Biochem. J. (1992) 288, 149–154 (Printed in Great Britain)

Inositol 1,3,4,5-tetrakisphosphate binding sites in neuronal and non-neuronal tissues

Properties, comparisons and potential physiological significance

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1. Ins(1,3,4,5)P_4 binding sites were studied in cerebellar and hepatic microsomes from rat, and in bovine adrenal-cortical microsomes. 2. At pH 7.0, all three tissues showed specific binding, with Ins(1,3,4,5)P_4 being the most potent competing ligand of those tested [which included Ins(1,4,5)P_3, Ins(1,3,4,5,6)P_5 and InsP_6] and Scatchard analysis suggested two sites; a site with high affinity and high specificity [K_d (1–6) $\times 10^{-9}$ M] and a site with low affinity and low specificity [K_d (2–6) $\times 10^{-7}$ M]. 3. At pH 5.5, cerebellar and bovine adrenal microsomes showed similar binding properties: two affinities with a similar specificity for Ins(1,3,4,5)P_4 as at pH 7.0. 4. However, when assayed in a low-ionic strength acetate-based buffer at pH 5.0, cerebellar microsomes retain specific Ins(1,3,4,5)P_4 binding sites, whereas bovine adrenal and hepatic microsomal binding sites lose much of their specificity, as InsP_6 and Ins(1,3,4,5,6)P_5 are equally as potent as Ins(1,3,4,5)P_4. 5. P_i (25 mM), which is frequently included in Ins(1,3,4,5)P_4 binding assays, had a small inhibitory effect on binding of cerebellar and adrenal microsomes at pH 5.5, but a large effect at pH 7.0, so that a considerable decrease occurs in the amount of specific binding at pH 5.5 compared with that at pH 7.0, if P_i is omitted from the binding assay. 6. Cerebellar and adrenal microsomes were used in a ligand-displacement mass assay (conducted under near-physiological conditions, at pH 7.0) on extracts of cerebral-cortex slices stimulated with agonists, and both preparations faithfully detected the increases in Ins(1,3,4,5)P_4 that occurred, implying that Ins(1,3,4,5)P_4 is the principal ligand on these binding sites in intact cells. 7. Apparent contradictions in the literature with regard to Ins(1,3,4,5)P_4 binding sites in neuronal and peripheral tissues can be largely accounted for by the data, and the properties of the binding sites detected at physiological pH are consistent with the possibility that they are putative receptors for the proposed second-messenger role for Ins(1,3,4,5)P_4.

INTRODUCTION

There has been a considerable increase over the last few years in the data and hypotheses concerning the possible intracellular role of Ins(1,3,4,5)P_4. An attempt to incorporate all this data into one unifying theory has been made (Irvine, 1990, 1991), and in this hypothesis it is envisaged that Ins(1,3,4,5)P_4 plays a pivotal role in regulation of extracellular Ca^{2+} entry into cells stimulated with agonists that increase inositol phosphate production (however, see Putney, 1990). One important step towards understanding the physiological role of Ins(1,3,4,5)P_4 will be the demonstration, characterization, purification and molecular-biological manipulation of an intracellular receptor.

Intracellular sites that specifically bind Ins(1,3,4,5)P_4 with high affinity have been described in a number of tissues. Bradford & Irvine (1987) were the first to demonstrate a saturable membrane binding site for Ins(1,3,4,5)P_4 (in HL60 cells), which was specific for Ins(1,3,4,5)P_4 over other inositol phosphates. Subsequently, Ins(1,3,4,5)P_4 binding sites have been characterized in other peripheral tissues, including bovine adrenal cortex (Enyedi & Williams, 1988) and bovine parathyroid (Enyedi *et al.*, 1989), and in neuronal tissues such as rat cerebellum (Theibert *et al.*, 1987; Challiss *et al.*, 1991) and pig cerebellum (Donié & Reiser, 1989). There are minor inconsistencies in the ligand-recognition properties reported by different groups, but most concur in showing that Ins(1,3,4,5)P_4 is the ligand with highest affinity, Ins(1,3,4,5,6)P_5 has relatively high affinity ($K_d \sim 200$ nM), and both Ins(1,4,5)P_3 and InsP_6 bind only weakly.

As with the Ins(1,4,5)P_3 receptor (Taylor & Richardson, 1991), Ins(1,3,4,5)P_4 binding sites have been shown to be present in relatively high amounts within cerebellum as compared with peripheral tissues. However, unlike Ins(1,4,5)P_3 binding, Ins(1,3,4,5)P_4 binding in cerebellum appears to be optimal under acidic conditions, and indeed a remarkably sharp pH-dependence of Ins(1,3,4,5)P_4 binding, with a maximum at pH 5.0 and barely detectable levels at pH 7.0, has been observed (Donié & Reiser, 1989; Challiss *et al.*, 1991). Donié & Reiser (1991) have recently purified a pH 5.0-dependent Ins(1,3,4,5)P_4 binding protein from pig cerebellum which appears to be a single protein of 42 kDa molecular mass. Further to this, Reiser *et al.* (1991) have recently developed an Ins(1,3,4,5)P_4 photoaffinity analogue which, when used to probe pig cerebellum under acidic conditions, binds specifically to a 42 kDa protein. In apparent contradiction to this, Theibert *et al.* (1991) have described and purified rat cerebellar proteins which bind Ins(1,3,4,5)P_4 at pH 7.4–8.3. These appear to be proteins of molecular-mass 182 kDa (plus a minor 123 kDa band) and 84 kDa (plus a minor 174 kDa band).

In view of the above observations, we wished to address the following questions concerning Ins(1,3,4,5)P_4 binding. (*a*) Are there any obvious major differences between neuronal and peripheral Ins(1,3,4,5)P_4 binding sites? (*b*) Can the apparent contradiction in rat cerebellum concerning the pH-dependence of Ins(1,3,4,5)P_4 binding be resolved by differences in assay conditions, or are there two separate Ins(1,3,4,5)P_4 binding proteins, one with a binding maximum under acidic conditions and the other under alkaline pH? (*c*) At a physiological pH, can neuronal and peripheral Ins(1,3,4,5)P_4 binding sites detect changes in Ins(1,3,4,5)P_4 in a stimulated cell extract in the presence of physiological levels of other inositol phosphates (i.e. do these binding sites have properties consistent with those expected of a physiological receptor for InsP_4)?

MATERIALS AND METHODS

Preparation of [^{3}H]Ins(1,3,4,5)P_4

A specific Ins(1,4,5)P_3 3-kinase partially purified from bovine brain (C. E. L. Spencer, N. Divecha & R. F. Irvine, unpublished work) has been used to phosphorylate commercially available [^{3}H]Ins(1,4,5)P_3 (Amersham International). Phosphorylation occurs in a buffer comprising 50 mM-Hepes/KOH (pH 7.5), 2 mM-EGTA, 1 mM-$MgCl_2$, 5 mM-ATP and 0.1% (w/v) BSA. After incubation at 37 °C for 15 min, phosphorylation was stopped by addition of ice-cold $HClO_4$ (final concn. 3%). The acid-soluble supernatant was neutralized by addition of a fixed volume of 2 M-KOH/0.1 M-Mes/10–20 mM-EDTA, such that the final pH was approx. 6. [^{3}H]Ins(1,3,4,5)P_4 was separated from residual [^{3}H]Ins(1,4,5)P_3 by h.p.l.c. as described previously (Cullen *et al.*, 1989).

Fractions containing [^{3}H]Ins(1,3,4,5)P_4 were freed of P_i on a Dowex column (Bio-Rad AG 1X8; 200–400 resin) (Irvine *et al.*, 1986) and the ammonium formate was removed by freeze drying. This procedure usually resulted in approx. 85–90% conversion of [^{3}H]Ins(1,4,5)P_3 into [^{3}H]Ins(1,3,4,5)P_4.

Preparation of membranes

Membranes from rat cerebellum were routinely prepared by the procedure of Thiebert *et al.* (1987). Briefly, 12 cerebella from adult male Sprague–Dawley rats (200–250 g) were homogenized (Polytron setting 7, for 14 s) in 10 vol. of ice-cold 50 mM-Tris/HCl (pH 7.4), 1 mM-EDTA and 1 mM fresh dithiothreitol. The membrane suspension was pelleted by centrifugation (36000 ***g*** for 10 min) and washed twice in homogenization buffer. The final pellet was resuspended to give approx. 10–15 mg/ml and could be stored at −20 °C without loss of binding activity.

Bovine adrenocortical microsomes were prepared as described by Palmer & Wakelam (1989). Approx. 20 bovine adrenal glands were cleared of fat and bisected. The medulla was removed with a scalpel, and the remaining cortex was weighed and placed in 2 vol. of ice-cold homogenization buffer (20 mM-$NaHCO_3$/1 mM-dithiothreitol, pH 7.5). The tissue was finely chopped in a blender, filtered through a colander, and then homogenized (Polytron setting 7, for 15 s). The homogenate was centrifuged (500 ***g*** for 15 min) with the resultant supernatant being pelleted at 35000 ***g*** for 20 min. After one wash the final pellet was resuspended at 20–30 mg/ml, and stored at −70 °C. Protein was determined as described by Bradford (1976), with BSA as standard.

Binding studies

For binding experiments, microsomes (for bovine adrenocortical microsomes 1.5–2.0 mg of protein/assay; for cerebellum 0.6–0.8 mg of protein/assay) were incubated at 4 °C in 400 μl of a solution containing 100 mM-KCl, 20 mM-NaCl, 10 mM-Hepes/KOH (pH 7.0) or 10 mM-Mes/KOH (pH 5.5), 1 mM-EDTA, 0.1% (w/v) BSA, 10–12 nCi of [^{3}H]Ins(1,3,4,5)P_4 (0.4–0.7 nM final concn.), and the appropriate concentration of unlabelled Ins(1,3,4,5)P_4 or other inositol phosphates. Binding was also carried out in 25 mM-sodium acetate/25 mM-KH_2PO_4/2 mM-EDTA, pH 5.0. Previous studies (Challiss & Nahorski, 1990; Enyedi & Williams, 1988) have shown that in the absence of Mg^{2+} less than 5% hydrolysis of Ins(1,3,4,5)P_4 occurs. Non-specific binding was defined by the addition of 20 μM unlabelled Ins(1,3,4,5)P_4. Samples were incubated for 30 min, after which time binding was stopped by filtration through pre-wetted Whatman GF/B filters. Filters were rapidly washed with 3 × 3 ml of ice-cold 100 mM-KCl/20 mM-NaCl/1 mM-EDTA/0.1% BSA and either 10 mM-Hepes/KOH (pH 7.0) or 10 mM-Mes/KOH (pH 5.5). Filters were left soaking overnight in 4 ml of scintillation fluid before determination of retained radioactivity by scintillation counting.

Ins(1,3,4,5)P_4 mass assay

Mass determinations of Ins(1,3,4,5)P_4 were performed under the assay conditions described above. Stimulated and control samples from cerebral-cortex slices were kindly provided by Dr. R. A. J. Challiss (for details of preparation of these samples see Challiss & Nahorski, 1990). Standard Ins(1,3,4,5)P_4 samples were diluted with a buffer blank taken through the inositol phosphate extraction procedure described by Challiss & Nahorski (1990). The standard Ins(1,3,4,5)P_4 samples were used to construct a displacement curve for each experiment. Where appropriate, cell samples were diluted in the prepared buffer blank to allow Ins(1,3,4,5)P_4 determination from the most sensitive part of the displacement curve.

Mathematical analysis

K_d values and the number of binding sites were estimated by using the EBDA LIGAND data-analysis computer program.

Materials

[^{3}H]Ins(1,4,5)P_3 (sp. radioactivity 33–51.4 Ci/mmol) was obtained from Amersham. All other reagents were obtained from Sigma.

RESULTS AND DISCUSSION

Adrenal cortex

Initial studies were conducted towards characterizing the binding of Ins(1,3,4,5)P_4 to microsomes derived from bovine adrenal cortex. Binding of [^{3}H]Ins(1,3,4,5)P_4 was rapid, with saturation reached by 20 min at 4 °C (results not shown). Under equilibrium binding conditions, 50% inhibition of the binding of labelled ligand was achieved with approx. 10 nM unlabelled Ins(1,3,4,5)P_4 (Fig. 1*a*). Scatchard analysis produced a curvilinear plot (Fig. 1*b*), which could be resolved into two specific components, a high-affinity site having an apparent K_d of $(1.9 \pm 0.7) \times 10^{-9}$ M with a $B_{max.}$ of 24 ± 12 fmol/mg of protein, and a low-affinity site with K_d $(3.2 \pm 2.3) \times 10^{-7}$ M with a $B_{max.}$ of 728 ± 122 fmol/mg of protein. Data were best described by a two-site model ($P < 0.05$ compared with a one-site fit). Specificity of Ins(1,3,4,5)P_4 binding was analysed by competition for binding by other unlabelled inositol phosphate isomers (Fig. 1*a*). Ins(1,3,4,5,6)P_5 showed the next highest potency, with an IC_{50} of 500 nM; Ins(1,4,5)P_3 showed an IC_{50} of 7 μM, and InsP_6 only competed for 20% of binding at a concentration of 1 μM. When looking more closely at this displacement curve, it can be seen that the low-affinity site shows a decreased specificity for Ins(1,3,4,5)P_4 as compared with the high-affinity site. Thus, at the lower-affinity site high concentrations of Ins(1,3,4,5,6)P_5 are shown to compete with a similar potency to that of Ins(1,3,4,5)P_4. These data are in good agreement with those obtained by Enyedi & Williams (1988) using the same tissue. In view of the Ins(1,3,4,5)P_4-binding sites characterized in other neuronal tissues, where the assays are carried out at pH 5.0 and in the presence of phosphate (see Donié & Reiser, 1989; Challiss *et al.*, 1991), we decided to examine the effects of both pH and phosphate on the non-neuronal site characterized in bovine adrenal cortex.

Firstly, addition of phosphate to the assay medium used to characterize the binding at pH 7.0 results in a marked inhibition of specific binding activity ($IC_{50} \sim 25$ mM-PO_4^{3-}; results not shown). Furthermore, when the pH 5.0 acetate-based assay buffer is used, there is an approx. 6-fold increase in binding activity, a result which has previously been described in bovine parathyroid

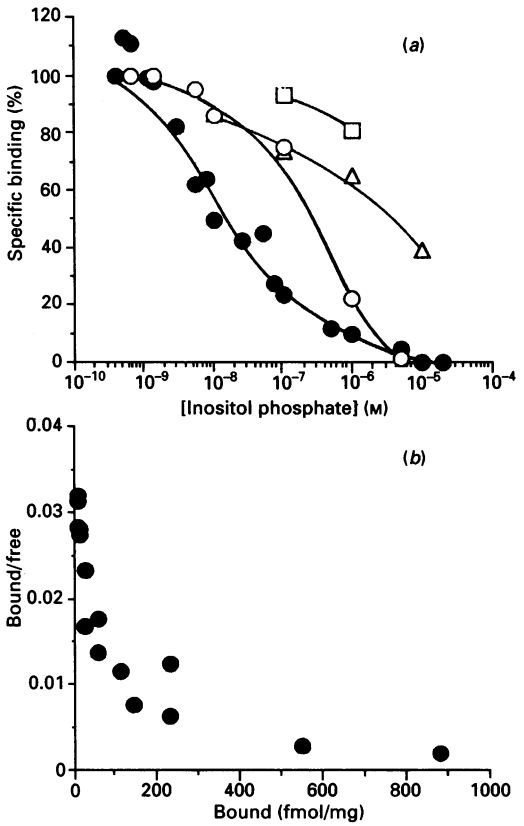

Fig. 1. Characterization of [^{3}H]Ins(1,3,4,5)P_4 binding sites in bovine adrenocortical microsomes at pH 7.0

(*a*) Displacement curve for Ins(1,3,4,5)P_4 and other competing ligands. Assays were carried out in 100 mM-KCl/20 mM-NaCl/10 mM-Hepes/KOH (pH 7.0)/1 mM-EDTA/0.1% BSA by the procedure described in the Materials and methods section. Each point is the mean of a duplicate determination; results from two further experiments showed essentially identical displacement. Data are expressed as the percentage of the total binding without unlabelled ligand (752±30 d.p.m./mg) and are corrected for non-specific binding (174±17 d.p.m./mg). Symbols: ●, Ins(1,3,4,5)P_4; △, Ins(1,4,5)P_3; ○, 2-hydroxy-InsP_5; □, InsP_6. (*b*) Scatchard analysis of Ins(1,3,4,5)P_4 displacement. Analysis was conducted by the EBDA-LIGAND binding program.

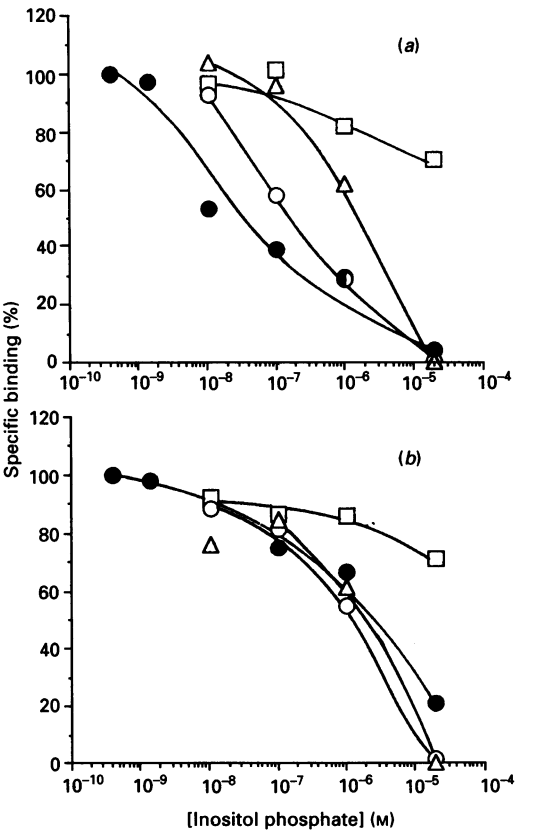

Fig. 2. Effect of an acetate-based pH 5.0 buffer on cerebellar and bovine adrenal microsomal [^{3}H]Ins(1,3,4,5)P_4 binding

(*a*) [^{3}H]Ins(1,3,4,5)P_4 binding to cerebellar microsomes. Assays were carried out in 25 mM-sodium acetate/25 mM-KH_2PO_4/2 mM-EDTA (pH 5.0). Each point is the mean of triplicate determinations. Non-specific binding was defined by 20 μM-InsP_6. Symbols: ●, Ins(1,3,4,5)P_4; □, Ins(1,4,5)P_3; ○, 2-hydroxy-InsP_5; △, InsP_6. (*b*) [^{3}H]Ins(1,3,4,5)P_4 binding to bovine adrenals. Assays were carried out as in (*a*).

glands (Enyedi *et al.*, 1989). However, those authors did not analyse the specificity of this site at pH 5.0. On looking at the specificity of binding at pH 5.0 in bovine adrenal cortex, there becomes apparent a dramatic loss in specificity of the Ins(1,3,4,5)P_4-binding site for Ins(1,3,4,5)P_4 (Fig. 2*b*). The non-specific binding increases from 20% at pH 7.0 to 40% at pH 5.0, and, more importantly, Ins(1,3,4,5,6)P_5 and InsP_6 are capable of competing for more bound [^{3}H]Ins(1,3,4,5)P_4 than is 20 μM unlabelled Ins(1,3,4,5)P_4. However, InsP_6 has a different IC_{50} value from that at pH 7.0 (cf. Fig. 1*a*), so the major difference in binding appears to result from an inability of low concentrations of Ins(1,3,4,5)P_4 to displace bound [^{3}H]Ins(1,3,4,5)P_4, coupled with an increased affinity of the binding site for InsP_6. A possible explanation for this observation could be due to the 'unmasking' of a low-affinity non-specific inositol polyphosphate binding site under these specific conditions.

This apparent loss in specificity may not, however, simply be a result of lowering the pH of the medium. If an assay medium at pH 5.5 containing constituents identical with that at pH 7.0 is used, Ins(1,3,4,5)P_4 binding to bovine adrenal cortex appears similar to that at pH 7.0, such that the IC_{50} values for Ins(1,3,4,5)P_4, Ins(1,3,4,5,6)P_5 and InsP_6 are approx. 9 nM, 450 nM and 900 nM respectively (results not shown). Furthermore, this pH 5.5 binding site is only slightly inhibited by 25 mM-phosphate (results not shown). Thus it would appear that the 'unmasking' of this inositol polyphosphate binding site is in some way dependent on the ionic environment in which the binding assay is conducted. This difference in Ins(1,3,4,5)P_4 binding to bovine adrenals highlights the problems of comparing binding characteristics when different assay conditions are employed. However, the point remains that, when a low-ionic-strength acetate-based buffer at pH 5.0 is used, there is a difference in Ins(1,3,4,5)P_4 binding characteristics between bovine adrenals and rat cerebellum (see below). This difference does not, however, become observable when using a relatively high salt buffer at pH 5.5. A similar effect of the ionic environment on [^{3}H]Ins(1,3,4,5)P_4 binding has been observed in isolated rat liver membrane fractions (P. J. Cullen & R. F. Irvine, unpublished work).

Cerebellum

Having characterized the bovine adrenal-cortex Ins(1,3,4,5)P_4 binding site by using a near-physiological medium, we became interested in seeing whether rat cerebellum would bind Ins(1,3,4,5)P_4 under identical assay conditions. Initial characterization showed that, at pH 7.0 and in the absence of

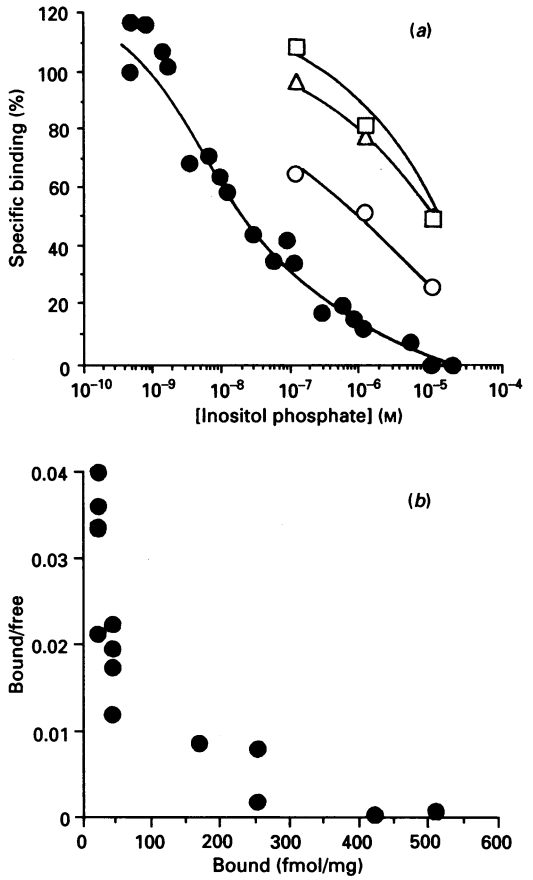

Fig. 3. Characterization of [^{3}H]Ins(1,3,4,5)P_4 binding sites in rat cerebellar microsomes at pH 7.0

(*a*) Displacement curve for Ins(1,3,4,5)P_4 and other competing ligands. Assays were carried out identically with that in Fig. 1(*a*) (total binding 790±33 d.p.m./mg; non-specific binding 266±59 d.p.m./mg). Symbols as in Fig. 1(*a*). (*b*) Scatchard analysis of Ins(1,3,4,5)P_4 displacement. Analysis was conducted as in Fig. 1(*b*).

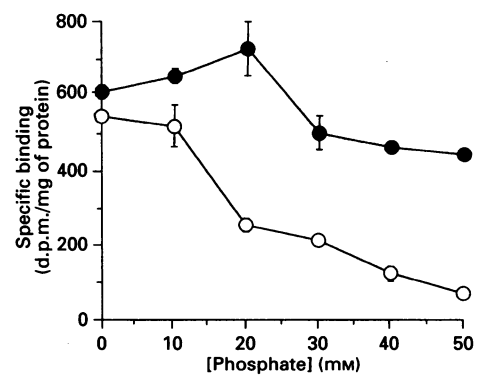

Fig. 4. Effect of phosphate on [^{3}H]Ins(1,3,4,5)P_4 binding in cerebellar microsomes when assayed at pH 7.0 (○) or pH 5.5 (●)

Assays were carried out in 100 mM-KCl/20 mM-NaCl/1 mM-EDTA/0.1% BSA and either 10 mM-Hepes/KOH (pH 7.0) or 10 mM-Mes/KOH (pH 5.5) plus the relevant amount of KH_2PO_4. Each point is the mean of triplicate determinations ($n = 2$). S.D. values are shown only for these points where the error is larger than the actual printed point. Specific binding is defined as amount of binding remaining after subtraction of non-specific binding from total binding. Similar data were obtained with bovine adrenocortical microsomes under identical conditions.

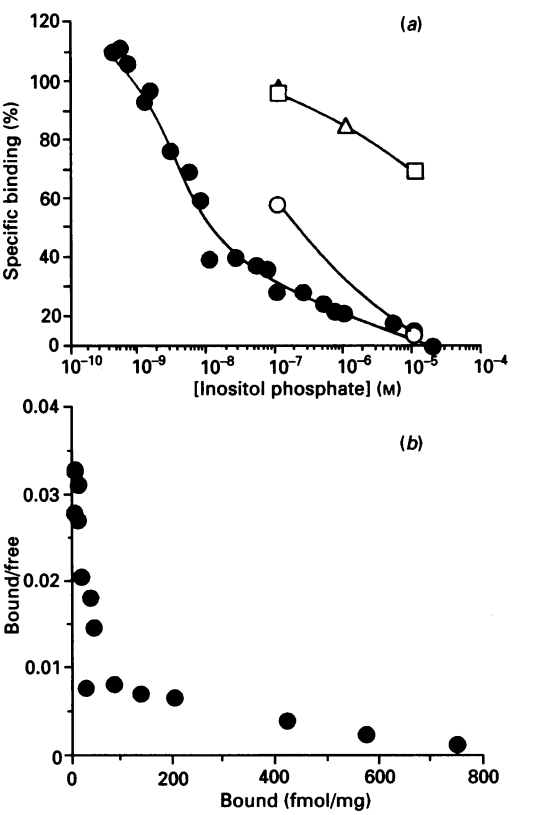

Fig. 5. Characterization of [^{3}H]Ins(1,3,4,5)P_4 binding sites in rat cerebellar microsomes at pH 5.5

(*a*) Displacement curve for Ins(1,3,4,5)P_4 and other competing ligands. Assays were carried out in 100 mM-KCl/20 mM-NaCl/10 mM-Mes/KOH (pH 5.5)/1 mM-EDTA/0.1% BSA, as described in the legend of Fig. 1(*a*) (total binding 1112±109 d.p.m./mg; non-specific binding 234±53 d.p.m./mg). Symbols as in Fig. 1(*a*). (*b*) Scatchard analysis of Ins(1,3,4,5)P_4 displacement. Analysis was carried out as in Fig. 1(*b*).

added phosphate, we could detect a saturable Ins(1,3,4,5)P_4 binding site in rat cerebellum. Under equilibrium conditions, 50% of bound [^{3}H]Ins(1,3,4,5)P_4 was displaced by approx. 10 nM unlabelled Ins(1,3,4,5)P_4 (see Fig. 3*a*). Like the bovine adrenal-cortex site, Scatchard analysis revealed two probable sites ($P < 0.02$ compared with a one-site fit), having high-affinity [K_d $(1.5 \pm 1.3) \times 10^{-9}$ M, $B_{max.}$ 13±10 fmol/mg] and low-affinity [K_d $(2 \pm 1.6) \times 10^{-7}$ M, $B_{max.}$ 404±278 fmol/mg] sites (Fig. 3*b*). Specificity of these binding sites was similar to that of the bovine adrenal cortex, with Ins(1,3,4,5,6)P_5 having an IC_{50} of 1 μM and both InsP_6 and Ins(1,4,5)P_3 being less efficacious ($IC_{50} \sim 10$ μM) (Fig. 3*a*). Interestingly, the addition of P_i to the assay medium at pH 7.0 again inhibited specific [^{3}H]Ins(1,3,4,5)P_4 binding with a similar potency to that in bovine adrenal cortex ($IC_{50} \sim 18$ mM-PO_4^{3-}) (see Fig. 4). Unlike the bovine adrenal cortex, however, when the pH 5.0 acetate-based assay medium is used rat cerebellum retains the specificity of Ins(1,3,4,5)P_4 binding (Fig. 2*a*). When assayed at pH 5.5, cerebellar Ins(1,3,4,5)P_4 binding sites are similar to the site characterized at pH 7.0 with respect to specificity and affinities (compare Figs. 3*a* and 5*a*). Again, a two-site model applies ($P < 0.01$ compared with a one-site fit), with high-affinity [K_d $(1.7 \pm 0.7) \times 10^{-9}$ M, $B_{max.}$ 28±6 fmol/mg of protein] and low-affinity [K_d $(6 \pm 0.5) \times 10^{-7}$ M, $B_{max.}$ 1±0.5 pmol/mg of protein] sites (Fig. 5*b*).

Under our pH 7.0 and pH 5.5 assay conditions we do not see a dramatic shift in specific binding on increasing the acidity of

Table 1. Ins(1,3,4,5)P_4 mass determinations on rat cerebellar and bovine adrenocortical microsomes

Assays were carried out in 100 mM-KCl/20 mM-NaCl/10 mM-Hepes/KOH (pH 7.0)/1 mM-EDTA/0.1% BSA as described in the Materials and methods section. Standard curves were constructed with known dilutions of Ins(1,3,4,5)P_4. Data for cerebellar microsomes at pH 5.0 was kindly provided by Dr. R. A. J. Challiss.

Sample	Microsomes used in mass determination		
	Cerebellar, pH 5.0 (nM)	Cerebellar, pH 7.0 (nM)	Bovine adrenal, pH 7.0 (nM)
Control	4.7	6.6±0.2	20.8±9.1
K^+ depolarization (25 mM final)	20	38.4±0.9	76.4±2.0
K^+ depolarization (25 mM final)+ noradrenaline (100 μM)	100	216±12.3	452±6.6

the medium, as was described by Donié & Reiser (1989) or Challiss *et al.* (1991). Those authors have shown that on increasing the acidity from pH 8 to 4 an increase of specific binding by between 4- and 10-fold is observed; we, however, at best only see an increase of between 1.5- and 2-fold over a similar pH range. Total specific binding at pH 5.5 is, as in bovine adrenal cortex, inhibited by addition of phosphate, but with a much decreased potency (see Fig. 4).

Comparison between cerebellum and adrenal cortex

Finally, we have characterized rat cerebellar Ins(1,3,4,5)P_4 binding site(s) at both pH 5.5 and pH 7.0, using the same composition of assay buffer, in an attempt to accommodate the apparently conflicting reports within the Ins(1,3,4,5)P_4-binding literature. As mentioned above, Challiss *et al.* (1991) and Donié & Reiser (1991) have described cerebellar Ins(1,3,4,5)P_4 binding sites which are most easily detected at pH 5.0 in an acetate-based buffer containing 25 mM-phosphate. Under these conditions they showed that at pH 7.0 there is comparatively little Ins(1,3,4,5)P_4 binding. On the other hand, Thiebert *et al.* (1991), using a Tris-based assay buffer lacking phosphate, have characterized and purified an Ins(1,3,4,5)P_4-binding protein at a more alkaline pH. Our results suggest that, in the same rat cerebellum preparation, specific Ins(1,3,4,5)P_4 binding can be detected at both pH 7.0 and pH 5.5 as long as the correct assay conditions are employed. Thus, for example, if 25 mM-phosphate is added to the assay buffer, this will bias detection of Ins(1,3,4,5)P_4 binding to more acidic pH, since this concentration of phosphate will inhibit 66% of binding at pH 7.0 but will hardly inhibit binding at pH 5.5 (see Fig. 4). This simple observation most probably accounts for most of the dramatic shift in specific Ins(1,3,4,5)P_4 binding observed by Challis *et al.* (1991) and Donié & Reiser (1989) on increasing the acidity of a phosphate-containing buffer. We have also prepared rat cerebellum as described by Challiss *et al.* (1991) and have obtained similar results (not shown) to those described above.

Physiological significance

The question which so far remains unaddressed is whether the binding site(s) described for bovine adrenal cortex and rat cerebellum have properties consistent with putative physiological receptors for Ins(1,3,4,5)P_4. In an attempt to answer this question, we have used neutralized acid extracts of agonist-stimulated cerebral-cortex slices (samples kindly provided by Dr. R. A. J. Challiss) to see whether these Ins(1,3,4,5)P_4 binding sites are capable of detecting changes in Ins(1,3,4,5)P_4 mass in the presence of physiological levels of other inositol phosphates, when the binding is performed under near-physiological conditions. Table 1 summarizes mass assays using both adrenal cortex and rat cerebellum at pH 7.0 (all assays in the absence of phosphate). The results are compared with mass determinations on the same samples carried out by Dr. R. A. J. Challiss using rat cerebellum at pH 5.0 in an acetate buffer containing 25 mM-phosphate (for experimental details, see Challiss *et al.*, 1991). The values for Ins(1,3,4,5)P_4 mass obtained with this particular mass assay have been shown to correlate closely with values of Ins(1,3,4,5)P_4 mass determined by isotopic labelling (see Challiss & Nahorski, 1990). As can be seen from the data, the two assay systems faithfully record the changes in Ins(1,3,4,5)P_4 mass that occur in the tissue under different stimulatory protocols, but the absolute values differ considerably. This difference is most apparent for bovine adrenal cortex, and the reason for this is that, because of the two binding affinities, the Ins(1,3,4,5)P_4 displacement curve is very shallow (8% displacement over 3 orders of magnitude), which therefore makes absolute determination of mass very difficult, since small errors in displacement values lead to large variations in the position of the standard curve [i.e. the values obtained from the 'standard' assay (Challiss *et al.*, 1991) can be accommodated within the errors of the assays when using adrenocortical membranes]. This makes these membranes not particularly suitable for a routine mass assay, whereas rat cerebellum produces much more accurate mass determination, since the displacement is more steep. However, once the standard curve is set, the proportional increases should not be prone to any more error in one tissue than in another, and this is borne out by the observation that the binding sites in both tissues accurately record the proportional changes in the tissue samples (Table 1). Thus both cerebellum and adrenal cortex contain binding sites which, under near-physiological conditions, are capable of detecting changes in physiological levels of Ins(1,3,4,5)P_4 in the presence of other inositol phosphates, implying that *in vivo* these binding sites recognize Ins(1,3,4,5)P_4.

Conclusions

We have described in this study Ins(1,3,4,5)P_4 binding sites in two different tissues. In each case the Ins(1,3,4,5)P_4 binding data are best analysed by using a two-site Scatchard displacement isotherm. This two-site fit comprises a high-affinity site [$K_d \sim (1–6) \times 10^{-9}$ M] and a low-affinity site with lower specificity for Ins(1,3,4,5)P_4 [$K_d \sim (2–6) \times 10^{-7}$ M). Our results do not, and cannot, address the question as to whether these two affinities are different affinity states of the same protein or whether they are due to two totally separate proteins. Some light may be shed on this by looking at the data on the purified Ins(1,3,4,5)P_4 binding proteins (see Theibert *et al.*, 1991; Donié & Reiser, 1991). Taking the Ins(1,3,4,5)P_4 competition curves for Peak 2 and Peak 3 of Theibert *et al.* (1991) and subjecting them to Scatchard analysis reveals for each peak a two-site fit ($P < 0.01$), with high-affinity ($K_d \sim 2 \times 10^{-9}$ M) and low-affinity ($K_d \sim 2 \times 10^{-7}$ M) components, and the stoichiometry of Ins(1,3,4,5)P_4 binding in these two peaks suggests that each peak is probably predominantly Ins(1,3,4,5)P_4-binding protein.

If the data of Reiser *et al.* (1991) at pH 5.0 are similarly treated by Scatchard analysis, a single Ins(1,3,4,5)P_4 binding site is resolved ($P < 0.01$) having a K_d of $\sim 2 \times 10^{-7}$ M (results taken from Fig. 2*a* in Reiser *et al.*, 1991). [Interestingly, but somewhat confusingly, Donié and co-workers state that their purified Ins(1,3,4,5)P_4 binding protein has a K_d of 12 nM (see Donié & Reiser, 1991; Reiser *et al.*, 1991)]. More importantly, however, the data in Fig. 2(*a*) of Reiser *et al.* (1991) show that this purified Ins(1,3,4,5)P_4 binding protein is not specific for Ins(1,3,4,5)P_4 in

so much as Ins(1,3,4,5,6)P_5 is equipotent as a competing ligand. Therefore it would appear either that Donié and co-workers have purified a single Ins(1,3,4,5)P_4 binding protein, or that their purified protein does have two affinity states but in their hands it is all in the low-affinity unspecific state. In fact, the purification of the Ins(1,3,4,5)P_4 binding protein(s) has, if anything, at present increased the general confusion, and it is to be hoped that sequencing and cloning data may resolve these problems more fully. Our results described above suggest that some of the confusion concerning Ins(1,3,4,5)P_4 binding studies on membrane fractions may be due to differences in assay conditions used by various groups (i.e. presence or absence of phosphate; ionic composition of medium). It would be interesting to know whether these conditions alter binding to the purified receptors described above.

Finally and most importantly, our data suggest that the properties of the binding sites described here, under near-physiological conditions, are not consistent with any enzymes known to interact with inositol polyphosphates (see Bradford & Irvine, 1987), but are entirely consistent with the described second-messenger actions of Ins(1,3,4,5)P_4 (Irvine, 1990, 1991). This allows us to suggest that both neural and peripheral tissues contain Ins(1,3,4,5)P_4 binding sites which are probably putative receptors for the proposed second-messenger function of this inositol phosphate.

We are most grateful to Catherine Spencer and Nullin Divecha for the supply of InsP_3 kinase, to Alan Richardson, Colin Taylor, John Challiss and Keith Wreggett for helpful discussions, and to John Challiss for his generous gift of cerebral-cortical slice extracts on which mass assays for InsP_4 had been performed. Finally, special thanks to our patient secretary, Linda Notton. P.J.C. is a Beit Memorial Fellow.

Received 9 March 1992/30 April 1992; accepted 14 May 1992

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