Biochem. J. (1990) 270, 227–232 (Printed in Great Britain)

Liver inositol 1,4,5-trisphosphate-binding sites are the Ca^{2+}-mobilizing receptors

David L. NUNN and Colin W. TAYLOR*
Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

Ins(1,4,5)P_3 is the intracellular messenger that in many cells mediates the effects of Ca^{2+}-mobilizing receptors on intracellular Ca^{2+} stores. An Ins(1,4,5)P_3 receptor from cerebellum has been purified and functionally reconstituted, but the relationship between this protein and the high-affinity Ins(1,4,5)P_3-binding sites of peripheral tissues is unclear. We compared the Ins(1,4,5)P_3-binding sites of liver and cerebellum by measuring inhibition of specific Ins(1,4,[^{32}P]5)P_3 binding by various ligands under equilibrium conditions, and find that each ligand binds with similar affinity in the two tissues. Earlier studies in which Ins(1,4,5)P_3 binding and Ca^{2+} mobilization were measured under different conditions demonstrated large differences between K_D values for binding and EC_{50} values (concn. giving half-maximal effect) for Ca^{2+} release. We show here that, when measured under identical conditions, K_D and EC_{50} values for four agonists are similar. Schild analysis of inhibition of Ins(1,4,5)P_3 binding by ATP demonstrates a competitive interaction between the two at the liver Ins(1,4,5)P_3-binding site, and this partly accounts for earlier discrepancies in binding and Ca^{2+}-release data. We conclude that the high-affinity Ins(1,4,5)P_3-binding site of hepatocytes is likely to be the receptor that mediates Ca^{2+} mobilization, and that this receptor is at present indistinguishable from that in cerebellum.

INTRODUCTION

In many cells Ins(1,4,5)P_3 stimulates release of intracellular Ca^{2+} from non-mitochondrial stores in response to a range of extracellular stimuli (Berridge & Irvine, 1984, 1989). The effects of Ins(1,4,5)P_3 are believed to be mediated by a specific intracellular receptor that regulates a Ca^{2+} channel controlling the passive emptying of intracellular Ca^{2+} stores (Muallem *et al.*, 1985). The existence of specific intracellular Ins(1,4,5)P_3 receptors was initially inferred from the structural specificity of inositol phosphate-induced Ca^{2+} release, and then supported by the demonstration of specific Ins(1,4,5)P_3-binding sites, first in liver and neutrophils (Spät *et al.*, 1986) and later in many other tissues (Worley *et al.*, 1987; Guillemette *et al.*, 1987; Nahorski & Potter, 1989; Nunn *et al.*, 1990). An Ins(1,4,5)P_3-binding protein has been purified from rat cerebellum (Supattapone *et al.*, 1988), and when reconstituted into lipid vesicles it mediates Ins(1,4,5)P_3-induced Ca^{2+} flux (Ferris *et al.*, 1989). The cDNA for the mouse cerebellar Ins(1,4,5)P_3-binding protein has been sequenced and is predicted to encode a protein with multiple potential membrane-spanning regions and *C*-terminal region that has some similarity to another Ca^{2+} channel, the ryanodine receptor of skeletal-muscle sarcoplasmic reticulum (Furuichi *et al.*, 1989).

Differences in the relative affinities of cerebellar and peripheral Ins(1,4,5)P_3-binding sites for inositol phosphates and the antagonist heparin have been interpreted as evidence in support of differences between them (Nahorski & Potter, 1989; Willcocks & Nahorski, 1989). One aim of the present project was to extend our earlier studies, which demonstrated that cerebellar and liver Ins(1,4,5)P_3-binding sites have similar molecular target sizes (Nunn *et al.*, 1990), to a comparison of the ligand-recognition properties of each of these proteins.

Functional reconstitution of the purified cerebellar Ins(1,4,5)P_3-binding protein (Ferris *et al.*, 1989; Furuichi *et al.*, 1989) is conclusive evidence that the specific Ins(1,4,5)P_3-binding site identified in that tissue by radioligand binding is the receptor that mediates the effects of Ins(1,4,5)P_3 on Ca^{2+} mobilization. In other tissues, where intracellular Ca^{2+} regulation is generally more thoroughly understood, there is only indirect evidence to suggest that the Ins(1,4,5)P_3-binding sites may be the functional receptors. The rank orders of potency of various inositol phosphates in inhibiting Ins(1,4,5)P_3 binding and in stimulating Ca^{2+} mobilization are similar (Nahorski & Potter, 1989; Nunn *et al.*, 1990). Ins(1,4,5)P_3-binding sites and Ins(1,4,5)P_3-induced Ca^{2+} mobilization are similarly enriched in subcellular fractions (Ghosh *et al.*, 1989) and in different brain regions (Joseph & Rice, 1989). Heparin antagonizes both Ins(1,4,5)P_3 binding and Ins(1,4,5)P_3-induced Ca^{2+} mobilization (Worley *et al.*, 1987; Ghosh *et al.*, 1988), and the liver Ins(1,4,5)P_3-binding site is clearly distinguishable from the substrate-binding site of both enzymes that are known to metabolize Ins(1,4,5)P_3 (Nunn *et al.*, 1990). A problem is that, in most studies where Ins(1,4,5)P_3 binding and Ca^{2+} mobilization have been compared, the affinity of the binding site is generally much higher than the concentration of inositol phosphate that causes half-maximal Ca^{2+} release (EC_{50}). A possible, though untested, explanation of this discrepancy is that the two measurements are generally made under different conditions: binding on ice in the absence of ATP, and Ca^{2+} mobilization at 37 °C in the presence of ATP. Here we compare Ins(1,4,5)P_3 binding and Ca^{2+} mobilization under identical conditions in permeabilized hepatocytes.

MATERIALS AND METHODS

Materials

Ins(1,4,[^{32}P]5)P_3 (about 1000 Ci/mmol) was from Amersham International. Ins(1,4,5)P_3 and its stable analogue DL-Ins(1,4,5)P_3[S]$_3$ (Taylor *et al.*, 1989) were generously given by Dr. R. F. Irvine (Babraham) and Dr. B. V. L. Potter (Leicester) respectively. Ins(2,4,5)P_3 and Ins(4,5)P_2 were from Boehringer Mannheim. L-α-Glycerophospho-D-*myo*-inositol 4,5-bisphosphate [L-α-Gro*P*Ins(4,5)P_2] was from Calbiochem. Heparin, de-*N*-sulphated heparin and chondroitin sulphate were from Sigma. Percoll was from Pharmacia.

Abbreviations used: DL-Ins(1,4,5)P_3[S]$_3$, DL-*myo*-inositol 1,4,5-trisphosphorothioate; L-α-Gro*P*Ins(4,5)P_2, L-α-glycerophospho-D-*myo*-inositol 4,5-bisphosphate; EC_{50}, concentration causing half-maximal effect; IC_{50}, concentration of unlabelled competitor that displaces half of specific binding.

* To whom correspondence should be addressed.

Preparation of rat liver and cerebellar membranes

A rat liver was perfused *in situ* with 40 ml of buffered saline [116 mM-NaCl, 5.4 mM-KCl, 0.96 mM-NaH_2PO_4, 0.8 mM-$MgCl_2$, 25 mM-$NaHCO_3$, 11 mM-glucose, under CO_2/O_2 (1:19), pH 7.4], chopped, and homogenized in buffered sucrose (250 mM-sucrose, 5 mM-Hepes, pH 7.4) with a glass Dounce homogenizer with ten strokes of a loose-fitting plunger and three with a tighter plunger. The homogenate was centrifuged (2500 *g*, 10 min), the pellet resuspended in 21 ml of buffered sucrose, and mixed thoroughly with 2.8 ml of Percoll (Prpić *et al.*, 1984). The mixture was centrifuged (35000 *g*, 30 min), and membranes were harvested as a fluffy band near the top of the tube and then washed in 40 ml of hypo-osmotic medium (5 mM-Hepes, 1 mM-EGTA, pH 7.4). After further centrifugation (48000 *g*, 10 min), the membranes were resuspended in binding buffer (20 mM-Tris, 1 mM-EDTA, pH 8.3) at approx. 2 mg of protein/ml and stored for up to 2 weeks in liquid nitrogen.

Cerebellar membranes were prepared exactly as described by Worley *et al.* (1987), and stored for up to 2 weeks in binding buffer at approx. 0.5 mg of protein/ml in liquid nitrogen. Protein was measured by the method of Bradford (1976), with BSA as standard.

Ins(1,4,[^{32}P]5)P_3 binding to liver and cerebellar membranes

Incubations, in a final volume of 0.5 ml of binding buffer, included membranes (0.1 mg of liver protein or 0.02 mg of cerebellar protein), 0.01 μCi of Ins(1,4,[^{32}P]5)P_3, 0.1 μCi of ^3H_2O to allow later correction for trapped volume, and the competing ligands (Nunn *et al.*, 1990). The reactions were incubated at 0 °C for 5 min, and the bound and free ligand were separated by centrifugation at 0 °C (20000 *g*, 5 min). There was negligible metabolism of Ins(1,4,[^{32}P]5)P_3 under these conditions: 94% of the added label was recovered unchanged from the liver incubations and 97% from the cerebellar incubations. Previous experiments (Nunn *et al.*, 1990) established that equilibrium binding was attained under these conditions. Despite the low concentration of Ins(1,4,[^{32}P]5)P_3 used (approx. 20 pM, i.e. 0.4% of K_D), the amount of radioligand bound was never more than 15% of that added.

When the effects of ATP on Ins(1,4,5)P_3 inhibition of Ins(1,4,[^{32}P]5)P_3 binding were examined, up to 4-fold more Ins(1,4,[^{32}P]5)P_3 was included in the incubation to provide detectable specific binding. However, the specific radioactivity of Ins(1,4,[^{32}P]5)P_3 is so high that, even when present at the highest radioactivity (0.04 μCi/500 μl), its total concentration never exceeded 1.5% of the K_D and would therefore have no significant effect on the IC_{50} (concn. that displaces half of specific binding).

Ins(1,4,5)P_3-induced Ca^{2+} release and Ins(1,4,[^{32}P]5)P_3 binding in permeabilized hepatocytes

Hepatocytes from male Wistar rats (about 200 g), prepared by collagenase perfusion of intact liver, were stored on ice in $NaHCO_3$-buffered Eagle's minimum essential medium (Taylor *et al.*, 1989). Cells were permeabilized by incubation with 75 μg of saponin/ml for 10 min at 37 °C in Ca^{2+}-free intracellular-type medium (140 mM-KCl, 20 mM-NaCl, 2 mM-$MgCl_2$, 1 mM-EGTA, 20 mM-Pipes, pH 6.8). Cells were washed and resuspended at very high cell density (8.5×10^6 cells/ml = approx. 2 mg of protein/ml) in the same medium but with the free Ca^{2+} adjusted to approx. 120 nM and with antimycin (10 μM), oligomycin (10 μM) and $^{45}CaCl_2$ (2 μCi/ml) added. After 3 min, ATP (1.5 mM), phosphocreatine (5 mM) and creatine kinase (1 unit/ml) were added to stimulate $^{45}Ca^{2+}$ uptake into non-mitochondrial intracellular stores. After a further 10 min, when cells had loaded to steady state with $^{45}Ca^{2+}$, they were simultaneously rapidly cooled and added to the test compound by dilution of cells (40 μl) into medium (360 μl) at 0 °C but otherwise identical with that in which they were loaded. Reactions were terminated after 5 min by addition of 2 ml of ice-cold 310 mM-sucrose/1 mM-EGTA plus ^3H_2O (0.6 μCi/ml), pH 7, and rapidly filtered through Whatman GF/C filters. The filters were washed with 5 ml of 310 mM-sucrose/1 mM-EGTA, pH 7, and then counted for radioactivity in Ecoscint-A liquid-scintillation fluid. After correction for trapped volume, $^{45}Ca^{2+}$ uptake was expressed as a fraction of ATP-dependent uptake (Taylor *et al.*, 1989).

Inhibition of specifically bound Ins(1,4,[^{32}P]5)P_3 was measured under conditions identical with those described for $^{45}Ca^{2+}$-flux measurements, except that $^{45}Ca^{2+}$ was omitted from the preincubation and the cells were rapidly diluted into medium that contained Ins(1,4,[^{32}P]5)P_3 (0.06 μCi/tube) and ^3H_2O (0.6 μCi/tube) in addition to the test compounds. After 5 min, during which equilibrium binding was attained, the reactions were terminated by rapid centrifugation (see above). Non-specific binding was estimated in the presence of 300 nM-Ins(1,4,5)P_3, and this value was used as an initial estimate for the iterative curve-fitting procedure described below. In these experiments, as in the radioligand experiments described above, there was negligible metabolism of Ins(1,4,5)P_3, because 95% of Ins(1,4,[^{32}P]5)P_3 was recovered unchanged.

Analysis of results

Results for both $^{45}Ca^{2+}$ release and inhibition of Ins(1,4,[^{32}P]5)P_3 binding were fitted to the logistic equation by using the non-linear least-squares curve-fitting routine, DRUG, in the EBDA computer program (McPherson, 1983):

$$R = \frac{(M-B)}{[1+(D/K)^h]} + B$$

where, for binding studies, R = specific Ins(1,4,[^{32}P]5)P_3 binding, M = maximum total binding in absence of competitor, B = non-specific binding, K = concn. of competitor that inhibits 50% of specific binding {= K_D in our experiments, because Ins(1,4,[^{32}P]5)P_3 is present at very low concentration; see above}, D = concn. of competitor, h = Hill coefficient; and for Ca^{2+}-release studies, R = size of remaining Ins(1,4,5)P_3-sensitive Ca^{2+} store, M = total Ca^{2+} store, B = Ins(1,4,5)P_3-insensitive Ca^{2+} store, K = concn. of inositol phosphate causing 50% release of the Ins(1,4,5)P_3-sensitive Ca^{2+} store, and h = Hill coefficient.

Inhibition of Ins(1,4,[^{32}P]5)P_3 binding to liver and cerebellar membranes was analysed by non-linear least-squares curve-fitting by the iterative computer program LIGAND (Munson & Rodbard, 1980).

RESULTS

Comparison of liver and cerebellum Ins(1,4,5)P_3-binding sites

We have previously shown that in both liver and cerebellar membranes Ins(1,4,5)P_3 binds reversibly to a single class of saturable high-affinity sites (Nunn *et al.*, 1990). In the present study we have confirmed those results and examined more closely the recognition properties of the sites in the two tissues by comparing inhibition of specific Ins(1,4,[^{32}P]5)P_3 binding by various ligands under identical conditions. In both tissues the most potent competitor was Ins(1,4,5)P_3 itself, and in both it bound with similar affinity [$K_D = 5.46 \pm 0.79$ nM ($n = 7$) in liver, and $K_D = 4.29 \pm 1.28$ nM ($n = 4$) in cerebellum (Fig. 1)], although, as previously reported (Worley *et al.*, 1987; Nunn *et al.*, 1990), there were substantially more binding sites in cerebellum ($B_{max.}$ = 13.7 pmol/mg of protein) than in liver membranes ($B_{max.}$ = 2.95 pmol/mg of protein). In both tissues the Hill coefficient for

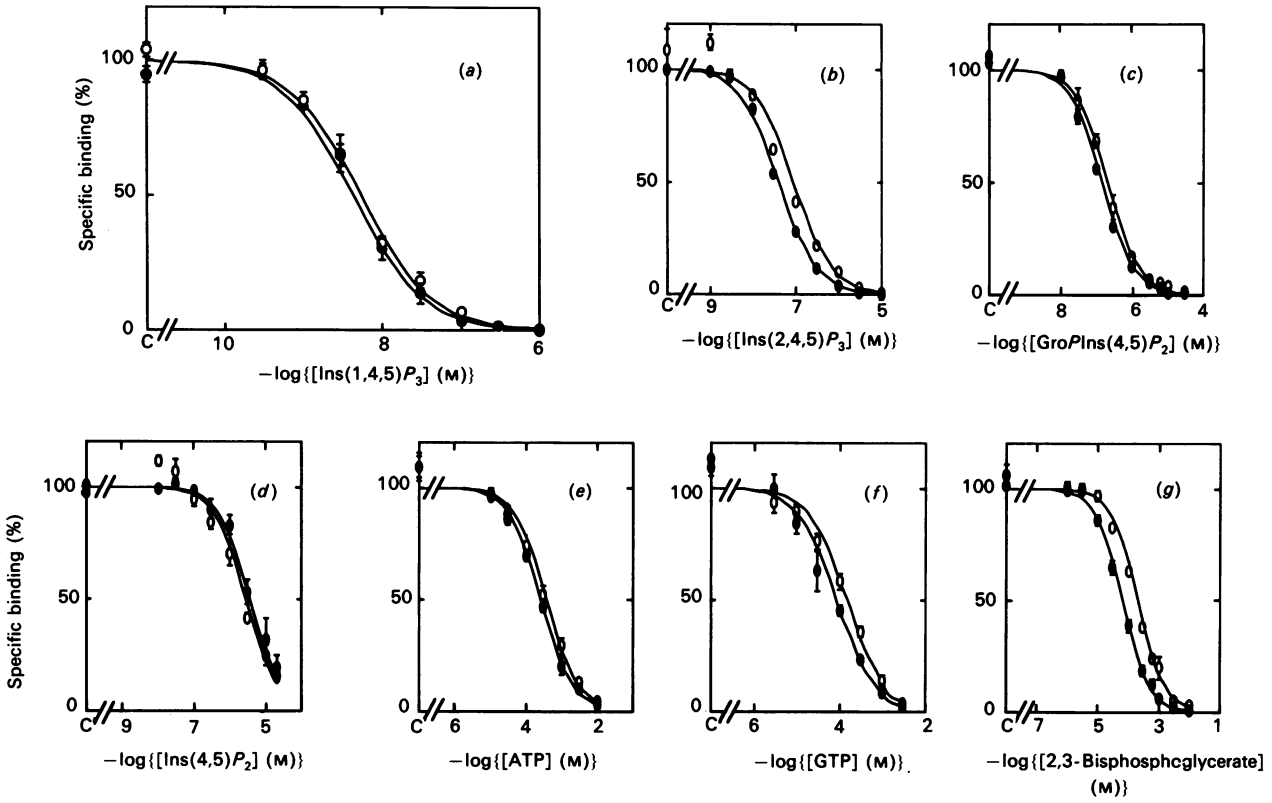

Fig. 1. Recognition properties of Ins(1,4,5)P_3-binding sites of liver and cerebellum

Specific Ins(1,4,[^{32}P]5)P_3 binding (mean ± S.E.M.; n = 3–7, see Table 1) was measured in liver (○) and cerebellum (●) as described in the text in the presence of various concentrations of (*a*) Ins(1,4,5)P_3, (*b*) Ins(2,4,5)P_3, (*c*) GroPIns(4,5)P_2 (*d*) Ins(4,5)P_2, (*e*) ATP, (*f*) GTP or (*g*) 2,3-bisphosphoglycerate. Curves were fitted by the iterative LIGAND program as described in the text. Abbreviation: C, control.

Table 1. Recognition properties of liver and cerebellar Ins(1,4,5)P_3-binding sites

The values shown are the means ± S.E.M. of the K_D values from the n individual experiments that are shown averaged in Fig. 1. Total binding was approx. 2000 c.p.m. and non-specific binding was 5–10% of total.

	K_D	
	Liver	Cerebellum
Ins(1,4,5)P_3	5.46 ± 0.79 nM (7)	4.29 ± 1.28 nM (4)
Ins(2,4,5)P_3	82.8 ± 11.9 nM (3)	33.6 ± 3.0 nM (3)
Ins(4,5)P_2	1.62 ± 0.28 μM (3)	4.13 ± 1.51 μM (3)
GroPIns(4,5)P_2	214.4 ± 40.0 nM (3)	125.4 ± 13.7 nM (3)
2,3-Bisphosphoglycerate	202.6 ± 26.7 μM (5)	59.7 ± 9.0 μM (5)
ATP	349.1 ± 46.8 μM (4)	219.1 ± 34.4 μM (4)
GTP	132.6 ± 20.0 μM (3)	70.5 ± 9.9 μM (3)

Ins(1,4,5)P_3 binding was close to 1 and further analysis with the LIGAND routine confirmed the presence of only a single class of binding site in each tissue.

In equilibrium binding studies (Fig. 1, Table 1) various inositol phosphates, Ins(2,4,5)P_3, Ins(4,5)P_2, L-α-GroPIns(4,5)P_2, and also 2,3-bisphosphoglycerate, ATP and GTP, inhibited specific high-affinity Ins(1,4,[^{32}P]5)P_3 binding in both liver and cerebellar membranes. Each of the ligands had very similar affinity for the cerebellar and liver Ins(1,4,5)P_3-binding sites (Table 1), and the Hill coefficient for each ligand in each tissue was close to 1. Heparin and low-molecular-mass heparin have previously been reported to compete with Ins(1,4,5)P_3 for its binding site, and its relatively higher affinity for the cerebellar site has been proposed to distinguish it from peripheral sites (Willcocks & Nahorski, 1989). In our experiments heparin (5–300 μg/ml) or low-molecular-mass heparin (5 μg/ml–1 mg/ml), but not desulphated heparin (1 mg/ml), caused a concentration-dependent decrease in the size of the membrane pellet formed after centrifugation. Whereas control liver membranes formed a solid pellet within 30 s of centrifugation, heparin (5 μg/ml) prevented formation of a solid pellet even when the centrifugation was increased to 15 min. Similar results were obtained with chondroitin sulphate (10 μg/ml–10 mg/ml). This unexpected problem, for which we have no explanation, prevented further analysis of the effects of glycosaminoglycans.

Effects of ATP on Ins(1,4,[^{32}P]5)P_3 binding

Previous reports (Guillemette *et al.*, 1988; Willcocks & Nahorski, 1988) and the results shown in Fig. 1(*e*) demonstrate that ATP inhibits Ins(1,4,[^{32}P]5)P_3 binding in both liver and cerebellar membranes. Equilibrium binding experiments in liver membranes, where the effects of various concentrations of ATP on Ins(1,4,5)P_3 inhibition of Ins(1,4,[^{32}P]5)P_3 binding were examined, showed that increasing concentrations of ATP did not affect the total number of Ins(1,4,5)P_3-binding sites ($B_{max.}$), but caused parallel shifts in the Ins(1,4,5)P_3 inhibition curves, indicating a competitive interaction between Ins(1,4,5)P_3 and ATP (Table 2). From the Schild equation, the affinity of ATP for the Ins(1,4,5)P_3-binding site was calculated to be 414 μM. This estimate of the affinity of ATP for the Ins(1,4,5)P_3-binding site agrees closely with the value of 349 μM determined from the equilibrium competition-binding results shown in Fig. 1(*e*). ATP (3 mM) did

Table 2. Effects of ATP on inhibition of Ins(1,4,[^{32}P]5)P_3 binding by Ins(1,4,5)P_3

The values shown are means ± S.E.M. for $K_D^{app.}$ and $B_{max.}$ for Ins(1,4,5)P_3 binding in n individual experiments.

	Ins(1,4,5)P_3 binding		
[ATP]	$K_D^{app.}$ (nM)	$B_{max.}$ (pmol/mg)	n
0	4.69 ± 0.78	2.54 ± 0.20	4
30 μM	5.99 ± 1.37	2.68 ± 0.22	4
300 μM	10.93 ± 2.45	2.53 ± 0.25	4
3 mM	40.41 ± 4.85	3.08 ± 0.29	3

not stimulate Ca^{2+} mobilization, though a much higher concentration (30 mM = 100 × K_D) stimulated some Ca^{2+} release. We have not established whether this small Ca^{2+} release is a non-specific effect of ATP or a reflection of very weak agonist activity at the Ins(1,4,5)P_3 receptor.

Ins(1,4,5)P_3-induced Ca^{2+} release and Ins(1,4,5)P_3 binding in permeabilized hepatocytes

The non-mitochondrial Ca^{2+} stores of permeabilized hepatocytes became loaded to steady state within 7 min of addition of ATP at 37 °C, and, when rapidly cooled by 10-fold dilution into medium at 0 °C, their $^{45}Ca^{2+}$ content decayed mono-exponentially ($t_{\frac{1}{2}}$ about 10 min) (results not shown). When cells were incubated at 0 °C in the presence of ATP, they did not accumulate $^{45}Ca^{2+}$, indicating that the protocol allows $^{45}Ca^{2+}$ efflux to be monitored with no contribution from ATP-dependent uptake. Cells preloaded with $^{45}Ca^{2+}$ and then diluted into ice-cold medium containing Ins(1,45)P_3, Ins(2,4,5)P_3 DL-Ins(1,4,5)P_3[S]$_3$ or Ins(4,5)P_2 released substantially more $^{45}Ca^{2+}$ than did controls (Fig. 2). The concentrations of inositol phosphates that caused half-maximal $^{45}Ca^{2+}$ release (EC_{50}) were 11.9 nM for Ins(1,4,5)P_3, 819 nM for Ins(2,4,5)P_3, 417 nM for DL-Ins(1,4,5)P_3[S]$_3$ and 7.79 μM for Ins(4,5)P_2, and the Hill coefficients were 1.57, 1.98, 0.78 and 1.67 respectively (Fig. 2, Table 3). When inhibition of Ins(1,4,[^{32}P]5)P_3 binding by these inositol phosphates was measured under identical conditions, the K_D values calculated from competition curves were 27.4 nM for Ins(1,4,5)P_3, 645 nM for Ins(2,4,5)P_3, 405 nM for Ins(1,4,5)P_3[S]$_3$ and 4.95 μM for Ins(4,5)P_2, and the Hill coefficients were 1.13, 0.86, 0.70 and 0.77 respectively (Fig. 2, Table 3). Clearly, when measured under identical conditions, for each of four inositol phosphates, the affinities of the binding site and the concentrations required for half-maximal Ca^{2+} mobilization were very similar.

Table 3. Inositol phosphate binding and Ca^{2+} mobilization in permeabilized hepatocytes

The values are derived from the results shown in Fig. 2 by non-linear least-squares curve-fitting to the logistic equation (see the text). Total binding was approx. 1500 c.p.m. and non-specific binding 30–50% of total. The considerable inhibition of specific Ins(1,4,[^{32}P]5)P_3 binding by the ATP needed to promote $^{45}Ca^{2+}$ accumulation (see the text) is the cause of the low ratio of total/non-specific binding. In non-Ins(1,4,5)P_3-treated samples, $^{45}Ca^{2+}$ content was approx. 1300 c.p.m. (after correction for trapped volume and pre-ATP-dependent $^{45}Ca^{2+}$ content), and Ins(1,4,5)P_3 maximally stimulated a release of about 55% of this value.

	Binding K_D (h)	Ca^{2+} release EC_{50} (h)
Ins(1,4,5)P_3	27.4 nM (1.17)	11.9 nM (1.72)
DL-Ins(1,4,5)P_3[S]$_3$	405 nM (0.96)	417 nM (1.47)
Ins(2,4,5)P_3	645 nM (1.05)	819 nM (2.22)
Ins(4,5)P_2	4.95 μM (1.18)	7.79 μM (1.25)

DISCUSSION

We have previously demonstrated that the molecular target sizes of the Ins(1,4,5)P_3-binding sites in liver and cerebellum are identical (250 kDa) (Nunn *et al.*, 1990) and very similar to the molecular size of the monomeric purified cerebellar Ins(1,4,5)P_3 receptor measured by SDS/polyacrylamide-gel electrophoresis (260 kDa) (Supattapone *et al.*, 1988). Here we have extended the comparison and have shown that seven different ligands, agonists and antagonists, bind with similar affinity to the Ins(1,4,5)P_3-binding sites of liver and cerebellum. Heparin, which others have

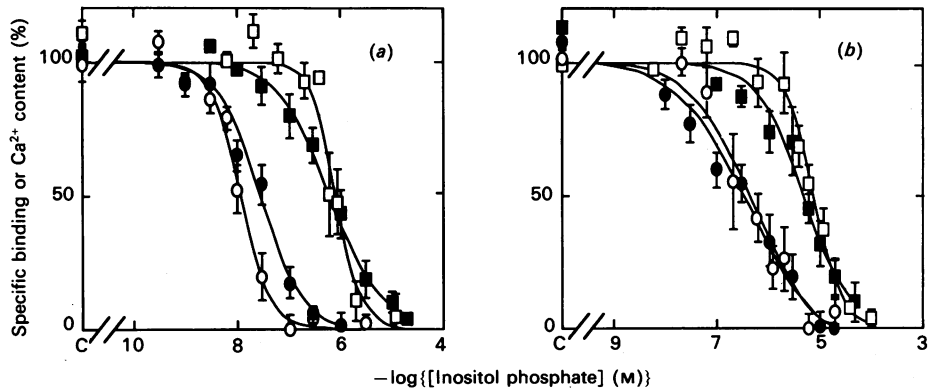

Fig. 2. Ins(1,4,5)P_3 binding and Ca^{2+} release under identical conditions

Permeabilized cells preincubated at 37 °C in the presence of 1.5 mM-ATP were diluted into ice-cold medium containing (*a*) Ins(1,4,5)P_3 (○, ●) or Ins(2,4,5)P_3 (□, ■) or (*b*) DL-Ins(1,4,5)P_3[S]$_3$ (○, ●) or Ins(4,5)P_2 (□, ■) as described in the text. Specific Ins(1,4,[^{32}P]5)P_3 binding (%) (●, ■) or the percentage of the Ins(1,4,5)P_3-releasable Ca^{2+} stores remaining (○, □) were analysed for each individual experiment by non-linear curve-fitting to the logistic equation. Results are shown as means ± S.E.M. (n = 6–8), and the curves are fitted to these means by the logistic equation as described in the text. Abbreviation: C, control.

convincingly shown to bind to the Ins(1,4,5)P_3 receptor (Worley *et al.*, 1987; Ghosh *et al.*, 1988), prevented formation of solid membrane pellets after centrifugation of both liver and cerebellar membranes in our experiments. Such non-specific effects suggest that apparent differences in the affinity of Ins(1,4,5)P_3-binding sites for heparin between tissues be interpreted with caution. We conclude from our earlier radiation-inactivation target-size analyses (Nunn *et al.*, 1990) and from this comparison of the ligand-recognition properties of cerebellar and liver Ins(1,4,5)P_3-binding sites that there is at present no convincing evidence to suggest differences between these two proteins.

ATP has been reported to inhibit Ins(1,4,5)P_3 binding in various tissues, including cerebellum (Willcocks & Nahorski, 1988; Ghosh *et al.*, 1989; but see Worley *et al.*, 1987), but the interaction between ATP and the Ins(1,4,5)P_3-binding site has not been further characterized. We have shown that in liver ATP is a competitive antagonist and have obtained very similar estimates of its affinity for the Ins(1,4,5)P_3-binding site when measured directly by inhibition of Ins(1,4,[^{32}P]5)P_3 binding (K_D = 349 μM) or indirectly by Schild analysis of its effects in shifting Ins(1,4,5)P_3 inhibition of Ins(1,4,[^{32}P]5)P_3 binding (K_D = 414 μM). GTP and 2,3-bisphosphoglycerate also inhibit Ins(1,4,[^{32}P]5)P_3 binding with similar affinities (K_D = 133 μM and 203 μM respectively in liver), but we have not proved that their interactions are competitive.

ATP, GTP and 2,3-bisphosphoglycerate are normal intracellular constituents; their interactions with the Ins(1,4,5)P_3 receptor may therefore substantially decrease its sensitivity to Ins(1,4,5)P_3. In many cells, estimates of the resting intracellular Ins(1,4,5)P_3 concentration are substantially higher than the concentration needed to stimulate Ca^{2+} mobilization *in vitro*. In pancreatoma cells, for example, the intracellular Ins(1,4,5)P_3 concentration increases from 2 μM at rest to about 25 μM after stimulation, yet in permeabilized cells the expected EC_{50} for Ins(1,4,5)P_3-induced Ca^{2+} mobilization is about 0.4 μM (Horstman *et al.*, 1988). Compartmentalization of intracellular Ins(1,4,5)P_3 could explain its relative inability to mobilize Ca^{2+}, but the effects of endogenous competing ligands such as ATP provide another explanation, because these could substantially shift the operating range of the Ins(1,4,5)P_3 receptor in intact cells. We conclude that, if measurements of intracellular Ins(1,4,5)P_3 concentration are to be related to mobilization of Ca^{2+} stores, it is essential to establish the sensitivity of the Ins(1,4,5)P_3 receptor *in vivo*.

We have attempted to establish whether the high-affinity Ins(1,4,5)P_3-binding sites of liver are the receptors that mediate Ins(1,4,5)P_3-induced Ca^{2+} mobilization, by measuring binding and Ca^{2+} release under identical conditions. In earlier work, where the measurements were made under very different conditions, the concentration of inositol phosphate needed to occupy half the binding sites (K_D) was substantially less than the concentration that caused half-maximal Ca^{2+} release (EC_{50}). Part of this discrepancy results from the absence of ATP from most binding assays (see above), and the results shown in Fig. 2 establish that the remaining discrepancy disappears when the two measurements are made under identical conditions. For four agonists, differing by almost 1000-fold in their relative potencies, the K_D for the specific Ins(1,4,5)P_3-binding site and the EC_{50} for Ca^{2+} mobilization were very similar. These results confirm and extend previous reports by Mauger *et al.* (1989), in which specific Ins(1,4,[^{32}P]5)P_3 binding and Ins(1,4,5)P_3-induced Ca^{2+} mobilization were measured under similar conditions and found to have similar sensitivity to Ins(1,4,5)P_3. Our present results, together with those of Mauger *et al.* (1989) and those reported previously (Nunn *et al.*, 1990), provide substantial evidence that high-affinity Ins(1,4,5)P_3-binding sites in liver are the recognition sites of the receptor that mediates the effects of Ins(1,4,5)P_3 on intracellular Ca^{2+} stores.

Although the concentrations of inositol phosphates required for half-maximal binding and half-maximal Ca^{2+} mobilization were similar when measured under identical conditions, the Hill coefficients of the two measurements were not. Inhibition of specific Ins(1,4,[^{32}P]5)P_3 binding by each of the four agonists had Hill coefficients close to 1, whereas Ca^{2+} mobilization was usually co-operative. It has been argued that the co-operative increases in net Ca^{2+} flux evoked by Ins(1,4,5)P_3 in other cells (Meyer *et al.*, 1988; Champeil *et al.*, 1989) may not result from co-operative opening of Ca^{2+} channels, but may be an artefact resulting from the proportionally greater ATP-dependent refilling of Ca^{2+} stores after stimulation with low Ins(1,4,5)P_3 concentrations (Champeil *et al.*, 1989). In our experiments most agonists evoked co-operative Ca^{2+} release under conditions where re-accumulation of Ca^{2+} by intracellular stores could not occur. We conclude, in agreement with the proposal by Meyer *et al.* (1988), that Ins(1,4,5)P_3 (and other inositol phosphates) co-operatively open a Ca^{2+} channel. A molecular understanding of how non-co-operative binding of Ins(1,4,5)P_3 to its receptor leads to co-operative opening of a Ca^{2+} channel may follow when electrophysiological recordings from reconstituted receptors provide the necessary temporal resolution.

Our earlier evidence demonstrated that the liver Ins(1,4,5)P_3-binding site is distinct from the substrate-binding sites of the two enzymes that metabolize Ins(1,4,5)P_3 (Nunn *et al.*, 1990). This, the earlier work by Mauger *et al.* (1989) and our present data, showing that similar concentrations of inositol phosphates cause half-maximal occupancy of the binding site and half-maximal Ca^{2+} mobilization, provide persuasive evidence that the Ins(1,4,5)P_3-binding site of liver is the physiological receptor that mediates the effects of Ins(1,4,5)P_3 on intracellular Ca^{2+} stores. Furthermore, we can at present find no evidence to distinguish the liver Ins(1,4,5)P_3-binding site from the cerebellar protein that has been purified and functionally reconstituted.

This work was supported by the Medical Research Council (M.R.C.). D.L.N. is supported by a M.R.C. Research Studentship. We thank Dr. Ian Martin and Dr. Mike Young for advice, and Dr. Barry Potter and Dr. Robin Irvine for gifts of DL-Ins(1,4,5)P_3[S]$_3$ and Ins(1,4,5)P_3.

REFERENCES

Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
Berridge, M. J. & Irvine, R. F. (1989) Nature (London) **341**, 197–205
Bradford, M. (1976) Anal. Biochem. **72**, 248–254
Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S. & Claret, M. (1989) J. Biol. Chem. **264**, 17665–17673
Ferris, C. D., Huganir, R. L., Supattapone, S. & Snyder, S. H. (1989) Nature (London) **342**, 87–89
Furuichi, T., Yoshikawa, S., Miyawake, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) Nature (London) **342**, 32–38
Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L. & Gill, D. L. (1988) J. Biol. Chem. **263**, 11075–11079
Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. (1989) Nature (London) **340**, 236–239
Guillemette, G., Balla, T., Baukal, A. J., Spät, A. & Catt, K. J. (1987) J. Biol. Chem. **262**, 1010–1015
Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1988) J. Biol. Chem. **263**, 4541–4548
Horstman, D. A., Takemura, H. & Putney, J. W., Jr. (1988) J. Biol. Chem. **263**, 15297–15303
Joseph, S. K. & Rice, H. L. (1989) Mol. Pharmacol. **35**, 355–359
Mauger, J.-P., Claret, M., Pietri, F. & Hilly, M. (1989) J. Biol. Chem. **264**, 8821–8826
McPherson, G. A. (1983) Comput. Programs Biomed. **17**, 107–114

Meyer, T., Holowka, H. & Stryer, L. (1988) Science **240**, 653–656
Muallem, S., Schoeffield, M., Pandol, S. & Sachs, G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4433–4437
Munson, P. J. & Rodbard, D. (1980) Anal. Biochem. **107**, 220–239
Nahorski, S. R. & Potter, B. V. L. (1989) Trends Pharmacol. Sci. **10**, 139–144
Nunn, D. L., Potter, B. V. L. & Taylor, C. W. (1990) Biochem. J. **265**, 393–398
Prpić, V., Green, K. C., Blackmore, P. F. & Exton, J. H. (1984) J. Biol. Chem. **259**, 1382–1385
Spät, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W., Jr. (1986) Nature (London) **319**, 514–516
Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988) J. Biol. Chem. **263**, 1530–1534
Taylor, C. W., Berridge, M. J., Cooke, A. M. & Potter, B. V. L. (1989) Biochem. J. **259**, 645–650
Willcocks, A. L. & Nahorski, S. R. (1988) Biochem. J. **255**, 1061
Willcocks, A. L. & Nahorski, S. R. (1989) Br. J. Pharmacol. **96**, 364*P*
Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987) J. Biol. Chem. **262**, 12132–12136

Received 15 March 1990/2 May 1990; accepted 4 May 1990