Conformational changes in plant Ins(1,4,5)P_3 receptor on interaction with different *myo*-inositol trisphosphates and its effect on Ca^{2+} release from microsomal fraction and liposomes

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The interaction of the only reported plant inositol trisphosphate receptor with different *myo*-inositol trisphosphates (InsP_3 species), namely Ins(1,4,5)P_3, Ins(1,3,4)P_3, Ins(1,5,6)P_3 and Ins(2,4,5)P_3, were studied to assess the extent of Ca^{2+} mobilization from microsomes/vacuoles as well as liposomes *in vitro*. Ins(1,4,5)P_3 and Ins(2,4,5)P_3 bind with the receptor with comparable affinities, as evidenced from their dissociation constants (K_d approx. 100 nM at 5 °C), whereas the interaction between Ins(1,3,4)P_3/Ins(1,5,6)P_3 and the receptor was not detected even with these ligands at 5 μM. Ins(1,3,4)P_3/Ins(1,5,6)P_3 isomers also do not elicit Ca^{2+} release from liposomes or microsomes/vacuoles. The ability of any InsP_3 to bind the receptor for Ins(1,4,5)P_3 is a prime requirement for Ca^{2+} release. However, the comparison of binding affinities at a single temperature does not help to correlate it directly with the extent of Ca^{2+} release from the intracellular stores, because the concentration of Ca^{2+} released by Ins(1,4,5)P_3 as estimated over a period of 20 s is 3500 ± 200 nM/mg of protein and is about 4-fold higher than that by Ins(2,4,5)P_3 under identical conditions. To understand the role of the receptor conformation in Ca^{2+} release by different isomers, we have probed the conformational change of the receptor when the different isomers bind to it. Accessibility of the tryptophan residues in the free and Ins(1,4,5)P_3/Ins(2,4,5)P_3-bound receptor was monitored by a neutral fluorescence quencher, acrylamide. The resulting Stern–Volmer-type quenching plots of the internal fluorescence indicate a change in the conformation of the receptor on binding to Ins(1,4,5)P_3 and Ins(2,4,5)P_3. It is also detected when far-UV CD spectra (205–250 nm) of the free and ligand [Ins(1,4,5)P_3/Ins(2,4,5)P_3]-bound receptor are compared. The results from CD spectroscopic studies further indicate that the conformational changes induced by the two isomers are different in nature. When thermodynamic parameters, such as enthalpy (ΔH), entropy (ΔS) and free energy (ΔG), for the formation of the two InsP_3–receptor complexes are compared, a major difference in the extent of changes in enthalpy and entropy is noted. All these findings taken together support the proposition that it is the overall interaction leading to the requisite conformational change in the receptor that determines the potency of the InsP_3 isomers in their abilities of Ca^{2+} mobilization from the intracellular stores or reconstituted liposomes.

INTRODUCTION

Considerable progress has been made in understanding the mechanism by which PtdIns(4,5)P_2 produces second-messenger molecules [1]. It has also been established that one of the key events in intracellular signalling is the receptor-mediated hydrolysis of PtdIns(4,5)P_2 by phospholipase C, generating Ins(1,4,5)P_3. It then binds to its receptor (InsP_3R), an Ins(1,4,5)P_3-gated Ca^{2+} channel located at the intracellular Ca^{2+} stores. As a result, Ca^{2+} is released into the cytoplasm [1]. Studies of the receptor from animal systems have characterized the structural domains of the receptor. The Ins(1,4,5)P_3-binding domain is formed largely by residues within the N-terminal region, which is separated by more than 1400 residues from the C-terminal Ca^{2+} channel [2]. Both the N-terminal and C-terminal tails are cytoplasmic, leaving only very short loops linking some of the membrane-spanning helices within the lumen of the Ca^{2+} stores. Comparatively little is known about the receptor in plant systems; there have been very few reports on the putative receptor from plants [3,4]. We reported for the first time the isolation and biochemical characterization of a receptor from the mung bean system [5]. The plant receptor (110 kDa per subunit) consists of four subunits, like its animal counterpart (250–300 kDa). It binds to both Ins(1,4,5)P_3 and Ins(2,4,5)P_3 with little difference in their affinities. In contrast, Ins(1,4,5)P_3 is about four times more effective than Ins(2,4,5)P_3 in Ca^{2+} mobilization from microsomes/vacuoles in mung bean. Specific recognition of the receptor by the second messenger and the subsequent release of Ca^{2+} have led to the question of whether there is any correlation between the release and the binding affinity of the receptor for different inositol phosphates. It seems that the receptor requires mainly the motif of the vicinal Ins(4,5)P_2 plus a free 6-OH group and one of the negative charges of the phosphate group at the C-1 position for binding [6]. However, a correlation between the binding affinities of different *myo*-inositol phosphates to the InsP_3R and effective Ca^{2+} mobilization has not been established [6,7].

The present work is aimed at finding out the nature and extent of conformational changes in the plant InsP_3R after it has bound to the InsP_3 isomers and correlating whether or not the affinity of the InsP_3R for different InsP_3 isomers is directly proportional to intracellular Ca^{2+} mobilization.

MATERIALS AND METHODS

Materials

[^{3}H]Ins(1,4,5)P_3 (40 Ci/mmol), was obtained from NEN–Dupont. Ins(1,4,5)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3, 2-[2-bis-

Abbreviations used: InsP_3, any of the isomers Ins(1,4,5)P_3, Ins(1,3,4)P_3, Ins(1,5,6)P_3 and Ins(2,4,5)P_3; InsP_3R, InsP_3 receptor; quin-2, 2-[2-bis-(carboxymethylphenoxy)-methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline.

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(carboxymethylphenoxy)methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline (quin-2), heparin–agarose, acrylamide (twice crystallized), polyvinylpyrrolidone, benzamidine and 2-mercaptoethanol were from Sigma Chemical Company. All other reagents were of analytical grade and obtained indigenously from different agencies. Mung bean (*Vigna radiata*) seeds were obtained from Seed Multiplication Farm (Behrampur, West Bengal, India).

Purification of InsP_3R from mung bean microsomes/vacuoles

InsP_3R was isolated from the microsomal/vacuolar fraction of mung bean hypocotyl and purified as described earlier [5]. In brief, the microsomes/vacuoles were prepared from 48 h-germinating mung bean hypocotyls by homogenizing the tissue in buffer A [25 mM Tris/HCl buffer (pH 8.0)/0.25 M sucrose/3 mM EDTA/10 mM 2-mercaptoethanol/1 mM PMSF/1 mM benzamidine/10 g/l polyvinylpyrrolidone/1 g/l BSA]. The homogenate was passed through two layers of cheese cloth. It was then centrifuged at 12000 ***g*** for 40 min and the supernatant was finally centrifuged at 80000 ***g*** for 1 h at 4 °C to pellet the microsomes/vacuoles. The pellet was suspended in 1 % (v/v) Triton X-100 plus buffer B [50 mM Tris/HCl buffer (pH 8.0)/1 mM EDTA/1 mM 2-mercaptoethanol] and the protein was solubilized by stirring the suspension for 1 h at 4 °C. This was then centrifuged at 100000 ***g*** for 2 h at 4 °C. To the supernatant was added sodium chloride to a final concentration of 0.2 M; it was then passed through the heparin–agarose column twice. The receptor was eluted with the buffer C [50 mM Tris/HCl (pH 8.0)/1 mM EDTA/1 mM 2-mercaptoethanol/1 mM PMSF/1 mM benzamidine/1 % (v/v) Triton X-100/0.6 M NaCl]. It was desalted, freeze-dried and subjected to PAGE [5 % (w/v) gel]. When this band was electroeluted and subjected to SDS/PAGE, one protein band (110 kDa) was visible after silver staining, and [^{3}H]Ins(1,4,5)P_3-binding activity coincided with this band, as assayed by slicing the gel. The electroeluted protein corresponding to the band also elicited Ca^{2+} efflux from the reconstituted proteoliposomes. The yield was approx. 0.08 mg/10 mg of microsomal/vacuolar protein and the purification at the final stage was approx. 1000-fold.

Preparation of proteoliposomes

The receptor obtained (80 μg) was reconstituted in 160 μg of phosphatidylcholine/phosphatidylethanolamine (1:1, w/w) in buffer [4 mM Tris/HCl (pH 8.0)/25 mM NaCl/23 mM KCl/100 μM $CaCl_2$] by sonication. The proteoliposomes were dialysed exhaustively. These vesicles were monitored for Ca^{2+}-release experiments as described below.

Evaluation of Ca^{2+} uptake and release

For the evaluation of Ca^{2+} influx and efflux, microsomal/vacuolar suspension (127 μg/ml) containing 100 μM quin-2, 3 mM NaN_3, 100 μM $CaCl_2$ and 2 mM ATP was placed in a cuvette for fluorescence measurements in Hitachi F-3010 spectrofluorimeter. The addition of ATP initiated the uptake of Ca^{2+} by the microsomes/vacuoles, which was monitored by the decrease in fluorescence of quin-2 (λ_{ex} 339 nm; λ_{em} 492 nm). After the process of uptake had reached a steady value, 1 μM of the InsP_3 isomer was added. The concentration of Ca^{2+} released over a period of 20 s was calculated from the following equation [8]:

$$[Ca^{2+}] = K_d(F-F_{min})/(F_{max}-F) \quad (1)$$

where K_d, F, F_{max} and F_{min} denote the dissociation constant (115 nM) for quin-2–Ca^{2+} interaction, the fluorescence of the sample, the maximum fluorescence measured in the presence of 2 mM Ca^{2+} and the minimum fluorescence measured in the presence of 5 mM EGTA respectively.

A suspension of proteoliposome (8 μg of protein) in a volume of 400 μl containing Ca^{2+} was incubated with 100 μM quin-2 for 3–4 min at 25 °C. To this were added various inositol phosphates; the release of Ca^{2+} was monitored as described above.

Evaluation of the binding stoichiometry, dissociation constant and thermodynamic parameters for the interaction of InsP_3 with the InsP_3R

The binding studies were performed in 50 mM Tris/HCl buffer (pH 8.0)/25 mM NaCl containing 0.05 % Triton X-100 (henceforth denoted buffer T). Uncorrected fluorescence spectra are reported here. Appropriate subtractions of the contribution from buffer T were made. The fluorescence of the receptor (λ_{ex} 295 nm) decreased on the addition of Ins(1,4,5)P_3/Ins(2,4,5)P_3. The addition of Ins(1,3,4)P_3/Ins(1,5,6)P_3 up to a concentration of 5 μM did not lead to any significant change in the fluorescence spectrum of the receptor (results not shown), thereby indicating the absence of association between them. A decrease in fluorescence of the receptor provides a method for determining the affinity parameters, binding stoichiometry and dissociation constant for its binding to the isomers Ins(1,4,5)P_3/Ins(2,4,5)P_3. It was plotted as a function of the input concentration of InsP_3. The binding stoichiometry was determined from the break-point in the straight lines as obtained. The dissociation constant (K_d) was evaluated from the ligand-induced quenching of the fluorescence of the receptor by means of the following equation [9]:

$$1/\Delta F = 1/\Delta F + K_d/(\Delta F_{max}[S]) \quad (2)$$

where [S] denotes the concentration of the ligand and ΔF is the extent of fluorescence quenching at 340 nm as a function of input concentration of the ligand [S]. A least-squares fit of the experimental points was done to get the best-fitting straight line. The ratio of the slope and intercept of the straight line from the plot of $1/\Delta F$ against 1/[S] gives the value of K_d. The association constant, K_{app} ($= 1/K_d$) determined at different temperatures was used for the calculation of the thermodynamic parameters.

The dissociation constant for the interaction of InsP_3 with InsP_3R was also determined by a filter-binding assay as follows. Different concentrations of [^{3}H]Ins(1,4,5)P_3 (specific radioactivity 0.677 μCi/nmol) were incubated with a fixed concentration (40 nM) of the receptor in buffer T at 5 °C. After 15 min the incubation mixture was passed through Whatman GF/C filter paper to separate the free ligand from the receptor-bound ligand. The filter paper was washed with the same buffer and dried for radioactivity measurements. Non-specific binding was checked in the presence of an excess of unlabelled Ins(1,4,5)P_3 (10 μM) and the corresponding value was subtracted from the total counts to obtain the binding isotherm.

Changes in enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) were estimated from the linear plot of $\ln K_{app}$ as a function of temperature (5, 14 and 25 °C), from the following equations [10]:

$$\ln K_{app} = -\Delta H/RT + \Delta S/R \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Conformations of InsP_3R alone and in the presence of InsP_3

The conformation of the receptor was ascertained in two ways as follows. In the first method the accessibility of its tryptophan residues under different conditions was checked by using acryl-

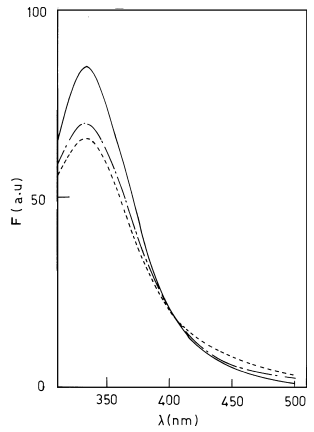

Figure 1 Change in fluorescence spectrum of InsP_3R due to binding with InsP_3

Fluorescence (F) emission spectrum (λ_{ex} 295 nm) of InsP_3R (40 nM) in 50 mM Tris/HCl buffer (pH 8.0)/25 mM NaCl containing 0.05% Triton X-100 at 14 °C under the following conditions: (i) alone (solid line), (ii) in the presence of 1 μM Ins(1,4,5)P_3 (broken line) and (iii) in the presence of 1 μM Ins(2,4,5)P_3 (dot–dashed line). The excitation and emission slit widths were 5 and 10 nm respectively. Abbreviation: a.u., arbitrary units.

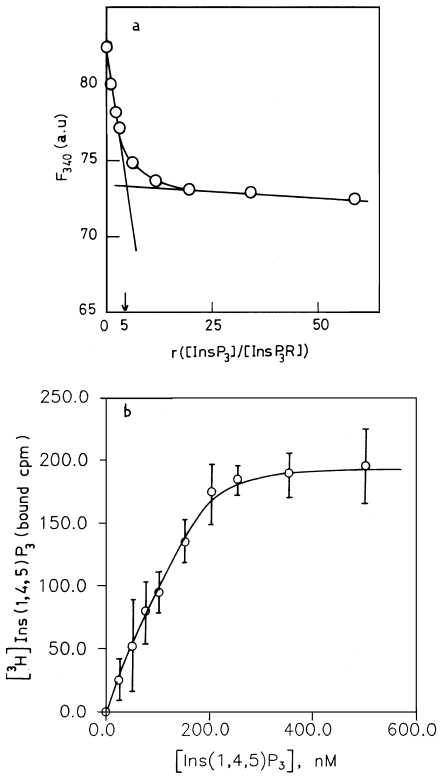

Figure 2 Binding isotherm for the association of InsP_3R with Ins(1,4,5)P_3

(**a**) Fluorescence titration (λ_{ex} 295 nm; λ_{em} 340 nm; excitation and emission slit widths 5 and 10 nm respectively) of 40 nM InsP_3R with Ins(1,4,5)P_3 in 50 mM Tris/HCl (pH 8.0)/25 mM NaCl containing 0.05% Triton X-100 at 5 °C. The variable r is the ratio of the molar concentrations of Ins(1,4,5)P_3 and InsP_3R. The arrow corresponds to the ratio at the intersection of the two straight lines and represents the binding stoichiometry. Abbreviation: a.u., arbitrary units. (**b**) Binding isotherm for the association of Ins(1,4,5)P_3 with 40 nM InsP_3R as estimated by the filter-binding assay in the same buffer as in (**a**) at 5 °C. The abscissa denotes the concentration of Ins(1,4,5)P_3 with a specific radioactivity of 0.677 μCi/nmol. The ordinate represents filter-bound radioactivity corresponding to each input concentration of Ins(1,4,5)P_3. The dissociation constant was the concentration of Ins(1,4,5)P_3 corresponding to half of the bound radioactivity. Representative error bars (S.E.M.) from three sets of estimates with different batches of the receptor are shown in the Figure.

amide, a tryptophan-specific fluorescence quencher [11] that is commonly used to assess the degree of exposure of an internal fluorophore such as tryptophan in protein. Quenching of the protein fluorescence by acrylamide was analysed by the Stern–Volmer equation [11]: $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are the initial and final fluorescence intensities of the receptor, [Q] denotes the input concentration of acrylamide, and K_{sv} is Stern–Volmer quenching constant. If the accessibility of the tryptophan residues in a protein changes, it leads to an alteration in K_{sv}. In the present case the value changes between the free receptor and the InsP_3-bound receptor. The change originates from an alteration in the conformation of the receptor as a result of the binding of the ligand. In the second method the CD spectra of the protein in the far-UV region (205–250 nm) were measured in the absence and presence of InsP_3. They were recorded with Jasco J-720 spectropolarimeter in a cuvette of 1 mm path length. The presence of Triton X-100 in the buffer prevented the recording of the CD spectrum below 205 nm. Limited solubility and non-specific aggregation of the receptor prevented us from studying the tertiary structure in the near-UV region because the longer wavelengths require a higher concentration of the protein to achieve a good signal-to-noise ratio.

RESULTS AND DISCUSSION

Affinity parameters for the interaction of InsP_3 isomers with InsP_3R

Addition of Ins(1,4,5)P_3 or Ins(2,4,5)P_3 (Figure 1) to the receptor leads to a quenching of the fluorescence of the receptor. Such quenching of tryptophan fluorescence could originate from two sources: an alteration in the conformation of the receptor on binding to the ligand, or the presence of the negatively charged trisphosphate in the vicinity of the indole fluorophore of the tryptophan residues. Considering the hydrophobic nature of this residue, the latter possibility seems remote. However, no significant change in the fluorescence of the receptor occurred in the presence of 5 μM Ins(1,3,4)P_3 or Ins(1,5,6)P_3, suggesting either an absence of its binding or binding with lower affinity (K_d $>$ 5 μM). The decrease in fluorescence on addition of Ins(1,4,5)P_3 or Ins(2,4,5)P_3 was dependent upon the input concentrations of InsP_3 until at a point at which the fluorescence reached a plateau (Figure 2a). This suggests that the quenching occurs as a result of the association between InsP_3R and InsP_3. Previously the association had been monitored by the filter-binding assay with radiolabelled *myo*-inositol phosphates [5,7]. The result of the filter-binding assay to evaluate the affinity constant for the association of Ins(1,4,5)P_3 with InsP_3R is shown in Figure 2(b). The observed quenching in fluorescence of the receptor provides an alternative method to evaluate the affinity parameters even for non-radiolabelled *myo*-inositol phosphates, e.g. Ins(2,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,5,6)P_3 in the present case. The dissociation constant and stoichiometry were calculated either directly from the linear binding isotherms (Figures 2a and 2b) or from a double-reciprocal plot [eqn. (2)] from fluorescence measurements (Figure 3a). Table 1 summarizes the relevant stoichiometry and dissociation constant values determined by the two methods. The internal consistency of the values for the dissociation constant for Ins(1,4,5)P_3 validates the fluorescence method as a means of evaluating the affinity constant for non-radiolabelled *myo*-

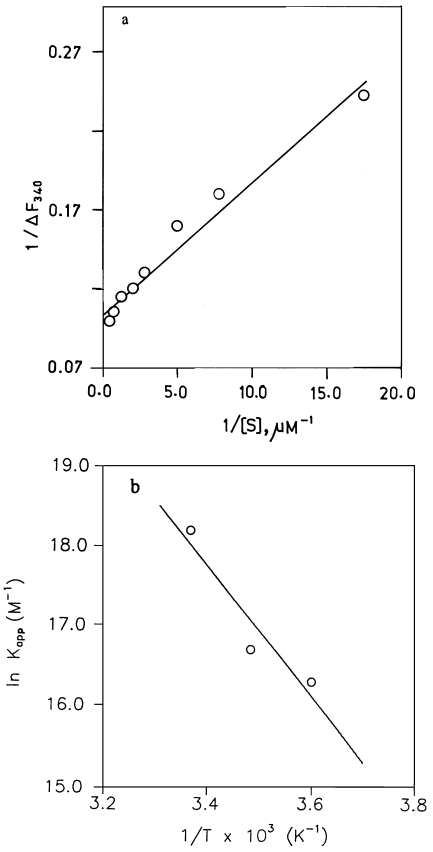

Figure 3 Evaluation of the apparent dissociation constant, K_d, and thermodynamic parameters for the InsP_3R–Ins(1,4,5)P_3 interaction

(**a**) Double-reciprocal plot of $1/\Delta F$ (λ_{ex} 295 nm; λ_{em} 340 nm) against 1/[S] to evaluate the dissociation constant for the interaction of Ins(1,4,5)P_3 with InsP_3R at 5 °C in 50 mM Tris/HCl buffer (pH 8.0)/25 mM NaCl containing 0.05% Triton X-100, by means of eqn. (2). The straight line obtained is from a least-squares fit of the experimental points as in Figure 2(a). (**b**) Van't Hoff plot of $\ln K_{app}$ ($= 1/K_d$) against the reciprocal of absolute temperature to determine ΔH and ΔS for the Ins(1,4,5)P_3–InsP_3R interaction. The straight line obtained is the least-squares fit of the three experimental points.

Table 1 Dissociation constant and binding stoichiometry for the interaction of InsP_3 isomers with InsP_3R

Results were determined from the fluorescence quenching data in 50 mM Tris/HCl buffer (pH 8.0)/25 mM NaCl containing 0.05% Triton X-100 at 5 °C. Each value is an average of two separate sets of preparations of the receptor. The stoichiometry was estimated as shown in the representative graph of Figure 2(a). The value for K_d in parenthesis was determined from the curve (Figure 2b) obtained from the filter-binding assay with [^{3}H]Ins(1,4,5)P_3. Abbreviation: n.d., not determined.

Ligand	Dissociation constant, K_d (nM)	Stoichiometry
Ins(1,4,5)P_3	82 ± 20 (100)	1.25
Ins(2,4,5)P_3	115 ± 30	1.1
Ins(1,3,4)P_3	> 5000*	n.d.
Ins(1,5,6)P_3	> 5000*	n.d.

* No change in fluorescence of the receptor at 5 μM InsP_3 isomers was obtained.

Table 2 Concentration of Ca^{2+} released by the InsP_3 isomers

The release of Ca^{2+} from liposomes and microsomes/vacuoles was monitored by the fluorescence assay as described in the Materials and methods section. The values for Ca^{2+} released from liposomes are the concentrations of Ca^{2+} released per 8 μg of receptor protein. It was measured over a period of 20 s. The concentration of ligand used was 1 μM in each case. The values for Ca^{2+} released from microsomes/vacuoles are the concentrations of Ca^{2+} released per mg of microsomal/vacuolar protein in 20 s. Abbreviation: n.d., not detected.

	Ca^{2+} (nM) released from:	
Ligand	Liposomes	Microsomes/ vacuoles
Ins(1,4,5)P_3	36 ± 3	3500 ± 200
Ins(2,4,5)P_3	12 ± 1.7	910 ± 100
Ins(1,3,4)P_3	n.d.	120 ± 30
Ins(1,5,6)P_3	n.d.	50 ± 10

Table 3 Thermodynamic parameters for the interaction of InsP_3 isomers with InsP_3R

Results were determined from a plot of $\ln K_{app}$ against $1/T$ (Figure 3b). ΔG was calculated by means of eqn. (4) at 25 °C and corresponds to the mean value of ΔH.

Ligand	ΔH (kJ/mol)	ΔS [J/(K · mol)]	ΔG (kJ/mol)
Ins(1,4,5)P_3	69.0 ± 14.6	380	−44.3
Ins(2,4,5)P_3	23.4 ± 6.3	221	−42.6

inositol phosphates. Furthermore the observed stoichiometry of one molecule of InsP_3 per subunit of the receptor is consistent with the results reported from the studies of InsP_3R in an animal system [12]. The value of the dissociation constant for Ins(1,4,5)P_3–InsP_3R interaction at 5 °C agrees with that reported from the filter-binding assay in our laboratory [5]. The affinities of the two isomers for InsP_3R are, however, comparable at this temperature. In a competitive filter-binding assay, prebound [^{3}H]Ins(1,4,5)P_3 (200 nM) to InsP_3R (40 nM) was challenged separately with unlabelled Ins(2,4,5)P_3, Ins(1,3,4)P_3 or Ins(1,5,6)P_3. Half of the total bound radioactivity could be displaced by 200 nM of Ins(2,4,5)P_3, whereas no displacement occurred even with 5 μM Ins(1,3,4)P_3 or Ins(1,5,6)P_3. This is in agreement with the results from the fluorescence studies.

Comparison of Ca^{2+} release from intracellular stores by the InsP_3 isomers

The functional properties of InsP_3 isomers were compared by their abilities to release Ca^{2+} from intracellular stores. It is clear from Table 2 that the extent of release of Ins(1,4,5)P_3 is about 4-fold higher than that for Ins(2,4,5)P_3. This ratio is not directly proportional to the InsP_3R binding affinities for the InsP_3 isomers. A similar trend in the extent of Ca^{2+} release was noticed with liposomes containing purified receptor (Table 2). Comparatively, Ins(1,3,4)P_3 and Ins(1,5,6)P_3 cause an insignificant release of Ca^{2+} because of their poor interactions with the receptor.

Thermodynamic parameters for InsP_3–InsP_3R interactions

A mutual compensation of ΔH and ΔS values might lead to a comparable ΔG value, i.e. affinity constant at a particular

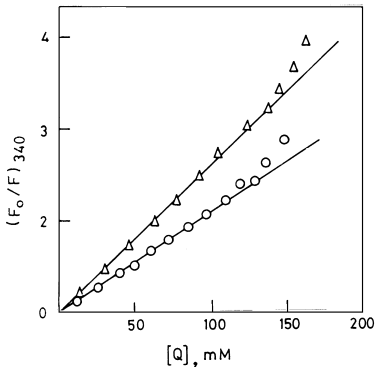

Figure 4 Acrylamide fluorescence-quenching assay to monitor the accessibility of tryptophan residues in the receptor

Stern–Volmer plots of fluorescence quenching (λ_{ex} 295 nm; λ_{em} 340 nm) of 40 nM InsP_3R alone (○) and in the presence of 0.54 μM Ins(1,4,5)P_3 (△) at 25 °C in 50 mM Tris/HCl buffer (pH 8.0)/25 mM NaCl containing 0.05% Triton X-100. [Q] denotes the molar concentration of the quencher, acrylamide. The value of K_{sv} in the absence of ligand was 22 M^{-1}; in the presence of Ins(1,4,5)P_3 it was 32 M^{-1} and in the presence of Ins(2,4,5)P_3 it was 34 M^{-1}.

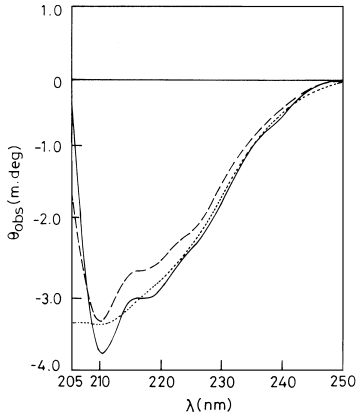

Figure 5 Far-UV CD spectra of the receptor

CD spectrum of InsP_3R (205–250 nm) at 25 °C in 50 mM Tris/HCl buffer (pH 8.0)/25 mM NaCl containing 0.05% Triton X-100 under different conditions: (i) alone (100 nM; solid line), (ii) in the presence of 1 μM Ins(1,4,5)P_3 (dotted line) and (iii) in the presence of 1 μM Ins(2,4,5)P_3 (broken line). Each spectrum represents the average of four runs. Appropriate subtraction of the buffer blank and smoothing of the subtracted spectrum without any distortion of the fine structure were done with a built-in program of the spectropolarimeter.

temperature ($\Delta G = -RT\ln K_{app}$). Therefore we measured the thermodynamic parameters to characterize and compare the interactions of the two isomers Ins(1,4,5)P_3 and Ins(2,4,5)P_3 in particular with InsP_3R. Changes in heat content and entropy are also useful parameters for comparing the conformational changes in the receptor as a result of its binding to the isomers. Figure 3(b) shows the representative Van't Hoff plot [eqn. (3)] for the Ins(1,4,5)P_3–InsP_3R interaction. The affinity constants were determined from the double-reciprocal plot such as that shown in Figure 3(a). From a comparison of similar types of report on the thermodynamics of interactions among nucleic acids and proteins with subunits [13], we suggest the following. The linear nature of the Van't Hoff plot within the temperature range 5–25 °C indicates that there is no major change in the quaternary structure of the receptor as a result of the association of each subunit with InsP_3. Table 3 summarizes the thermodynamic parameters. The association with InsP_3R in both cases [Ins(1,4,5)P_3/Ins(2,4,5)P_3] leads to a positive change in the enthalpy and is therefore entropy-driven. It could be ascribed to two factors: electrostatic interaction between the negative phosphate group of InsP_3 and the positively charged side chain of an amino acid residue in the N-terminal domain of InsP_3R, and an alteration in the conformation of the InsP_3R. The contribution of the first factor is usually of the order of 8–20 kJ/mol (2–5 kcal/mol). It indicates that the rest of the value for ΔH is contributed by the conformational change of the receptor on binding to Ins(1,4,5)P_3. It implies that Ins(1,4,5)P_3 and Ins(2,4,5)P_3 induce different degrees and/or natures of conformational change in InsP_3R. Similarly, the positive entropy change could be the sum of two factors: $\Delta S = \Delta S_{water} + \Delta S_{configuration}$ [13]. The first term stands for the entropy change due to the release of bound water from the receptor and the second arises mostly from the configurational entropy change of the receptor due to association with InsP_3. A comparison of the entropy change in the two cases (Table 3) shows that there is an additional entropy change [159 J/(K·mol)] when Ins(1,4,5)P_3 binds to the receptor. The different extent or nature of the conformational change in the receptor in the two cases is its plausible source. It also supports the earlier proposition made from the trend in enthalpy change.

Acrylamide-quenching probe for conformational change in InsP_3R as a result of binding to InsP_3

The results from the acrylamide quenching studies are shown in the Figure 4. The Stern–Volmer plots show an upward curvature that is characteristic of static quenching [11]. It might occur because the fluorophoric tryptophan residues of the receptor are easily accessible to the quencher. The addition of InsP_3 does not change this trend in static quenching; however, an alteration in K_{sv} results, suggesting that an alteration in the conformation of the receptor occurs without a radical change in the environment of the tryptophan residues. As a second but remote possibility, it could be that K_{sv} increases because bound InsP_3 hinders the access of acrylamide to the tryptophan residues.

Conformational probe for free and InsP_3-bound InsP_3R by CD spectroscopy

Far-UV CD spectra of InsP_3R, alone and in the presence of two InsP_3 isomers, are shown in Figure 5. The spectrum of free InsP_3R indicates the presence of ordered α-helix and β-sheet structures from the characteristic bands at 207, 215 and 222 nm [14]. However, it is different from the normal spectrum typical of a model α-helix or β-sheet conformation, possibly because of the transmembrane nature of the protein. Also, the presence of Triton X-100 in the buffer prevented the recording of spectra below 205 nm. Therefore we did not attempt to estimate the relative percentages of the different conformations and confined ourselves to a comparison of the spectrum of free and bound InsP_3R. Our suggestion of helical structure in free InsP_3R is consistent with the prediction of the presence of transmembrane helical domains in the InsP_3R reported from rat liver [2]. Binding of either isomer [Ins(1,4,5)P_3 or Ins(2,4,5)P_3] alters the CD spectrum of InsP_3R. The helical domain, mostly consisting of hydrophobic residues, undergoes a conformational change leading to an alteration in the far-UV CD spectrum. The nature of the alteration in the presence of Ins(1,4,5)P_3 is not comparable with that of Ins(2,4,5)P_3. Addition of Ins(2,4,5)P_3 to InsP_3R leads to a decrease in the ellipticity without any significant change in the spectral shape. In contrast, the spectrum of InsP_3R shows a

change in the line shape in the 205–230 nm region in the presence of Ins(1,4,5)P_3, which implies that the nature of the conformational changes in InsP_3R induced by Ins(1,4,5)P_3 and Ins(2,4,5)P_3 are different. In accordance with the results from the fluorescence and filter-binding assays, 5 μM Ins(1,3,4)P_3 or Ins(1,5,6)P_3 do not induce any change in the CD spectrum of the receptor (results not shown). It could be suggested from CD data that the conformational change might involve a membrane-spanning helical domain in InsP_3R, as required for its property as a Ca^{2+} channel. The difference in the extent and nature of the conformational change of the receptor in the presence of, particularly, the two isomers Ins(1,4,5)P_3 and Ins(2,4,5)P_3 are clearly indicated from a comparison of the far-UV CD spectra, K_{sv} values and relative values of ΔH and ΔS characterizing their associations with InsP_3R. We reasoned that this difference might be the origin of the trend reflected in the relative abilities of the isomers to release Ca^{2+} from the liposomes and microsomes/vacuoles.

In an exhaustive study [6] the efficiency of different *myo*-inositol phosphates to elicit Ca^{2+} release in *Xenopus* oocytes was shown to follow a rank order as given: Ins(1,4,5)P_3 > Ins(2,4,5)P_3 > Ins(1,2,4,5)P_4 > Ins(4,5)P_2 > Ins(1,4,6)P_3 > Ins(1,2,4,6)P_4 $\geqslant$ other InsP. This rank order does not necessarily follow that observed in their binding abilities to InsP_3R. The present findings support this observation and indicate that it is not only the binding affinity of InsP_3 for the receptor but the overall interaction leading to the requisite conformational change in the receptor that determines the potency of the isomers of InsP_3 in their ability to release Ca^{2+} from the intracellular stores. It does, however, predict that if the interaction of Ins(1,3,4)P_3/Ins(1,5,6)P_3 with the receptor is promoted by any means, the potentiation of its Ca^{2+} mobilization from intracellular stores is possible. It was demonstrated recently in our laboratory that Ins(1,3,4)P_3, when complexed with the phytase enzyme, can interact with the receptor, eliciting Ca^{2+} efflux from the intracellular stores [15].

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