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Characterisation of stereospecific binding sites for inositol 1,4,5-trisphosphate in airway smooth muscle

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1 A 'P_2' membrane fraction of bovine tracheal smooth muscle displays high affinity (K_D 3.8 ± 0.2 nM), saturable (B_{max} 1003 ± 170 fmol mg^{-1} protein) and reversible binding of D-*myo*[^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-Ins(1,4,5)P_3).

2 This binding site shows strict stereo- and positional specificity for the D-Ins(1,4,5)P_3 isomer with L-Ins(1,4,5)P_3, DL-Ins(1,3,4,5)P_4 and D-Ins(1,3,4)P_3 displacing [^{3}H]-Ins(1,4,5)P_3 with K_i values of 20 μM, 0.35 μM and 2.4 μM, respectively.

3 Specific binding of [^{3}H]-Ins(1,4,5)P_3 is enhanced at alkaline pH values (maximal at pH 7.75) and, in distinct contrast to [^{3}H]-Ins(1,4,5)P_3 binding in rat cerebellum membranes, is not inhibited by Ca^{2+} (5–500 μM).

4 Heparin displaces [^{3}H]-Ins(1,4,5)P_3 specific binding with an IC_{50} of 7.6 ± 1.0 μg ml^{-1}.

5 Comparative studies demonstrated specific [^{3}H]-Ins(1,4,5)P_3 binding in bovine cardiac atrial preparations (B_{max} 75 ± 5 fmol mg^{-1} protein) and very low specific [^{3}H]-Ins(1,4,5)P_3 binding in bovine cardiac ventricle and skeletal muscle membranes ($\leqslant$ 25 fmol mg^{-1} protein).

Introduction

It is now well recognised that the activation of cell surface receptors that mobilize intracellular Ca^{2+} results in the release of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) into the cell through the action of phosphoinositidase C on phosphatidylinositol 4,5-bisphosphate (Downes & Michell, 1985; Berridge, 1987). In a wide variety of tissues, including airway smooth muscle (ASM), Ins(1,4,5)P_3 has been shown to release Ca^{2+} from non-mitochondrial, intracellular stores and in the latter tissue this results directly in the generation of tension (Hashimoto *et al.*, 1985). On the basis of limited structure-activity studies, it has been proposed that Ins(1,4,5)P_3 is recognised by a receptor protein which is linked either directly, or indirectly, to a Ca^{2+} channel located on a discrete portion of the endoplasmic reticulum (Berridge & Irvine, 1984; Nahorski & Potter, 1989). Indeed, a number of studies have demonstrated high affinity, saturable Ins(1,4,5)P_3 binding sites in a variety of peripheral and central tissues (Baukal *et al.*, 1985; Spät *et al.*, 1986a; Guillemette *et al.*, 1987). In cerebellum these sites have been shown to display strict stereo- and positional specificity for the D-Ins(1,4,5)P_3 isomer (Willcocks *et al.*, 1987) that corresponds to that associated with Ca^{2+} release (Strupish *et al.*, 1988; Nahorski, 1988). More recently it has been shown that in brain, submicromolar concentrations of Ca^{2+} potently inhibit Ins(1,4,5)P_3 binding (Worley *et al.*, 1987), an action possibly mediated via a neutral membrane protein termed 'calmedin' (Danoff *et al.*, 1988). This feedback inhibition of Ins(1,4,5)P_3 binding by calcium has been proposed as a mechanism allowing the generation of intracellular Ca^{2+} oscillations, which are observed in many cells following addition of agonists (Joseph *et al.*, 1989).

Although Ins(1,4,5)P_3 has now been shown to open single Ca^{2+} channels in aortic smooth muscle sarcoplasmic reticulum incorporated into planar lipid bilayers (Ehrlich & Watras, 1988), direct binding of Ins(1,4,5)P_3 has not been demonstrated previously in a smooth muscle preparation. Here we describe the binding of [^{3}H]-Ins(1,4,5)P_3 to a bovine tracheal smooth muscle preparation which displays high affinity and strict stereo- and positional specificity for the D-Ins(1,4,5)P_3 isomer. In addition, we demonstrate that, in marked contrast to cerebellum, Ca^{2+} in concentrations up to 1 mM does not inhibit [^{3}H]-Ins(1,4,5)P_3 binding in this tissue and discuss the importance of this observation in terms of the regulation of Ca^{2+} release in peripheral and central tissues.

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Methods

Preparation of tracheal smooth muscle membranes

The cervical trachealis muscle from freshly slaughtered cattle was dissected free of epithelium and surrounding connective tissue in ice-cold Krebs-Henseleit buffer. Pieces of tissue (15–20 g) were cross-chopped at 300 μm by a McIlwain tissue chopper. Tissue slices were washed in 20 mM $NaHCO_3$, 1 mM dithiothreitol (DTT), homogenised in 10 vol ice-cold buffer (Polytron, setting 6, 15 s) and centrifuged at 5000g for 15 min at 4°C. The supernatant was decanted and kept on ice and the pellet rehomogenised in 5 vol $NaHCO_3$/DTT buffer and recentrifuged as above. The pooled supernatants were then centrifuged at 38,000g for 20 min at 4°C and the pellet resuspended in homogenisation buffer at a protein concentration of ~5 mg ml^{-1}. This crude 'P_2' membrane fraction, which contains sarcoplasmic reticular and sarcolemmal membranes (Grover *et al.*, 1980) was used in binding studies. For the comparative studies, bovine adrenal cortex, cardiac atria and ventricle, skeletal muscle (sterno-maxillaris) and rat cerebella obtained from male Wistar rats were prepared in an identical manner (Challiss *et al.*, 1988). Protein concentrations were determined according to Lowry *et al.* (1951).

[^{3}H]-Ins(1,4,5)P_3 binding

Assays were all performed at 4°C in a final volume of 120 μl, containing 30 μl membranes (~200 μg protein), 30 μl D-*myo*[^{3}H]-Ins(1,4,5)P_3 (~2 nM), 30 μl 100 mM Tris-HCl, 4 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8 and 30 μl water or water containing 40 μM DL-Ins(1,4,5)P_3 to define non-specific binding (Challiss *et al.*, 1988). Assays were intermittently vortex-mixed and kept on ice for 30 min. Separation of bound and free [^{3}H]-Ins(1,4,5)P_3 was achieved by rapid dilution (3 ml) and filtration through Whatman GF/B glass-fibre filters followed by 3 × 3 ml washes using ice-cold 25 mM Tris-HCl, 5 mM $NaHCO_3$, 1 mM EDTA, pH 8.0. Radioactivity was subsequently determined after 12 h extraction in scintillation fluid.

Determination of [^{3}H]-Ins(1,4,5)P_3 metabolism

The metabolism of [^{3}H]-Ins(1,4,5)P_3 was determined by incubating ~2 nM [^{3}H]-Ins(1,4,5)P_3 with active or heat-inactivated (15 min, 100°C) ASM membranes (200 μg protein) at 4°C; reactions were terminated after 30 min by addition of an equal volume of 1 M trichloroacetic acid. Neutralized extracts were applied to Dowex AG1-X8 anion exchange columns and [^{3}H]-$InsP_x$ fractions eluted (Berridge *et al.*, 1983; Batty *et al.*, 1985).

Materials

D-*myo*[^{3}H]-Ins(1,4,5)P_3 (17.0 Ci mmol^{-1}) was a kind gift from Du Pont-New England Nuclear. D-Ins(1,4,5)P_3 was purchased from Amersham. L-Ins(1,4,5)P_3 and DL-Ins(1,3,4,5)P_4 were produced synthetically in the Department of Chemistry, University of Leicester, and D-Ins(1,3,4)P_3 was generously provided by Dr R. Irvine (Department of Biochemistry, Babraham, Cambridge). Adenosine 5′-triphosphate (ATP) and heparin (Sodium salt: Grade I porcine intestinal mucosal) were obtained from Sigma. All other reagents were of analytical grade obtained from commercial sources.

Data analysis

Dissociation equilibrium constant (K_D) and maximal binding capacity (B_{max}) of [^{3}H]-Ins(1,4,5)P_3 were determined by Scatchard analysis of [^{3}H]-Ins(1,4,5)P_3 displacement with unlabelled D-Ins(1,4,5)P_3, following correction for isotopic dilution and non-specific binding. IC_{50} and slope factor values of displacement data were determined by computer-assisted curve fitting (ALLFIT) (De Lean *et al.*, 1978) following correction of data for non-specific binding. K_i values were calculated from IC_{50} values by the equation derived by Cheng & Prusoff (1973).

Results

Although binding experiments were performed in the absence of Mg^{2+}, which is essential for phosphorylation and dephosphorylation of Ins(1,4,5)P_3, preliminary experiments were performed to determine whether significant metabolism of [^{3}H]-Ins(1,4,5)P_3 occurred under the assay conditions described (see Methods section). Under normal incubation conditions, in the presence of 1 mM EDTA, >98% of the radioactivity remained in the $InsP_3$ fraction with <1.5% and <0.5% eluting as inositol bisphosphate ($InsP_2$) or inositol monophosphate ($InsP_1$) respectively.

In contrast to the rat cerebellum and bovine adrenal cortex preparations freezing the ASM 'P_2' fraction for subsequent use caused significant (>60%) loss of specific binding. This occurred irrespective of whether samples were frozen at −20°C or plunged into liquid nitrogen. Therefore all studies were performed on freshly prepared tissue. Comparison of centrifugation and rapid filtration techniques for separating bound and free radioligand revealed essentially similar total binding with the two methods. However, filtration gave lower and more consistent values for non-specific binding.

Saturation analysis of [^{3}H]-Ins(1,4,5)P_3 binding in ASM membranes

Scatchard analysis of [^{3}H]-D-Ins(1,4,5)P_3 displacement by unlabelled D-Ins(1,4,5)P_3 indicates a single binding site with a

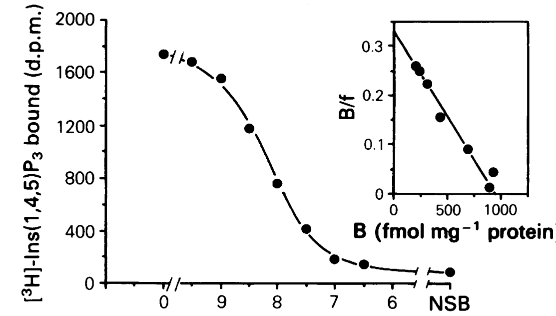

Figure 1 Saturation analysis of [^{3}H]-Ins(1,4,5)P_3 binding to airway smooth muscle (ASM) membranes. Assays were performed using 2 nM [^{3}H]-Ins(1,4,5)P_3 and 200 μg ASM membrane protein in a 25 mM Tris-HCl, 1 mM EDTA buffer (pH 7.8) together with increasing concentrations of unlabelled D-Ins(1,4,5)P_3 and incubated for 30 min at 4°C before rapid filtration. Non-specific binding (NSB) was determined with 10 μM DL-Ins(1,4,5)P_3. Values represent means of duplicate determinations from a single experiment with near identical results obtained in a further 2 separate experiments. The inset shows a Scatchard plot of bound/free (B/f) versus bound (B) ligand.

K_D of 3.8 ± 0.2 nM (mean ± s.e.mean) and B_{max} of 1003 ± 170 fmol mg^{-1} protein (n = 3) (Figure 1). Comparative data for [^{3}H]-D-Ins(1,4,5)P_3 binding to 'P_2' membrane fractions prepared in an identical manner from rat cerebellum, bovine adrenal cortex, cardiac atria and ventricle, and skeletal muscle are shown in Table 1. Binding of [^{3}H]-Ins(1,4,5)P_3 was also shown to be extremely rapid ($t_{1/2}$ on = 2 min) and reversible with a $t_{1/2}$ off of 5 min, determined by addition of D-Ins(1,4,5)P_3 (Figure 2).

Specificity of D-myo*[^{3}H]-Ins(1,4,5)P_3 binding*

The $InsP_3$ binding site in ASM appears highly selective for D-Ins(1,4,5)P_3 (Table 2). The pure L-enantiomer has a 4700 fold lower affinity for the site (Figure 3) which indicates that the binding site, as in cerebellum (Willcocks *et al.*, 1987), shows strict stereospecificity for the D-Ins(1,4,5)P_3 isomer. DL-Ins(1,3,4,5)P_4, which in its pure D-form has been shown to be

Table 1 Comparison of [^{3}H]-Ins(1,4,5)P_3 binding between tissues

Tissue	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n
Bovine			
Tracheal smooth muscle	3.8 ± 0.2	1003 ± 170	3
Adrenal cortex	3.7 ± 0.2	872 ± 70	4
Cardiac atria	4.3 ± 1.2	75 ± 5	3
Cardiac ventricle	—	≤25†	3
Skeletal muscle	—	≤25†	3
Rat			
Cerebellum	29.7 ± 4.1	14700 ± 1600	3

'P_2' membrane fractions from tissue homogenates were prepared as detailed in the Methods section with the cardiac, skeletal and smooth muscle cross-chopped (300 × 300 μm) on a McIlwain tissue chopper before homogenization. Incubation conditions were identical to those described in Figure 1 legend. B_{max} and K_D values were determined by Scatchard analysis of [^{3}H]-Ins(1,4,5)P_3 displacement by D-Ins(1,4,5)P_3 after correction for isotopic dilution and non-specific binding assessed in the presence of 10 μM DL-Ins(1,4,5)P_3. Values represent mean ± s.e.mean of n experiments.

† In the assay described using 2 nM [^{3}H]-Ins(1,4,5)P_3 and 250–350 μg membrane protein, specific binding was 45–70 d.p.m. This approximates to B_{max} values ≤25 fmol mg^{-1} protein.

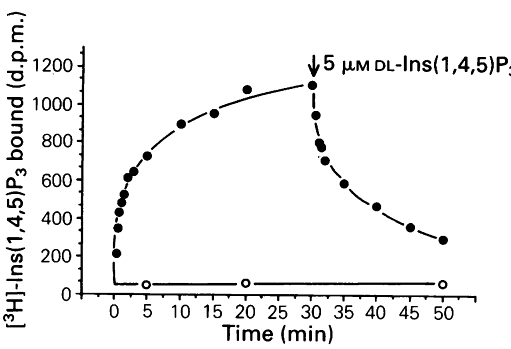

Figure 2 Rates of association and dissociation for [^{3}H]-Ins(1,4,5)P$_3$ binding to airway smooth muscle (ASM) membranes. Assay conditions were identical to those described in the legend to Figure 1. Reactions were started by the addition of 200 µg ASM membranes at 0 min and specific (●) and non-specific (○) [^{3}H]-Ins(1,4,5)P$_3$ binding determined at various times thereafter. The dissociation curve was obtained by adding D-Ins(1,4,5)P$_3$ (final concentration of 5 µM) to ASM membranes which had been incubated with [^{3}H]-Ins(1,4,5)P$_3$ for 30 min. Data from a single representative experiment are shown.

the most potent naturally occurring competitor for [^{3}H]-Ins(1,4,5)P$_3$ binding in other tissues, displayed an 80 fold lower affinity for this binding site. We have previously demonstrated that in bovine tracheal smooth muscle labelled with

Table 2 Inhibition of specific [^{3}H]-Ins(1,4,5)P$_3$ binding in airway smooth muscle (ASM) membranes

	IC_{50} (µM)	n
D-Ins(1,4,5)P$_3$	0.0062 ± 0.0004	4
L-Ins(1,4,5)P$_3$	29 ± 8	3
DL-Ins(1,3,4,5)P$_4$	0.49 ± 0.04	3
D-Ins(1,3,4)P$_3$	3.7, 3.0	2
ATP	1900 ± 300	3

Incubations were performed exactly as detailed in legend to Figure 1. Non-specific binding was determined using 10 µM DL-Ins(1,4,5)P$_3$. Values represent mean ± s.e.mean for n experiments, except for D-Ins(1,3,4)P$_3$ where, due to limited availability, $n = 2$ and hence individual values are given. The concentration of [^{3}H]-Ins(1,4,5)P$_3$ was 1.58 ± 0.17 nM for the 4 experiments performed to obtain all data-sets. Therefore IC$_{50}$ values can be divided by (1 + 1.58/3.8) to obtain K_i values.

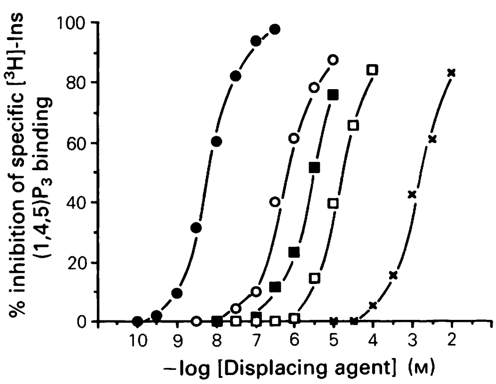

Figure 3 Specificity of [^{3}H]-Ins(1,4,5)P$_3$ binding to airway smooth muscle (ASM) membranes. Incubation conditions were identical to those in Figure 1 with specific binding determined by addition of 10 µM DL-Ins(1,4,5)P$_3$ to assess non-specific binding. Displacement of specific [^{3}H]-Ins(1,4,5)P$_3$ binding due to the presence of increasing concentrations of D-Ins(1,4,5) P$_3$ (●), DL-Ins(1,3,4,5) P$_4$ (○), D-Ins(1,3,4) P$_3$ (■), L-Ins(1,4,5)P$_3$ (□) and ATP (×) is shown, with data points representing means of 3 separate experiments each performed in duplicate, with the exception of Ins(1,3,4)P$_3$ where availability limited the number of experiments to 2; s.e.mean values (< 5% of mean) were omitted for clarity.

[^{3}H]-inositol and stimulated with the muscarinic receptor agonist carbachol, that [^{3}H]-Ins(1,3,4)-P$_3$ is the major [^{3}H]-InsP$_3$ accumulating in this tissue, even at relatively early time-points (> 85% at 1 min) (Chilvers *et al.*, 1988 and unpublished observations). It was therefore of considerable importance to assess the affinity of this InsP$_3$ isomer for the D-Ins(1,4,5)P$_3$ binding site in the same preparation. With a K_i value for Ins(1,3,4)P$_3$ of 2.4 µM it proved to have a 550 fold lower affinity for the binding site. Since incubations were performed at 4°C in the presence of 1 mM EDTA, and in the absence of added Mg^{2+} or ATP it is unlikely that any significant metabolism of the competing inositol polyphosphates occurred (Shears, 1989). Figure 3 also demonstrates that ATP had very weak displacing activity at this site.

Earlier studies have indicated that the glycosaminoglycan heparin is a potential Ins(1,4,5)P$_3$ receptor antagonist (Worley *et al.*, 1987; Cullen *et al.*, 1988; Ghosh *et al.*, 1988); furthermore, heparin-agarose affinity chromatography has been used to purify Ins(1,4,5)P$_3$ binding sites from rat cerebellum (Supattapone *et al.*, 1988). Therefore the ability of heparin to displace [^{3}H]-Ins(1,4,5)P$_3$ binding in ASM was examined. Figure 4 shows that heparin inhibited [^{3}H]-Ins(1,4,5)P$_3$ binding with a IC$_{50}$ of 7.6 ± 1.0 µg ml^{-1} ($n = 4$), a value directly comparable to that seen in the rat cerebellum (6.6 ± 1.2 µg ml^{-1}; $n = 3$).

pH dependence and effect of Ca^{2+} on [^{3}H]-Ins(1,4,5)P$_3$ binding

The effect of pH on [^{3}H]-Ins(1,4,5)P$_3$ binding in ASM membranes was assessed by Tris-maleate (pH values 6.0–7.5) and Tris-HCl (pH values 7.5–9.0) buffers. Figure 5 demonstrates the marked increase in [^{3}H]-Ins(1,4,5)P$_3$ binding at pH values between 6 and 8. Assays were hence routinely conducted at pH 7.8.

Previous studies have indicated that the [^{3}H]-Ins(1,4,5)P$_3$ binding site in brain is potently inhibited by Ca^{2+} (EC$_{50}$ ~ 300 nM) (Worley *et al.*, 1987; Joseph *et al.*, 1989). The inhibition by Ca^{2+} appears to be mediated by a protein termed 'calmedin' (Danoff *et al.*, 1988), since the purified cerebellum Ins(1,4,5)P$_3$ receptor is not Ca^{2+}-sensitive (Supattapone *et al.*, 1988). In the study by Danoff and colleagues it was also indicated that 'calmedin' activity is much lower in peripheral tissues, since 500 µM Ca^{2+} completely inhibited [^{3}H]-Ins(1,4,5) P$_3$ binding in various rat brain regions including spinal cord, yet caused only minor (15–20%) inhibition in testis, lung, liver and kidney preparations. Initial experiments, in which the

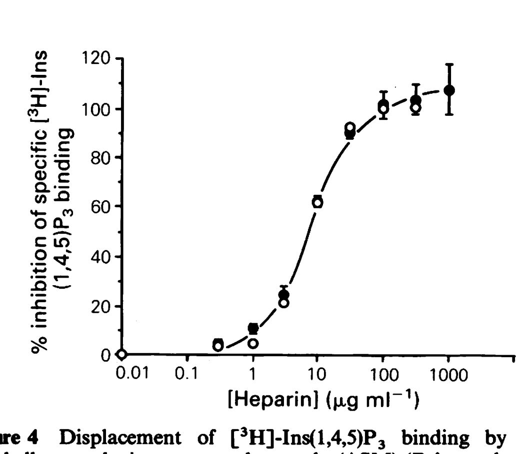

Figure 4 Displacement of [^{3}H]-Ins(1,4,5)P$_3$ binding by heparin. Cerebellum and airway smooth muscle (ASM) 'P$_2$' membrane fractions were prepared in an identical manner in 20 mM NaHCO$_3$, 1 mM dithiothreitol (pH 7.8) as described in the Methods section. With the exception that heparin (0.1–1000 µg ml^{-1}) was included instead of Ins(1,4,5)P$_3$, assays were performed as detailed in the legend for Figure 1. Values represent mean of 3 separate experiments each performed in duplicate; vertical lines show s.e.mean. (●) Airway smooth muscle; (○) cerebellum.

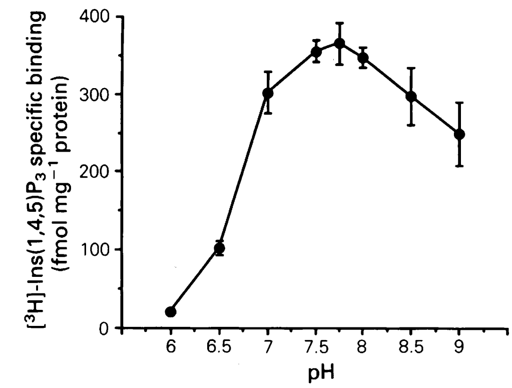

Figure 5 pH dependence of [^{3}H]-Ins(1,4,5)P_3 binding to airway smooth muscle (ASM) membranes. [^{3}H]-Ins(1,4,5)P_3 binding in ASM membranes was determined using 2 nM [^{3}H]-Ins(1,4,5)P_3 and 200 μg membrane protein in a range of 25 mM Tris (pH 7.5–9) and Tris-maleate (pH 6–7.5), 1 mM EDTA buffers incubated on ice for 30 min. Values represent mean of 3 separate experiments each performed in duplicate; vertical lines show s.e.mean.

effect of Ca^{2+} on [^{3}H]-Ins(1,4,5)P_3 binding to ASM membranes (prepared as described above) was examined, demonstrated that in the absence of EDTA substantial metabolism of the [^{3}H]-Ins(1,4,5)P_3 occurred during the 30 min incubation period (53 ± 3%; mean ± s.e.mean for 3 separate experiments). This was despite assays being performed at 4°C and the extent of metabolism clearly prevented interpretation of [^{3}H]-Ins(1,4,5)P_3 binding data. However, with further washing of the ASM membranes (2 × 50 ml 20 mM $NaHCO_3$, 1 mM DTT, pH 7.8), metabolism of the [^{3}H]-Ins(1,4,5)P_3, both in the absence of EDTA or in the presence of added $CaCl_2$ (1 mM) was considerably reduced (Table 3) with only minor inhibition of specific [^{3}H]-Ins(1,4,5)P_3 binding observed. In the presence of 1 mM EDTA the free [Ca^{2+}], measured with a Ca^{2+}-sensitive electrode (Sigel & Affolter, 1987) was <50 nM, whereas in the absence of EDTA the free [Ca^{2+}] was 5–10 μM. In the presence of 1 mM $CaCl_2$ (no EDTA) the free [Ca^{2+}] did not differ significantly from 1 mM.

Discussion

These data provide the first direct evidence for an Ins(1,4,5)P_3 binding site in smooth muscle. The binding site appears to represent the true physiological Ins(1,4,5)P_3 receptor rather than the inositol polyphosphate 5-phosphatase or 3-kinase enzymes since the affinity of Ins(1,4,5)P_3 binding in ASM membranes is at least 5000 fold greater than the K_m of the 5-phosphatase (Downes *et al.*, 1982; Connolly *et al.*, 1987), and at least 100 fold greater than the K_m of the 3-kinase enzyme which is also predominantly soluble (Irvine *et al.*, 1986). In addition, the selectivity of the binding site for the various inositol polyphosphates tested, together with the affinity of heparin for this site, mirrors the relative potencies of these compounds in initiating/inhibiting Ca^{2+} release, as demonstrated in other tissues (Strupish *et al.*, 1988; Nahorski, 1988; Ghosh *et al.*, 1988).

The stereo- and positional specificity of [^{3}H]-Ins(1,4,5)P_3 binding in ASM membranes is very similar to that obtained in cerebellum (Willcocks *et al.*, 1987), with the use of synthetic pure DL-Ins(1,3,4,5)P_4 and L-Ins(1,4,5)P_3 uncontaminated by D-Ins(1,4,5)P_3 greatly assisting in these determinations. The high affinity binding displayed by these sites in ASM (K_D 3.8 nM) is in close agreement with that demonstrated in particulate fractions of adrenal cortex (Baukal *et al.*, 1985), anterior pituitary cells (Guillemette *et al.*, 1987), liver (Spät *et al.*, 1986b) and permeabilized hepatocytes and neutrophils (Spät *et al.*, 1986a), but is of approximately 10 fold higher affinity than that obtained in cerebellum (K_D 20–40 nM) (Worley *et al.*, 1987; Willcocks *et al.*, 1987). The clear disparity in K_D values between neuronal and non-neuronal Ins(1,4,5)P_3 binding sites, together with the differences in Ca^{2+} regulation discussed above and preliminary findings that heparin may also have different affinities at these two sites (Willcocks & Nahorski, 1989), suggest possible heterogeneity of the Ins(1,4,5)P_3 receptor.

Joseph *et al.* (1989) have recently shown that the effects of Ca^{2+} on Ins(1,4,5)P_3 binding in cerebellum microsomes are exerted through a change in the apparent affinity of the receptor for Ins(1,4,5)P_3 rather than through effects on maximal binding. The sensitivity of [^{3}H]-Ins(1,4,5)P_3 binding in neuronal tissues to submicromolar Ca^{2+} concentrations is in marked contrast to the Ca^{2+} insensitivity of binding in ASM membranes, a property shared by a number of other peripheral tissues (Danoff *et al.*, 1988). These different effects of Ca^{2+} on Ins(1,4,5)P_3 binding in different tissues may reflect the heterogeneous distribution of calmedin (Danoff *et al.*, 1988) and may explain Ca^{2+}-induced inhibition of Ins(1,4,5)P_3-induced Ca^{2+} release, which is observed in neuronal (Joseph *et al.*, 1989) but not peripheral tissue preparations (Burgess *et al.*, 1984). These findings suggest that in ASM, Ca^{2+} release should parallel Ins(1,4,5)P_3 concentration in the cell, whereas in neuronal tissue Ca^{2+}, once released from intracellular stores, may inhibit further Ins(1,4,5)P_3-induced Ca^{2+} release. In line with this proposal, we have demonstrated recently that the muscarinic agonist carbachol induces a sustained increase in D-Ins(1,4,5)P_3 mass in cerebral cortex slices, compared to a transient increase in tracheal smooth muscle slices despite ongoing Ins(1,4,5)P_3 formation (Challiss *et al.*, 1988; Chilvers *et al.*, 1989). It appears therefore that the differential effect of Ca^{2+} on Ins(1,4,5)P_3 binding may be of fundamental importance in terms of differences in $[Ca^{2+}]_i$ regulation in neuronal and non-neuronal tissues.

In contrast to the different effects of Ca^{2+} on [^{3}H]-Ins(1,4,5)P_3 binding, the pH profile appears very similar between

Table 3 Effect of Ca^{2+} on [^{3}H]-Ins(1,4,5)P_3 binding and metabolism in airway smooth muscle (ASM) membranes

Incubation conditions	*Relative inhibition of specific [^{3}H]-Ins(1,4,5)P_3 binding* (% EDTA value)	n	*Metabolism of [^{3}H]-Ins(1,4,5)P_3* (% control)	n
+EDTA (1 mM)	0	3	0.6 ± 0.3	6
−EDTA	14.0 ± 3.3	3	24 ± 3	3
+$CaCl_2$ (1 mM)	8.0 ± 1.7	3	6 ± 1	3

Incubations were performed as described in the legend to Figure 1, except that ASM 'P_2' membrane fractions were washed in 2 × 50 ml 20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 7.8 before use and 1 mM EDTA or 1 mM $CaCl_2$ included as indicated. Free [Ca^{2+}] in the absence of EDTA, measured using a Ca^{2+}-sensitive electrode, was 5–10 μM. [^{3}H]-Ins(1,4,5)P_3 metabolism was determined in parallel incubations by terminating reactions with 1 M trichloroacetic acid and sequential elution of [^{3}H]-Ins to [^{3}H]-InsP_3 in neutralised extracts by anion exchange chromatography. Values are means ± s.e.mean for *n* experiments. Control values for the metabolism experiments were determined by incubations containing heat-inactivated (100°C, 15 min) ASM membranes.

tissues at physiological pH values. Joseph *et al.* (1989) cite provisional data suggesting that pH-induced changes in ionization of the $Ins(1,4,5)P_3$ molecule closely parallel the effects of pH on $Ins(1,4,5)P_3$ binding. Since activation of Ca^{2+} mobilizing receptors on the surface of cells frequently leads to an increase in intracellular pH through protein kinase C stimulation of Na^+/H^+ exchange (Rozengurt, 1986; Frelin *et al.*, 1988), and given such a relationship between pH and $Ins(1,4,5)P_3$ binding, then even subtle changes in intracellular pH may have a physiological role in regulating $Ins(1,4,5)P_3$-induced Ca^{2+} release.

The IC_{50} values obtained for heparin at the $Ins(1,4,5)P_3$ binding site in ASM and cerebellum are directly comparable to values obtained previously in rat cerebellum ($5\,\mu g\,ml^{-1}$, Worley *et al.*, 1987, and $16\,\mu g\,ml^{-1}$, Willcocks *et al.*, 1989) and rat liver microsomes (approx. $5\,\mu g\,ml^{-1}$, Tones *et al.*, 1989) and these correlate closely with the potency of heparin in inhibiting $Ins(1,4,5)P_3$-induced Ca^{2+} release (IC_{50} 5–16 $\mu g\,ml^{-1}$, Hill *et al.*, 1987; Cullen *et al.*, 1988). The activity of the heparin molecule appears to depend on an interaction between the negatively charged N-hexosamine sulphated residues and the $Ins(1,4,5)P_3$ receptor since the de-N-sulphated heparin is unable to compete with $Ins(1,4,5)P_3$ binding or affect $Ins(1,4,5)P_3$-induced Ca^{2+} release (Tones *et al.*, 1989). In permeabilized DDT_1MF-2 smooth muscle cells, heparin has been shown to be a competitive, reversible and potent antagonist of $Ins(1,4,5)P_3$-induced Ca^{2+} release (Ghosh *et al.*, 1988), with a heparin concentration of $10\,\mu g\,ml^{-1}$ completely inhibiting the response. In cerebellum membranes, $Ins(1,4,5)P_3$ binding has been shown to be approximately five times more sensitive to low molecular weight heparin (mol. wt. 4–6000) used in the above study compared with the non-cleaved heparin (mol. wt. >20,000) used in this study.

$Ins(1,4,5)P_3$ has been shown to result in Ca^{2+} release in permeabilized cardiac muscle (Suematsu *et al.*, 1984) and a range of contractile agonists, including noradrenaline, have been shown to increase $[^3H]$-$InsP_3$ formation in $[^3H]$-inositol prelabelled tissue (Poggioli *et al.*, 1986; Heathers *et al.*, 1988). However, the physiological importance of this second messenger in regulating contraction in this tissue remains controversial (Volpe *et al.*, 1988). Ehrlich & Watras (1988) failed to demonstrate $Ins(1,4,5)P_3$ activation of Ca^{2+} channels in cardiac sarcoplasmic reticulum vesicles incorporated into lipid bilayers and this, together with our demonstration of low specific $[^3H]$-$Ins(1,4,5)P_3$ binding in bovine cardiac atria and very low binding to cardiac ventricle membranes, supports the view that phosphoinositide hydrolysis may not play a central role in excitation-contraction coupling in this tissue. Indeed, it is possible that the very low specific $[^3H]$-$Ins(1,4,5)P_3$ binding detected in these cardiac muscle preparations reflects binding in contaminating vascular and neuronal elements.

In summary, we have identified a high affinity, stereo- and positionally specific binding site for D-*myo*$[^3H]$-$Ins(1,4,5)P_3$ in ASM membranes. This binding is maximal at pH 7.75, is displaced by heparin and is unaffected by physiological and supraphysiological Ca^{2+} concentrations. These findings provide an important link in the hypothesis that receptor-mediated increases in $Ins(1,4,5)P_3$ may, through interaction of this second messenger with specific intracellular receptors which mediate release of Ca^{2+} from intracellular stores, play a central role in pharmacomechanical coupling in smooth muscle (Somlyo *et al.*, 1988). In the airway, the development of an $Ins(1,4,5)P_3$ receptor antagonist, that can gain access to the interior of the ASM cell following topical administration via the inhaled route, may have therapeutic importance in diseases characterised by airways hyperreactivity.

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