shown) from mung-bean microsomes confirmed that the PE/PC ratio is 1:1. The addition of 2 μM Ins(1,4,5)P_3 triggered an optimal Ca^{2+} release in the range 210–230 nM (Table 7). Heparin (10 μg/ml) inhibited Ca^{2+} efflux from the proteoliposomes.

Table 7 Ca^{2+} release from proteoliposome reconstituted with the purified receptor protein

Ins(1,4,5)P_3 (2 μM) was used for Ca^{2+} release. Heparin was used at a concentration of 10 μg/ml.

Ratio of PE/PC in proteoliposome	Ca^{2+} released (nM)	
	By InsP_3	By InsP_3 + heparin
2:1	45–55	15–21
1:1	210–230	3–5
1:2	40–50	4–8

Comparison of InsP_3 receptors from animal and plant origin

This report demonstrates the presence of a receptor from the microsomal/vacuolar membrane fraction from plant (mung-bean) cells for Ins(1,4,5)P_3/Ins(2,4,5)P_3. The binding of InsP_3 is specific as other Ins$P$ molecules bind with lower affinity and do not cause Ca^{2+} release from the microsomal fraction (Table 5). It is also apparent that Ins(1,4,5)P_3 has the highest affinity; the IC_{50} values for a variety of Ins$P$ molecules were shown to be different (Table 2). Ins(2,4,5)P_3 (IC_{50} 0.4 μM) is one-third as effective as Ins(1,4,5)P_3 not only with respect to binding but also in stimulating Ca^{2+} release from the internal stores (Figure 1). When the receptor was purified it showed similar patterns of binding with Ins(1,4,5)P_3 and Ins(2,4,5)P_3.

Ca^{2+} mobilization from microsomes was inhibited by heparin in a concentration-dependent manner (Figure 3), and the $K_{\frac{1}{2}}$ for heparin binding was found to be 0.26 μM which is in the same range as that reported for red beet microsomal extract [17].

The K_d for Ins(1,4,5)P_3 binding was found to be 1.5 nM, suggesting that it binds to high-affinity sites. However, the affinity of InsP_3 for animal receptors varies from 1 to 100 nM. The abundance of apparent Ins(1,4,5)P_3-specific binding sites in mung bean (1.1 pmol/mg of protein) is two orders of magnitude lower than that in cerebellum [2], suggesting that the mung-bean microsomal fraction has a lower receptor density [18]. However, this value is also comparable with that from red beet storage root microsomes [17].

The molecular mass of the subunit was recorded as 110 kDa under denaturing conditions, which is similar to that of the receptor isolated from olfactory cilia [19], although the receptor from animal systems is normally around 260 kDa [2,4,20]. Animal InsP_3 receptors appear to function as tetrameters in the membrane [21] which has also been found to be the case for mung-bean microsomal receptor. The apparent molecular mass of native protein is 400 kDa, as determined from gel-exclusion studies and also from native PAGE.

That the receptor isolated is associated with Ca^{2+} release has been confirmed by its inclusion in proteoliposomes and subsequent Ca^{2+} release from them after addition of InsP_3 (Table 7). This protein binds Ins(1,4,5)P_3, as is evident from the data given in Figure 4(b). The present InsP_3 receptor shows similarity to animal receptors with respect to InsP_3-binding affinity, selectivity for binding and heparin binding. Whether the same receptor is also present in the plasma membrane has yet to be ascertained. The link between the microsomal receptor activity/Ca^{2+} release and the signal-transduction network needs to be fairly established in the plant cell, even though evidence is now accumulating that enhanced turnover of PtdIns(4,5)P_2 and an associated increase in

$Ins P_3$ level is a primary event in a variety of responses in plants [22].

Thanks are due to the Council of Scientific and Industrial Research and the Department of Biotechnology, Government of India for financial support. We also thank Dr. P. C. Sen, Department of Chemistry, Bose Institute, for the proteoliposome preparations.

REFERENCES

1 Berridge, M. J. and Irvine, R. F. (1989) Nature (London) **341**, 197–205
2 Supattapone, S., Worley, P. F., Baraban, J. M. and Snyder, S. H. (1988) J. Biol. Chem. **263**, 1530–1534
3 Mignery, G. A. and Sudhof, T. C. (1990) EMBO J. **9**, 3893–3898
4 Maeda, N., Niinobe, M. and Mikoshiba, K. (1990) EMBO J. **9**, 61–67
5 Mignery, G. A., Newton, C. L., Archer III, B. T. and Sudhof, T. C. (1990) J. Biol. Chem. **265**, 12679–12685
6 Miyawaki, A., Furichi, T., Ryon, Y., Yoshikawa, S., Nakagawa, T., Saitoh, T. and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4911–4915
7 Ross, C. A., Danoff, S. K., Schell, M. J., Snyder, S. H. and Ullrich, A. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4265–4273
8 Schumaker, K. S. and Sze, H. (1987) J. Biol. Chem. **262**, 3944–3946
9 Samanta, S., Dalal, B., Biswas, S. and Biswas, B. B. (1993) Biochem. Biophys. Res. Commun. **191**, 427–434
10 Maitra, R., Samanta, S., Mukherjee, M., Biswas, S. and Biswas, B. B. (1988) Indian J. Biochem. Biophys. **25**, 655–659
11 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
12 Mazorow, D. L. and Miller, D. B. (1990) Anal. Biochem. **186**, 28–30
13 Shumaker, K. S. and Sze, H. (1986) J. Biol. Chem. **261**, 12172–12178
14 Aducci, P. and Marra, M. (1990) Biochem. Biophys. Res. Commun. **168**, 1041–1046
15 Davis, B. J. (1964) Ann. N. Y. Acad. Sci. **121**, 404–410
16 Laemmli, U. K. (1990) Nature (London) **227**, 680–685
17 Brosnan, J. M. and Sanders, D. (1993) Plant Cell **5**, 931–940
18 Challis, J., Willcocks, A. L., Mulloy, B., Potter, B. V. and Nahorski, S. R. (1991) Biochem. J. **274**, 861–867
19 Kalinoski, D. L., Aldinger, S. B., Boyle, A. G., Huque, T., Marecek, J. F., Prestwich, G. D. and Restrepo, D. (1992) Biochem. J. **281**, 449–456
20 Ross, C. A., Meldolesi, J., Milner, T. A., Sato, T., Supattapore, S. and Snyder, S. H. (1989) Nature (London) **339**, 468–470
21 Chadwick, C. C., Saito, A. and Fleischer, S. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2132–2136
22 Drobak, B. K. (1992) Biochem. J. **288**, 697–712

Received 22 July 1994/14 October 1994; accepted 3 November 1994