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# Identification and Preliminary Characterization of a $Ca^{2+}$-Dependent High-Affinity Binding Site for Inositol-1,4,5-Trisphosphate from *Chenopodium rubrum*[1]

**Chris H. Scanlon*, Jan Martinec, Ivana Macháčková, Carole E. Rolph, and Peter J. Lumsden**

Department of Applied Biology, University of Central Lancashire, Preston, PR1 2HE, United Kingdom (C.H.S., C.E.R., P.J.L); and Institute for Experimental Botany (J.M.), and Norman Borlaugh Institute for Plant Sciences, De Monfort University (I.M.), Institute for Experimental Botany, Czech Academy of Sciences, Ke dvoru 16, 166 30 Prague 6, Czech Republic

**Using a radioligand-binding assay we have identified a $Ca^{2+}$-dependent high-affinity D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) binding site in a membrane vesicle preparation from *Chenopodium rubrum*. Millimolar concentrations of $Ca^{2+}$ were required to observe specific binding of [$^3$H]$InsP_3$. A stable equilibrium between bound and free ligand was established within 5 min and bound [$^3$H]$InsP_3$ could be completely displaced by $InsP_3$ in a time- and concentration-dependent manner. Displacement assays indicated a single class of binding sites with an estimated dissociation constant of 142 ± 17 nM. Other inositol phosphates bound to the receptor with much lower affinity. The glycosaminoglycan heparin was an effective competitor for the binding site (inhibitor concentration for 50% displacement = 534 nM). ATP at higher, although physiologically relevant, concentrations (inhibitor concentration for 50% displacement = 241 μM) also displaced [$^3$H]$InsP_3$ from the receptor. Recent studies in animals have highlighted the importance of $Ca^{2+}$ regulation of $InsP_3$-induced $Ca^{2+}$ release. The potential for the operation of similar regulatory mechanisms in plants is discussed.**

In animals, $InsP_3$ is firmly established at the center of an increasingly complex, yet seemingly ubiquitous, $Ca^{2+}$-mobilizing transduction pathway (Berridge, 1993; Hughes and Michell, 1993). The evidence is not yet conclusive in plant systems but supports an analogous signaling role for $InsP_3$ (see reviews by Drøbak, 1992, 1993; Coté and Crain, 1993, 1994). Increases in intracellular $InsP_3$ have been reported in response to stimuli such as light (Morse et al., 1987), ABA (MacRobbie, 1992), and phytoalexins (Walton et al., 1993). Furthermore, photolysis of caged $InsP_3$ microinjected into stomatal guard cells resulted in a rapid increase in cytoplasmic $Ca^{2+}$ concentration and was followed by stomatal closure (Gilroy et al., 1990, 1991). Other evidence supportive of a signaling role for $InsP_3$ has come from studies of its ability to elicit $Ca^{2+}$ release from intracellular stores. For example, $InsP_3$-induced $Ca^{2+}$ release has been demonstrated in tonoplast vesicles prepared from oat roots (Schumaker and Sze, 1987) and suspension-cultured carrot cells (Canut et al., 1993) and from isolated vacuoles of sycamore cells (Ranjeva et al., 1988). Oscillations in intracellular $Ca^{2+}$ concentrations in response to extracellular stimuli have been reported in plant cells (McAinsh et al., 1992) and it may be that $Ca^{2+}$ regulation of $InsP_3$-induced $Ca^{2+}$ release contributes to these responses. Electrophysiological measurements have also confirmed the presence of $InsP_3$-gated $Ca^{2+}$ channels in tonoplast from red beet storage root (Alexandré et al., 1990; Allen and Sanders, 1995). In addition to supporting a signaling role for $InsP_3$, these studies suggest that, in plants, the vacuole may represent the major $InsP_3$-sensitive $Ca^{2+}$ store.

Our interest in phosphoinositide signaling has focused on its possible role in transducing light signals involved in flowering responses in the SD plants *Pharbitis nil* and *Chenopodium rubrum*. These species can be induced to flower by exposure of leaves to a single period of darkness and have been widely used to investigate the photoperiodic control of this process (Vince-Prue, 1973). A feature of both species is that short exposure to light at certain times during an otherwise inductive dark period results in inhibition of floral induction. This phenomenon, known as the night-break response, exhibits red/far-red light reversibility, thereby implicating phytochrome as the photoreceptor. The involvement of $Ca^{2+}$ in transducing phytochrome-mediated responses is well documented (Shacklock et al., 1992; Neuhaus et al., 1993; Bowler et al., 1994) and in certain cases it appears that the phosphoinositide pathway may be involved in $Ca^{2+}$ mobilization (Guron et al., 1992). A phosphoinositide-specific phospholipase C has previously been identified in both *C. rubrum* (Crespi et al., 1993) and *P. nil* (Bonner et al., 1992), and evidence exists that correlates inhibitory night-break light treatments with increases in $InsP_3$ (Prior, 1993). An important component of the transduction pathway would be a receptor for $InsP_3$; therefore, to further elucidate the coupling of light signals

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* Corresponding author; e-mail c.scanlon@uclan.ac.uk; fax 44–1772–892903.

Abbreviations: $B_{max}$, concentration of binding sites; BTP, bis-Tris propane; CaM, calmodulin; $EC_{50}$, effector concentration for 50% response; $IC_{50}$, inhibitor concentration for 50% displacement; Ins, D-*myo*-inositol; $InsP_3$, D-*myo*-inositol 1,4,5-trisphosphate; $K_d$, dissociation constant; NSB, nonspecific binding; $R_{av}$, average rotor radius.

to flowering inhibition we have attempted to characterize an $InsP_3$ receptor in the leaves of *C. rubrum*.

The $InsP_3$ receptor in animals exists as a homotetramer containing an integral $Ca^{2+}$ channel and an $InsP_3$-binding site on each subunit. Binding of $InsP_3$ and opening of the $Ca^{2+}$ channel are regulated by factors such as $Ca^{2+}$, pH, and ATP (Taylor and Marshall, 1992; Berridge, 1993). To date, at least three separate genes coding for $InsP_3$ receptors have been identified in mammals (Furuichi et al., 1994). Furthermore, tissue-specific splicing variants have been described, which suggests that a family of structurally heterogeneous $InsP_3$ receptors exists (Iida and Bourguignon, 1994). Evidence supporting differential expression (Ross et al., 1992) and posttranscriptional processing (Danoff et al., 1991) of this family of gene products is beginning to suggest mechanisms through which the specificity of $InsP_3$-induced $Ca^{2+}$ signals might be achieved.

To date, only two reports have presented direct evidence for $InsP_3$ receptors in higher plants. Brosnan and Sanders (1993) described a selective, high-affinity binding site for $InsP_3$ in a detergent-solubilized membrane preparation from red beet. They found a single class of binding site at a concentration of 841 fmol $mg^{-1}$ protein. More recently, an $InsP_3$-binding protein from mung bean hypocotyls was identified and purified (Biswas et al., 1995). This receptor had a $K_d$ of 1.5 nM and was present at a concentration of 1.1 pmol $mg^{-1}$ protein in a microsomal membrane fraction. Significantly, when reconstituted into liposomes preloaded with $Ca^{2+}$, the purified protein was able to effect $InsP_3$-gated $Ca^{2+}$ efflux. Both of these receptors have properties broadly similar to $InsP_3$ receptors previously described in mammals (Furuichi et al., 1994, and refs. therein).

To better understand the role of phosphoinositide signaling in plant physiological responses, further information concerning the properties, regulation, and localization of $InsP_3$ receptors is required. The work presented here describes the identification and preliminary characterization of a putative $Ca^{2+}$-dependent $InsP_3$ receptor from *C. rubrum*.

## MATERIALS AND METHODS

### Plant Material

*Chenopodium rubrum* seeds were sown on moist compost and germinated under a 36-h regime consisting of 12 h of light, 30°C; 12 h of dark, 8°C; 12 h of light, 30°C. Seedlings were then thinned and transferred to constant fluorescent white light (100 $\mu$mol $m^{-2}$ $s^{-1}$) at 25°C. Leaves were harvested after 3 weeks of growth.

### Preparation of Membrane Fractions

Membrane preparations were carried out at 4°C. Leaves were ground in homogenization buffer (0.25 M Suc, 3 mM EDTA, 0.2% [w/v] BSA, 5 mM DTT, 70 mM Tris adjusted to pH 8.0 with Mes) using a pestle and mortar. The ratio of homogenization buffer to tissue was 2 mL $g^{-1}$ fresh weight. The homogenate was filtered through four layers of Miracloth (Calbiochem) and the filtrate centrifuged at 6,000*g* ($R_{av}$) for 15 min (Beckman 70 Ti rotor). The supernatant was decanted and centrifuged at 150,000*g* ($R_{av}$) for 45 min (Beckman 70 Ti rotor). The resulting pellet, the microsomal fraction, was resuspended in suspension buffer (1.1 M glycerol, 1 mM DTT, 5 mM Tris adjusted to pH 8.0 with Mes). Further fractionation of the microsomes was achieved by Suc density gradient centrifugation. Routinely, the microsomal fraction was layered onto a discontinuous gradient (4 mL each of 10, 20, and 35% [w/v] Suc) and centrifuged at 150,000*g* ($R_{av}$) for 3 h (Beckman SW 40 rotor). Membrane vesicles equilibrating between 20 and 35% (w/v) Suc were removed using an automated gradient sampler (Auto Densi-flow II C; Haakebuchler, Saddlebrook, NJ), diluted in suspension buffer, and centrifuged at 150,000*g* ($R_{av}$) for 45 min (Beckman 70 Ti rotor). Pellets (Suc density gradient purified membrane fraction) were resuspended in a minimal volume of suspension buffer, stored at 0 to 4°C, and used within 4 d of preparation.

### Assay of $InsP_3$-Binding Sites

Binding of $InsP_3$ to membrane vesicles was quantified using a radioligand-binding assay based on the principles outlined by Hulme and Birdsall (1992). A working stock of [$^3$H]$InsP_3$ (Amersham TRK999, specific activity 0.77–1.15 TBq $mmol^{-1}$) was prepared by drying 100 $\mu$L (37 kBq) of the supplied source under $N_2$ to remove ethanol, and resuspending it in 4 mL of $H_2O$ (exact radioactive concentration was verified by scintillation counting). Routine assays (final volume 100 $\mu$L) contained 20 $\mu$L of stock [$^3$H]$InsP_3$ (11,000–12,000 dpm), 20 mM BTP (adjusted to pH 9.0 with Mes), 10 mM $CaCl_2$, and 20 $\mu$L of a suitable dilution of Suc density gradient purified membrane fraction (40 $\mu$g of protein). Following a brief incubation (4°C, 5 min) assays were initiated by addition of membranes. After a further 15-min incubation, bound and free [$^3$H]$InsP_3$ were separated by rapid filtration through 0.4-$\mu$m-pore-diameter nitrocellulose membranes, which were immediately washed with 2 mL of ice-cold wash buffer (20 mM BTP, 10 mM $CaCl_2$, pH 9.0). NSB was quantified by parallel experiments that included 15 $\mu$M unlabeled $InsP_3$ (synthetic hexasodium salt; LC Laboratories, Woburn, MA). Filter discs were transferred to plastic minivials and dissolved in 4 mL of scintillant (Filter count; Packard, Meriden, CT), and radioactivity was determined by scintillation spectroscopy (Beckman LS 5801). In some assays, separation of bound and free ligand was achieved by microfugation at 13,000 rpm for 15 min. The supernatant was then aspirated, pellets were dissolved in 100 $\mu$L of Soluene 350 (Packard), and radioactivity was quantified as described above. These latter experiments yielded similar results except that NSB was routinely 3 to 4 times greater. In the case of association and dissociation experiments, reaction media were made up in larger volumes and 50-$\mu$L aliquots were removed at the times indicated.

### HPLC Analysis of Inositol Phosphates

Radiolabeled inositol phosphates were analyzed using a 420 HPLC pump (Kontron, Watford, UK) and 425 gradient former equipped with a Partisphere Sax cartridge column system (0.47 cm i.d. × 12.5 cm; Whatman). Elution of

different inositol phosphates was achieved using a modification of the isocratic method described by Wreggett and Irvine (1989). A stepwise gradient of 3, 60, 300, and 700 mM $NaH_2PO_4$ (pH 3.8), delivered at a flow rate of 1.5 mL $min^{-1}$, was used to elute mono-, bis-, tris-, and tetrakis-inositol phosphate isomers, respectively. Any possible metabolism of [$^3$H]$InsP_3$ was investigated by analyzing binding assay filtrates by HPLC. Binding assays were carried out as described above, except that the amount of each component was proportionally increased such that the final assay volume was 250 μL. Following incubation with membrane vesicles (100 μg of protein), bound and free ligands were separated by rapid filtration and immediately rinsed with 2 mL of wash buffer. An aliquot (2 mL) of the resulting filtrate was then injected onto the column. Fractions of eluate were collected at 2-min intervals. Samples (100 μL) were removed from each fraction and diluted 10-fold with $H_2O$, and 10 mL of scintillant (Ecoscint A; National Diagnostics, Manville, NJ) were added. Radioactivity was determined by scintillation spectroscopy. Peaks were identified by reference to the retention times of authentic, tritiated standards (Amersham).

## RESULTS

### Validation of Binding Assay

Protocols to characterize the interactions between $InsP_3$ and its receptor(s) in membrane fractions from animal tissues have most frequently used either glass fiber filtration or centrifugation to separate bound from free ligand. We found glass fiber filters unsuitable for the present investigation because a large proportion of the membrane vesicles were not retained. The requirement for a smaller pore diameter (0.4 μm) necessitated the use of cellulose nitrate/acetate filters but also resulted in longer washing times and the consequent danger of perturbing the receptor-ligand equilibrium. Investigation of the washing procedure revealed that a 2-mL wash (which was completed within 5 s) removed 66% of the nonspecifically bound but only 7% of the specifically bound [$^3$H]$InsP_3$. Increasing the washing volume to 4 mL removed little further nonspecifically associated ligand but resulted in displacement of 22% of the specifically bound [$^3$H]$InsP_3$ (Fig. 1). As a means to separate bound from free ligand we have found this filtration technique to have advantages over centrifugation on grounds of data reproducibility, specific binding to NSB ratio, scintillation counting efficiency, and ease of sample processing.

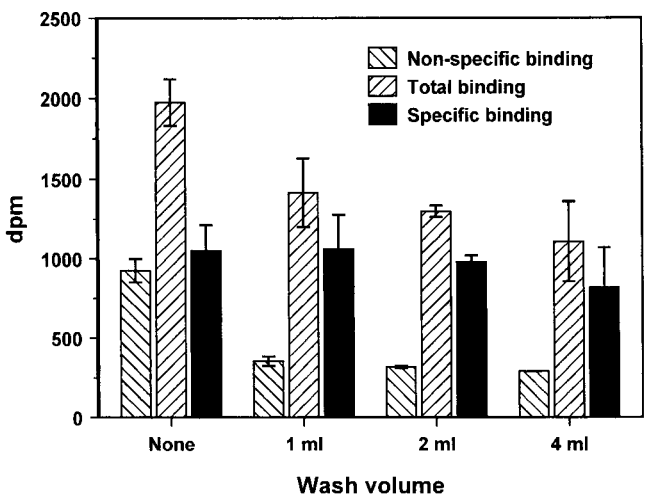



**Figure 1.** Optimization of filtration procedure. Membrane vesicles were incubated (15 min, 4°C) with [$^3$H]$InsP_3$ (1.6 nM) before separation of bound and free ligand by vacuum filtration. Filters were immediately rinsed with the indicated volume of ice-cold wash buffer. NSB was established in the presence of 15 μM $InsP_3$. Specific binding was calculated as the difference between total binding and NSB. Data points are means (±SE) of triplicates from a representative experiment that was repeated on two occasions, yielding similar results.

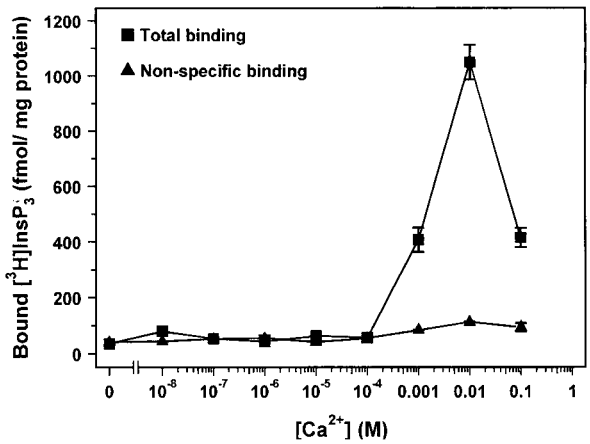



**Figure 2.** Effect of $Ca^{2+}$ on [$^3$H]$InsP_3$ binding. Membrane vesicles (40 μg of protein) were incubated (4°C, 15 min) with [$^3$H]$InsP_3$ (1.6 nM) and the indicated concentrations of $Ca^{2+}$. NSB was established in the presence of 15 μM $InsP_3$. Data points are means (±SE) of triplicates from a representative experiment that was repeated on further occasions, yielding similar results.

In all experiments, NSB of [$^3$H]$InsP_3$ was established in the presence of 15 μM unlabeled $InsP_3$. NSB generally represented 20 to 30% of the total binding but in different membrane preparations varied from less than 10% to more than 50%.

### Effect of $Ca^{2+}$ on Binding

Millimolar concentrations of $Ca^{2+}$ were required to observe specific binding of [$^3$H]$InsP_3$ to membrane vesicle preparations from *C. rubrum*. Binding was detectable at 1 mM $Ca^{2+}$, reached a maximum at 10 mM $Ca^{2+}$, and declined on further increases in $Ca^{2+}$ concentration (Fig. 2). Over similar concentration ranges, in both the presence and absence of $Ca^{2+}$, other cations ($Na^+$, $K^+$, $Mg^{2+}$) had no effect on binding (data not shown). This suggests that the enhancement of $InsP_3$ binding is a specific effect of $Ca^{2+}$. The significance of the $Ca^{2+}$ dependence displayed by the *C. rubrum* $InsP_3$ receptor is explored in "Discussion." At the pH 9.0 used in these binding assays, the commonly used $Ca^{2+}$ buffers (EDTA, EGTA, etc.) provide effective buffering over only a limited range of very low free $Ca^{2+}$ concentrations (Föhr et al., 1993). Furthermore, in common with Richardson and Taylor (1993), we have found that

EGTA itself acts as an antagonist of $InsP_3$ binding (data not shown). For both of these reasons, we have carried out our investigations using unbuffered solutions of $Ca^{2+}$.

### Effect of pH on Binding

In common with previous reports for both plant (Brosnan and Sanders, 1993) and animal species (Worley et al., 1987; Chadwick et al., 1990), specific binding of [$^3$H]$InsP_3$ to *C. rubrum* membrane vesicles was enhanced at alkaline pH. A 4.3-fold increase in specific binding was observed in the pH 7.5 to 9.0 range (Fig. 3). NSB did not vary with pH. Responses to pH above 9.0 are not depicted because the buffer system used (BTP-Mes) is not effective in this range. However, by using alternative buffer systems (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid-Mes) we have observed a decline in specific binding above pH 9.5 (data not shown).

### Kinetics of Association and Dissociation

The association of [$^3$H]$InsP_3$ with *C. rubrum* membrane vesicles was rapid, but a linear decline (3.4 dpm $min^{-1}$) from the maximum level of binding (2527 dpm, 5.5 min) was clearly evident (Fig. 4). NSB was constant over the period investigated. These data suggest that either [$^3$H]$InsP_3$ or the putative receptor was subject to degradation. To further investigate the stability of [$^3$H]$InsP_3$ under the conditions used in the binding assay, parallel incubations were performed in which inositol phosphates recovered in the filtrates were separated by HPLC. Such an approach is valid because less than 10% of the total [$^3$H]$InsP_3$ is retained by the filters and, therefore, any phosphatase or kinase activity will be revealed by analysis of the filtrates. These analyses revealed no significant depletion of radioactivity in the $InsP_3$ peak or accumulation of mono-, bis-, or tetraphosphorylated inositol esters (Fig. 5).

Binding of [$^3$H]$InsP_3$ to membrane preparations was reversible by both dilution (data not shown) and competition

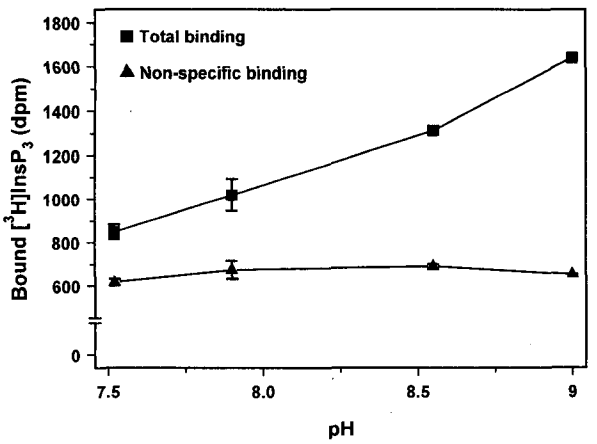



**Figure 3.** Effect of pH on [$^3$H]$InsP_3$ binding. Membrane vesicles (40 $\mu$g of protein) were incubated (4°C, 15 min) with [$^3$H]$InsP_3$ (1.6 nM) and the indicated pH. NSB was established in the presence of 15 $\mu$M $InsP_3$. Data points are means (±SE) of triplicates from a representative experiment that was repeated on two occasions, yielding similar results.

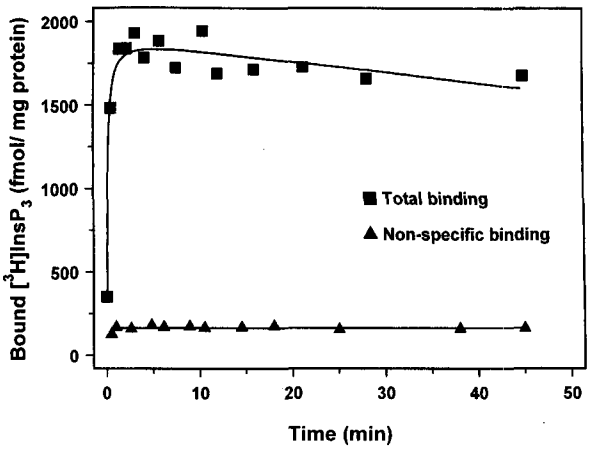



**Figure 4.** Time course of [$^3$H]$InsP_3$ association. Membrane vesicles (final protein concentration, 0.4 mg $mL^{-1}$) were incubated (4°C) with [$^3$H]$InsP_3$ (1.6 nM). Aliquots (50 $\mu$L) were removed at the times indicated and bound and free [$^3$H]$InsP_3$ were separated as described in "Materials and Methods." NSB was established in the presence of 15 $\mu$M $InsP_3$. Data are from a representative experiment that was repeated on two occasions, yielding similar results.

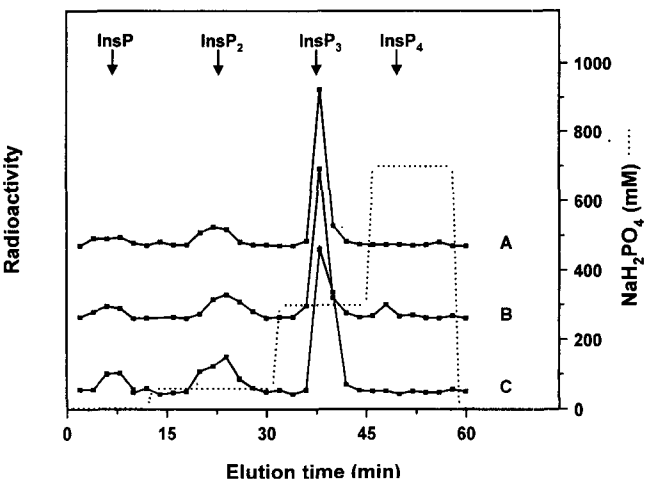



**Figure 5.** Validation of [$^3$H]$InsP_3$ stability by HPLC analysis. Binding assays were performed as described in "Materials and Methods" except that the volume of each component was proportionally increased such that the final assay volume was 250 $\mu$L (30,000 dpm). Following incubation with membrane vesicles, bound and free ligand were separated by rapid filtration and immediately rinsed with 2 mL of wash buffer. An aliquot (2 mL) of the filtrate was injected onto a Partisphere Sax HPLC column and inositol phosphates were separated by degree of phosphorylation with a stepwise $NaH_2PO_4$ gradient. The figure depicts elution profiles for standard [$^3$H]$InsP_3$ (A) and filtrates previously incubated with membrane fractions for 5 (B) and 30 (C) min. Data are normalized to the height of the $InsP_3$ peak. In all experiments [$^3$H]$InsP_3$ retained by the filters represented less than 10% of the total. Data are from a representative experiment that yielded similar results when repeated. InsP, $InsP_2$, and $InsP_4$ are mono-, bis-, and tetraphosphorylated inositol esters, respectively.

by $InsP_3$ (Fig. 6). Addition of $InsP_3$ to membrane fractions preincubated with [$^3$H]$InsP_3$ resulted in a time- and $InsP_3$ concentration-dependent dissociation of [$^3$H]$InsP_3$.

### Effect of Ligand and Receptor Concentration on Binding

The amount of specifically bound [$^3$H]$InsP_3$ was directly proportional to the concentration of membrane protein used in the assay, and heat treatment (100°C, 5 min) of the membrane preparation prior to assaying resulted in a complete loss of specific binding (data not shown). These observations are consistent with the binding sites being located on a protein.

The effect of ligand concentration on binding was quantified by displacement assays in which membrane vesicles were incubated with a constant amount of [$^3$H]$InsP_3$ in the presence of increasing concentrations of unlabeled $InsP_3$. This approach assumes that both the labeled and unlabeled ligand are uncontaminated and have identical affinities for the receptor sites but is generally accepted to yield valid estimates of the binding parameters (Hulme and Birdsall, 1992). Data were fitted to models of single- and multiple-site receptor populations using computer-calculated nonlinear regression (FigP; Biosoft, Milltown, NJ). In all experiments, only the single-site model (represented by Eq. 1) was consistent with the data. Because the concentration of [$^3$H]$InsP_3$ used in these experiments was less than $0.1\times K_d$, the resulting estimate for $IC_{50}$ can be considered equivalent to the $K_d$. A simple, noncooperative binding mechanism is also implied because the estimated Hill coefficients were not significantly different from unity.

$$B_{sp} = \frac{B_{nsp} + (B_{tot} - B_{nsp})}{1 + \left(\frac{IC_{50}}{[InsP_3]}\right)^{-nH}}, \quad (1)$$

where $B_{sp}$ = specific binding, $B_{nsp}$ = NSB, $B_{tot}$ = total binding, $n$H = Hill coefficient, and $IC_{50}$ = concentration of competing ligand displacing 50% of the [$^3$H]$InsP_3$.

In Figure 7 the above model is shown fitted to data from a typical experiment and, in this case, an estimated $K_d$ of 142 ± 17 nM was obtained. We have observed deviations of up to 20% from this value in separate membrane preparations (range 112–166 nM, $n$ = 5). To obtain greater reproducibility in $K_d$ estimations we found it important to include a relatively high concentration of reductant (DTT) in homogenization and suspension buffers.

An estimation of $B_{max}$ was calculated using the equation:

$$B_{max} = B_{sp}\left(1 + \frac{K_d}{[[^3H]InsP_3]}\right) \quad (2)$$

By substitution of values obtained for the preparation depicted in Figure 7, a value of 47 pmol mg$^{-1}$ protein for $B_{max}$ was obtained.

### Specificity of the Binding Sites

Specificity was investigated by comparing the efficacy with which a range of competing ligands displaced [$^3$H]$InsP_3$ from the binding sites. $IC_{50}$ values for each of the competing ligands were estimated using an approach similar to that described above for determination of $K_d$. The binding site was found to exhibit stereospecific selectivity for the D enantiomer, since L-inositol-1,4,5-trisphosphate ($IC_{50}$ = 691 ± 114 nM) was 5 to 6 times less effective in displacing [$^3$H]$InsP_3$. Other inositol phosphates were even less potent competitors. Ins-1,4-$P_2$ displaced [$^3$H]$InsP_3$ with an $IC_{50}$ of 24.8 ± 6.9 μM (Fig. 8), whereas Ins-4-P and Ins-$P_6$ were ineffective at concentrations of up to 4 mM (data not shown). Low-molecular-weight heparin, which has been shown to be an antagonist of both animal (Supattapone et al., 1988) and plant (Johannes et al., 1991; Brosnan and Sanders, 1993) $InsP_3$-induced $Ca^{2+}$ release, was also found to inhibit $InsP_3$ binding ($IC_{50}$ = 534 ± 142 nM). ATP has been reported to compete for the $InsP_3$-binding site at higher, although physiologically relevant, concentrations (Taylor and Marshall, 1992). This is also the case in *C. rubrum*, since ATP displaces [$^3$H]$InsP_3$ with an $IC_{50}$ of 241 ± 25 μM (Fig. 8).

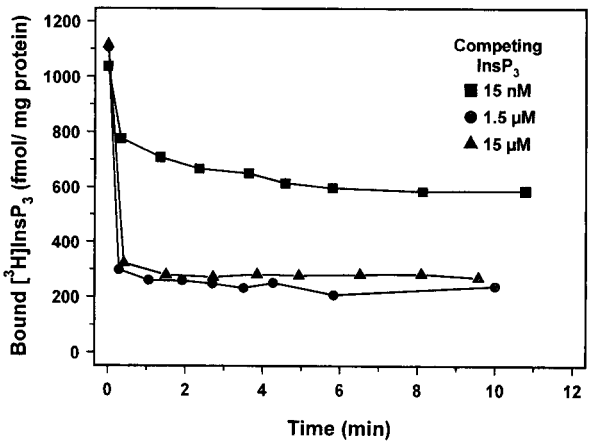



**Figure 6.** Time course of [$^3$H]$InsP_3$ dissociation. Membrane vesicles (final protein concentration, 0.4 mg mL$^{-1}$) were preincubated (4°C, 15 min) with [$^3$H]$InsP_3$ (concentration after addition of competing ligand, 1.6 nM) before addition (taken as time = 0) of $InsP_3$ to the concentrations indicated. Aliquots (50 μL) were removed at the times indicated and bound and free [$^3$H]$InsP_3$ were separated as described in "Materials and Methods." Data are from a single experiment that was repeated on two occasions, yielding similar results.

## DISCUSSION

The data presented in this paper demonstrate the presence of $Ca^{2+}$-dependent high-affinity-binding sites for $InsP_3$ in membrane preparations from *C. rubrum*. Whether these sites represent an authentic $InsP_3$ receptor, which would be expected to form an $InsP_3$-gated $Ca^{2+}$ channel, is as yet unproven but the circumstantial evidence supports this hypothesis: (a) The stability of [$^3$H]$InsP_3$ under the assay conditions argues against the binding site being an $InsP_3$ phosphatase or kinase. (b) Binding of [$^3$H]$InsP_3$ to the membrane preparation is strongly inhibited by heparin. In animals, this glycosaminoglycan has been shown to competitively inhibit binding of [$^3$H]$InsP_3$ to its receptor but to have no effect on the activity of $InsP_3$ kinases or phosphatases (Supattapone et al., 1988). (c) The specificity of the

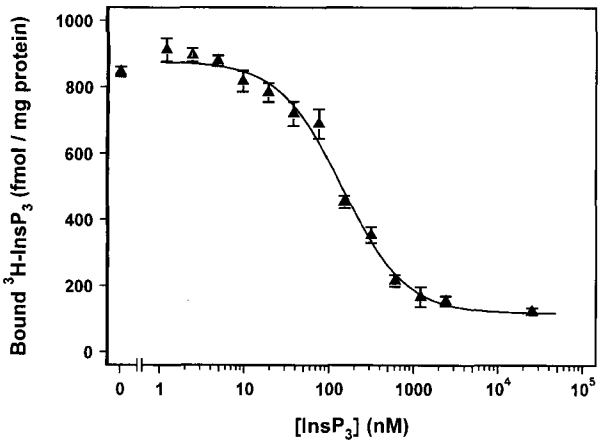



**Figure 7.** Analysis of binding parameters by competitive displacement assay. Membrane vesicles (37 $\mu$g of protein) were incubated with [$^3$H]InsP$_3$ (2.32 nM) and the indicated concentrations of InsP$_3$. Data points are means (±SE) of duplicates and were fitted to Equation 1 using mean values weighted by the inverse of the variance. The resulting estimates (±SE) obtained were: specific binding in the absence of competing InsP$_3$, 763 fmol mg$^{-1}$ protein; $K_d$, 142 ± 17 nM; $n$H, −1.16 ± 0.14.

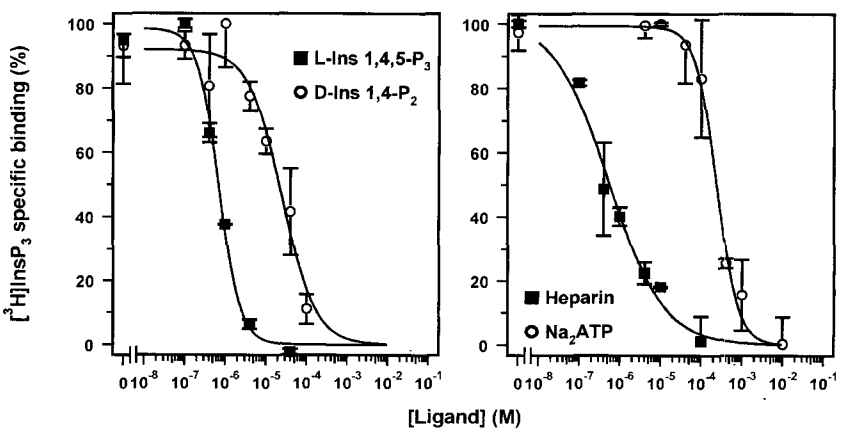
binding sites for InsP$_3$ as compared to other inositol phosphates is similar to that displayed by authentic InsP$_3$ receptors characterized in animals.

The properties of the InsP$_3$ receptor that we have identified in *C. rubrum* are broadly similar to those from red beet storage root (Brosnan and Sanders, 1993) and mung bean hypocotyls (Biswas et al., 1995). Features such as an alkaline pH optimum, rapid association and dissociation kinetics, and competitive inhibition by heparin and ATP appear to be conserved in both plant and animal InsP$_3$ receptors. In contrast to the present report, previous studies of InsP$_3$ receptors in plants have not investigated the effects of $Ca^{2+}$ on the interactions between InsP$_3$ and its receptor. Calcium dependence of InsP$_3$ binding has been reported for receptors isolated from a number of animal sources, including rat liver (Pietri et al., 1990; Marshall and Taylor, 1994), rat cerebellum (Worley et al., 1987), and *Xenopus laevis* oocytes (Callamaras and Parker, 1994). The precise response to $Ca^{2+}$ is, however, both tissue and species specific. For example, similar $Ca^{2+}$ concentrations ($EC_{50} \cong 300$ nM) increased receptor affinity in liver (Pietri et al., 1990; Marshall and Taylor, 1994) but decreased it in cerebellum (Worley et al., 1987). In contrast, in *X. laevis* oocytes increasing $Ca^{2+}$ concentration ($EC_{50} = 60$ nM) did not affect receptor affinity but resulted in a 10-fold increase in the apparent receptor concentration (Callamaras and Parker, 1994).

As well as modulating InsP$_3$ binding to its receptor, $Ca^{2+}$ can also affect $Ca^{2+}$ channel conductance (Mauger et al., 1994). In combination, these effects result in InsP$_3$-induced $Ca^{2+}$ release typically displaying a biphasic response to $Ca^{2+}$ concentration (Iino and Tsukioka, 1994). Based on studies of the receptor from rat liver, Marshall and Taylor (1994) have proposed a model in which interconversion of three conformations (low-affinity conducting, high-affinity conducting, high-affinity nonconducting) is mediated by the binding of $Ca^{2+}$ at two different sites. It has been suggested that this feedback control of InsP$_3$-induced $Ca^{2+}$ release may play a role in the generation of $Ca^{2+}$ spikes and oscillations that are a feature of $Ca^{2+}$ signaling (Irvine, 1990; Clapham, 1995).

Binding of InsP$_3$ to *C. rubrum* membranes also displays a dependency on $Ca^{2+}$; however, the much higher concentrations of $Ca^{2+}$ required to potentiate binding makes it difficult to see how the effect could be manifested in vivo. One possibility is that accessory proteins that were lost during isolation may mediate the effects of $Ca^{2+}$. Bethke and Jones (1994) recently described a potassium channel from vacuoles of barley aleurone cells that is modulated by $Ca^{2+}$ in the 10 to 100 $\mu$M range. Addition of CaM sensitized the channel to $Ca^{2+}$, suggesting that $Ca^{2+}$-CaM may be responsible for modulating the channel activity in vivo. A similar $Ca^{2+}$-CaM-regulated ion channel has also been described in *C. rubrum* (Weiser et al., 1991). There is accumulating evidence for a plant CaM-activated protein kinase analogous to the animal $Ca^{2+}$-CaM-dependent protein kinase II (Poovaiah and Reddy, 1993). It is intriguing that this enzyme is known to phosphorylate the animal InsP$_3$ receptor in vitro (Ferris et al., 1991). Further evidence for the interaction of accessory proteins with the InsP$_3$ receptor comes from studies in animals. Supattapone et al. (1988) found that the rat cerebellar receptor lost its sensitivity to

**Figure 8.** Displacement of [$^3$H]InsP$_3$ by potentially completing ligands. Membrane vesicles (40 $\mu$g of protein) were incubated with [$^3$H]InsP$_3$ (1.6 nM) and the indicated concentrations of a range of other ligands. Data points are means (±SE) of duplicates and were fitted to Equation 1 using mean values weighted by the inverse of the variance. Resulting estimates for $IC_{50}$ values were: L-*myo*-inositol-1,4,5-trisphosphate (L-Ins 1,4-P$_3$), 691 ± 114 nM; Ins-1,4-P$_2$, 24.8 ± 6.9 $\mu$M; heparin, 534 ± 142 nM; ATP, 241 ± 25 $\mu$M. In the same preparation, the estimated $K_d$ for InsP$_3$ (Fig. 7) was 142 ± 17 nM.



$Ca^{2+}$ upon purification and that the activity of the factor conferring this sensitivity was destroyed by trypsin. Such interactions could provide mechanisms for the regulation of $InsP_3$-induced $Ca^{2+}$ release and certainly warrant further investigation.

Comparison of the binding parameters of the *C. rubrum* receptor with those from red beet (Brosnan and Sanders, 1993) and mung bean (Biswas et al., 1995) reveals it to be of somewhat lower affinity but present at greater concentration. The latter is perhaps most clearly illustrated by comparing the 40 $\mu$g of membrane protein utilized per assay in the present study to the 1 to 2 mg used in the reports cited above. Although these data may reflect species and/or tissue differences in the binding parameters, other factors such as the membrane fractions used are also likely to be of significance. Brosnan and Sanders (1993) characterized $InsP_3$-binding sites in a Triton X-100-solubilized microsomal preparation from the storage root of red beet and used a PEG precipitation technique to separate bound and free ligand. Although such methods are routinely used in the study of the interactions between ligands and soluble receptors, it is possible that the precipitation and/or solubilization procedure may influence the observed properties of the receptor population (Hulme and Birdsall, 1992). For example, if the equilibrium between receptor and ligand is re-established on addition of PEG, an underestimation of $B_{max}$ will be obtained. Biswas et al. (1995) carried out binding experiments using microsomal membrane vesicles but used [$^3$H]$InsP_3$ of relatively low specific activity (37 GBq $mmol^{-1}$) and consequently had to include much higher concentrations in their binding assays. It is likely that this approach would have led to significant depletion of free ligand during establishment of equilibrium. Such depletion introduces complications into the analysis and can result in erroneous estimations of binding parameters. To obtain valid estimates of the binding parameters of the receptor preparation, we were particularly careful to demonstrate that an equilibrium between receptor and ligand had been established and that free ligand depletion (due to specific and NSB) was less than 20% of the total. We have found that relatively high concentrations of reductant (5 mM DTT) are required to improve the stability of $InsP_3$-binding sites in *C. rubrum* membrane preparations. This may well be related to previous demonstrations that $InsP_3$ binding is sensitive to treatment with reagents that specifically modify sulfhydryl groups (Supattapone et al., 1988; Brosnan and Sanders, 1993). The inhibitory effects of such treatments are attenuated by the presence of reductants such as DTT and $\beta$-mercaptoethanol (Supattapone et al., 1988) or $InsP_3$ (Brosnan and Sanders, 1993), and these data have been interpreted as implying the involvement of Cys residues in maintaining the integrity of the $InsP_3$-binding site.

Evidence is now accumulating to suggest that plants possess $InsP_3$ receptors that are similar to those characterized in animals. Recent studies of animal $InsP_3$ receptors have highlighted the importance of $Ca^{2+}$ regulation of $InsP_3$-induced $Ca^{2+}$ release in generation of stimulus-specific responses. The data presented here demonstrate that $Ca^{2+}$ modulates binding of $InsP_3$ to a potential receptor in *C. rubrum* and may point to the operation of similar mechanisms in plants. Work is now in progress to elucidate both the mechanism of this regulation and the subcellular location(s) of these binding sites.

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