Purification and characterization of the inositol 1,4,5-trisphosphate receptor protein from rat vas deferens

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Among rat peripheral tissues examined, Ins(1,4,5) P_3 receptor binding is highest in the vas deferens, with levels about 25 % of those of the cerebellum. We have purified the Ins P_3 receptor binding protein from rat vas deferens membranes 600-fold. The purified protein displays a single 260 kDa band on SDS/PAGE, and the native protein has an apparent molecular mass of 1000 kDa, the same as in cerebellum. The inositol phosphate specificity, pH-dependence and influence of various reagents are the same for purified vas deferens and cerebellar receptors. Whereas particulate Ins P_3 binding in cerebellum is potently inhibited by Ca^{2+} , particulate and purified vas deferens receptor binding of Ins P_3 is not influenced by Ca^{2+} . Vas deferens appears to lack calmodin activity, but the Ins P_3 receptor is sensitive to Ca^{2+} inhibition conferred by brain calmodin. The vas deferens may prove to be a valuable tissue for characterizing functional aspects of Ins P_3 receptors.

INTRODUCTION

Inositol 1,4,5-trisphosphate (Ins P_3) is a final common modulator of the phosphoinositide second messenger system, triggering the release of intracellular Ca^{2+} in numerous tissues (for review, see [1,2]). Ins P_3 acts by binding to a receptor protein which has been shown by reconstitution experiments to contain both the recognition site for Ins P_3 and the associated Ca^{2+} channel [3]. Ca^{2+} is released by Ins P_3 from a non-mitochondrial pool associated with the endoplasmic reticulum [4]. In the brain, the Ins P_3 receptor has been shown by electron-microscopic immunohistochemistry to be localized to subdivisions of the rough and smooth endoplasmic reticulum as well as the nuclear membrane [5]. The Ins P_3 receptor purified from mammalian brain consists of 260 kDa monomers, with the native protein comprising a homotetramer [6]. The cDNA for the rat cerebellar Ins P_3 receptor protein has been cloned [7]. Very recently, the isolation and characterization of an Ins P_3 receptor from bovine aorta has been described [8].

Although Ins P_3 binding sites were initially demonstrated both in peripheral tissues [9–12] and in the brain [13,14], their great density in brain tissue, especially the cerebellum, resulted in a focus upon the brain as a source for purification and characterization of physiological Ins P_3 receptors. A growing body of evidence implicates Ins P_3 as a mediator of pharmacomechanical coupling in smooth muscle, although the role of Ins P_3 in skeletal and cardiac muscle contraction is still controversial (for reviews, see [15–17]). Ca^{2+} -induced Ca^{2+} release mediated via the ryanodine receptor junctional foot protein appears to be a major determinant of excitation–contraction coupling in skeletal and cardiac muscle (for reviews, see [18,19]). Interestingly, the cloned Ins P_3 receptor protein displays marked sequence similarity with the cloned ryanodine receptor protein [7,20,21]. We wondered whether Ins P_3 binding sites in peripheral tissues involve the same protein as in the brain or are different, conceivably more closely resembling ryanodine receptors. Because of high densities of Ins P_3 receptor binding sites in the vas deferens and the extensive studies characterizing differential roles of Ins P_3 and ryanodine in the vas deferens and other smooth

muscle [22–25], we have purified and characterized the Ins P_3 receptor protein from rat vas deferens.

MATERIALS AND METHODS

Materials

[3H]Ins P_3 (20 Ci/mmol) and Formula 963 scintillation cocktail were obtained from NEN/du Pont. NB2 photographic emulsion was purchased from Kodak. Ins P_3 and other unlabelled inositol phosphates were obtained from Calbiochem. Concanavalin A (Con A)–Sephacrose, Mono Q and Superose 6 analytical columns were purchased from Pharmacia. All other reagents were from Sigma.

Ins P_3 autoradiography

Ins P_3 binding sites were visualized as described previously [26], with minor modifications. Frozen 10 μ m-thick rat vas deferens sections were incubated for 15 min at 4 °C (equilibrium conditions) in buffer containing 50 mM-Tris/HCl, pH 8.0, 2 mM-EDTA, 1 mM- β -mercaptoethanol (β -ME), 5 μ M-digitonin and 50 nM-[3H]Ins P_3 . Sections were then washed for 2 $\times$ 1 min in the same buffer minus radioligand, dried under a cool airstream and exposed to NB2-emulsion-coated coverslips for 3 weeks for preparation of autoradiograms. Non-specific ligand binding, determined in the presence of 5 μ M unlabelled Ins P_3 , was typically less than 5 % of the total binding.

Preparation of membranes

Tissue from freshly killed male Sprague–Dawley rats (200–300 g) was homogenized (50 mg wet wt./ml of buffer) with a Brinkmann Polytron (setting 6; 10 s) at 4 °C in buffer A containing 50 mM-Tris/HCl, pH 7.7, 150 mM-NaCl, 1 mM-EDTA, 1 mM- β -ME, phenylmethanesulphonyl fluoride (100 μ g/ml), *N* $^{\alpha}$ -*p*-tosylarginine methyl ester (10 μ g/ml) and leupeptin and aprotinin (each 5 μ g/ml). Homogenates were centrifuged (15 min, 30000 *g*), the supernatants were discarded and the pellets were resuspended in the same volume of buffer. Homogenization, centrifugation and resuspension of the mem-

Abbreviations used: Con A, concanavalin A; PKA, cyclic AMP-dependent protein kinase; PEG, poly(ethylene glycol); NEM, *N*-ethylmaleimide; β -ME, β -mercaptoethanol.

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brane pellets were repeated twice to provide the washed membrane fraction.

$[^3\text{H}]\text{InsP}_3$ binding to membranes

InsP_3 binding to washed membranes was measured essentially as described [14]. Binding reactions consisted of 200 μl of washed membranes, 5 nM- $[^3\text{H}]\text{InsP}_3$ and buffer A to a final volume of 0.5 ml. Reactions were incubated to equilibrium at 4 °C for 10 min and terminated by centrifugation (5 min, 12000 g). Unbound ligand in the supernatant was removed by aspiration and bound radioactivity in the pellets was measured by liquid scintillation chromatography in 5 ml of Formula 963 following membrane pellet solubilization with 200 μl of 1% SDS. Specific binding represented less than 10% of added radioactivity. Non-specific binding was determined in the presence of 5 μM unlabelled InsP_3 .

Purification of InsP_3 receptor sites from vas deferens

The InsP_3 binding site in rat vas deferens was purified by a procedure similar to the purification of the cerebellar InsP_3 receptor [6]. Fresh rat vas deferens (10 g) was minced with a razor blade, and washed membranes were prepared as described above. All procedures were carried out at 4 °C. Membrane proteins were solubilized in Triton X-100 detergent (final concentration 1.5%) with gentle stirring for 1 h followed by centrifugation (45 min, 45000 g). The supernatant was collected, adjusted to 0.25 M-NaCl and passed over a heparin-agarose column (7 ml packed volume) that had been equilibrated with buffer A containing 0.25 M-NaCl and 0.1% Triton X-100. The column was washed twice with 5 column vol. of buffer A containing 0.25 M-NaCl/0.1% Triton X-100 and eluted with 4 column vol. of buffer A/0.5 M-NaCl/0.1% Triton X-100. The heparin eluate was adjusted to 1 mM- MgCl_2 and 1 mM- CaCl_2 and incubated with 3 ml of packed glutaraldehyde-fixed Con A-Sepharose for 1 h on a rotator. Glutaraldehyde fixation of the Con A-Sepharose prevents Con A leaching from the column [27]. After 1 h, the resin was poured into a column, washed twice with 5 column vol. of buffer A containing 0.5 M-NaCl/1 mM- MgCl_2 /1 mM- CaCl_2 /0.1% Triton X-100, and eluted batchwise for at least 2 h with 20 ml of buffer A containing 2 mM-EDTA, 1 M- α -methylmannoside and 0.1% Triton X-100. The Con A eluate was diluted 5-fold with buffer A and applied to an analytical f.p.l.c. 5/5 Mono Q anion-exchange column at a flow rate of 1 ml/min. The column was washed with 5 bed vol. of buffer A and eluted with a linear gradient of 0–400 mM-NaCl at 0.5 ml/min. InsP_3 binding activity reached a peak at 250–300 mM-NaCl.

$[^3\text{H}]\text{InsP}_3$ binding to the solubilized receptor

InsP_3 binding to detergent-solubilized fractions and purified InsP_3 receptor was determined by poly(ethylene glycol) (PEG) precipitation assay as follows. Samples were incubated to equilibrium (10 min) at 4 °C with 5 nM- $[^3\text{H}]\text{InsP}_3$ in a reaction volume of 0.5 ml. Protein containing bound InsP_3 was precipitated with 50 μl of 0.5% γ -globulin and 0.8 ml of ice-cold 30% PEG. After an additional 7 min, the samples were centrifuged (10 min, 12000 g), supernatants were removed, the pellets were solubilized with 0.1 ml 1% SDS and radioactivity was measured in 5 ml of Formula 963. Non-specific binding was determined in the presence of 5 μM unlabelled InsP_3 .

Assessment of calmodin activity

$[^3\text{H}]\text{InsP}_3$ binding was assayed as described above. Calmodin activity was measured according to Danoff *et al.* [33]. Purified cerebellar or vas deferens InsP_3 receptor was incubated with calmodin-enriched fractions (heparin flow-through, 100 μl) in (a)

the absence of Ca^{2+} , (b) the presence of 500 μM - Ca^{2+} and (c) the presence of 500 μM - Ca^{2+} followed by 15 min of treatment with 1 mM-EDTA. Calmodin activity is defined as the EDTA-reversible Ca^{2+} -dependent inhibition of $[^3\text{H}]\text{InsP}_3$ binding.

Other methods

Protein was determined by a modification of the Lowry method [28]. SDS/PAGE was performed by the method of Laemmli [29], and the gels were stained with silver [30]. InsP_3 5-phosphatase and InsP_3 3-kinase activities were assayed as previously described [31].

RESULTS

Tissue distribution and autoradiographic localization of InsP_3 binding sites

In a screen of numerous rat tissues, highest levels of $[^3\text{H}]\text{InsP}_3$ binding occurred in the cerebellum (Table 1). Of 12 peripheral tissues examined, high binding levels were apparent in the vas deferens (25% of cerebellum) and testis, with moderate levels in immune tissue (spleen and thymus), endocrine tissue (adrenal gland and pancreas), kidney and forebrain. Low levels of specific InsP_3 binding were observed in liver, epididymis, skeletal muscle and heart muscle. To ascertain whether binding sites in peripheral tissues are the same as in brain, we examined the relative potencies of various inositol phosphates. In all tissues examined, $\text{Ins}(1,4,5)\text{P}_3$ was most potent, but $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and InsP_6 had negligible affinity (results not shown).

To localize InsP_3 binding sites within the vas deferens, we conducted autoradiography with $[^3\text{H}]\text{InsP}_3$ (Fig. 1). InsP_3 binding was abolished in the presence of 5 μM unlabelled InsP_3 . Binding sites were densely concentrated throughout the muscle layer, and substantial binding was evident in the epithelial layer adjacent to the lumen. No binding was apparent in the lamina propria or adventitia. Interestingly, substantial binding was evident in the smooth muscle layer of blood vessels of the adventitia surrounding the vas deferens.

Purification of InsP_3 binding protein from the vas deferens

We purified an InsP_3 binding site from rat vas deferens by a procedure resembling the protocol we employed previously in purifying rat cerebellar InsP_3 receptor (Table 2) [6]. InsP_3 binding

Table 1. Distribution of $[^3\text{H}]\text{InsP}_3$ binding sites in rat tissues

Membranes from various rat organs were prepared, and $[^3\text{H}]\text{InsP}_3$ binding was assayed as described in the Materials and methods section. InsP_3 binding is expressed as fmol of $[^3\text{H}]\text{InsP}_3$ bound/mg of protein. This experiment was conducted twice in duplicate, and data represent mean values which varied by less than 20%.

Tissue	$[^3\text{H}]\text{InsP}_3$ specific binding (fmol/mg)
Cerebellum	5500
Vas deferens	1300
Testis	731
Thymus	352
Adrenal gland	206
Spleen	195
Kidney	178
Forebrain	151
Pancreas	143
Liver	67
Epididymis	31
Skeletal muscle	29
Heart muscle	27

Fig. 1. Autoradiographic localization of *InsP₃* binding sites in rat vas deferens

[³H]*InsP₃* binding sites were visualized by autoradiography as described in the Materials and methods section. (a) Bright-field view of vas deferens (cross-section) stained with 0.5 % Toluidine Blue. Autoradiographs of (b) total [³H]*InsP₃* binding and (c) non-specific [³H]*InsP₃* binding viewed with dark-field optics are also shown. The micrograph depicts adventitia (ad) containing blood vessels (bv), outer longitudinal muscle (olm), circular muscle (cm), inner longitudinal muscle (ilm), epithelium (e), lumen (lu) and lamina propria (lp).

sites in the vas deferens are more resistant to solubilization by Triton X-100 detergent than those in the cerebellum. However, by increasing the Triton X-100 concentration to 1.5 % and solubilizing for 1 h, 80 % of the binding sites were solubilized. Solubilized *InsP₃* binding sites adhered to a heparin-agarose column and were eluted with 0.5 M-NaCl, providing a 73-fold increase in specific activity with about a 50 % recovery of receptor binding. The partially purified receptor adhered to a Con A-Sepharose column, from which it was eluted with 1 M- α -methylmannoside. The final purification step employed f.p.l.c. Mono Q anion-exchange chromatography. This purification procedure provided a 600-fold enhancement of specific activity, with a 20 % yield. Throughout the course of the purification, the *K_d* for *InsP₃* was unchanged, whereas the *B_{max}* of the binding sites increased 600-fold. The extent of purification to obtain homogeneous receptor protein for the vas deferens was somewhat less than that required to fully purify the rat cerebellar *InsP₃* receptor we described previously [6]. The difference in results appears to reflect an improved yield associated with modifications that we have introduced into the original purification technique, such as the use of proteinase inhibitors and a more rapid execution of the purification procedure. Thus, in parallel purification experiments of the vas deferens and cerebellar *InsP₃*

Fig. 2. SDS/PAGE analysis of purified vas deferens *InsP₃* receptor

The purity of the vas deferens *InsP₃* receptor was examined and its mobility compared with that of the purified cerebellar *InsP₃* receptor by SDS/PAGE in a 7.5 % acrylamide gel stained with silver. Lane A, 100 ng of purified vas deferens *InsP₃* receptor; lane B, 100 ng of purified cerebellar *InsP₃* receptor; lane C, molecular mass markers: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

receptors, we have been able to obtain homogeneous *InsP₃* receptor protein from the rat cerebellum after a 200–300-fold purification (results not shown).

The purified *InsP₃* receptor protein from vas deferens displayed the same mobility on SDS/PAGE as receptor purified in parallel experiments from rat cerebellum (Fig. 2). Moreover, gel filtration revealed the same apparent molecular masses for the native proteins from cerebellum and vas deferens (Fig. 3), slightly in excess of 1000 kDa.

InsP₃ 5-monophosphatase and *InsP₃* 3-kinase both display high affinity for *InsP₃*. To rule out the possibility that the *InsP₃* binding protein purified from vas deferens represents one of these enzymes, we assayed the purified *InsP₃* binding protein for these two enzymic activities (results not shown). No *InsP₃* 5-monophosphatase or *InsP₃* 3-kinase activity was demonstrable with the purified vas deferens *InsP₃* binding protein. To further ensure that the *InsP₃* binding activity does not reflect either of these two enzymes, we assayed for these two enzymic activities throughout the purification procedure (results not shown). *InsP₃* 5-monophosphatase and *InsP₃* 3-kinase activities were fully resolved from *InsP₃* binding activity in the course of purification of the vas deferens receptor in a pattern closely resembling our

Table 2. Purification of the rat vas deferens *InsP₃* receptor

The *InsP₃* binding site from rat vas deferens was purified using the purification steps outlined in the Materials and methods section. This purification scheme was replicated four times and the data presented are from a typical experiment.

Fraction	Protein (mg)	[³ H] <i>InsP₃</i> binding (pmol)	Specific activity (pmol/mg)	Purification (-fold)	Yield (%)
Washed membranes	250	93	0.37	1	100
Solubilization (1.5 % Triton X-100)	131	74	0.56	1.5	80
Heparin-agarose	1.6	44	27	73	47
Con A-Sepharose	0.18	26	144	389	28
f.p.l.c. Mono Q	0.084	20	238	643	21

Fig. 3. Estimation of native molecular mass of the purified vas deferens InsP_3 receptor

The native molecular mass of the purified vas deferens InsP_3 receptor (●) was compared with that of the cerebellar InsP_3 receptor (□) by gel-filtration chromatography. Purified receptor, concentrated 20-fold, was injected (200 μl) on to an f.p.l.c. Superose 6 column and resolved at a flow rate of 0.3 ml/min in buffer A containing 200 mM-NaCl, 5% glycerol and 0.1% Triton X-100. Identical runs were performed for both vas deferens and cerebellar InsP_3 receptors. The column was calibrated with Blue Dextran (V_0), thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). Data shown are from a typical experiment, which was replicated twice.

previous separation of these enzymes from the brain InsP_3 receptor [31]. Specifically, the InsP_3 5-monophosphatase failed to adhere to the heparin column, and the InsP_3 3-kinase did not adhere to the Con A-Sepharose column.

Properties of purified InsP_3 receptor protein from vas deferens

Earlier, we observed a single homogeneous population of InsP_3 binding sites in brain membranes and with purified cerebellar InsP_3 receptor [6]. Mauger *et al.* [32] reported distinct

Fig. 4. Scatchard analysis of $[\text{H}]\text{InsP}_3$ receptor

Binding of $[\text{H}]\text{InsP}_3$ to purified vas deferens was measured by PEG precipitation as described in the Materials and methods section. Data obtained from the displacement of $[\text{H}]\text{InsP}_3$ by unlabelled InsP_3 were plotted. Assays were conducted with 0.1 μg of pure InsP_3 receptor. This experiment was conducted twice in duplicate and data shown are mean values which varied by less than 20% (K_d 9.6 nM; B_{max} 1.9 nmol/mg of protein). $[\text{H}]\text{InsP}_3$ binding in washed vas deferens membranes showed a similar K_d value of 10.4 nM (B_{max} of 3.7 pmol/mg of protein), indicating that no change in binding affinity occurred during purification.

high- and low-affinity binding sites for InsP_3 in rat liver membranes. We conducted saturation analysis of $[\text{H}]\text{InsP}_3$ binding to purified vas deferens receptor protein (Fig. 4). Scatchard analysis indicated only a single population of binding sites with a K_d of about 10 nM and a B_{max} for the purified protein of 2 nmol/mg of protein. This B_{max} value is similar to the B_{max} of the purified cerebellar InsP_3 receptor in parallel purification experiments with the two tissues (results not shown).

The inositol phosphate specificity of the purified vas deferens receptor appears identical with that of the InsP_3 binding site in crude vas deferens membrane and InsP_3 binding sites in particulate and purified cerebellar protein (Table 3). $\text{Ins}(1,4,5)\text{P}_3$

Table 3. Inhibition of $[\text{H}]\text{InsP}_3$ binding by inositol phosphates and nucleotides

$[\text{H}]\text{InsP}_3$ binding to vas deferens membranes and purified InsP_3 receptor was assayed as described in the Materials and methods section and compared with the membrane and purified forms of the cerebellar InsP_3 receptor. Data are means of four determinations which varied by less than 10%.

Displacer (1 μM)	$[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding (% of control)			
	Vas deferens		Cerebellum	
	Membrane	Pure receptor	Membrane	Pure receptor
Inositol	100	100	100	101
$\text{Ins}(2)\text{P}_1$	100	100	103	100
$\text{Ins}(1,4)\text{P}_2$	96	96	86	90
$\text{Ins}(1,4,5)\text{P}_3$	6	5	10	8
$\text{Ins}(1,3,4)\text{P}_3$	90	95	88	90
$\text{Ins}(1,3,4,5)\text{P}_4$	22	16	37	40
InsP_6	68	62	72	68
$\text{Ins}(\text{SO}_4)_6$	100	98	96	100
ATP	101	98	110	101
Cyclic AMP	99	93	98	97
GTP	95	98	92	95
Cyclic GMP	98	94	98	96

Table 4. Effects of various agents on $[^3\text{H}]\text{InsP}_3$ binding

Substances known to inhibit the cerebellar InsP_3 receptor were examined for their effect on $[^3\text{H}]\text{InsP}_3$ binding to the membrane and purified vas deferens InsP_3 receptor. $[^3\text{H}]\text{InsP}_3$ binding was assayed as described in the Materials and methods section. Ca^{2+} was buffered by EDTA. Inhibition by Ca^{2+} was defined by reversibility with 1 mM-EDTA. The experiment was performed twice in triplicate, and data presented are mean values which varied by less than 20%.

Treatment	$[^3\text{H}]\text{InsP}_3$ binding (% of control)			
	Vas deferens		Cerebellum	
	Membrane	Pure receptor	Membrane	Pure receptor
Ca^{2+} (10 μM)	100	100	7	100
Ca^{2+} (100 μM)	99	100	5	100
Ca^{2+} (1 mM)	96	99	0	100
Heparin (5 $\mu\text{g}/\text{ml}$)	60	54	57	48
Heparin (20 $\mu\text{g}/\text{ml}$)	0	0	0	0
NEM (1 mM)	15	4	14	0
NEM(1 mM) + β -ME (2 mM)	99	100	95	102
Heat (1 h, 60 $^{\circ}\text{C}$)	0	0	0	0
Trypsin (0.1 unit, 5 min, 25 $^{\circ}\text{C}$)	99	100	100	98
Trypsin (0.1 unit, 15 min, 25 $^{\circ}\text{C}$)	37	39	40	42
Trypsin (0.1 unit, 40 min, 25 $^{\circ}\text{C}$)	0	0	0	20

was the most potent inhibitor in all preparations, while $\text{Ins}(1,3,4)\text{P}_3$ was essentially inactive at 1 μM concentration. At 1 μM , $\text{Ins}(1,3,4,5)\text{P}_4$ produced substantial inhibition of binding in all preparations, with somewhat greater inhibition in vas deferens. However, using multiple lower concentrations, $\text{Ins}(1,3,4,5)\text{P}_4$ was 20–30-fold less potent than InsP_3 (results not shown). InsP_6 (1 μM) inhibited binding by 30–35% in all preparations. $\text{Ins}(2)\text{P}_1$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(\text{SO}_4)_6$, ATP, cyclic AMP, GTP and cyclic GMP were inactive at 1 μM .

Heparin is a potent inhibitor of InsP_3 binding to cerebellar receptors [14]. Heparin displayed about the same potency in inhibiting particulate and purified vas deferens as cerebellar receptors, with an IC_{50} (concn. causing 50% inhibition) of approx. 5 $\mu\text{g}/\text{ml}$ (Table 4). As with the cerebellar receptor, InsP_3 binding to the vas deferens receptor in particulate and purified preparations was inhibited by *N*-ethylmaleimide (NEM), with the inhibition reversed by β -ME. Receptor binding in particulate and purified preparations of both vas deferens and cerebellum was abolished by heating for 1 h at 60 $^{\circ}\text{C}$ and by trypsin treatment (Table 4).

A striking property of the cerebellar InsP_3 receptor is its sensitivity to physiological intracellular concentrations of Ca^{2+} in membranes and crude solubilized preparations, with 50% inhibition of binding at 0.3 μM - Ca^{2+} [14]. Purification of cerebellar InsP_3 receptor protein results in a dramatic loss of sensitivity to inhibition of binding by Ca^{2+} , with sensitivity being restored by a particulate protein activity designated 'calmodin' [33]. We confirmed the almost complete and reversible inhibition of cerebellar particulate InsP_3 binding by 10 μM - Ca^{2+} , whereas the purified cerebellar receptor showed no inhibition with 1 mM- Ca^{2+} (Table 4). Surprisingly, the vas deferens particulate and purified InsP_3 binding protein was resistant to inhibition by 1 mM- Ca^{2+} .

Failure of Ca^{2+} to inhibit particulate InsP_3 binding in vas deferens may reflect a lack of calmodin in peripheral tissues [33] or inability of the smooth muscle receptor to associate with calmodin. To address this issue, calmodin-enriched fractions [33] prepared from cerebellum or vas deferens were added to purified cerebellar and vas deferens InsP_3 receptors, and sensitivity to calcium-dependent inhibition of InsP_3 binding was examined (Table 5). The calmodin-enriched fraction from vas deferens

Table 5. Sensitivity of vas deferens InsP_3 receptor to calmodin activity

The sensitivity of purified vas deferens and cerebellar InsP_3 receptor to calmodin-enriched heparin column flow-through fractions from cerebellum or vas deferens was examined as described in the Materials and methods section. Triplicate samples were assayed in parallel.

	Inhibition of InsP_3 binding (%)	
	CaCl_2 (500 μM)	CaCl_2 (500 μM) + EDTA (1 mM)
Cerebellum		
– Calmodin	0	0
+ Brain calmodin	91	10
+ Vas deferens calmodin	14	12
Vas deferens		
– Calmodin	0	0
+ Brain calmodin	79	3
+ Vas deferens calmodin	6	7

failed to produce EDTA-reversible Ca^{2+} -dependent inhibition of InsP_3 binding to purified cerebellar or vas deferens receptors. Calmodin-enriched fractions from cerebellum, however, did produce pronounced EDTA-reversible Ca^{2+} -dependent inhibition of InsP_3 binding in both purified vas deferens and cerebellar InsP_3 receptors. Approx. 10% of InsP_3 binding was not reversible by EDTA, possibly due to Ca^{2+} activation of proteinases. These experiments indicate that the vas deferens InsP_3 receptor apparently lacks an endogenous calmodin activity, but is similar to the brain InsP_3 receptor in its sensitivity to brain calmodin.

The cerebellar InsP_3 receptor is highly sensitive to pH, with a doubling of InsP_3 binding between pH 7.5 and 8.5 [14]. A similar pH-dependence of InsP_3 binding at the purified vas deferens receptor was apparent (Fig. 5). Maximal binding reached a plateau at pH 8.5–9, with values double those at pH 7.5. The very steep pH-dependence curve within the physiological pH ranges fits with suggestions that actions of the phosphoinositide

Fig. 5. Effect of pH on [^3H]InsP₃ binding to vas deferens membranes

[^3H]InsP₃ binding to vas deferens membranes was assayed as described in the Materials and methods section, except for the substitution of the following buffers: 50 mM-sodium acetate (pH values < 7.0), 50 mM-Tris/HCl (pH values 7.0–8.8) and sodium borate (pH values > 9.0). The experiment was performed twice in duplicate and the data shown are mean values differing by less than 20%.

system may vary functionally as intracellular pH changes under circumstances such as mitogenesis [34].

The cerebellar InsP₃ receptor is phosphorylated by cyclic AMP-dependent protein kinase (PKA) which decreases the potency of InsP₃ to release Ca²⁺ while increasing the overall amount of Ca²⁺ released, presumably by enhancing the Ca²⁺ pump activity by phosphorylation [35]. Cyclic AMP also increases InsP₃-induced mobilization of intracellular calcium in aortic smooth muscle cells [36]. We have observed that the purified vas deferens InsP₃ receptor protein is also stoichiometrically phosphorylated by PKA (results not shown).

To ascertain a potential relationship between the InsP₃ receptor and the ryanodine-binding junctional foot protein, we evaluated the effects of ryanodine on [^3H]InsP₃ binding to purified vas deferens InsP₃ receptor (results not shown). No effect of ryanodine was observed up to a concentration of 10 μM .

DISCUSSION

In the present study we have purified the InsP₃ receptor protein from rat vas deferens and defined some of its properties. We have also compared the relative [^3H]InsP₃ binding levels in rat peripheral tissues (Table 1). We observed high-affinity InsP₃ binding ($K_d = 5\text{--}10\text{ nM}$) in vas deferens and other peripheral tissues, which is similar to the InsP₃ binding affinities reported in liver (1.7 nM, [11]), adrenal gland (5 nM, [9]) and pituitary gland (1.5 nM, [12]). Vas deferens InsP₃ binding levels are by far the highest of rat peripheral tissues. Our InsP₃ binding levels in adrenal gland, liver and spleen resemble those observed by others in adrenal gland (186 fmol/mg of protein [9], liver (33 fmol/mg [32]) and neutrophils (194 fmol/mg [10]). Our InsP₃ binding levels in liver are somewhat lower than those reported by Guillemette *et al.* (239 fmol/mg of protein [11]).

The purified vas deferens protein appears to be identical with the protein purified in parallel experiments from rat cerebellum, and is of similar molecular mass to other InsP₃ receptors detected in rat peripheral tissues using rabbit antisera raised to purified cerebellar InsP₃ receptors (A. H. Sharp, R. J. Mourey, C. D.

Ferris & S. H. Snyder, unpublished work). Nunn *et al.* [37] have also identified a 260 kDa protein in liver and cerebellum using radiation inactivation. Thus in the rat we have little evidence for heterogeneity of the InsP₃ receptor protein in any tissues. Chadwick *et al.* [8] recently purified and characterized an InsP₃ receptor from bovine aorta. This protein has similar properties to the rat vas deferens InsP₃ receptor, namely similar abundance, K_d , B_{max} , and inhibition by heparin. The bovine aorta InsP₃ receptor has an apparently lower molecular mass (224 kDa) than the cerebellar and vas deferens InsP₃ receptor (260 kDa). Possible explanations for discrepancies in molecular mass could be explained by species variations or differences in electrophoretic properties.

The substantial levels of nearly identical InsP₃ receptor proteins in numerous peripheral tissues of the rat fit with the postulated role of InsP₃ in regulating the activities of organs throughout the body, and suggest that similar molecular mechanisms of InsP₃ action in releasing Ca²⁺ are involved in all tissues. Unlike the cerebellar InsP₃ receptor, however, we did not see Ca²⁺-dependent inhibition of InsP₃ binding to vas deferens membrane preparations. Similarly, Nahorski and associates [38] found no effects of Ca²⁺ on InsP₃ binding in airway smooth muscle membranes. Failure of Ca²⁺ to inhibit particulate InsP₃ binding in vas deferens and other smooth muscle tissue may reflect a lack of calmodin in peripheral tissues or, alternatively, the smooth muscle receptor may not associate with calmodin. We have addressed this question by conducting reconstitution experiments using calmodin from either vas deferens or cerebellum with purified cerebellar and vas deferens InsP₃ receptor. We observe that the vas deferens InsP₃ receptor lacks an endogenous supply of calmodin activity, although it is still sensitive to Ca²⁺ inhibition by brain calmodin. Our inability to demonstrate calmodin activity in vas deferens extracts does not rule out the presence of low, undetectable levels. Our earlier studies [33] suggested that calmodin may possess additional roles besides regulating InsP₃ receptors, since the regional distribution of calmodin in the brain differs from that of InsP₃ receptors.

The purified vas deferens InsP₃ receptor specifically recognizes Ins(1,4,5)P₃, whereas other inositol phosphates are either inactive or 20–30-fold less potent. At 1 μM concentrations, InsP₆ inhibits 30–35% of [^3H]InsP₃ binding. InsP₆ levels in animal tissues have been reported to be 5–15 μM [39], suggesting a potential physiological role at InsP₃ receptors. It is unlikely that inhibition of binding by InsP₆ is due to InsP₃ impurities, because our InsP₆ was determined to be > 99% pure by thin-layer chromatography (P. Pingerelli, unpublished work).

The high density of InsP₃ receptors in the vas deferens and their localization to smooth muscle agrees with evidence indicating a role for InsP₃ in pharmacomechanical coupling of smooth muscle [15–17]. A similar role for the ryanodine receptor in smooth muscle is suggested by the ability of ryanodine to abolish phasic contractions of smooth muscle [24,25]. Perhaps both InsP₃ and ryanodine receptors are involved. Release of Ca²⁺ by InsP₃ would be expected to elevate intracellular Ca²⁺ levels from basal values of about 0.3 μM to about 3 μM . The threshold for stimulation of the Ca²⁺-induced Ca²⁺ release associated with ryanodine receptors is in the low micromolar range [19]. Functional interactions of InsP₃ and ryanodine receptors might represent a novel relationship, as ryanodine receptor binding levels in the brain are very low (S. Supattapone, unpublished work), while skeletal muscle has extremely low levels of InsP₃ binding, though influences of InsP₃ on skeletal muscle contraction have been reported [40]. Such an interaction between InsP₃ and ryanodine receptors has been described by Iino *et al.* [24]. They describe two classes of Ca²⁺ pools in smooth muscle, one containing both Ca²⁺-induced Ca²⁺ release and InsP₃-

mediated Ca^{2+} release, and the other possessing only InsP_3 -gated Ca^{2+} channels.

Because InsP_3 receptors in vas deferens and peripheral tissues are similar to the brain InsP_3 receptor, peripheral organs may be of particular value in studying physiological actions of InsP_3 . Direct correlations of functional and molecular events are more feasible in peripheral than in central tissues. The vas deferens, whose contractile features are well characterized, is an excellent model for studying functional aspects of the phosphoinositide cycle. The vas deferens contains high concentrations of phosphoinositide-linked α_1 -adrenergic receptors [41]. Moreover, co-transmitter interactions of neurotransmitters, such as noradrenaline and ATP [42], have been well delineated in the vas deferens.

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REFERENCES

- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341**, 197–205
- Ferris, C., Haganir, R., Supattapone, S. & Snyder, S. H. (1989) *Nature (London)* **342**, 87–89
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–68
- Ross, C. A., Meldolesi, J., Milner, T. A., Sato, T., Supattapone, S. & Snyder, S. H. (1989) *Nature (London)* **468**–470
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988) *J. Biol. Chem.* **263**, 1530–1534
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) *Nature (London)* **342**, 32–38
- Chadwick, C. C., Saito, A. & Fleischer, S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2132–2136
- Baukal, A. J., Guillemette, G., Rubin, R., Spat, A. & Catt, K. J. (1985) *Biochem. Biophys. Res. Commun.* **133**, 532–538
- Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W. (1986) *Nature (London)* **319**, 514–516
- Guillemette, G., Balla, T., Baukal, A., Spat, A. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1010–1015
- Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8195–8199
- Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. (1987) *Nature (London)* **325**, 159–161
- Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987) *J. Biol. Chem.* **262**, 12132–12136
- Somlyo, A. P., Walker, J. W., Goldman, Y. E., Trentham, D. R., Kobayashi, S., Kitazawa, T. & Somlyo, A. V. (1988) *Philos. Trans. R. Soc. London B* **340**, 399–414
- VanBreeman, C. & Saida, K. (1989) *Annu. Rev. Physiol.* **51**, 315–329
- Karaki, H. & Weiss, G. B. (1988) *Life Sci.* **42**, 111–122
- Fill, M., Ma, J., Knudson, C. M., Imagawa, T., Campbell, K. P. & Coronado, R. (1989) *Ann. N.Y. Acad. Sci.* **560**, 115–162
- Meissner, G. & Lai, F. A. (1988) *J. Bioenerg. Biomembr.* **21**, 227–246
- Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. & Numa, S. (1989) *Nature (London)* **339**, 439–445
- Mighery, G. A., Sudhof, T. C., Takei, K. & DeCamilli, P. (1989) *Nature (London)* **342**, 192–195
- Hashimoto, T., Hirata, M., Itoh, T., Kaninura, Y. & Kuriyama, H. (1986) *J. Physiol. (London)* **370**, 605–618
- Erlach, B. E. & Watras, J. (1988) *Nature (London)* **336**, 583–586
- Iino, M., Kobayashi, T. & Endo, M. (1988) *Biochem. Biophys. Res. Commun.* **152**, 417–422
- Vesperinas, G., Feddersen, M., Lewin, J. & Huidobro-Toro, J. P. (1989) *Eur. J. Pharmacol.* **165**, 309–313
- Worley, P. F., Baraban, J. M. & Snyder, S. H. (1989) *J. Neurosci.* **9**, 339–346
- Kamra, A. & Gupta, M. N. (1988) *Biochem. Int.* **16**, 679–687
- Dulley, J. R. & Grieve, P. A. (1975) *Anal. Biochem.* **64**, 136–144
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Wray, W., Bouliskas, T., Wray, V. P. & Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203
- Theibert, A. B., Supattapone, S., Ferris, C. D., Danoff, S. K., Evans, R. K. & Snyder, S. H. (1990) *Biochem. J.* **267**, 441–445
- Mauger, J., Claret, M., Pietri, F. & Hilly, M. (1989) *J. Biol. Chem.* **264**, 8821–8826
- Danoff, S. K., Supattapone, S. & Snyder, S. H. (1988) *Biochem. J.* **254**, 701–705
- Macara, J. G. (1985) *Am. J. Physiol.* **248**, C3–C11
- Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Steiner, J. & Snyder, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8747–8750
- Tawada, Y., Furukawa, K. & Shigekawa, M. (1988) *J. Biochem. (Tokyo)* **104**, 795–800
- Nunn, D. L., Potter, B. V. L. & Taylor, C. W. (1990) *Biochem. J.* **265**, 393–398
- Chilvers, E. R., Challiss, R. A. J., Willcocks, A. L., Potter, B. V. L. & Nahorski, S. R. (1990) *Br. J. Pharmacol.* **99**, 297–302
- Szwergold, B. S., Graham, R. A. & Brown, T. R. (1987) *Biochem. Biophys. Res. Commun.* **149**, 874–881
- Volpe, P., Di Virgilio, F., Pozzan, T. & Salviati, G. (1986) *FEBS Lett.* **197**, 1–4
- Minneman, K. P. (1988) *Pharmacol. Rev.* **40**, 87–119
- Huidobro-Toro, J. P. & Parada, S. (1988) *Neurosci. Lett.* **85**, 339–344

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