Preparation and characterization of a D-*myo*-inositol 1,4,5-trisphosphate-specific antibody

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Antibodies against Ins(1,4,5) P_3 were raised by immunizing rabbits with two types of Ins P_3 -BSA conjugates which were synthesized by covalently coupling Ins(1,4,5) P_3 to the carrier protein via alkyl linkages. The anti-Ins(1,4,5) P_3 antibody was detected by a novel ELISA using Ins(1,4,5) P_3 -immobilized microtitre plates. Both antiserum preparations showed specific binding with Ins(1,4,5) P_3 , with titres of 1:4000. Most inositol phosphates, including Ins1 P , Ins(4,5) P_2 , Ins(1,3,4) P_3 , Ins(1,5,6) P_3 , Ins(1,2,5,6) P_4 , Ins(3,4,5,6) P_4 , Ins(1,3,4,5,6) P_5 , Ins P_6 , and PtdIns(4,5) P_2 , did not exhibit significant molecular interactions with the antibodies. Ins(1,3,4,5) P_4 , however, cross-reacted with these antibodies with one-third of the affinity as that

of Ins(1,4,5) P_3 , in part due to the largely shared structural motifs. The differential affinity was significantly improved by affinity purification on Ins(1,4,5) P_3 -agarose. The affinity-purified antibody displayed IC_{50} values of 12 nM and 730 nM for Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 respectively, according to a competitive ELISA; these values are in line with those reported for the Ins(1,4,5) P_3 receptor. The modes of ligand recognition at the binding sites of these two types of biomolecules are, however, different. Moreover, although the ligand binding was interfered with by multivalent anions such as ATP^{4-} , HPO_4^{3-} and SO_4^{2-} at high concentrations, no inhibition was noted with heparin, an antagonist of the Ins(1,4,5) P_3 receptor.

INTRODUCTION

The crucial role of D-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5) P_3] as a second messenger acting by initiating intracellular Ca^{2+} mobilization is well recognized [1–3]. In the cytosol, Ins(1,4,5) P_3 and its metabolites undergo rapid metabolism by sequential actions of phosphatases and kinases, generating a plethora of endogenous inositol phosphates [4]. Although the intracellular distribution and biological roles of most of these congeners remain unclear, the rich diversity of phosphoinositols generated from such a complex metabolic network implies the physiological relevance of these compounds. A body of evidence indicates that some of these inositol phosphate metabolites are capable of eliciting Ca^{2+} release by interacting with the Ins(1,4,5) P_3 receptor, albeit at lower potencies [5–9]. These include Ins(1,3,4,6) P_4 , Ins(1,3,4,5) P_4 , Ins(1,4,5,6) P_4 and Ins(4,5) P_2 . One school of thought is that these inositol phosphates exert a concerted effort to maintain Ca^{2+} oscillations in the course of signal transduction. Alternatively, one may argue that the Ca^{2+} -mobilizing activity of these polyphosphates is coincidental, due to the sharing of common structural motifs. Evidently, these two theories bear vastly different implications in the context of intracellular Ca^{2+} signalling and warrant close examination.

To address this issue, one of our strategies is to develop specific antibodies against inositol phosphates, particularly Ins(1,4,5) P_3 as biochemical probes. In the literature, antibodies to PtdIns and PtdIns(4,5) P_2 have been prepared through immunizing animals with the corresponding phospholipids [10] or with liposomes

containing these compounds [11–14], and have shown utility in probing signal-transducing mechanisms. These techniques, however, cannot be applied to inducing antibodies against Ins(1,4,5) P_3 due to the difference in physical nature between inositol lipids and phosphoinositols. Another impediment to the antibody preparation is the difficulties associated with the synthesis of chiral analogues of Ins P_3 . Consequently, in the literature, reports on the preparation of Ins(1,4,5) P_3 -specific antibodies are still lacking. In this account, we report the successful use of two types of Ins(1,4,5) P_3 -protein conjugates as antigens to induce anti-Ins(1,4,5) P_3 serum in rabbits. Moreover, an affinity matrix for antibody purification and a unique ELISA system, developed in the course of this investigation, are described.

MATERIALS AND METHODS

Materials

6-*O*-(ω -Aminoethyl)-D-*myo*-inositol-1,4,5-trisphosphate [(–)-1] and 6-*O*-(5',6'-dihydroxyethyl)-2:3-*O*-cyclohexylidene-D-*myo*-inositol-1,4,5-trisphosphate [(–)-2] were used for the preparation of Ins P_3 -C₆NC₅-BSA and Ins P_3 -C₅-BSA, respectively (Figure 1), of which the synthesis has been reported elsewhere [15]. Ins(1,4,5) P_3 , Ins(1,3,4) P_3 , Ins(1,3,4,5) P_4 , Ins(4,5) P_2 , Ins(1,5,6) P_3 , Ins(1,2,5,6) P_4 and Ins(3,4,5,6) P_4 were synthesized from optically active 1,2:5,6-di-*O*-cyclohexylidene-inositol (optical purity 98 % enantiomeric excess) according to previously described procedures [8,16]. The chemical purity of these chiral inositol

Abbreviations used: Ins(1,4,5) P_3 , D-*myo*-inositol 1,4,5-trisphosphate; other inositol phosphates are abbreviated accordingly, with the positional isomerism of phosphoesters in parentheses and with the number of phosphate groups in subscript behind P ; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; (–)-1, 6-*O*-(ω -aminoethyl)-D-*myo*-inositol-1,4,5-trisphosphate; (–)-2, 6-*O*-(5',6'-dihydroxyethyl)-2:3-*O*-cyclohexylidene-D-*myo*-inositol-1,4,5-trisphosphate.

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Figure 1 Preparation of InsP₃-C₆NC₅-BSA and InsP₃-C₅-BSA from (–)-1 and (–)-2

phosphates was greater than 98% according to ¹H- and ³¹P-NMR spectroscopy. The amounts of isomeric impurities were not detectable, as indicated by these NMR spectra. Phytic acid, PtdIns(4,5)P₂, and Ins1P were purchased from Sigma. Other chemicals and biochemicals were supplied from Sigma or Aldrich unless otherwise mentioned.

Preparation of Ins(1,4,5)P₃-agarose

Ins(1,4,5)P₃-agarose was prepared by reacting (–)-1 with 1,1'-carbonyldi-imidazole-activated 6% cross-linked beaded agarose (Reacti-Gel; Pierce) according to a protocol recommended by Pierce [17]. In brief, activated Reacti-Gel (6 ml) was quickly washed with ice-cold distilled water and was added to 15 ml of 10 mM borate buffer, pH 9.5, containing the InsP₃ ligand (–)-1 (28 mg). The suspension was incubated at room temperature with gentle shaking for 25 h. The reaction was terminated by adding 10 ml of 1 M Tris/HCl, pH 8.0, to the reaction mixture. The gel was recovered by filtration, thoroughly washed, and stored in 10 mM Tris/HCl, pH 7.5, containing 0.1% NaN₃ at 4 °C.

Preparation of InsP₃-BSA conjugates and immunization

InsP₃-C₆NC₅-BSA (Figure 1a)

Glutaraldehyde (10%; 4 ml) was added dropwise to a solution of BSA (20 mg) in 10 mM NaHCO₃/Na₂CO₃ buffer, pH 9.5. The mixture was stirred at 5 °C for 40 min, and dialysed against the same buffer overnight with at least three buffer changes. (–)-1 (20 mg) was then added, and the mixture was incubated at 15 °C. After 3 h, NaBH₄ (100 mg) was added, and the incubation continued at 15 °C for an additional 3 h. The solution was dialysed against 10 mM NaHCO₃/CO₂ buffer, pH 7.0, overnight, and lyophilized to afford InsP₃-C₆NC₅-BSA.

InsP₃-C₅-BSA (Figure 1b)

NaIO₄ (12 mg, 0.06 mmol), dissolved in water (1 ml), was added in several portions to a solution of (–)-2 (50 mg, 0.06 mmol) in distilled water (1 ml), pH 7.5, at 0 °C. The mixture was stirred at 20 °C for 2.5 h, and 1 M HCl (100 μl) was added. After stirring for an additional 2 h, the solution was extracted with diethyl ether to remove cyclohexanone, adjusted to pH 8 with NaHCO₃, and lyophilized to afford 6-O-(4'-formylbutyl)-D-myoinositol-1,4,5-trisphosphate (3). The aldehyde intermediate, without purification, was incubated with BSA (20 mg) in 4 ml of 10 mM NaHCO₃/Na₂CO₃ buffer, pH 9.5, at 5 °C for 2 h. NaBH₄ (20 mg)

was then added and the incubation continued at room temperature for an additional 2 h. The reaction mixture was dialysed against 15 mM NaHCO₃/CO₂ buffer, pH 7.4, overnight, and lyophilized to give InsP₃-C₅-BSA.

New Zealand White female rabbits were immunized with 1 mg of the InsP₃-BSA conjugate in 0.5 ml of saline emulsified with an equal volume of Freund's complete adjuvant by multiple subcutaneous injections into the back and both flanks. Booster injections were given, at monthly intervals, with the same amounts of the InsP₃-BSA prepared with incomplete Freund's adjuvant. After the second boost, the animals were bled from the ear vein 2 weeks after each booster injection. The antisera thus prepared were stored at –20 °C until required for use.

ELISA

The presence of anti-Ins(1,4,5)P₃ antibodies in rabbit serum was detected by an ELISA. Ins(1,4,5)P₃ was covalently attached to microtitre plates through a C-6 linker by adding (–)-1 (1.25 μg in 0.1 ml of PBS per well) to maleic anhydride-activated polystyrene plates (Pierce). This anhydride-activated plate allows direct coupling of amine-bearing molecules to the well surface [18], and obviates the need for a carrier protein to immobilize the hapten. After overnight incubation at room temperature, the plate was decanted, and each well was blocked by adding 0.12 ml of the SuperBlock blocking solution prepared in PBS (Pierce). After 10 min at room temperature, the plate was washed twice with TBS (0.05% Tween-20 in 10 mM Tris/HCl containing 0.85% NaCl, pH 7.2), followed by two further washes in TBS buffer. Rabbit antiserum diluted in TBS buffer (200–300-fold) containing 0.1% gelatin was added (95 μl per well). The plate was incubated at 37 °C for 2 h, and washed as stated above. Specific antibody binding was assessed by adding goat anti-(rabbit IgG)-horseradish peroxidase conjugate (1/200 dilution in TBS containing 0.1% gelatin; 90 μl per well), and the plate was incubated for another 2 h at 37 °C. After the plate was washed again, the peroxidase substrate solution (90 μl per well) containing 2 mM 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid), 2.5 mM hydrogen peroxide and 50 mM citrate buffer, with a final pH of 5.0, was added. The reaction was incubated at room temperature for 15–20 min and terminated by adding 20 μl of 5% SDS to each well. The absorbance at 415 nm was measured by a microtitre plate reader. Rabbit serum taken before immunization was used as a control in all the assays, from which the absorbance values obtained served as a blank for the correction of experimental data. For the competitive ELISA, the diluted antiserum was incubated with different amounts of

inositol phosphates at room temperature for 30 min with gentle shaking. The mixtures (95 μ l) were added to the plate, and the assay was carried out by following the aforementioned procedures.

Purification of Ins(1,4,5) P_3 -specific antibody

Protein A column chromatography (step 1)

The antiserum (5.2 ml) was applied to an Econo-Pac Protein A cartridge (5 ml; Bio-Rad) equilibrated with 100 mM Tris/HCl, pH 8.0. The column was washed with, in tandem, 20 ml of the equilibrating buffer and 25 ml of 10 mM Tris/HCl, pH 8.0. The bound IgG was eluted with 25 ml of 100 mM glycine buffer, pH 3.0, at a flow rate of 0.5 ml/min. Fractions of 1.7 ml were collected in tubes containing 100 μ l of 1 M Tris/HCl, pH 8.0. Fractions 36–38, exhibiting anti-Ins(1,4,5) P_3 antibody activity, were collected, concentrated by ultrafiltration and dialysed against 10 mM Tris/HCl, pH 7.5.

Ins(1,4,5) P_3 -agarose chromatography (step 2)

The dialysed solution from step 1 was applied to a Ins(1,4,5) P_3 -agarose column (5.8 ml bed volume) equilibrated with 10 mM Tris/HCl, pH 7.5. The column was washed with the equilibration buffer followed by 15 ml of 500 mM NaCl in the same buffer. The absorbed proteins were eluted with, in sequence, 30 ml of 100 mM glycine buffer, pH 3.5, 15 ml of 10 mM Tris/HCl, pH 8.8, and 35 ml of 100 mM NaHCO₃/Na₂CO₃ buffer, pH 10.5. Fractions of 1.7 ml were collected. For the eluate with the glycine buffer or with the NaHCO₃/Na₂CO₃ buffer, fractions were collected in tubes containing 100 μ l of 1 M Tris/HCl, pH 8.0, and 700 μ l of 1 M Tris/HCl, pH 7.6, respectively. Fractions 66–69, exhibiting anti-Ins(1,4,5) P_3 antibody activity, were pooled, concentrated and dialysed against 10 mM Tris/HCl, pH 7.5. This solution, with appropriate dilutions (50–100-fold), was used directly for the ELISA.

RESULTS

Preparation of antigens

Design of the Ins(1,4,5) P_3 analogues, (–)-1 and (–)-2, as haptens was based on two considerations: (a) the strategic importance of the C-2,3-*cis*-dihydroxyl groups, especially the axial 2-OH, in recognizing the microenvironments surrounding the phosphate functions, and (b) the potential steric effect of the linker on epitope recognition. The amine-bearing derivative (–)-1 was cross-linked to BSA using glutaraldehyde as a coupling agent, followed by *in situ* NaBH₄ reduction, to afford Ins P_3 -C₆NC₅-BSA. The vicinal diol (–)-2 was subjected to sodium periodate oxidation to yield the aldehyde intermediate, which, without purification, was coupled to the amino functions of the carrier protein. *In situ* NaBH₄ reduction of the Schiff base yielded Ins P_3 -C₅-BSA. The phosphorus contents of Ins P_3 -C₆NC₅-BSA and Ins P_3 -C₅-BSA, determined by elemental analysis, were 2.9% and 0.67% respectively. Accordingly, the molar ratios of the bound Ins(1,4,5) P_3 to BSA were estimated to be 22 and 5 respectively. The lengths of the linkers for Ins P_3 -C₆NC₅-BSA and Ins P_3 -C₅-BSA were 19.7 Å and 9.0 Å respectively. Presumably, in the former conjugate the carrier protein imposed less steric impact on the Ins P_3 moiety due to the longer linker.

Anti-Ins(1,4,5) P_3 sera

Three New Zealand rabbits were immunized with Ins P_3 -C₆NC₅-

BSA, and a fourth one was injected with Ins P_3 -C₅-BSA. The antibodies in the rabbit sera were detected by an ELISA where Ins(1,4,5) P_3 was covalently attached to the well surface through a C-6 linkage by reacting (–)-1 with maleic anhydride-activated polystyrene plates. This antibody capture immunoassay provided a straightforward and consistent analysis of the antisera, and obviated the use of radioactive material and tedious receptor preparation associated with the radioligand-binding assay. Moreover, this strategy avoided interference caused by non-specific antibodies. For instance, analyses using conventional EIA plates coated with Ins(1,4,5) P_3 -C₆NC₅-casein were interfered with by concurrent binding of the C₆NC₅-linker-directed antibodies to the exposed spacer between the carrier protein and Ins(1,4,5) P_3 . Consequently, this conventional assay failed to respond to competitive inhibition by free Ins(1,4,5) P_3 in a quantitative manner.

After 2–3 sets of booster injections, one of the rabbits immunized with Ins P_3 -C₆NC₅-BSA and the rabbit receiving Ins P_3 -C₅-BSA were found to produce antibodies against Ins(1,4,5) P_3 , both of which showed similar titres of about 1:4000. This antibody formation seemed to be independent of the spacer length and Ins P_3 content. The antibody titres increased only moderately in both rabbits after subsequent booster injections. It appeared that the remaining two rabbits that received Ins P_3 -C₆NC₅-BSA immunization generated antibodies predominantly directed against the C₆NC₅-linker.

The avidity and specificity of these antisera were examined by a competitive ELISA between immobilized Ins(1,4,5) P_3 and various inositol phosphates. As indicated from the concentrations at half-maximal absorbance ($B/B_0 = 0.5$) or IC₅₀ (Table 1), for the Ins P_3 -C₆NC₅-BSA-induced antiserum, the affinity toward various inositol phosphates was in the order: Ins(1,4,5) P_3 > Ins(1,3,4,5) P_4 $\geq$ Ins(1,3,4) P_3 > Ins(3,4,5,6) P_4 > Ins(1,5,6) P_3 , PtdIns(4,5) P_2 , Ins(1,2,5,6) P_4 > Ins(4,5) P_2 > Ins P_6 > Ins1 P .

The recognition of inositol phosphates by the Ins P_3 -C₅-BSA-induced antiserum qualitatively paralleled that aforementioned (Table 1). However, the latter antibodies exhibited higher affinity for Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 and a 10-fold decrease in affinity with Ins(1,3,4) P_3 .

Although both antisera were highly specific for Ins(1,4,5) P_3 , they also cross-reacted with Ins(1,3,4,5) P_4 . The ratios of IC₅₀ [$^{[Ins(1,3,4,5)P_4]}$] to IC₅₀ [$^{[Ins(1,4,5)P_3]}$] were 2.2 and 2.3 for the antisera against Ins P_3 -C₆NC₅-BSA and Ins P_3 -C₅-BSA respectively.

Table 1 Avidity of antisera towards various *D*-myo-inositol phosphates and PtdIns P_2

The avidity is expressed as the concentrations at half-maximal absorbance ($B/B_0 = 0.5$) in the competitive ELISA experiments. Abbreviation: N.D., not determined.

Competitor	[Competitor] (M) at half-maximal absorbance	
	Ins P_3 -C ₆ NC ₅ -BSA-induced antibodies	Ins P_3 -C ₅ -BSA-induced antibodies
Ins(1,4,5) P_3	8.9×10^{-8}	3.1×10^{-8}
Ins(1,3,4,5) P_4	2.0×10^{-7}	7.1×10^{-8}
Ins(1,3,4) P_3	1.7×10^{-6}	1.0×10^{-5}
Ins(3,4,5,6) P_4	4.5×10^{-6}	6.3×10^{-6}
PtdIns P_2	1.6×10^{-5}	3.2×10^{-5}
Ins(1,2,5,6) P_4	2.1×10^{-5}	7.1×10^{-5}
Ins(4,5) P_2	3.8×10^{-5}	2.5×10^{-5}
Ins P_6	1.5×10^{-4}	8.0×10^{-4}
Ins1 P	4.2×10^{-4}	N.D.

Figure 2 Affinity purification of Ins(1,4,5) P_3 -specific antibodies on (a) immobilized Protein A and (b) Ins(1,4,5) P_3 -agarose

(a) The Ins P_3 -C₅-BSA-induced antiserum (5.2 ml) was applied to an Econo-Pac Protein A cartridge (5 ml; Bio-Rad) equilibrated with 100 mM Tris/HCl buffer, pH 8.0. The column was washed, in sequence, with (A) 20 ml of the equilibrating buffer; (B) 25 ml of 10 mM Tris buffer, pH 8.0; and (C) 25 ml of 100 mM glycine buffer, pH 3.0, at a flow rate of 0.5 ml/min. Fractions of 1.7 ml were collected in tubes containing 100 μ l of 1 M Tris/HCl buffer, pH 8.0. Fractions 36–38, exhibiting anti-Ins(1,4,5) P_3 antibody activity, were pooled, concentrated, and dialysed against 10 mM Tris buffer, pH 7.5. (b) The dialysed solution was applied to a Ins(1,4,5) P_3 -agarose column (5.8 ml bed volume) equilibrated with 10 mM Tris buffer, pH 7.5. The column was washed with the equilibrating buffer followed by, in sequence, (A) 15 ml of 500 mM NaCl in the same buffer; (B) 30 ml of 100 mM glycine buffer, pH 3.5; (C) 15 ml of 10 mM Tris buffer, pH 8.8; and (D) 35 ml of 100 mM NaHCO₃/Na₂CO₃ buffer, pH 10.5. Fractions of 1.7 ml were collected in tubes containing 100 μ l of 1 M Tris/HCl buffer, pH 8.0. Fractions 66–69, exhibiting anti-Ins(1,4,5) P_3 antibody activity, were pooled, concentrated, and dialysed against 10 mM Tris buffer, pH 7.5.

Purification of the Ins(1,4,5) P_3 -specific antibody

The level of discrimination between Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 by these antisera appeared to be lower than that reported for Ins(1,4,5) P_3 receptors [8,19]. The lack of specificity could be attributed to the heterogeneity in the antigen-binding sites, which prompted us to subject the Ins P_3 -C₅-BSA-induced antiserum to chromatographic purification on immobilized Protein A and Ins(1,4,5) P_3 -agarose (Figures 2a and 2b). The utility of the Ins P_3 affinity column is especially noteworthy. The Ins(1,4,5) P_3 -specific antibody showed strong binding with the affinity matrix, and could only be eluted under alkaline conditions.

Figure 3 Inositol phosphate specificity of the affinity-purified antibodies

The displacement curves were generated from competitive ELISA experiments between immobilized Ins(1,4,5) P_3 and various inositol phosphates shown in the key. The antibodies were preincubated with individual competitors at various concentrations for 30 min at room temperature before they were added to the Ins(1,4,5) P_3 -immobilized EIA plates. The assay for the competitive binding was the same as that described for the ELISA in the Materials and methods section. Percentage total specific binding is expressed as B/B_0 , where B = absorbance with competitor, and B_0 = absorbance without competitor. Each data point represents the mean of three determinations after background correction. For clarity, the standard deviations are not shown.

These affinity-purified antibodies exhibited much improved selectivity between Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , with IC_{50} values of 12 nM and 730 nM respectively (Figure 3). The differential affinity, as indicated by the ratios of IC_{50} values, increased from 2- to 60-fold after purification.

IC_{50} values for other inositol phosphates were: Ins(4,5) P_2 , 14 μ M; PtdIns P_2 , 16 μ M; Ins(1,3,4) P_3 , 39 μ M; Ins(1,3,4,5,6) P_5 , 45 μ M; these were three orders of magnitude higher than that of Ins(1,4,5) P_3 (Figure 3). Moreover, except PtdIns P_2 , the range of concentrations for complete displacement of immobilized Ins(1,4,5) P_3 binding was approximately 1 log unit, which is a good indication of specific binding. The aberrant behaviour of PtdIns P_2 may be caused by the 'sticky' nature of the phospholipid.

Non-specific inhibition of Ins(1,4,5) P_3 -antibody interactions by multivalent ions

In view of the electrostatic nature of Ins(1,4,5) P_3 -antibody recognition, the interaction might be interfered with by anions that competitively bound to the positively charged binding domain of the antibodies in a non-specific manner. Figure 4 shows that the binding between the affinity-purified antibodies and the immobilized Ins(1,4,5) P_3 was inhibited by a number of polyanionic substances at concentrations in the millimolar range (Figure 4). The IC_{50} values for individual inhibitors were: ATP⁴⁻, 0.21 mM; HPO₄²⁻, 2.61 mM; SO₄²⁻, 11.6 mM.

However, no appreciable inhibition was noted with heparin up to 0.3 mM. Monovalent anions such as N₃⁻ and Cl⁻ or cations such as K⁺, Na⁺, NH₄⁺ and Tris at concentrations up to 100 mM did not cause significant inhibition to the antibody binding.

Figure 4 Inhibition of antibody binding to immobilized Ins(1,4,5) P_3 by various multivalent ions

The affinity-purified antibodies were preincubated with individual multivalent ions at the indicated concentrations for 30 min at room temperature before they were added to the Ins(1,4,5) P_3 -immobilized EIA plates. The counter ion for all the anions tested was Na^+ , which showed no inhibiting effect on the binding. The assay for the binding inhibition was the same as that described for the ELISA in the Materials and methods section. Percentage maximal antibody binding is expressed by B/B_0 , where B = absorbance with inhibitor and B_0 = absorbance without inhibitor. Each data point represents the means of three determinations after background correction.

DISCUSSION

We here report the first preparation of highly specific anti-Ins(1,4,5) P_3 antibody through immunizing rabbits with covalent Ins(1,4,5) P_3 -BSA conjugates, followed by affinity purification. Both Ins P_3 - C_6 NC $_5$ -BSA and Ins P_3 - C_5 -BSA were able to generate antibodies that displayed discriminative affinity for Ins(1,4,5) P_3 . Most inositol phosphates, including Ins1 P , Ins(4,5) P_2 , Ins(1,3,4) P_3 , Ins(1,5,6) P_3 , Ins(1,2,5,6) P_4 , Ins P_6 , Ins(3,4,5,6) P_4 and PtdIns P_2 , failed to effect sufficient molecular interactions with the antibodies. The differential binding affinity ranged from two to four orders of magnitude. These antisera, however, cross-reacted with Ins(1,3,4,5) P_4 with affinity in the same order of magnitude as that of Ins(1,4,5) P_3 , which might be accounted for by the largely shared structural motifs between these two polyphosphates.

The problem of cross-reactivity could be overcome by affinity purification on Ins(1,4,5) P_3 -agarose. This affinity matrix is different from other types of Ins(1,4,5) P_3 -based affinity absorbents reported in the literature [20,21]. The three phosphate functions of the immobilized Ins(1,4,5) P_3 are freely exposed, enabling optimal interaction with the binding proteins to occur. As a result, the degree of discrimination among various inositol phosphates by the affinity-purified antibody (Figure 3) was comparable with that of the Ins(1,4,5) P_3 receptor. For example, in the competitive ELISA assay, the IC_{50} value for Ins(1,3,4,5) P_4 was 60-fold higher than that of Ins(1,4,5) P_3 , which is similar to that reported for the receptor binding [8,19]. However, it is worthy of note that the modes of ligand recognition at the binding sites of these two types of biomolecules are somewhat different.

Since the ligand binding arises from complementary interactions involving ion pairing and hydrogen bonding between the antigen-binding domain and the hapten, the binding could be disrupted by a number of unrelated multivalent anions such as ATP^{4-} , HPO_4^{2-} or SO_4^{2-} at high concentrations, whereas no significant inhibition was observed with monovalent ions. As indicated from the concentration spans for complete inhibition (Figure 4), these multivalent anions bound to and neutralized the charges of basic amino acid residues inside the Ins P_3 binding domain in a non-specific manner. This inhibition is noteworthy because use of any of these anions during the antibody preparation or immunoassay would lead to loss of the antibody activity. In our study, attempts to enrich the antibodies using $(NH_4)_2SO_4$ precipitation resulted in a complete loss of binding activity even after extensive dialysis. In the literature, inhibition of ligand binding by multivalent anions has also been reported for Ins(1,4,5) P_3 receptors. The antagonistic effects of heparin, decavanadate, nucleotides and phosphate at the Ins(1,4,5) P_3 binding site is well understood [22–27]. However, unlike Ins(1,4,5) P_3 receptors, the binding of the ligand to the antibody was not affected by heparin up to 0.3 mM. Moreover, cations such as Tris, K^+ , Na^+ , etc. did not cause any inhibition.

A technical point worth mentioning concerns the ELISA developed in this study. In our initial experiments, Ins P_3 - C_6 NC $_5$ -casein, prepared in a manner similar to that described for the BSA counterpart, was coated to regular microtitre plates through adsorption. These plates failed to capture anti-Ins P_3 antibodies, and did not respond to competitive binding experiments by free Ins P_3 in a concentration-dependent fashion. This lack of specific binding may be attributed to (a) the presence of antibodies directed against the C_6 NC $_5$ -linker, and/or (b) the lack of adsorption of Ins P_3 - C_6 NC $_5$ -casein to the polystyrene surface due to the high charge density of the Ins P_3 moiety. As a result, the Ins P_3 -immobilized microtitre plates were used to circumvent these interferences.

The utility of the anti-Ins(1,4,5) P_3 antibody is 2-fold. First, the ELISA developed here provides a sensitive, quantitative analysis of Ins(1,4,5) P_3 along with many other advantages compared with the radioligand-binding assay. The detection limit of this assay was in the range of picograms. Secondly, antibodies directed against phosphatidylinositol and PtdIns P_2 have been applied to probing the intracellular transduction mechanism in various types of cells involving PtdIns P_2 as a second-messenger precursor [10,13,28,29]. This anti-Ins(1,4,5) P_3 antibody will add a new line of biological probes for examining the actions of Ins(1,4,5) P_3 and its metabolites. Examinations of these potential applications with the anti-Ins(1,4,5) P_3 antibody are currently underway in this laboratory.

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