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Binding sites for α-trinositol (inositol 1,2,6-trisphosphate) in porcine tissues; comparison with Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4-binding sites

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1 The molecular mechanism of action of the inositol trisphosphate isomer, α-trinositol (Ins(1,2,6)P_3) which has potential therapeutic use in treatment of inflammation and burn oedema, is still unclear. Therefore we have studied binding sites for α-trinositol in different tissues.

2 In membranes from pig cerebellum, liver, kidney, heart, and spleen, the density of specific [^{3}H]-α-trinositol binding sites was maximal at pH 5.0. Cerebellum and spleen showed only one binding site (cerebellum K_D = 9.1 μM, spleen K_D = 7.3 μM). In the other tissues, there were a high-affinity site (heart K_D = 70 nM, liver K_D = 790 nM and kidney K_D = 1800 nM), besides a low-affinity site with a K_D ranging between 32 and 120 μM. In cerebellar membranes, the affinity and density (107 pmol mg^{-1} protein) of α-trinositol binding sites were not affected by phosphate (0 to 25 mM).

3 Binding of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 to membranes from different porcine tissues was also determined. Ins(1,3,4,5)P_4, the isomer stereochemically related to α-trinositol, binds with an affinity of 1.2 nM in cerebellum, but in the other tissues the binding site density was too low to determine the affinity. With cerebellar membranes heterologous displacement of [^{3}H]-Ins(1,3,4,5)P_4 by α-trinositol yielded a K_I of 11 μM. The Ins(1,4,5)P_3 receptor displayed an affinity of 15 nM in cerebellum and of 5 to 7 nM in the other tissues investigated.

4 The solubilized Ins(1,3,4,5)P_4 receptor preparation from cerebellum did not show Ins(1,2,6)P_3 binding. Ins(1,2,6)P_3 binding was found in the pellet obtained after solubilization of the membranes with the detergent Brij 58.

5 Thus, in different tissues α-trinositol binds to proteins with different affinity. They are obviously not related to binding sites for Ins (1,4,5)P_3 or for Ins(1,3,4,5)P_4. Future experiments have to unravel the identity of the binding protein(s) for α-trinositol.

Keywords: Inositolphosphate; inositolphosphate binding; α-trinositol

Introduction

Inositololigophosphates are thought to have a function only as intracellular signal molecules (Berridge, 1993; Shears, 1992). There is, however, evidence, albeit sparse, for an extracellular role of inositolphosphates. $InsP_5$ (Inositol 1,3,4,5,6-pentakisphosphate) and $InsP_6$ have been described as modulating Ca^{2+} influx in cultured neurones (Nicoletti *et al.*, 1989; Regunathan *et al.*, 1992) and it has been suggested that they influence cardiovascular regulation in the central nervous system (Vallejo *et al.*, 1987). Ins(1,2,6)P_3 (α-trinositol; D-*myo*-inositol 1,2,6-trisphosphate), which is produced by partial degradation of phytic acid by yeast phytase (Goldschmidt, 1990), also has physiological effects. For example, α-trinositol blocks vasoconstriction produced by neuropeptide Y, but not by other constrictors (Potter *et al.*, 1992). α-Trinositol appears to be a functional antagonist of neuropeptide Y (Wahlestedt *et al.*, 1992), but certainly does not act as a competitive antagonist (Wahlestedt & Reis, 1994). The effects of α-trinositol on cardiac and regional hemodynamics have been studied in conscious rats (Gardiner *et al.*, 1994).

In addition, α-trinositol has potent anti-inflammatory activity, and has been shown to reduce inflammation in several animal models (Claxson *et al.*, 1990; Lund & Reed, 1994; Nakazawa *et al.*, 1994). So far, the molecular mechanism of action of α-trinositol is unclear. It is to be expected that one receptor protein exists (or several different types) specific for α-trinositol-mediated actions. Even though α-trinositol has not been shown to be a natural constituent of mammalian cells, interactions with intracellular receptors for endogenous inositololigophosphates are possible.

Here we attempted to determine α-trinositol binding sites in membranes from various pig tissues, and to compare these with the receptors for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. The steric orientation of the phosphate groups of Ins(1,2,6)P_3 resembles that of Ins(1,3,4,5)P_4. Therefore the α-trinositol binding characteristics were studied mainly in the context of properties of Ins(1,3,4,5)P_4 receptors identified by our (Reiser *et al.*, 1991; Reiser, 1993) and other laboratories (Theibert *et al.*, 1992; Cullen *et al.*, 1995a,b) and with regard to the possible physiological functions of Ins(1,3,4,5)P_4.

Since α-trinositol has physiological functions when tested in animal models, we can assume that some of the binding sites identified in different tissues represent functional receptors. This issue will only be resolved when the physiological response or, at least the second messenger system activated by α-trinositol, has been identified.

Methods

Membrane preparation

The tissues (pig cerebellum, spleen, liver, kidney and heart) were collected within 15 min after death of the animal, dissected free of connective tissue and stored at −80°C. For

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membrane preparation the tissue samples were thawed and homogenized in buffer containing 50 mM Tris/HCl, pH 7.7, 1 mM EDTA, and 1 mM mercaptoethanol, at a ratio of 4 ml of buffer per g of tissue, with a Waring Blendor (medium setting, 3 × 20 s). The pellet obtained by centrifugation (2,000 *g*, Kontron A8.24, 10 min) was re-homogenized and centrifuged (2,000 *g*, 10 min). Both supernatants were combined and centrifuged (35,000 *g*, 30 min). After resuspension of the pellet in homogenization buffer containing 400 mM NaCl, the homogenate was poured through cheese cloth and the microsomes were sedimented by centrifugation (35,000 *g*, 30 min). The resulting membranes were washed twice in homogenization buffer by resuspension and centrifugation (30 min, 35,000 *g*). The final pellet was suspended in homogenization buffer by use of a Potter-Elvehjem homogenizer at a protein concentration of 5 to 20 mg ml^{-1} and kept at −20°C.

Solubilization of inositolphosphate binding proteins

The Ins(1,3,4,5)P_4 receptor protein was solubilized as described (Donié *et al.*, 1990; Reiser, 1993). Frozen membranes were thawed and centrifuged (10 min, 4°C, 27,000 *g*). The pellet was mixed with Tris-buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM mercaptoethanol) supplemented with 15 g l^{-1} Brij 58 and 400 mM NaCl in a ratio of 4 ml per g wet weight of membranes and stirred for 1 h at 4°C. After centrifugation for 1 h at 100,000 *g* (Kontron TST 28.38), the supernatant was dialyzed overnight twice against 2,000 ml Tris-buffer. In some experiments, the resulting pellet was resuspended inTris-buffer at a protein concentration of 5 to 10 mg ml^{-1} and also used for binding experiments. Protein was determined by the Lowry method with bovine serum albumin (BSA) as standard.

Binding assays

For determining binding to membranes, incubations were carried out in a final volume of 400 μl in microcentrifuge tubes at 4°C. Microsomes (0.3 to 1 mg of protein/assay) were incubated in a solution containing 2.4 to 3.2 nM [^{3}H]-Ins(1,2,6)P_3 or 0.3 to 0.6 nM [^{3}H]-Ins(1,3,4,5)P_4, in three different buffers (A, B and C). These buffers have been used by different laboratories for assaying Ins(1,3,4,5)P_4 binding activity. The purpose of the present study was not a systematical analysis of influence of pH on binding. This has already been described for Ins(1,3,4,5)P_4 binding activity (Donié *et al.*, 1990). We checked for the alledged optimum binding at a pH close to a physiological value and at salt concentrations mimicking the intracellular milieu (Cullen & Irvine, 1992). For these reasons, buffers B and C (Cullen & Irvine, 1992) were employed as well as buffer A, found optimal by Donié *et al.* (1990) and by Walsh *et al.* (1995). Buffer A contained 25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.25% (w/v) BSA, 1 mM EDTA (Donié & Reiser, 1989; Challiss *et al.*, 1991). Buffer B contained 100 mM KCl, 20 mM NaCl, 10 mM HEPES/KOH, pH 7.0, 0.25% (w/v) BSA, 1 mM EDTA. Buffer C contained 100 mM KCl, 20 mM NaCl, 10 mM MES/KOH, pH 5.5, 0.25% (w/v) BSA, 1 mM EDTA (Cullen & Irvine, 1992).

For Ins(1,4,5)P_3, binding, microsomes (0.4 to 1.5 mg of protein/assay) were incubated in buffer containing 0.2 to 0.4 nM [^{3}H]-Ins(1,4,5)P_3, 0.1% (w/v) BSA, 1 mM EDTA and 25 mM Tris/HCl, pH 9.0. Nonspecific binding was determined in the presence of 1 μM Ins(1,3,4,5)P_4, 1 μM Ins(1,4,5)P_3, or 0.5 to 1 mM Ins(1,2,6)P_3, respectively. The samples were incubated in the presence of different concentrations of unlabelled Ins(1,2,6)P_3, Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 for 20 to 30 min, and bound ligand was separated from free ligand by centrifugation (10,000 *g*, 5 min). The supernatants were discarded and the pellets were solubilized with 2% (w/v) SDS before transferring into scintillation vials for measuring the bound radioactive tracer.

Binding of Ins(1,3,4,5)P_4 to the solubilized receptor protein was determined as described previously (Donié *et al.*, 1990; Reiser, 1993). Solubilized membrane protein (350 to 600 μg) was assayed in 280 μl binding medium composed of 0.4 nM [^{3}H]-Ins(1,3,4,5)P_4, 25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05% (w/v) Brij 58, 1 mM EDTA, and 0 to 200 nM unlabelled Ins(1,3,4,5)P_4. Nonspecific binding was determined in the presence of 1 μM Ins(1,3,4,5)P_4. Binding was allowed to proceed for 20 min at 4°C. Then an aliquot of the reaction mixture was centrifuged through columns containing 1 ml packed resin of BioGel P-4 (grade fine 65 μm; Bio-Rad, München, Germany) to separate bound from free ligand. In both cases (assaying binding either to membranes or to solubilized proteins) 4 ml of scintillation fluid (Ultima Gold, Packard) was added for measurement of the bound radioactivity (Rackbeta 1214, LKB). For each experiment all determinations were made in triplicate and showed less than 10% deviation.

Materials

The sodium salt of [^{3}H]-Ins(1,2,6)P_3 (7 Ci $mmol^{-1}$, or 45 Ci $mmol^{-1}$) and Ins(1,2,6)P_3 (sodium salt) was provided by Perstorp Pharma (Lund, Sweden). [^{3}H]-Ins(1,3,4,5)P_4 (40 Ci $mmol^{-1}$), [^{3}H]-Ins(1,4,5)P_3 (54 Ci $mmol^{-1}$) and Ins(1,4,5)P_3 (potassium salt) were obtained from Amersham (Braunschweig; Germany). Ins(1,3,4,5)P_4 (potassium salt) was purchased from Calbiochem (Bad Soden; Germany). Brij 58 was from Serva (Heidelberg, Germany).

Data analysis

K_D values and the number of binding sites were estimated by use of the RADLIG data-analysis computer programme (Version 4; BIOSOFT, Cambridge, U.K.). Free ligand was calculated from the concentration of total bound ligand and curve fitting was done by using total binding (i.e. nonspecific binding is not subtracted by the programme before curve-fitting and can be estimated by the programme). The data were weighted assuming a constant percentage of error in the measured binding (i.e. $1/Y^2$). For statistical analysis the *F*-test was employed.

Results

The first aim of the present study was a comparison of binding sites for α-trinositol and for Ins(1,3,4,5)P_4. Binding of Ins(1,3,4,5)P_4 has been investigated by different authors using buffers with different pH and ionic composition (Cullen & Irvine, 1992; Donié & Reiser, 1989; Theibert *et al.*, 1987). In initial experiments we used these three buffer systems to investigate their influence on binding of [^{3}H]-Ins(1,3,4,5)P_4 and [^{3}H]-Ins(1,2,6)P_3 in membranes from different porcine tissues. A comparison using three different buffer systems shown in Figure 1a demonstrates that binding of [^{3}H]-Ins(1,3,4,5)P_4 to membranes from cerebellum and spleen was strongly influenced by the ionic composition and pH of the buffer system, whereas in the other tissues tested there was only a marginal effect. The apparently high density of binding sites in spleen was further investigated. We found that there was no displacement at concentrations of unlabelled Ins(1,3,4,5)P_4 up to 200 nM. Moreover, 70–80% of total [^{3}H]-Ins(1,3,4,5)P_4 bound could be displaced by 50 μM of the unlabelled compound (data not shown). Displacement curves indicated the presence of one binding site with a medium affinity in spleen (K_D = 970 nM, B_{max} = 251 ± 96 pmol mg^{-1} of protein; 3 experiments). Because in liver, heart and kidney only low densities of Ins(1,3,4,5)P_4-binding sites could be detected (Figure 1a), no attempts at further characterization were made.

[^{3}H]-Ins(1,2,6)P_3 binding to membranes was highest when a low-ionic strength acetate-based buffer, pH 5.0 was used (Figure 1b). In contrast to the results obtained with Ins(1,3,4,5)P_4, in all tissues investigated, a high density of α-trinositol binding sites in the range of 30 to 60 fmol mg^{-1} protein was detected. Therefore, characterization of the un-

derlying binding sites was possible. In Figure 2 the displacement curves obtained for membranes from cerebellum (a), spleen (b), liver (c), kidney (d) and heart (e) are shown.

In Table 1, the analysis of α-trinositol binding characteristics in different pig tissues is summarized. The results from a one-site analysis and from a two-site analysis using the same set of data are juxtaposed to give the statistical significance of one or the other model. Displacement of binding of radioactive α-trinositol by unlabelled α-trinositol shows that in spleen (Figure 2b) there was only one class of binding sites with an affinity which was between 5 and 15 μM. In cerebellum (Figure 2a) there was one class of binding sites with $K_D = 9.1$ μM. The second binding site derived from the analysis with 2 binding sites cannot be accepted because it had a K_D above the concentration used for determining non-specific binding (cf. Table 2). However, the binding data obtained for membranes from heart (Figure 2e), liver (Figure 2c) and kidney (Figure 2d) were best described by a two-site model ($P < 0.05$ compared with a one-site fit; Table 1). In heart there was clearly a high-affinity binding site with a K_D in the range of 70 nM. In kidney and liver the affinity of the first binding site ranged between 0.70 and 1.8 μM. In these tissues a second, low-affinity binding site with a K_D in the upper micromolar range was found (Table 1).

For comparison, Ins(1,4,5)P_3 binding was also assessed using the membranes from porcine cerebellum, spleen, liver, and kidney (Figure 3) in which α-trinositol and $InsP_4$-binding have also been studied. The data obtained with Ins(1,4,5)P_3 are best described by a one-binding site model for the membranes from all tissues investigated (Figure 3). The dissociation constant for Ins(1,4,5)P_3 in cerebellar membranes (15.5 ± 0.6 nM) was approximately three times higher than the values obtained for spleen, liver and kidney (6.5 ± 0.65, 5.3 ± 0.48 and 6.4 ± 0.64 nM respectively). Another striking difference between the various tissues was that in cerebellum the density of Ins(1,4,5)P_3-binding sites (3.64 ± 0.15 pmol mg^{-1}) was significantly higher than in spleen (787 ± 71 fmol mg^{-1}), liver (343 ± 24 fmol mg^{-1}) and in kidney (345 ± 28 fmol mg^{-1}).

A detailed comparison of the binding of [^{3}H]-Ins(1,3,4,5)P_4 and [^{3}H]-Ins(1,2,6)P_3 was carried out with membranes from cerebellum. In Figure 4 the homologous displacement of [^{3}H]-Ins(1,3,4,5)P_4 and [^{3}H]-Ins(1,2,6)P_3 by the unlabelled compound and the heterologous displacement of [^{3}H]-Ins(1,3,4,5)P_4 by Ins(1,2,6)P_3 are shown. The experimental incubations were carried out using the same batch of microsomes in acetate-based buffer, pH 5.0, which yielded maximal binding capacity (Figure 1). Analysis of the Ins(1,3,4,5)P_4

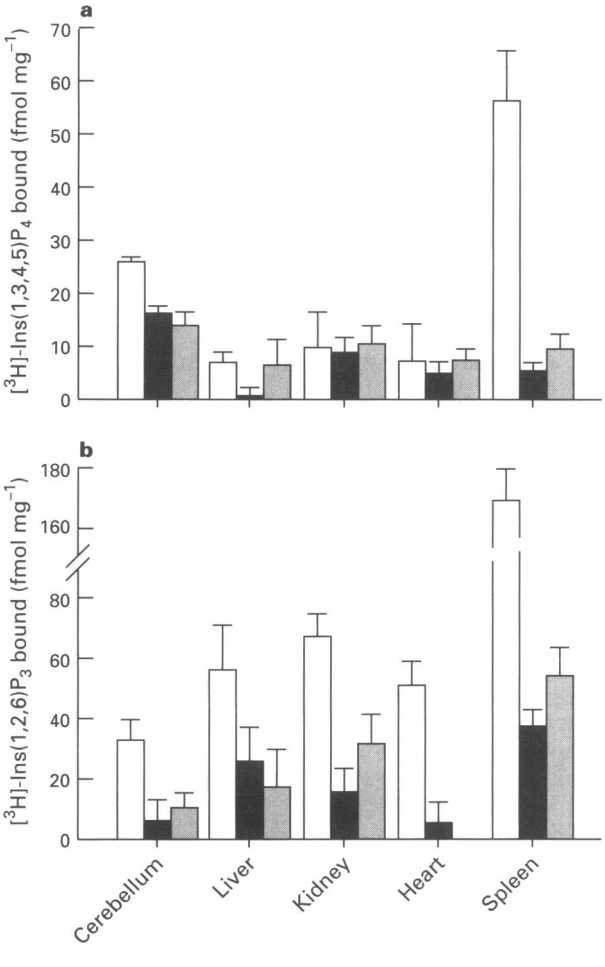

Figure 1 Specific binding of [^{3}H]-Ins(1,3,4,5)P_4 (a) and [^{3}H]-Ins(1,2,6)P_3 (b) in various tissues. Membranes prepared from the different tissues indicated (500 μg of protein each) were incubated in the presence of 0.7 nM [^{3}H]-Ins(1,3,4,5)P_4 (25000 d.p.m. per assay) or 2.8 nM [^{3}H]-Ins(1,2,6)P_3 (18000 d.p.m. per assay) at pH 5.0 (open columns), pH 5.5 (solid columns) or pH 7.0 (cross-hatched columns) using the buffer systems described under Methods. Nonspecific binding was determined in the presence of 1 μM Ins(1,3,4,5)P_4 and 1 mM Ins(1,2,6)P_3, respectively. Error bars are unusually large because they represent the combined errors of total binding and of non-specific binding.

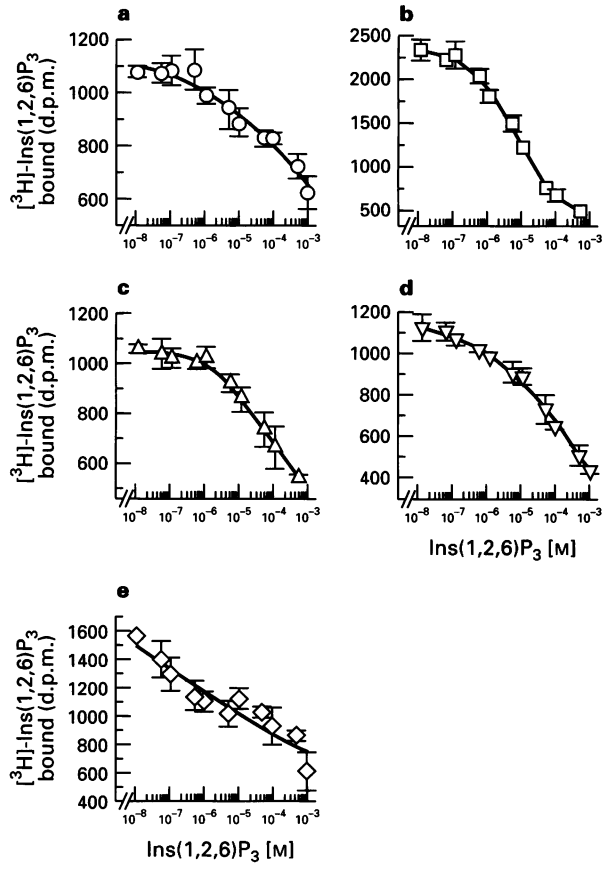

Figure 2 α-Trinositol-binding in membranes from pig cerebellum (a), spleen (b), liver (c), kidney (d) and heart (e). [^{3}H]-Ins(1,2,6)P_3 (α-trinositol, 2.9 nM) was used with membranes from different tissues (500 μg of protein per assay) in 25 mM sodium acetate/25 mM potassium-phosphate buffer, pH 5.0. For non-specific binding, 1 mM (a, d, e) or 0.5 mM Ins(1,2,6)P_3 (b, c) were added, respectively. Data analysis of such displacement curves by the Radlig programme for the presence of one or two binding sites is shown in Table 1. All determinations were made in triplicate. The standard deviations (usually less than 10%) are indicated by the error bars only when they exceed the size of the symbol used. The curves are drawn in the plots according to 4-parameter logistic fits to the data points shown, assuming one binding site.

Table 1 Binding of Ins(1,2,6)P_3 to microsomes from different tissues

	1 Binding site		*2 Binding sites*				
Tissue	K_d (μM)	B_{max} (pmol mg^{-1})	K_{d1} (μM)	B_{max1} (pmol mg^{-1})	K_{d2} (μM)	B_{max2} (nmol mg^{-1})	P
Cerebellum	9.1±2.1	106.7±27.1	5.1±2.8	48.6±36.3	1268*	16.9*	$P=0.014$
Spleen	7.3±3.2	203±91	0.57*	7.8*	11.7±19.9	0.25±0.25	$P=0.683$
Heart	1.6±0.7	39.6±16.2	0.07±0.05	1.4±0.9	32.0±21.7	0.52±0.36	$P=0.002$
Kidney	12.7±4.0	325±71	1.8±0.9	26.4±16.2	100.8±16.2	1.7±0.8	$P=0.000$
Liver	47.8±11.2	1241±391	0.79±0.56	6.9±5.7	122.7±44.1	2.7±1.0	$P=0.000$

Displacement of binding of [^{3}H]-Ins(1,2,6)P_3 (1.1 nM, 45 Ci $mmol^{-1}$ or 2.1 to 3.3 nM, 7 Ci $mmol^{-1}$) by unlabelled Ins(1,2,6)P_3, using membranes from different tissues (0.5 to 1 mg per assay) in 25 mM sodium acetate/25 mM potassium phosphate buffer, pH 5.0. Nonspecific binding was determined in the presence of 1 mM Ins(1,2,6)P_3, in some cases 0.5 mM. The data were analyzed for the presence of one or two binding sites with the LIGAND component of the Radlig programme by a combined fit of the results from 4 (cerebellum) or 3 (all other tissues) independent experiments (cf. Figure 2). For each experiment at least 10 concentrations were tested in triplicate. The values given are the final estimates of the parameters K_D and B_{max} and the associated standard errors calculated by the curve-fitting programme. *gives a statistical standard error exceeding the estimated parameter by more than 200%.

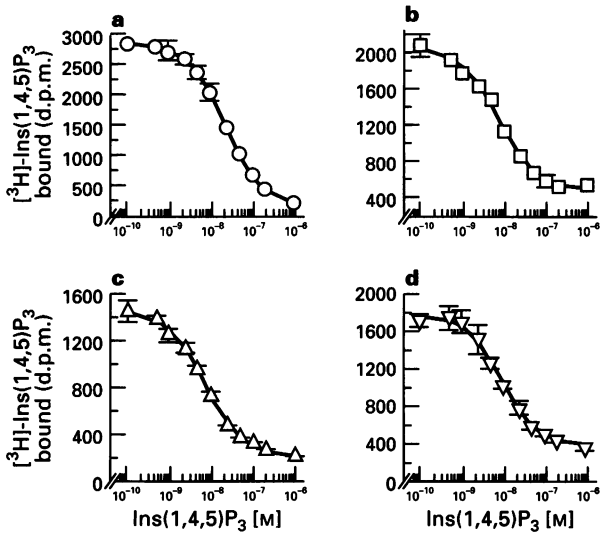

Figure 3 Binding of Ins(1,4,5)P_3 to microsomes from pig cerebellum (a), spleen (b), liver (c) and kidney (d). Displacement of [^{3}H]-Ins(1,4,5)P_3 binding to membranes from various tissues by unlabelled Ins(1,4,5)P_3 was determined in Tris-buffer, pH 9.0. Membranes (0.45 to 1.3 mg of protein) were incubated with 0.22 to 0.27 nM [^{3}H]-Ins(1,4,5)P_3 (11000–13000 d.p.m. per assay) and varying concentrations of unlabelled Ins(1,4,5)P_3. Nonspecific binding was determined in the presence of 1 μM Ins(1,4,5)P_3. The data were analyzed with the Radlig programme for the presence of one or two binding sites. In all experiments, there was no evidence for a two binding site model ($P>>0.05$). It was not possible to analyze the displacement data for heart microsomes because the difference between the amount of maximally bound tracer (approx. 3.8 fmol) and nonspecific bound tracer (approx. 2.3 fmol) in the presence of 1.5 mg protein was too small.

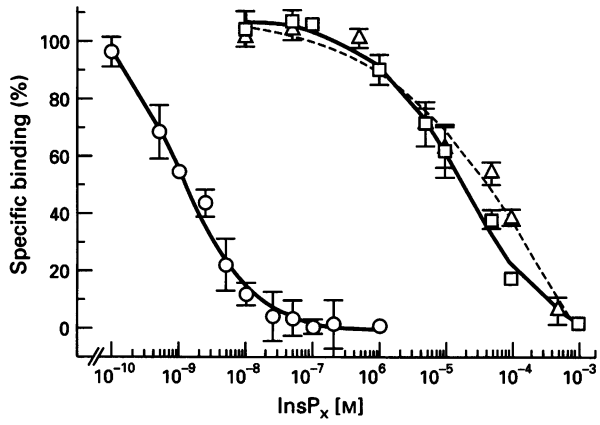

Figure 4 Displacement of binding of [^{3}H]-Ins(1,3,4,5)P_4 by unlabelled Ins(1,3,4,5)P_4 (○) or Ins(1,2,6)P_3 (□), and displacement of binding of [^{3}H]-Ins(1,2,6)P_3 by unlabelled Ins(1,2,6)P_3 (Δ) in pig cerebellum. Membranes from pig cerebellum (380 to 570 μg of protein) were incubated with 0.36 nM [^{3}H]-Ins(1,3,4,5)P_4 or 2.1 nM [^{3}H]-Ins(1,2,6)P_3 corresponding to ≈ 13000 d.p.m. per assay and the indicated concentrations of ligands in acetate-phosphate buffer, pH 5.0, on ice for 20 min. The binding reaction was terminated by centrifugation. Nonspecific binding was determined in the presence of 1 μM Ins(1,3,4,5)P_4 (○, 5 experiments) or 1 mM Ins(1,2,6)P_3 (□, 3 experiments; Δ, 4 experiments). Total binding and nonspecific binding were 1110 d.p.m./399 d.p.m. (○); 927 d.p.m./208 d.p.m. (□), and 991 d.p.m./707 d.p.m. (Δ), respectively. The curves shown were calculated according to a 4-parameter logistic function, assuming one binding site.

binding data in Figure 4 gave one class of binding sites with a dissociation constant of 1.2±0.2 nM and B_{max} of 96.5±11.6 fmol mg^{-1}. Using these values, we obtained a K_I of 10.9±3.4 μM for the heterologous displacement of [^{3}H]-Ins(1,3,4,5)P_4 by Ins(1,2,6)P_3. As shown in Figure 2a and Table 1, the homologous displacement of [^{3}H]-Ins(1,2,6)P_3 by the unlabelled compound in cerebellar membranes (Figure 4) gave one class of binding sites with K_D of 5.2±1.6 μM and B_{max} of 56.4±18.6 pmol mg^{-1} of protein.

Yoo *et al.* (1994) showed that inorganic phosphate interferes with [^{3}H]-Ins(1,2,6)P_3 binding in membranes from rat heart. They reported that binding was inhibited at 1 mM orthophosphate by 70%, and totally abolished at 25 mM. Therefore, to study the influence of the phosphate concentration on [^{3}H]-Ins(1,2,6)P_3 binding in pig cerebellar membranes, binding experiments were carried out, using the acetate based buffer, pH 5.0, which contained phosphate between 0 and 25 mM. Table 2 demonstrates that neither the appearance of a second binding site for [^{3}H]-Ins(1,2,6)P_3 nor the affinity and binding site density were influenced by varying the potassium phosphate concentration in the buffer between 0 and 25 mM. In those cases where a two-binding-site model seemed to fit the data with a $P<?10.05$ significantly better than a one-binding-site model, the K_D values obtained for the low-affinity binding sites were comparable in size to the highest concentration of α-trinositol used in the displacement experiments, and cannot therefore be relied on. Moreover, for technical reasons, in these cases the standard deviations estimated for K_D and B_{max} were

Table 2 Binding of Ins(1,2,6)P_3 to membranes from pig cerebellum in the presence of various concentrations of phosphate

	1 Binding site		*2 Binding sites*				
[Pi]	K_d (μM)	B_{max} (pmol mg^{-1})	K_{d1} (μM)	B_{max1} (pmol mg^{-1})	K_{d2} (μM)	B_{max2} (nmol mg^{-1})	P
0	6.1 ± 2.4	102 ± 43	2.5 ± 1.7	32.0 ± 28.8	504*	6.8*	$P=0.032$
5 mM	5.9 ± 2.1	66.3 ± 25.2	4.4 ± 2.4	46.4 ± 28.8	1.100*	5*	$P=0.720$
15 mM	4.1 ± 1.7	59.3 ± 25.5	1.9 ± 1.1	22.3 ± 15.8	475*	5*	$P=0.041$
25 mM	5.2 ± 1.6	56.4 ± 18.6	2.3 ± 1.7	18.1 ± 17.6	235*	3*	$P=0.250$

Membranes (500 μg of protein) were incubated with 2.1 to 2.8 nM [^{3}H]-Ins(1,2,6)P_3 (13000 to 18000 d.p.m. per assay) in sodium acetate buffer, pH 5.0 containing different concentrations of potassium phosphate. Displacement of [^{3}H]-Ins(1,2,6)P_3 by unlabelled Ins(1,2,6)P_3 was determined as described under Methods. For measuring nonspecific binding, 1 mM Ins(1,2,6)P_3 was employed. Sample size as described in legend to Table 1. The resulting displacement curves were analyzed for the presence of one or two binding sites. *denotes standard errors >200%.

too high to allow a reliable interpretation by a two-site model. Thus, the phosphate concentration had no effect on binding of [^{3}H]-Ins(1,2,6)P_3 to pig cerebellar membranes.

Solubilization of an Ins(1,3,4,5)P_4 binding protein with the nonionic detergent Brij 58 has been described by Donié *et al.* (1990). Therefore, we tested whether the Ins(1,2,6)P_3 binding protein was solubilized under these conditions. Figure 5 exemplifies the results. In the membrane sample employed for solubilization (2,733 mg of total protein), we found only one class of binding sites for each ligand. For Ins(1,3,4,5)P_4 the K_D was 1.6 nM and B_{max} was 62 fmol mg^{-1}. This gave a total amount of binding sites of 169.2 pmol. For α-trinositol, the corresponding data were $K_D = 9.2$ μM, $B_{max} = 69.7$ pmol mg^{-1} and a total binding sites of 190 nmol. These values were comparable to those obtained in the experiments shown in Figure 4. After solubilization, the resulting pellet bound Ins(1,3,4,5)P_4 with a K_D of 0.26 nM ($B_{max} = 95.6$ fmol mg^{-1}; total 42 pmol) and Ins(1,2,6)P_3 with a K_D of 6.1 μM ($B_{max} = 209.7$ pmol mg^{-1}; total 95 nmol). The solubilized proteins bound Ins(1,3,4,5)P_4 with a K_D of 1.7 nM ($B_{max} = 169$ fmol mg^{-1}; total 348 pmol). However, no [^{3}H]-Ins(1,2,6)P_3 binding could be detected in the solubilized sample with the assay conditions used.

During solubilization the total number of binding sites for Ins(1,3,4,5)P_4 was more than duplicated (i.e. 169 pmol before vs. 389 pmol after solubilization), indicating that there were binding sites within the microsomes which were not accessible to the ligand. Alternatively, the existence of endogenous inhibitors which were released during solubilization has also to be considered.

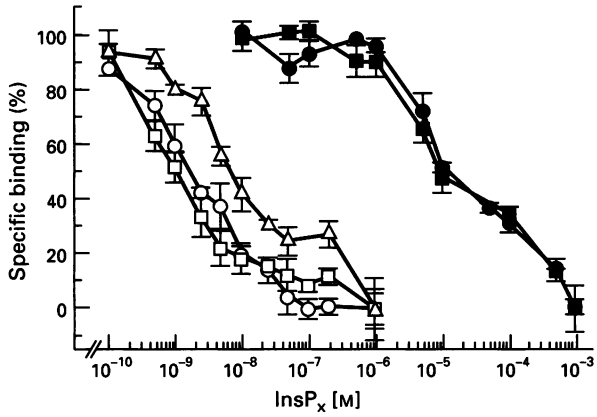

Figure 5 Binding of [^{3}H]-Ins(1,3,4,5)P_4 and [^{3}H]-Ins(1,2,6)P_3 in different fractions obtained during solubilization of proteins from cerebellum membranes. Following solubilization of membranes from pig cerebellum (2,733 mg of protein) and centrifugation, the resulting dialyzed supernatant (2,055 mg of protein) and the resuspended pellet (453 mg of protein) were analyzed for Ins(1,3,4,5)P_4 and Ins(1,2,6)P_3 binding. Membranes (500 to 900 μg, ○), the pellet (300 μg, □) and the solubilized proteins (500 μg, Δ) were incubated with 0.4 to 0.5 nM [^{3}H]-Ins(1,3,4,5)P_4 (14000–18000 d.p.m. per assay; open symbols) or 3.0 to 3.3 nM [^{3}H]-Ins(1,2,6)P_3 (19000–20000 d.p.m. per assay; solid symbols) and the indicated concentrations of unlabelled ligands were added in acetate-phosphate buffer, pH 5.0. After separation of bound ligand from free ligand the resulting displacement curves were analyzed as described under Methods. Results shown are typical for at least 2 further experiments. Total binding and nonspecific binding were 2297 d.p.m./1375 d.p.m. (○); 2560 d.p.m./996 d.p.m. (□); 2697 d.p.m./1462 d.p.m. (Δ); 875 dp.m./298 d.p.m. (■), and 1474 d.p.m./1037 d.p.m. (●) respectively.

Discussion

Here we have demonstrated in different tissues, binding sites for the therapeutically interesting inositoltrisphosphate, α-trinositol. The highest affinity for these binding sites was found for heart membranes (70 nM) followed by the liver and the kidney. Most physiological effects of α-trinositol are related to mechanisms involving blood vessels (see Introduction), and a binding study using autoradiography in tissue sections from various species has shown that α-trinositol binding density was highest in blood vessel wall structures (Walsh *et al.*, 1995). That binding (maximal at pH 5, $K_D = 10$ to 20 nM) has properties comparable to those described here.

The characteristics of receptor sites for inositololigophosphates can significantly depend on the method used for membrane preparation. Yoo *et al.* (1994), for example used detergent treatment to condition membranes from rat heart in order to improve specific α-trinositol binding. In those membrane preparations, one apparently homogeneous binding site population was found (K_D of 159 nM), the pH-dependence of [^{3}H]-α-trinositol binding was minimal, binding was severely hampered by the presence of phosphate ions, and there was great similarity with [^{3}H]-Ins(1,3,4,5)P_4 binding (Yoo *et al.*, 1994).

Here we have used a conventional protocol for membrane preparation and have validated the results obtained for α-trinositol binding by an analogous analysis of binding for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 with the same batches of membranes. The data obtained for Ins(1,4,5)P_3 binding are in close agreement with the data published previously (Challiss *et al.*, 1991; Joseph & Samanta, 1993). Binding of Ins(1,3,4,5)P_4 reached the highest level at pH 5.0 in cerebellar microsomes from rat (Challis *et al.*, 1991) and pig (Donié & Reiser, 1989) when assayed in acetate-based, phosphate-containing buffer (Figure 1a). Similarly, α-trinositol binding was found here to be maximal with this pH 5.0 buffer.

The literature concerning the binding characteristics of the Ins(1,3,4,5)P_4 receptor protein(s) is still controversial, partly due to inconsistencies in some of the reports. Cullen & Irvine (1992) showed that in membranes from rat cerebellum and

from bovine adrenal cortex, binding of Ins(1,3,4,5)P_4 was influenced more significantly by the ionic strength than by the pH of the buffer used when assayed in the range of pH 5.0 to 7.0. In contrast to these results, Theibert *et al.* (1987) demonstrated that Ins(1,3,4,5)P_4 binding to membranes from different rat tissues was maximal when measured in a Tris-based buffer at pH 8.5 which contained no further salts.

A similar uncertainty applies to ligand binding characteristics of the solubilized and purified Ins(1,3,4,5)P_4 receptor protein. In detergent-solubilized membranes from canine smooth muscle, Zhang *et al.* (1993) found that binding was maximal at pH 5.0, similar to the finding of Donié *et al.* (1990). The data presented by Zhang *et al.* (1993) are in close agreement with those described by Cullen *et al.* (1995a,b) for a purified Ins(1,3,4,5)P_4 receptor from pig platelets. This protein showed a decrease in specific Ins(1,3,4,5)P_4 binding when assayed between pH 5.5 to pH 9.0. But in this case no clear pH maximum was described. With the purified Ins(1,3,4,5)P_4 binding protein from pig cerebellum, Donié *et al.* (1990) found the same pH-dependency as in membranes, whereas Theibert *et al.* (1992), using photoaffinity labelling, identified two Ins(1,3,4,5)P_4 binding protein complexes with different pH-dependencies. The first complex displayed a broad pH optimum between 7 and 9. The second complex was resolved into two components, one with a pH-dependency comparable to that of the first complex, and a second protein with a maximal binding at acidic pH values of 5 to 6.

The platelet Ins(1,3,4,5)P_4 binding protein molecularly cloned by Cullen *et al.* (1995b) with a molecular mass of 94.6 kDa was identified as a GTPase activating protein. This protein however, has no structural similarity with the 42 kDa Ins(1,3,4,5)P_4 receptor protein identified in our laboratory (Donié & Reiser, 1991). This is obvious from amino acid sequences (Stricker *et al.*, 1995; Reiser *et al.*, 1995) and from cDNA clones obtained from the 42 kDa receptor protein (Hülser and Reiser, unpublished observation).

We found the same influence of pH and buffer composition on Ins(1,3,4,5)P_4 and on α-trinositol binding in all tissues studied. Therefore these data do not allow us to distinguish between separate binding sites for these inositolphosphates. The obvious difference in the number of binding sites for Ins(1,2,6)P_3 (pmol mg^{-1}) and for Ins(1,3,4,5)P_4 (fmol mg^{-1}) within all tissues tested indicates that these inositolphosphates do not bind to the same class of receptor protein(s). It has been suggested by Walsh *et al.* (1995) that specific [^{3}H]-α-trinositol binding sites may represent binding to low affinity Ins(1,3,4,5)P_4 receptors.

Further evidence for the fact that the high-affinity Ins(1,3,4,5)P_4 receptor does not represent the Ins(1,2,6)P_3 binding site is provided by the solubilization experiments (Figure 5). The solubilized proteins showed no α-trinositol binding, in contrast to the sizeable Ins(1,3,4,5)P_4 binding activity. In summary, here we have clearly established the existence of α-trinositol binding sites with different characteristics in different tissues possibly mediating the physiological effects of the drug. Future experiments will have to determine whether or not these are receptor sites for α-trinositol.

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References

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–325.

CHALLISS, R.A.J., WILLCOCKS, A.L., MULLOY, B., POTTER, B.V.L. & NAHORSKI, S.R. (1991). Characterization of inositol 1,4,5-trisphosphate- and inositol 1,3,4,5-tetrakisphosphate-binding sites in rat cerebellum. *Biochem. J.*, **274**, 861–867.

CLAXSON, A., MORRIS, C., BLAKE, D., SIREN, M., HALLIWELL, B., GUSTAFSSON, T., LÖFKVIST, B. & BERGELIN, I. (1990). The anti-inflammatory effects of D-*myo*-inositol 1,2,6-trisphosphate (PP56) on animal models of inflammation. *Agents Actions*, **29**, 68–70.

CULLEN, P.J., DAWSON, A.P. & IRVINE, R.F. (1995a). Purification and characterization of an Ins(1,3,4,5)P_4 binding protein from pig platelets: possible identification of a novel non-neuronal Ins(1,3,4,5)P_4 receptor. *Biochem. J.*, **305**, 139–143.

CULLEN, P.J., HSUAN, J.J., TRUONG, O., LETCHER, A.J., JACKSON, T.R., DAWSON, A.P. & IRVINE, R.F. (1995b). Identification of a specific Ins(1,3,4,5)P_4-binding protein as a member of the GAP1 family. *Nature*, **376**, 527–530.

CULLEN, P.J. & IRVINE, R.F. (1992). Inositol 1,3,4,5-tetrakisphosphate binding sites in neuronal and non-neuronal tissues. *Biochem. J.*, **288**, 149–154.

DONIÉ, F., HÜLSER, E. & REISER, G. (1990). High-affinity inositol 1,3,4,5-tetrakisphosphate receptor from cerebellum: solubilization, partial purification and characterization. *FEBS Lett.*, **268**, 194–198.

DONIÉ, F. & REISER, G. (1989). A novel, specific binding protein assay for quantitation of intracellular inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) using a high-affinity $InsP_4$ receptor from cerebellum. *FEBS Lett.*, **254**, 155–158.

DONIÉ, F. & REISER, G. (1991). Purification of a high-affinity inositol 1,3,4,5-tetrakisphosphate receptor from brain. *Biochem. J.*, **275**, 453–457.

GARDINER, S.M., KEMP, P.A., FALLGREN, B. & BENNET, T. (1994). Effects of chronic infusion of α-trinositol on regional and cardiac haemodynamics in conscious rats. *Br. J. Pharmacol.*, **113**, 129–136.

GOLDSCHMIDT, B. (1990). Preparative separation of myo-inositol bis- and trisphosphate isomers by anion-exchange chromatography. *Carbohyd. Res.*, **208**, 105–110.

JOSEPH, S.K. & SAMANTA, S. (1993). Detergent solubility of the inositol trisphosphate receptor in rat brain membranes. *J. Biol. Chem.*, **268**, 6477–6486.

LUND, T. & REED, R.K. (1994). α-Trinositol inhibits edema generation and albumin extravasation in thermally injured skin. *J. Trauma*, **36**, 761–765.

NAKAZAWA, H., GUSTAFSSON, T.O., TRABER, L.D., HERNDON, D.N. & TRABER, D.L. (1993). α-Trinositol decreases lung edema formation after smoke inhalation in an ovine model. *J. Appl. Physiol.*, **76**, 278–282.

NICOLETTI, F., BRUNO, V., FIORE, L., CAVALLARO, S. & CANONICO, P.L. (1989). Inositol hexakisphosphate (phytic acid) enhances Ca^{2+} influx and D-[^{3}H]aspartate release in cultured cerebellar neurons. *J. Neurochem.*, **53**, 1026–1030.

POTTER, E.K., EDVINSSON, L. & GUSTAFSSON, T. (1992). Antagonismof pre- and postjunctional responses to nemopeptide Y and sympathetic stimulation by D- myo- inositol- 1,2,6- trisphoshate in the anaesthetised dog. *Eur. J. Pharmacol.*, **221**, 307–314.

REGUNATHAN, S., REIS, D.J. & WAHLESTEDT, C. (1992). Specific binding of inositol hexakisphosphate (phytic acid) to adrenal chromaffin cell membranes and effects on calcium-dependent catecholamine release. *Biochem. Pharmacol.*, **43**, 1331–1336.

REISER, G. (1993). High-affinity inositol 1,3,4,5-tetrakisphosphate receptor from cerebellum. In *Methods in Neurosciences*, ed. Fain, J.N., Vol. 18, pp. 280–297. San Diego, London: Academic Press.

REISER, G., KUNZELMANN, U., HÜLSER, E., STRICKER, R., HOPPE, J., LOTTSPEICH, F. & KALBACHER, H. (1995). Peptide-specific antibodies demonstrate species heterogeneity of a 42-kDa high-affinity inositol 1,3,4,5-tetrakisphosphate receptor protein from brain. *Biochem. Biophys. Res. Commun.*, **214**, 20–27.

REISER, G., SCHÄFER, R., DONIÉ, F., HÜLSER, E., NEHLS-SAHA-BANDU, M. & MAYR, G.W. (1991). A high-affinity inositol 1,3,4,5-tetrakisphosphate receptor protein from brain is specifically labelled by a newly synthesized photoaffinity analogue, N-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-myo-inositol 3,4,5-trisphosphate. *Biochem. J.*, **280**, 533–539.

SHEARS, S.B. (1992). Metabolism of inositol phosphates. *Adv. Second Messenger Phosphoprotein Res.*, **26**, 63–92.

STRICKER, R., KALBACHER, H., LOTTSPEICH, F. & REISER, G. (1995). Characterization of a high-affinity Ins-P_4 (inositol 1,3,4,5-tetrakisphosphate) receptor from brain by an anti-peptide antiserum. *FEBS Lett.*, **370,** 226–240.

THEIBERT, A.B., ESTEVEZ, V.A., MOUREY, R.J., MARECEK, J.F., BARROW, R.K., PRESTWICH, G.D. & SNYDER, S.H. (1992). Photoaffinity labeling and characterization of isolated inositol 1,3,4,5-tetrakisphosphate- and inositol hexakisphosphate-binding proteins. *J. Biol. Chem.*, **267,** 9071–9079.

THEIBERT, A.B., SUPATTAPONE, S., WORLEY, P.F., BARABAN, J.M., MEEK, J.L. & SNYDER, S.H. (1987). Demonstration of inositol 1,3,4,5-tetrakisphosphate receptor binding. *Biochem. Biophys. Res. Commun.*, **148,** 1283–1289.

VALLEJO, M., JACKSON, T., LIGHTMAN, S. & HANLEY, M.R. (1987). Occurrence and extracellular actions of inositol pentakis- and hexakisphosphate in mammalian brain. *Nature*, **330,** 656–658.

WAHLESTEDT, C. & REIS, D.J. (1993). Neuropeptide Y (NPY)-related peptides and their receptors: are the receptors potential therapeutic drug targets? *Annu. Rev. Pharmacol. Toxicol.*, **32,** 309-352.

WAHLESTEDT, C., REIS, D.J., YOO, H., ADAMSON, M., ANDERSSON, D. & EDVINSSON, L. (1992). A novel inositol phosphate selectively inhibits vasoconstriction evoked by the sympathetic co-transmitters neuropeptide Y (NPY) and adenosine triphosphate (ATP). *Neurosci. Lett.*, **143,** 123–126.

WALSH, D.A., MAPP, P.L., POLAK, J.M. & BLAKE, D.R. (1995). Autoradiographic localization and characterization of [^{3}H]-α-trinositol (1D-myo-inositol 1,2,6-trisphosphate) binding sites in human and mammalian tissues. *J. Pharmacol. Exp. Ther.*, **273,** 461–469.

YOO, H., FALLGREN, B., LINDAHL, A. & WAHLESTEDT, C. (1994). Characterization of specific binding sites for α-trinositol (D-myo-inositol 1,2,5-trisphosphate) in rat heart. *Eur. J. Pharmacol.*, **268,** 55–63.

ZHANG, L., BRADLEY, M.E., KHOYI, M., WESTFALL, D.P. & BUXTON, I.L.O. (1993). Inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate binding sites in smooth muscle. *Br. J. Pharmacol.*, **109,** 905–912.

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