Characterization of inositol 1,4,5-trisphosphate- and inositol 1,3,4,5-tetrakisphosphate-binding sites in rat cerebellum

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1. The properties of specific Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -binding sites have been compared in a crude ' P_2 ' cerebellar membrane fraction. 2. A homogeneous population of [3H]Ins(1,4,5) P_3 -binding sites was present (K_D 23.1 $\pm$ 3.6 nM) at high density (B_{max} 11.9 $\pm$ 1.8 pmol/mg of protein); whereas data obtained for [^{32}P]Ins(1,3,4,5) P_4 specific binding were best fitted to a two-site model, the high-affinity binding component (K_D 2.6 $\pm$ 0.7 nM) constituted 64.2 $\pm$ 4.3 % of the total population and was present at relatively low density (B_{max} 187 $\pm$ 27 fmol/mg of protein). 3. The two high-affinity inositol polyphosphate-binding sites exhibited markedly different pH optima for radioligand binding, allowing the two sites to be independently investigated. At pH 8.0, [3H]Ins(1,4,5) P_3 binding was maximal, whereas [^{32}P]Ins(1,3,4,5) P_4 specific binding was very low; conversely, at pH 5.0, [^{32}P]Ins(1,3,4,5) P_4 binding was maximal, whereas [3H]Ins(1,4,5) P_3 binding was undetectably low. 4. Both inositol polyphosphate-binding sites exhibited marked positional and stereo-specificity. Of the analogues studied, only phosphorothioate substitution to form inositol 1,4,5-trisphosphorothioate was tolerated at the Ins(1,4,5) P_3 -binding site, with only a 2–3-fold loss of binding activity. Addition of a glyceroyl moiety at the 1-phosphate position or addition of further phosphate substituents at the 3- or 6- positions caused dramatic losses in displacing activity. Similarly, complete phosphorothioate substitution of Ins(1,3,4,5) P_4 caused an approx. 6-fold loss of binding activity at the [^{32}P]Ins(1,3,4,5) P_4 -binding site, whereas Ins(1,4,5,6) P_4 , Ins(1,3,4,6) P_4 , Ins(1,4,5) P_3 and Ins(1,3,4,5,6) P_5 were bound at least 100-fold weaker at this site. Therefore, only the phosphorothioate derivatives retained high affinity and selectivity for the two inositol polyphosphate-binding sites. 5. Heparin and pentosan polysulphate were potent but non-selective inhibitors at Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -binding sites. *N*-Desulphation (with or without *N*-reacetylation) of heparin decreased inhibitory activity at the Ins(1,4,5) P_3 -, but not at the Ins(1,3,4,5) P_4 -binding site; however, the selectivity of this effect was only about 4-fold. *O*- and *N*-desulphated *N*-reacetylated heparin was essentially inactive at both sites. 6. The results are discussed with respect to the separate identities of the inositol polyphosphate-binding sites.

INTRODUCTION

The phosphoinositide cascade plays a central role in the transduction of many extracellular stimuli. Interaction with cell-surface receptors causes the activation of phosphoinositidase C to generate Ins(1,4,5) P_3 , which in turn effects Ca^{2+} mobilization from intracellular stores (Berridge & Irvine, 1989; Joseph & Williamson, 1989). Metabolism of Ins(1,4,5) P_3 can proceed to either Ins(1,4) P_2 or Ins(1,3,4,5) P_4 (Irvine *et al.*, 1988). Since the initial discovery of Ins(1,3,4,5) P_4 (Batty *et al.*, 1985), considerable work has been undertaken to ascribe a distinct second-messenger function to this molecule (for review see Irvine, 1990); proposals have included activation of Ca^{2+} entry and modulation of the Ins(1,4,5) P_3 -sensitive Ca^{2+} pool (Irvine & Moor, 1987; Hill *et al.*, 1988; Pittet *et al.*, 1989), and a direct effect on intracellular Ca^{2+} mobilization has also been reported (Parker & Miledi, 1987; Crossley *et al.*, 1988; Joseph *et al.*, 1989a; Ely *et al.*, 1990; Gawler *et al.*, 1990).

Inositol polyphosphate binding sites have been identified in a number of tissues, including microsomal preparations of cerebellum (Willcocks *et al.*, 1987; Worley *et al.*, 1987; Theibert *et al.*, 1987; Donié & Reiser, 1989; Challiss & Nahorski, 1990). The rat cerebellum contains a high density of stereospecific Ins(1,4,5) P_3 -binding sites (Willcocks *et al.*, 1987), and this has allowed the purification (Supattapone *et al.*, 1988) and cloning

(Furiuchi *et al.*, 1989) of the Ins(1,4,5) P_3 -receptor protein. A distinct Ins(1,3,4,5) P_4 -binding site has also been demonstrated in cerebellum by radioligand binding (Donié & Reiser, 1989; Challiss & Nahorski, 1990), and preliminary results were obtained linking this to Ca^{2+} -releasing activity distinct from that induced by Ins(1,4,5) P_3 (Joseph *et al.*, 1989a; Gawler *et al.*, 1990).

Further progress in this active area of research is becoming increasingly dependent on the development of selective pharmacological tools to enable characterization of the distinct and interactive actions of Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -receptor activation. A recent advance has been the synthesis of phosphorothioate analogues of the inositol polyphosphates (Nahorski & Potter, 1989), allowing the investigation of the mechanism and control of Ca^{2+} mobilization from intracellular Ca^{2+} pools in permeabilized cells independently of metabolism (Wojcikiewicz *et al.*, 1990).

In the present study, we have investigated the ability of various inositol polyphosphates and inositol phosphorothioate analogues to interact with distinct Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -binding sites in membrane preparations of rat cerebellum. In addition, in view of the clear reports of the ability of heparin, chemically modified heparins and heparin-like glucosaminoglycans to inhibit Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 binding (Worley *et al.*, 1987; Ghosh *et al.*, 1988; Guillemette *et al.*, 1989; Donié & Reiser,

Abbreviations used: Ins(1,4,5) PS_3 , inositol 1,4,5-trisphosphorothioate; Ins(1,3,4,5) PS_4 , inositol 1,3,4,5-tetrakisphosphorothioate; IC_{50} , concn. of displacing agent causing 50 % displacement of specific binding; GroPIns P_2 , 1- α -glycerophosphoinositol 4,5-bisphosphate.

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1989; Tones *et al.*, 1989; Willcocks *et al.*, 1990), we also evaluate their potential as selective Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -receptor antagonists.

MATERIALS AND METHODS

Materials

[^3H]Ins(1,4,5) P_3 (17–20 Ci/mmol), [^{32}P]Ins(1,3,4,5) P_4 (100–200 Ci/mmol), inositol 1,4,5-trisphosphorothioate [Ins(1,4,5) PS_3 ; Cooke *et al.* (1987a)] and DL-inositol 1,3,4,5-tetrakisphosphorothioate [Ins(1,3,4,5) PS_4] were gifts from NEN–DuPont Biotechnology (Stevenage, Herts., U.K.). Ins(1,4,5) P_3 was obtained from Amersham International. DL-Ins(1,4,5) P_3 , L-Ins(1,4,5) P_3 and DL-Ins(1,4,5,6) P_4 were synthesized as described previously (Cooke *et al.*, 1987b; Gawler *et al.*, 1990). Ins(1,3,4,6) P_4 was kindly provided by Dr. R. Gigg (N.I.M.R., London N.W.7, U.K.). L- α -Glycerophosphoinositol 4,5-bisphosphate (GroPIns P_2) and Ins(1,3,4,5,6) P_5 were purchased from Boehringer-Mannheim. Pig (intestinal mucosal) heparin, 'low molecular weight' heparin, ATP, 2,3-bisphosphoglyceric acid, chondroitin sulphate A, phytic acid (Ins P_6) and (pig) *N*-desulphated heparin were obtained from Sigma.

Pentosan polysulphate was generously given by Benechemie G.m.b.H. (Munich, Germany). The *N*-desulphated *N*-reacetylated heparin and *O*- and *N*-desulphated *N*-reacetylated heparin analogues were prepared from bovine lung heparin (2nd International Standard) as described by Mulloy & Johnson (1987).

Membrane preparations for [^3H]Ins(1,4,5) P_3 binding

Rat cerebella were dissected on a chilled plate and homogenized with a Polytron (setting 3, 2 $\times$ 10 s) in 'cytosolic' buffer, consisting of 100 mM-KCl, 20 mM-NaCl, 20 mM-Tris/HCl, 1 mM-EDTA, 1 mg of BSA/ml, pH 7.7. Membranes were harvested by centrifugation (50000 *g*, 20 min 4 °C) and the pellet was resuspended in the same buffer and the centrifugation repeated; the final pellet was diluted to 5 mg of protein/ml and used freshly in the binding assay.

Membrane preparation for [^{32}P]Ins(1,3,4,5) P_4 binding

Rat cerebella were dissected and homogenized as described above in 20 vol. of 20 mM-NaHCO₃/1 mM-dithiothreitol, pH 8.0. After centrifugation (5000 *g*, 10 min, 4 °C), the supernatant was re-centrifuged (38000 *g*, 20 min, 4 °C) and the resultant pellet washed twice by homogenization in the same medium. The final pellet was resuspended at a protein concentration of 7–8 mg/ml in NaHCO₃/dithiothreitol and stored at –20 °C until use.

Binding of [^3H]Ins(1,4,5) P_3 to cerebellar membranes

Binding assays for [^3H]Ins(1,4,5) P_3 were performed in Microfuge tubes in 0.25 ml of 'cytosolic' medium as described for the membrane preparation. A 0.1 ml portion of membrane preparation was incubated with 1 nM-[^3H]Ins(1,4,5) P_3 and various displacing agents for 10 min on ice. Non-specific binding was estimated with 20 μM -DL-Ins(1,4,5) P_3 . After incubation, assays were terminated by rapid centrifugation in a Microfuge (10000 *g*, 4 min), and the pellets were rapidly rinsed with water and solubilized in 0.1 ml of Lumasolve (May and Baker). Solubilized pellets were taken up in 1 ml of scintillation cocktail, and radioactivity was determined.

Binding of [^{32}P]Ins(1,3,4,5) P_4 to cerebellar membranes

Binding assays for [^{32}P]Ins(1,3,4,5) P_4 were performed by a modification (Challiss & Nahorski, 1990) of the method described by Donié & Reiser (1989). Each assay, unless indicated, contained 25 mM-sodium acetate, 25 mM-KH₂PO₄, 1 mM-EDTA, pH 5.0,

0.1 nM-[^{32}P]Ins(1,3,4,5) P_4 (12000–20000 d.p.m.) and various concentrations of competing ligand as indicated in the Results section. Non-specific binding was defined by inclusion of 10 μM -DL-Ins(1,3,4,5) P_4 . Reactions were initiated by addition of cerebellar binding protein (0.2–0.3 mg of protein), and samples were intermittently vortex-mixed and maintained at 4 °C for 30 min. Separation of bound and free [^{32}P]Ins(1,3,4,5) P_4 was achieved by rapid filtration through Whatman GF/B filters and washing with 3 $\times$ 3 ml of 25 mM-sodium acetate/25 mM-KH₂PO₄/5 mM-NaHCO₃/1 mM-EDTA, pH 5.0. Filters were extracted in scintillation cocktail for > 4 h and radioactivity was determined.

Data analysis

Displacement curves were constructed by using at least six concentrations of each agent, with each experiment being performed at least three times. Displacement data, in the form of percentage inhibition of specific binding, were analysed by using the computer-assisted iterative curve-fitting programme ALLFIT (DeLean *et al.*, 1978) to determine IC₅₀ (the concentration of displacing agent causing 50% displacement of specific binding) and slope factor.

RESULTS

Previous studies have demonstrated that optimal [^3H]Ins(1,4,5) P_3 binding to receptors isolated from a range of tissues occurs at pH 7.5–8.5 (Worley *et al.*, 1987; Enyedi *et al.*, 1989; Rossier *et al.*, 1989; Chilvers *et al.*, 1990). This finding is confirmed by the data shown in Fig. 1. At pH < 6, essentially no

Fig. 1. pH-dependence of [^3H]Ins(1,4,5) P_3 and [^{32}P]Ins(1,3,4,5) P_4 specific binding to rat cerebellar membranes

Ins(1,4,5) P_3 binding (●) was determined with 3 nM-[^3H]Ins(1,4,5) P_3 (13000 d.p.m.) and 75 μg of membrane protein in a range of 25 mM-Tris/maleate (pH 6.0–7.5) and 25 mM-Tris/HCl (pH 7.5–8.5) buffers containing 1 mM-EDTA (final vol. 120 μl) incubated on ice for 30 min with separation of bound and free radioligand by centrifugation. Non-specific binding was determined in the presence of 20 μM -DL-Ins(1,4,5) P_3 and was 350–400 d.p.m. for all pH values. Ins(1,3,4,5) P_4 binding (■) was determined with 0.25 nM-[^{32}P]Ins(1,3,4,5) P_4 (21000 d.p.m.) and 250 μg of membrane protein in a range of 25 mM-sodium acetate (pH 4.2–5.7) and 25 mM-Tris/maleate (pH 5.7–7.2) buffers containing 25 mM-KH₂PO₄ and 1 mM-EDTA (final vol. 160 μl) incubated on ice for 30 min with separation of bound and free radioligand by centrifugation. Non-specific binding was determined in the presence of 40 μM -DL-Ins(1,3,4,5) P_4 and was 650–700 d.p.m. for all pH values > 5.2 (see the Results section). Values represent means of triplicate determinations from a single experiment, with similar results obtained in a further two experiments.

Fig. 2. Saturation analysis of [^3H]Ins(1,4,5) P_3 binding to cerebellar membranes

Assays were performed with 3.3 nM-[^3H]Ins(1,4,5) P_3 (15000 d.p.m.), 75 μg of cerebellar ' P_2 ' membrane protein and increasing concentrations of unlabelled Ins(1,4,5) P_3 as described in the Materials and methods section. Non-specific binding, determined in the presence of 20 μM -DL-Ins(1,4,5) P_3 , was 295 ± 12 d.p.m. Saturation analysis of the data is shown in the inset. Values are means of duplicate determinations. K_D and B_{max} values from this experiment and four similar data-sets are given in the Results section.

specific binding of [^3H]Ins(1,4,5) P_3 to cerebellar membranes could be detected. Specific [^3H]Ins(1,4,5) P_3 binding increased dramatically as the assay pH was increased to pH 8. At this pH, specific binding was maximal and represented $92 \pm 3\%$ of total binding.

In contrast, specific [^{32}P]Ins(1,3,4,5) P_4 binding to cerebellar membranes was low at pH > 6, but increased dramatically at more acidic pH (Fig. 1). This finding is in agreement with Enyedi *et al.* (1989), who have demonstrated similar changes in [^{32}P]Ins(1,3,4,5) P_4 specific binding to bovine parathyroid membranes at acidic pH, and Donié & Reiser (1989), who have reported saturable high-affinity [^3H]Ins(1,3,4,5) P_4 binding to pig cerebellar membranes at pH 5. In the present study, maximal specific [^{32}P]Ins(1,3,4,5) P_4 binding was observed at pH 4.7 [although the non-specific binding component, defined in the presence of 10 μM -DL-Ins(1,3,4,5) P_4 , was significantly increased at this and lower pH: as percentage of total binding, at pH 5.2, $24 \pm 5\%$; at pH 4.7, $41 \pm 10\%$], but at pH 4.2 specific binding was markedly decreased, possibly owing to receptor instability at increasingly acidic pH.

Displacement of specifically bound [^3H]Ins(1,4,5) P_3 from cerebellar membranes by unlabelled Ins(1,4,5) P_3 is shown in Fig. 2. Saturation analysis of these data (Fig. 2 inset) demonstrated that Ins(1,4,5) P_3 bound to an apparently homogeneous receptor population with a K_D of 23.1 ± 3.6 nM and a B_{max} of 11.9 ± 1.8 pmol/mg of protein (mean $\pm$ S.E.M., $n = 5$). Displacement of specifically bound [^{32}P]Ins(1,3,4,5) P_4 from cerebellar membranes by unlabelled Ins(1,3,4,5) P_4 is shown in Fig. 3. In

Fig. 3. Saturation analysis of [^{32}P]Ins(1,3,4,5) P_4 binding to cerebellar membranes

Assays were performed with 0.09 nM-[^{32}P]Ins(1,3,4,5) P_4 (8000 d.p.m.), 240 μg of cerebellar ' P_2 ' membrane protein and increasing concentrations of unlabelled Ins(1,3,4,5) P_4 as described in the Materials and methods section. Non-specific binding, determined in the presence of 20 μM -DL-Ins(1,3,4,5) P_4 , was 132 ± 13 d.p.m. Saturation analysis of the data is shown in the inset. Values are means of duplicate determinations. K_D and B_{max} values from this experiment and five similar data-sets were obtained by computer-assisted two-site analysis of the displacement isotherms and are given in the Results section.

contrast with the data reported above for Ins(1,4,5) P_3 , analysis of the Ins(1,3,4,5) P_4 isotopic-dilution data yielded a slope factor (nH) significantly less than unity (0.68 ± 0.10 ; $n = 6$), and saturation analysis (Fig. 3 inset) demonstrated a curvilinear Scatchard transformation. Computer-assisted two-site analysis of isotope-dilution data demonstrated a high-affinity binding component (K_D 2.6 ± 0.7 nM), which constituted $64.2 \pm 4.3\%$ of the sites, and a lower-affinity component (K_D 234 ± 73 nM). The B_{max} of the high-affinity site was 187 ± 27 fmol/mg of protein ($n = 6$).

The abilities of various stereo- and positional inositol polyphosphate isomers and analogues to interact with the Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -binding sites in rat cerebellar membranes were studied. The IC_{50} values of specific [^3H]Ins(1,4,5) P_3 or [^{32}P]Ins(1,3,4,5) P_4 binding are shown in Table 1. As reported previously, the Ins(1,4,5) P_3 -binding site exhibited dramatic stereo-selectivity for the D-isomer of Ins(1,4,5) P_3 (Willcocks *et al.*, 1987, 1990). Addition of the glyceroyl moiety at the 1-position of Ins(1,4,5) P_3 , giving GroPIns P_2 , caused a 30-fold decrease in displacing activity (Table 1). The effects of Ins P_4 and Ins P_5 were also examined. Addition of phosphate groups at the 3- and/or 6-positions of Ins(1,4,5) P_3 [to give Ins(1,3,4,5) P_4 , Ins(1,4,5,6) P_4 and Ins(1,3,4,5,6) P_5] dramatically decreased the binding affinity at the [^3H]Ins(1,4,5) P_3 -binding site by at least 100-fold. Further

Table 1. Inhibition of specific [^3H]Ins(1,4,5) P_3 and [^{32}P]Ins(1,3,4,5) P_4 binding to cerebellar membranes by inositol phosphate analogues and related compounds

Displacement isotherms were constructed by using at least six concentrations of each displacing agent, and curves were analysed by using ALLFIT. IC_{50} values are expressed as means $\pm$ S.E.M. for at least three independent experiments performed in duplicate. N/D, not determined; ^a < 10% displacement at 10 μM ; ^b < 10% displacement at 1 μM ; ^c < 10% displacement at 100 μM .

	IC_{50} (μM)	
	[^3H]Ins(1,4,5) P_3	[^{32}P]Ins(1,3,4,5) P_4
Ins(1,4,5) P_3	0.056 $\pm$ 0.002	19.1 $\pm$ 6.7
L-Ins(1,4,5) P_3	100 $\pm$ 14	N/D
GroPIns P_2	1.83 $\pm$ 0.19	11.6 $\pm$ 1.6
Ins(1,3,4,5) P_4	21.0 $\pm$ 4.4	0.005 $\pm$ 0.001
DL-Ins(1,4,5,6) P_4	35.0 $\pm$ 4.5	0.87 $\pm$ 0.05
Ins(1,3,4,6) P_4	3.44 $\pm$ 0.27	0.80 $\pm$ 0.06
Ins(1,3,4,5,6) P_5	> 10 ^a	1.38 $\pm$ 0.39
Ins P_6	36.0 $\pm$ 1.3	3.62 $\pm$ 0.22
Ins(1,4,5) PS_3	0.13 $\pm$ 0.02	4.45 $\pm$ 0.59
DL-Ins(1,3,4,5) PS_4	> 1 ^b	0.069 $\pm$ 0.014
ATP	790 $\pm$ 67	129 $\pm$ 7
2,3-Bisphosphoglyceric acid	> 100 ^c	97 $\pm$ 6

addition of a 2-phosphate substitution to give Ins P_6 (phytic acid) had no further significant effect on displacing activity (Table 1). Ins(1,3,4,5) P_4 was about 60-fold less potent than Ins(1,4,5) P_3 at causing displacement of specific [^3H]Ins(1,4,5) P_3 binding. Phosphorothioate substitution of Ins(1,4,5) P_3 to yield D-Ins(1,4,5) PS_3 caused only a 2–3-fold decrease in [^3H]Ins(1,4,5) P_3 -displacing activity.

The concentrations of various inositol polyphosphate analogues which cause 50% displacement of specific [^{32}P]Ins(1,3,4,5) P_4 binding to cerebellar membranes are also shown in Table 1. The absolute requirement for the precise arrangement of phosphate substituents of the inositol moiety was even more graphically illustrated at this site; addition of a phosphate group at the 6-position [to give Ins(1,3,4,5,6) P_5] or rearrangement of the phosphate substituents to form DL-Ins(1,4,5,6) P_4 or Ins(1,3,4,6) P_4 resulted in > 100-fold decreases in [^{32}P]Ins(1,3,4,5) P_4 -displacing activity. Similarly, removal of the 3-phosphate caused a > 1000-fold decrease in [^{32}P]Ins(1,3,4,5) P_4 -displacing activity. Total phosphorothioate substitution of Ins(1,3,4,5) P_4 to produce DL-Ins(1,3,4,5) PS_4 caused an approx. 12-fold decrease in displacing activity at the [^{32}P]Ins(1,3,4,5) P_4 -binding site.

Both D-Ins(1,4,5) PS_3 and DL-Ins(1,3,4,5) PS_4 appeared to retain a high degree of selectivity between Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -binding sites (Table 1). For D-Ins(1,4,5) PS_3 the discrimination between sites was 35-fold [compared with 340-fold selectivity of D-Ins(1,4,5) P_3], and, although an IC_{50} value could not be precisely determined for Ins(1,3,4,5) PS_4 displacement of specific [^3H]Ins(1,4,5) P_3 binding, owing to limited availability of the compound, no significant displacement of [^3H]Ins(1,4,5) P_3 binding was evident in the presence of 1 μM -DL-Ins(1,3,4,5) PS_4 [IC_{50} for DL-Ins(1,3,4,5) PS_4 displacement of [^3H]Ins(1,4,5) P_3 in bovine adrenal-cortical membranes was 1.8 $\pm$ 0.2 μM ; R. A. J. Challiss, unpublished work], suggesting at least 100-fold selectivity of DL-Ins(1,3,4,5) PS_4 for the cerebellar Ins(1,3,4,5) P_4 -binding site.

Heparin has been shown to displace specific binding of Ins(1,4,5) P_3 to membranes from a number of tissues (Worley *et al.*, 1987; Supattapone *et al.*, 1988; Guillemette *et al.*, 1989;

Table 2. Inhibition of specific [^3H]Ins(1,4,5) P_3 and [^{32}P]Ins(1,3,4,5) P_4 binding to cerebellar membranes by heparin analogues and related compounds

Displacement isotherms were constructed by using at least six concentrations of each displacing agent, and curves were analysed by using ALLFIT. IC_{50} values are expressed as means $\pm$ S.E.M. for at least three independent experiments performed in duplicate. ^a < 20% displacement at 1000 $\mu\text{g/ml}$; ^b 40% displacement at 1000 $\mu\text{g/ml}$.

	IC_{50} ($\mu\text{g/ml}$)	
	[^3H]Ins(1,4,5) P_3	[^{32}P]Ins(1,3,4,5) P_4
Heparin	17.0 $\pm$ 6.0	31.2 $\pm$ 1.1
'Low-molecular-weight' heparin	10.2 $\pm$ 2.3	19.3 $\pm$ 2.2
N-Desulphated heparin	145 $\pm$ 12	33.7 $\pm$ 2.8
N-Desulphated re-N-acetylated heparin	72.5 $\pm$ 13.2	20.6 $\pm$ 1.8
O- and N-desulphated re-N-acetylated heparin	> 1000 ^a	> 1000 ^a
Pentosan polysulphate	11.3 $\pm$ 1.1	12.6 $\pm$ 0.8
Chondroitin sulphate A	> 1000 ^a	> 1000 ^b

Tones *et al.*, 1989; Chilvers *et al.*, 1990) and to act as a potent antagonist of Ins(1,4,5) P_3 -stimulated Ca^{2+} release from intracellular stores (Guillemette *et al.*, 1989; Joseph & Rice, 1989; Kobayashi *et al.*, 1989; Yamamoto *et al.*, 1990). The IC_{50} values for the effects of heparin, chemically modified heparin and related glycosaminoglycans on [^3H]Ins(1,4,5) P_3 and [^{32}P]Ins(1,3,4,5) P_4 specific binding to cerebellar membranes are shown in Table 2. Both heparin and 'low-molecular-weight' heparin were potent inhibitors at both binding sites. IC_{50} values for 'low-molecular-weight' heparin were approx. 2 and 4 μM for inhibition of Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 binding respectively. Similarly, pentosan polysulphate was equally effective in displacing specific [^3H]Ins(1,4,5) P_3 and [^{32}P]Ins(1,3,4,5) P_4 binding. O- and N-desulphation of heparin dramatically decreased the potency of the glycosaminoglycan at both inositol polyphosphate binding sites. N-Desulphation decreased the activity of this modified heparin at the Ins(1,4,5) P_3 -binding site about 4–8-fold; however, this modification had only a modest effect on the ability of this molecule to inhibit [^{32}P]Ins(1,3,4,5) P_4 binding (Table 2).

DISCUSSION

In the present study, the properties of the cerebellar Ins(1,4,5) P_3 receptor have been compared with those of an Ins(1,3,4,5) P_4 -binding site present in the same crude subcellular-membrane fraction. In common with previous reports by a number of groups (Willcocks *et al.*, 1987; Worley *et al.*, 1987; Joseph & Rice, 1989), the cerebellar membrane preparation contains a high density (B_{max} 11.9 $\pm$ 1.8 pmol/mg of protein) of apparently homogeneous Ins(1,4,5) P_3 receptors (K_D 23.1 $\pm$ 3.6 nM). In contrast, Ins(1,3,4,5) P_4 binding to cerebellar membranes appears to be heterogeneous at pH 5.0, with a high-affinity site (K_D 2.6 $\pm$ 0.7 nM) present at relatively low density (B_{max} 187 $\pm$ 27 fmol/mg of protein) and a second, lower-affinity, site (K_D 234 $\pm$ 73 nM) defined by computer-assisted two-site analysis of the isotopic-dilution data. Similar heterogeneous Ins(1,3,4,5) P_4 binding has been reported previously in membrane preparations of adrenal cortex (Enyedi & Williams, 1988) and parathyroid (Enyedi *et al.*, 1989). From previously reported data on the interaction of Ins(1,3,4,5) P_4 with the 3-kinase and the

5-phosphomonoesterase (Polokoff *et al.*, 1988; Nahorski & Potter, 1989), and data presented by other groups on the identification of Ins(1,3,4,5) P_4 -binding sites in cerebellum and other cell types (Bradford & Irvine, 1987; Theibert *et al.*, 1987; Enyedi & Williams, 1988; Enyedi *et al.*, 1989; Donié & Reiser, 1989; Challiss & Nahorski, 1990), it would seem likely from the specificity of the binding that the high-affinity site represents an Ins(1,3,4,5) P_4 receptor; however, whether the lower-affinity site is a different conformation of the same binding site or is accounted for by Ins(1,3,4,5) P_4 binding to a distinct enzyme/receptor (e.g. a 3-phosphatase activity) is unknown. In this context it is noteworthy that Ins(1,3,4,5) P_4 has been reported to displace specific Ins P_6 binding with an IC_{50} of 5–10 μ M in brain preparations (Hawkins *et al.*, 1990; Nicoletti *et al.*, 1990).

The distinct pH-dependencies of the Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 receptor binding are in broad agreement with previous findings by other groups (Worley *et al.*, 1987; Donié & Reiser, 1989; Enyedi *et al.*, 1989). The pH-dependence is likely to relate to the required ionization state of the phosphate substituents of the ligand and the critical amino acid residues of the binding site within the receptor (Nahorski & Potter, 1989), and clearly these interactive requirements are radically different for stabilization of high-affinity binding to the two inositol polyphosphate receptors. Evidence in support of this has been provided by two studies which have demonstrated that increasing the assay-buffer pH increases the affinity of the cerebellar Ins(1,4,5) P_3 receptor (Bredt *et al.*, 1989; Joseph *et al.*, 1989b). Therefore, it is possible that an ambient pH which leads to the Ins(1,4,5) P_3 molecule carrying a maximal negative charge will result in high-affinity binding to the Ins(1,4,5) P_3 receptor. The acidic pH necessary for demonstration of high-affinity Ins(1,3,4,5) P_4 binding reported here agrees precisely with a number of other studies (Donié & Reiser, 1989; Enyedi *et al.*, 1989; Challiss & Nahorski, 1990). It is possible that the high-affinity conformation of the Ins(1,3,4,5) P_4 -binding site is stabilized at acidic pH, facilitating its observation by radioligand-binding studies, whereas at physiological pH the affinity of the receptor may be in the micromolar range. Thus, other groups have demonstrated specific Ins(1,3,4,5) P_4 binding at neutral and slightly alkaline assay pH, with lower receptor-binding affinities (90–300 nM) being reported (Bradford & Irvine, 1987; Theibert *et al.*, 1987). The pH-dependency of high-affinity binding is maintained for the solubilized Ins(1,3,4,5) P_4 -binding site from rat cerebellum (Donié *et al.*, 1990; Theibert *et al.*, 1990). The radical difference between pH profiles for the inositol polyphosphate-binding sites may alternatively relate to the solution conformation of these second messengers.

Limited information is available at present concerning the conformation(s) adopted by Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 in solution, and there is no information relating to the conformations adopted by these molecules in the bound state. An early study on pH effects on the conformation of Ins P_6 in solution demonstrated that at neutral and alkaline pH Ins P_6 adopts the expected conformation of five equatorial and one axial phosphate groups, whereas at acidic pH the cyclohexane ring 'flips' to give a reverse conformation with one equatorial and five axial phosphate substituents stabilized by intramolecular transannular hydrogen-bonding interactions (Emsley & Niazi, 1981). This unusual conformation may be adopted at acidic pH by other highly charged phosphorylated inositol derivatives, and the likelihood of this occurrence will presumably increase with the increasing degree of phosphorylation. However, a more recent study of pH effects on the solution conformation of Ins(1,4,5) P_3 did not support this idea (Lindon *et al.*, 1987). Therefore, we cannot explain the dramatic difference between the pH profiles of the inositol polyphosphate receptors on the basis

of the predicted physical properties of the ligands. Furthermore, the differences may reside in the protein conformation and ionization of titratable groups within the ligand-binding site of the receptors.

A number of studies have reported data on the stereo- and positional requirements for high-affinity ligand binding to the Ins(1,4,5) P_3 receptor (Willcocks *et al.*, 1987; Worley *et al.*, 1987; Polokoff *et al.*, 1988; Hirata *et al.*, 1989; Challiss *et al.*, 1990) and the correlation between interaction with the Ins(1,4,5) P_3 -binding site and Ca^{2+} release from microsomes (see Joseph & Williamson, 1989), permeabilized cell preparations (Spät *et al.*, 1986; Strupish *et al.*, 1988; Hirata *et al.*, 1989) and the purified Ins(1,4,5) P_3 receptor reconstituted into a lipid bilayer (Ferris *et al.*, 1989). In common with these studies, the present results emphasize the importance of D-4,5-vicinal bisphosphate and the poor tolerance of other phosphate substitutions. Modification of the Ins(1,4,5) P_3 molecule by complete phosphorothioate substitution caused only a 2-fold decrease in receptor binding affinity, in agreement with the reported potency of this compound in Ca^{2+} -mobilization experiments (Strupish *et al.*, 1988; Taylor *et al.*, 1989). Furthermore, substitution of Ins(1,4,5) P_3 or Ins(1,4,5) PS_3 by phosphate or phosphorothioate groups respectively at the 3-position dramatically decreases potency of the Ins P_4 /Ins PS_4 molecules (Table 1). It should be noted that the inositol (poly)-phosphorothioates are intrinsically less stable than the corresponding (poly)phosphates to acidic or oxidizing conditions; inclusion of thiol redox agents such as dithiothreitol or 2-mercaptoethanol is therefore important in optimizing long-term stability [D. F. Moakley & R. Shaw (DuPont de Nemours & Co., Boston, MA, U.S.A.), personal communication]. It is therefore likely that some studies demonstrating that Ins(1,4,5) PS_3 is considerably less potent than Ins(1,4,5) P_3 in competitive binding assays (e.g. Willcocks *et al.*, 1990) are due to the use of Ins(1,4,5) PS_3 where some decomposition of the compound has occurred.

With respect to the structure-activity profile of the Ins(1,3,4,5) P_4 -binding site, only total phosphorothioate substitution to yield Ins(1,3,4,5) PS_4 is tolerated. Ins(1,3,4,5) PS_4 is a racemic mixture containing 50% L-Ins(1,3,4,5) PS_4 , which we can assume with some confidence is substantially less active and does not interfere with the binding of the D-isomer to the receptor. D-Ins(1,3,4,5) PS_4 may be therefore assumed to be only about 7-fold less potent than Ins(1,3,4,5) P_4 at this binding site (Table 1). Recent studies have suggested that the Ins(1,3,4,5) P_4 -binding site may be a Ca^{2+} -mobilizing receptor, releasing Ca^{2+} from a Ca^{2+} pool perhaps distinct from that susceptible to Ins(1,4,5) P_3 (Joseph *et al.*, 1989a; Ely *et al.*, 1990; Gawler *et al.*, 1990). Whether the Ins(1,3,4,5) P_4 receptor is a second-messenger-operated Ca^{2+} channel, as is the Ins(1,4,5) P_3 receptor (Ferris *et al.*, 1989; Furiuchi *et al.*, 1989; Mignery *et al.*, 1990), is not known at present, but recent reports of the solubilization and (partial) purification of the Ins(1,3,4,5) P_4 -binding site (Donié *et al.*, 1990; Theibert *et al.*, 1990) should eventually resolve this important issue. The radioligand-binding data presented here for [^{32}P]Ins(1,3,4,5) P_4 displacement by DL-Ins(1,4,5,6) P_4 and Ins(1,3,4,5,6) P_5 are in good agreement with those of Donié & Reiser (1989) and our data on the Ca^{2+} -releasing activity of these analogues in permeabilized SH-SY5Y neuroblastoma cells (Gawler *et al.*, 1990).

A number of studies have reported the effects of heparin on Ins(1,4,5) P_3 binding (Worley *et al.*, 1987; Guillemette *et al.*, 1989; Willcocks *et al.*, 1990) and inhibition of Ca^{2+} release from microsomal or permeabilized-cell preparations (Ghosh *et al.*, 1988; Guillemette *et al.*, 1989; Joseph & Rice, 1989). Furthermore, there are also some data on structure-activity relationships for chemically modified glucosaminoglycan

derivatives (Ghosh *et al.*, 1988; Tones *et al.*, 1989; Yamamoto *et al.*, 1990). Analysis in these studies of the interaction of various glycosaminoglycans with the $\text{Ins}(1,4,5)\text{P}_3$ - and $\text{Ins}(1,3,4,5)\text{P}_4$ -binding sites proved disappointing, as none of the agents investigated discriminated sufficiently between the binding sites to be pharmacologically useful, although both *N*-desulphated and *N*-desulphated *N*-reacetylated heparin did show an approx. 4–5-fold difference between the two sites. *N*-Desulphation decreases the net negative charge of the heparin molecule, and also exposes positively charged amino groups; *N*-reacetylation removes the free amino groups. A specific role of the *N*-sulphate groups in the interaction with the inositol polyphosphate-binding sites seems unlikely, as the synthetic *O*-sulphated polyxylan, pentosan polysulphate, is an equally effective inhibitor of $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ and $[\text{^32P}]\text{Ins}(1,3,4,5)\text{P}_4$ specific binding.

Contradictory reports have been published about the ability of heparin to inhibit $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} release, with Joseph *et al.* (1989a) demonstrating that heparin was a potent inhibitor in rat cerebellar microsomes, but Ely *et al.* (1990) providing evidence for heparin-insensitive $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} release from adrenal microsomes. Furthermore, the ability of $\text{Ins}(1,3,4,5)\text{P}_4$ to re-sequester Ca^{2+} in 261B rat liver epithelial cells is also insensitive to heparin (Hill *et al.*, 1988). The report by Ely *et al.* (1990) appears to contradict the findings reported for radioligand-binding studies to cerebellum (Doni & Reiser, 1989; Challiss & Nahorski, 1990; the present results) and the fact that the solubilized cerebellar $\text{Ins}(1,3,4,5)\text{P}_4$ -binding protein is retained by heparin-agarose columns (Doni *et al.*, 1990; Theibert *et al.*, 1990). However, it remains possible that central and peripheral $\text{Ins}(1,3,4,5)\text{P}_4$ -binding sites may differ in their ability to recognize heparin. In this respect we have recently reported differences in the apparent affinity of heparin at cerebellar and adrenal-cortical $\text{Ins}(1,4,5)\text{P}_3$ receptors (Willcocks *et al.*, 1990).

Finally, further studies to develop a specific, selective, inhibitor which differentiates between the inositol polyphosphate-binding sites, such as the possibility of defining a low-molecular-mass oligosaccharide unit of heparin or a related compound may prove untenable, as decreases in polysaccharide chain length causes a decrease in activity in $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ radioligand-binding assays (Tones *et al.*, 1989). This suggests that the size of the polysaccharide, as well as the degree of sulphation, is important for the inhibitory action. Therefore, the fact that heparin is not only a potent non-selective inhibitor of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ binding in cerebellum, but also inhibits the 3-kinase enzyme (Guillemette *et al.*, 1989) and other processes important in signal transduction (Willuweit & Aktories, 1988; Knaus *et al.*, 1990), may severely limit the usefulness of this group of agents to dissect inositol polyphosphate second-messenger function.

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