Inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate binding sites in smooth muscle

Lubo Zhang, Michael E. Bradley, Mohammad Khoyi, David P. Westfall & ¹Iain L.O. Buxton

Department of Pharmacology/318, University of Nevada School of Medicine, Reno, Nevada 89557, U.S.A.

1 We have previously demonstrated that activation of M₃ muscarinic receptors increases inositol 1,4,5-trisphosphate (InsP₃) and inositol 1,3,4,5-tetrakisphosphate (InsP₄) accumulation in colonic smooth muscle.

2 In the present study, we demonstrate the existence of InsP₃ and InsP₄ binding sites in colonic circular smooth muscle by use of radioligand binding methods. Both [³H]-InsP₃ and [³H]-InsP₄ bound rapidly and reversibly to a single class of saturable sites in detergent-solubilized colonic membranes with affinities of 5.04 ± 1.03 nM and 3.41 ± 0.78 nM, respectively. The density of [³H]-InsP₃ binding sites was 335.3 ± 19.3 fmol mg⁻¹ protein which was approximately 2.5 fold greater than that of [³H]-InsP₄ sites (127.3 ± 9.1 fmol mg⁻¹ protein).

3 The two high affinity inositol phosphate binding sites exhibited markedly different pH optima for binding of each radioligand. At pH 9.0, specific [³H]-InsP₃ binding was maximal, whereas [³H]-InsP₄ binding was only 10% that of [³H]-InsP₃. Conversely, at pH 5.0, [³H]-InsP₄ binding was maximal, while [³H]-InsP₃ binding was reduced to 15% of its binding at pH 9.0.

4 InsP₃ was about 20 fold less potent ($K_1 = 50.7 \pm 8.3$ nM) than InsP₄ in competing for [³H]-InsP₄ binding sites and could compete for only 60% of [³H]-InsP₄ specific binding. InsP₄ was also capable of high affinity competition with [³H]-InsP₃ binding ($K_1 = 103.5 \pm 1.5$ nM), and could compete for 100% of [³H]-InsP₃ specific binding.

5 [³H]-InsP₃ binding in subcellular fractions separated by discontinuous sucrose density gradients followed NADPH-cytochrome c reductase activity, suggesting an intracellular localization for the majority of InsP₃ receptors in this tissue, whereas [³H]-InsP₄ binding appeared to be equally distributed between plasma membrane and intracellular membrane populations.

6 These results suggest the existence of distinct and specific InsP₃ and InsP₄ binding sites which may represent the physiological receptors for these second messengers; differences in the subcellular distribution of these receptors may contribute to differences in their putative physiological roles.

Keywords: InsP₃ receptor; InsP₄ receptor; radioligand binding; smooth muscle; colon (canine)

Introduction

It has been well established that the phosphoinositide cascade plays a central role in the transduction of many extracellular stimuli. Activation of phosphoinositide-specific phospholipase C generates inositol 1,4,5-trisphosphate (InsP₃), which in turn releases Ca²⁺ from an intracellular store (Berridge & Irvine, 1989). In most tissues, InsP₃ is metabolized by either 5-dephosphorylation to inositol 1,4-bisphosphate (InsP₂) or 3-phosphorylation to inositol 1,3,4,5-tetrakisphosphate (InsP₄) (Irvine *et al.*, 1986; Shears, 1989). InsP₄ has also been implicated in Ca²⁺ homeostasis. Proposals for the action of InsP₄ have included activation of extracellular Ca²⁺ entry (Irvine & Moor, 1986; Changya *et al.*, 1989), modulation of the InsP₃-sensitive Ca²⁺ pool (Gawler *et al.*, 1991), and a direct effect on intracellular Ca²⁺ mobilization (Hill *et al.*, 1988).

InsP₃ binding sites have been identified in a number of tissues (Worley *et al.*, 1987; Enyedi *et al.*, 1989; Varney *et al.*, 1990; Van Delden *et al.*, 1992), including smooth muscle (Chadwick *et al.*, 1990). Comparison of the smooth muscle InsP₃ receptor with that from cerebellum indicates that the two receptors are similar both structurally and functionally (Marks *et al.*, 1990). The InsP₃ receptor has been purified from cerebellum and its primary structure has been determined (Furuichi *et al.*, 1989; Maeda *et al.*, 1990). Functional identity of the InsP₃ receptor as an intracellular Ca²⁺ release channel has been indicated by incorporation of the cerebellar and smooth muscle InsP₃ receptors into phospholipid vesicles and observing an InsP₃-activated Ca²⁺ conductance (Ferris *et al.*, 1989; Mayrleitner *et al.*, 1991). Compared to the InsP₃

receptor, however, our understanding of the InsP₄ receptor is poor. InsP₄ receptors have been described by radioligand binding methods in cerebellum, HL-60 cells, and parathyroid glands (Bradford & Irvine, 1987; Enyedi *et al.*, 1989; Donie *et al.*, 1990). Recently, a putative high-affinity InsP₄ receptor was purified from pig cerebellar membranes (Donie & Reiser, 1991). While a role for InsP₄ action in smooth muscle has not been established, generation of InsP₄ in smooth muscle has been described by ourselves (Zhang & Buxton, 1991; Schiemann *et al.*, 1991) and others (Bielkiewicz-Vollrath *et al.*, 1987; Pijuan & Litosch, 1988; Salmon & Bolton, 1988) although the location and nature of smooth muscle InsP₄ receptors have not been described. It is still unknown whether InsP₄ acts at the plasma membrane, on internal Ca²⁺ stores, or on a structural element which connects the plasma membrane and the Ca²⁺ store. Subcellular localization along with functional studies and reconstitution experiments of InsP₄ receptors should shed light on these questions.

We have recently demonstrated the coexistence of muscarinic receptors of the M₂ and M₃ subtypes in canine colonic circular smooth muscle (Zhang *et al.*, 1991). Activation of M₃ muscarinic receptors leads to a rapid accumulation of InsP₃ while InsP₄ accumulation is delayed. The rapid accumulation of InsP₃ is likely to play a direct role in initiation of the early tonic phase of contraction of the muscle following activation of muscarinic receptors since removal of extracellular Ca²⁺ does not abolish this phase of contraction or the M₃-mediated Ca²⁺-transient seen in single colonic myocytes loaded with fura-2, while the phasic contractions that follow are entirely dependent on extracellular Ca²⁺ (unpublished observation). The role of InsP₄ in regulating intracellular

¹ Author for correspondence.

Ca^{2+} oscillations, which correlate well with phasic contractions in colon smooth muscle is not known.

To clarify the role of InsP_3 and InsP_4 as second messengers in colonic smooth muscle, we have examined the binding of these two inositol polyphosphates to detergent-solubilized colonic smooth muscle membranes. We have resolved distinct, high affinity binding sites for InsP_3 and InsP_4 and have determined the intracellular distribution of these sites by means of subcellular fractionation techniques.

Methods

Solubilized membrane preparations

Canine colonic circular smooth muscle was prepared as described previously (Zhang *et al.*, 1991). The smooth muscle was minced and suspended in 10 volumes of Buffer A (composition, mM: Tris/HCl 20, NaCl 20, KCl 100, EDTA 1, BSA 1 mg ml⁻¹, NaN₃ 0.02%, pH 7.7). Tissue was then homogenized in a Polytron tissue homogenizer at setting 3.5 in two bursts of 15 s each. The homogenate was centrifuged at 100,000 g for 30 min. Pellets were re-homogenized in half the original volume of Buffer A at setting 3.5 for 15 s, and re-centrifuged using the same conditions. The resulting supernatant was discarded and the pellet was solubilized as described by Hingorani & Agnew (1991). Briefly, the pellet was resuspended in Buffer B (composition mM: Tris/HCl 20, KCl 100, NaCl 20, EDTA 1, NaN₃ 0.02%, pH 8.5) containing 2% (w/v) Lubrol-PX. The suspension was gently homogenized with 20 strokes in a glass-glass homogenizer on ice, followed by incubation on ice with stirring for 20 min. The detergent extract was then centrifuged at 80,000 g for 40 min. For [³H]- InsP_4 binding, the supernatant pH was adjusted to 5.0 with 1 mM HCl.

Radioligand binding studies

Binding of the [³H]- InsP_3 and [³H]- InsP_4 to solubilized colonic smooth muscle membranes was measured by the syringe assay as previously described (Hingorani & Agnew, 1991). The syringe ion-exchange column was prepared with 0.5–0.6 ml of Dowex AG 1-X2 resin equilibrated in 2 volumes of Buffer C (composition mM: Tris/HCl 50, EDTA 1, NaN₃ 0.02%, pH 8.5) containing BSA 10 mg ml⁻¹ for 2 h. A small Spectra/Mesh nylon disk (10 µm pore size) was inserted into the barrel of a 3 ml plastic syringe, followed by a disk of Whatman No. 1 paper filter and then Dowex AG 1-X2 resin. Radioligand binding assays were carried out in a final volume of 200 µl consisting of 175 µl solubilized membrane supernatant, 20 µl radioligand, and 5 µl buffer or unlabelled inositol phosphates. Saturation binding experiments employed concentrations of [³H]- InsP_3 or [³H]- InsP_4 from 0.15 nM to 80 nM and nonspecific binding was determined by the addition of the appropriate unlabelled ligand (12 µM). Equilibrium binding was carried out at 4°C for 15 min. Bound and free radioligand were separated by rapidly pushing equilibrated samples through the syringe columns. The separation could be completed reliably within approximately 1 s. The labelled receptor-ligand complex was collected directly into a vial for counting at 45% efficiency in a Beckman LS 6000IC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, U.S.A.). All determinations were made in triplicate. Specific binding of [³H]- InsP_3 or [³H]- InsP_4 at 10 nM was examined in competition studies employing increasing concentrations of unlabelled inositol phosphates. Protein was determined by the method of Bradford (1976).

Preparation of subcellular fractions

Freshly isolated colonic circular smooth muscle was minced and homogenized on ice in Buffer D (sucrose 300 mM, Tris/base 20 mM, DTT 5 mM, pH 7.2) by 20 strokes in a Dual

No. 20 tissue grinder. All samples were kept at 4°C throughout purification. The homogenate was centrifuged at 2,500 g for 20 min, after which the pellet was again homogenized, diluted, and centrifuged. A postnuclear supernatant was obtained by centrifuging the combined supernatants at 15,000 g for 20 min. The resulting supernatant was then centrifuged at 40,000 g for 90 min, and the pellet was resuspended in 4 ml Buffer E (NaCl 80 mM, Na-oxalate 5 mM). This suspension was separated on a discontinuous sucrose density gradient (24%, 28%, 33%, 45%, w/v) by centrifuging at 64,000 g for 3 h in a swinging bucket rotor as described by Carsten & Miller (1980). PMSF (0.2 mM final concentration) was added to gradient media to inhibit proteinase. Three bands of decreasing densities were identified: a heavy-density band (interface 45% and 33% sucrose), an intermediate-density band (interface 33% and 28% sucrose) and a low-density band (interface 28% and 24% sucrose). The gradient was separated into 8 fractions including the pellet, which was discarded. Collected fractions were diluted 7 fold in Buffer E and centrifuged at 70,000 g for 2 h. The resulting pellets were resuspended in 1 ml of Buffer D and 0.1 ml of the suspensions was reserved for marker enzyme and protein assay. The remaining samples were diluted in binding buffer A and centrifuged at 70,000 g for 2 h, after which pellets were solubilized as described above.

Biochemical marker determinations

K⁺-dependent *p*-nitrophenyl phosphatase (*p*-NPPase) was assayed by the method of Murer *et al.* (1976) and NADPH-cytochrome c reductase by the method of Mircheff *et al.* (1983). Protein was determined by the method of Bradford (1976) with BSA as the standard.

ATP-dependent calcium accumulation

Pellets following fractionation were resuspended in 350 µl of a solution of the following composition (mM): KCl 150, HEPES 20 (pH 7.0, adjusted with KOH). Calcium uptake was measured according to the method of Moore *et al.* (1975) in the following medium (mM): KCl 130, HEPES 20, MgCl₂ 5, ammonium oxalate 5 (to precipitate intravesicular Ca²⁺), CaCl₂ 0.02 and ⁴⁵CaCl₂ 0.6 µCi ml⁻¹, with or without ATP 5 (pH adjusted to 7.0 with KOH). The incubation was carried out at 37°C for 10 min and the vesicle accumulated ⁴⁵Ca was separated from medium by rapid filtration through 0.45 µm millipore membrane filters. The filter was washed twice with 4 ml ice cold solution of KCl (150 mM) and HEPES (20 mM, pH 7.0) and left in a scintillation vial overnight at room temperature to dry. ⁴⁵Ca was determined by liquid scintillation spectrometry. The ratio of ⁴⁵Ca uptake in the presence of ATP to that in the absence of ATP was determined.

Data analysis

Saturation binding data were analyzed by both Scatchard plot and computer assisted non-linear least-squares regression to fit binding data (GraphPad Inplot v. 4.0, Graphpad Software, San Diego, CA, U.S.A.). Both methods yielded similar *K_D* and *B_{max}* values (Schiemann *et al.*, 1990). For competition binding data, the non-linear least-squares approach fits the data to either one or two classes of binding sites and assists in determining if the two site model is significantly better than the one-site fit of the data (*F* test). Data are expressed as the mean ± s.e.

Materials

The materials and their sources were as follows: [³H]-inositol 1,4,5-trisphosphate ([³H]- InsP_3 , 17 Ci mmol⁻¹), [³H]-inositol 1,3,4,5-tetrakisphosphate ([³H]- InsP_4 , 17 Ci mmol⁻¹) and ⁴⁵CaCl₂ (18 Ci g⁻¹) were from Dupont-NEN (Boston, MA,

U.S.A.); inositol 1,4,5-trisphosphate (InsP₃), inositol 1,3,4,5-tetrakisphosphate (InsP₄) and bovine serum albumin (BSA) were from Calbiochem (La Jolla, CA, U.S.A.); Lubrol-PX, Dowex AG 1-X2 resin (chloride form, 200–400 mesh), sucrose, reduced β -nicotinamide adenine dinucleotide phosphate (NADPH), *p*-nitrophenyl phosphate, adenosine triphosphate (ATP) Sigma 104, EDTA, Tris/HCl, NaN₃, phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Spectra/Mesh nylon filters (10 μ m pore size) were from Spectrum (Los Angeles, CA, U.S.A.); Whatman No. 1 paper filters were from Whatman (Hillsboro, OR, U.S.A.).

Results

Radioligand binding characteristics

Studies of the binding of [³H]-InsP₃ and [³H]-InsP₄ to detergent-solubilized membranes prepared from colon circular smooth muscle indicated that the binding of these two inositol polyphosphates was specific and exhibited markedly different pH optima (Figure 1). By increasing the pH from 4 to 9, specific binding of [³H]-InsP₃ increased 8 fold. At pH 5.0, little specific binding of [³H]-InsP₃ to solubilized membranes could be detected. In contrast, specific binding of [³H]-InsP₄ was maximal at pH 5.0, but decreased dramatically as the assay pH was increased, and was minimal at pH 9.0. This finding is in agreement with previous studies which demonstrated that optimal binding of [³H]-InsP₃ and [³H]-InsP₄ in cerebellum occurred at pH 8.0 and pH 5.0, respectively (Donie & Reiser, 1989; Challiss *et al.*, 1991).

At pH 8.5, binding of [³H]-InsP₃ to detergent-solubilized membranes of colon circular smooth muscle was specific and saturable (Figure 2). Non-specific binding represented approximately 10% of the total binding at the concentration of [³H]-InsP₃ near the K_D (5 nM). Saturation isotherms employing [³H]-InsP₃ consistently yielded linear transforms (bound/free vs bound) and were best described by an interaction of the radioligand with a single class of binding sites with an apparent dissociation constant (K_D) of 5.04 ± 1.03 nM and

Figure 1 Effect of pH on [³H]-InsP₃ and [³H]-InsP₄ specific binding to detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Binding of [³H]-InsP₃ (15 nM; ○) and [³H]-InsP₄ (15 nM; ●) at different pH values was determined as described in Methods. Non-specific binding was determined in the presence of 12 μ M unlabelled ligand in each case. Values represent means of triplicate determinations from a single representative experiment. Standard deviations were less than 20%.

Figure 2 Saturation binding of [³H]-InsP₃ to detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Increasing concentrations of [³H]-InsP₃ (0.15 to 80 nM) were incubated with the solubilized sarcolemmal membrane at pH 8.5 as described in Methods. Specific [³H]-InsP₃ binding, defined as the difference between total binding and nonspecific binding observed in the presence of 12 μ M unlabelled InsP₃, is presented as fmol of receptor bound per mg of solubilized protein. Nonspecific binding represented about 10% of total binding at 5 nM [³H]-InsP₃. Analysis of the specific binding data by Scatchard plot (inset) confirmed that [³H]-InsP₃ bound to a single class of binding sites in the solubilized membranes. The receptor affinity (K_D) and density (B_{max}) are 4.8 nM and 345 fmol mg⁻¹ protein respectively for the experiment shown. Data points represent the means of triplicate determinations in a single experiment. Mean data obtained from several such experiments ($n = 6$) are presented in the text.

B_{max} of 335.3 ± 19.3 fmol mg⁻¹ protein ($n = 6$). Saturation binding of [³H]-InsP₄ to solubilized membranes was performed at pH 5.0 (Figure 3). Consistent with the data reported above for [³H]-InsP₃, analysis of [³H]-InsP₄ binding data demonstrated that [³H]-InsP₄ bound to an apparently homogeneous receptor population with a K_D of 3.41 ± 0.78 nM and a B_{max} of 127.3 ± 9.1 fmol mg⁻¹ protein ($n = 7$). Non-specific binding of [³H]-InsP₄ was slightly higher than that of [³H]-InsP₃ and was 40% of total binding at 4 nM.

In order to investigate the specificity of [³H]-InsP₃ and [³H]-InsP₄ binding to solubilized membranes, unlabelled InsP₃ and InsP₄ were employed in radioligand binding competition studies. In a manner consistent with the saturation binding data, InsP₃ and InsP₄ competed with their radioligand form with high affinity (Figure 4). InsP₃ vs [³H]-InsP₃ competition gave a K_i of 4.4 ± 1.2 nM and InsP₄ vs [³H]-InsP₄ of 2.8 ± 0.9 nM ($n = 3$). Computer assisted analysis of the competition curves indicated that both inositol polyphosphates bound to a single class of sites. Despite marked differences in pH optima between [³H]-InsP₃ and [³H]-InsP₄ binding, there is significant cross-specificity of each ligand. As shown in Figure 4, the specific binding of [³H]-InsP₃ was not only competed for by InsP₃ but also by InsP₄. InsP₄ was about 20 fold less potent than InsP₃ in competition for specific [³H]-InsP₃ binding with K_i being 103.5 ± 1.5 nM ($n = 3$). Similar results were obtained with InsP₃ competitions for [³H]-InsP₄ specific binding ($K_i = 50.7 \pm 8.3$ nM, $n = 3$). Unlike InsP₄ competition for [³H]-InsP₃ binding however, InsP₃ was not able to compete for some 40% of [³H]-InsP₄ binding sites. While the reason for this discrepancy is not clear, it is possible that some InsP₄ binding sites are not capable of binding InsP₃. Such a possibility suggests the presence of two disparate InsP₄ receptors.

Figure 3 Saturation binding of [^3H]-InsP $_4$ to detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Increasing concentrations of [^3H]-InsP $_4$ (0.15 to 80 nM) were incubated with the solubilized sarcolemmal membrane at pH 5.0 as described in Methods. Specific [^3H]-InsP $_4$ binding, defined as the difference between total binding and nonspecific binding observed in the presence of 12 μM unlabelled InsP $_4$, is presented as fmol of receptor bound per mg of solubilized protein. Nonspecific binding represented about 40% of total binding at 4 nM [^3H]-InsP $_4$. Analysis of the specific binding data by Scatchard plot (inset) confirmed that [^3H]-InsP $_4$ bound to a single class of binding sites in the solubilized membranes. The receptor affinity (K_D) and density (B_{max}) are 3.9 nM and 129 fmol mg $^{-1}$ protein respectively for the experiment shown. Data points represent the means of triplicate determinations in a single experiment. Mean data obtained from several such experiments ($n = 7$) are presented in the text.

Subcellular fractionation

In order to delineate the intracellular distribution of inositol phosphate binding in colonic circular smooth muscle, tissue homogenates were subjected to a combination of differential sedimentation and equilibrium density gradient centrifugations, followed by assays for membrane marker enzymes and total protein. Figure 5 depicts the results of a typical density gradient distribution of marker activities including *p*-NPPase (known to be equivalent to the activity of ouabain-sensitive Na,K-ATPase). *p*-NPPase activity has traditionally been used as a marker for plasma membranes, but is also known to be present in significant quantities within intracellular membranes such as the Golgi complex (Yiu *et al.*, 1991). Also depicted in Figure 5 are the distributions of membrane-associated protein and the endoplasmic reticulum marker NADPH-cytochrome *c* reductase; while the latter activity is also present within the membranes of the mitochondria, the vast majority of this potentially contaminating activity was removed from the preparation along with intact mitochondria by means of a differential sedimentation step, prior to density gradient separation (not shown). The activities present within the density gradients suggest the presence of at least 3 major membrane populations with distribution modes in density fractions II, IV, and V (Figure 5). The distribution of *p*-NPPase activity is distinctly bimodal, with peaks of activity present within fractions II and V; this observation is consistent with the localization of sodium pump proteins on intracellular as well as plasma membrane populations (Yiu *et al.*, 1991). In contrast, NADPH-cytochrome *c* reductase exhibits a major mode only within density fraction II, and is only nominally present within fractions IV–VII; the latter activities could be due to a skewed distribution of a single population, or might represent minor activities present on the

Figure 4 Competition of [^3H]-InsP $_3$ and [^3H]-InsP $_4$ by unlabelled inositol phosphates in detergent-solubilized membranes prepared from canine colonic circular smooth muscle. The solubilized membranes were incubated with 10 nM [^3H]-InsP $_3$ (b) or 10 nM [^3H]-InsP $_4$ (a) in the presence and absence of increasing concentrations of unlabelled InsP $_3$ (●) and InsP $_4$ (■). Data, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 12 μM unlabelled InsP $_3$ or InsP $_4$, are expressed as the percentage of maximal [^3H]-InsP $_3$ or [^3H]-InsP $_4$ binding. Data points represent the means of triplicate determinations in a single representative experiment. Binding parameters determined in several such experiments ($n = 3$) are presented in the text.

p-NPPase-containing membranes in the population localized to fraction V. Correlating well with the peaks of *p*-NPPase and NADPH-cytochrome *c* reductase activities in fraction II is a large peak of membrane-bound protein, a result which has been found to be consistent with the presence of intracellular (eg. Golgi complex and endoplasmic reticulum) membranes (Bradley & Mircheff, unpublished observation). These data, taken together, suggest an intracellular origin for the membranes equilibrating within density fraction II.

When ratios of percentage recovered marker enzyme activity to recovered protein are calculated, an indication of the enrichments of the enzyme activities as a result of density gradient separations is obtained. As can be seen in Table 1, small amounts of protein recovered in density fraction V provide for large enrichments for all activities present in this fraction, and the large peak of *p*-NPPase activity in fraction V, when normalized to protein, results in the highest observed enrichment factor (6.8) obtained from any of the markers. The enrichment for *p*-NPPase, coupled with the large (at least 25%) amount of this activity recovered in fraction V, as well as the relatively low amounts of intracel-

Figure 5 Density distribution of membrane markers in circular smooth muscle membrane. Colonic circular smooth muscle homogenates were first subjected to low-speed and then high-speed differential centrifugation procedures, and then loaded onto discontinuous sucrose density gradients for equilibrium density centrifugation as described in Methods. Values given are representative of results from 3 different preparations, and are presented as percentages of the total activity recovered from the gradient.

lular markers present, provide strong evidence for the plasmalemmal origin of the membranes in this region of the gradient.

In accordance with the enzyme markers, ATP-dependent calcium accumulation was found highest within density fraction II and very weak in density fraction V (Figure 6). There was four times as much ATP-dependent calcium accumulation in density fraction II as in the fraction V. Although thapsigargin inhibited 50% of calcium uptake into the microvesicles before sucrose density gradient separation (data not shown), the inhibition was not seen in the fractions from the density gradient.

Subcellular distribution of inositol phosphate binding

Figure 7 presents the results of inositol phosphate binding assays performed on vesicles separated by the procedures summarized above and subsequently solubilized as described in Methods. The largest peak of recovered InsP_3 activity was found in fraction II of the density gradient, and therefore appears to co-equilibrate with intracellular membrane marker

Table 1 Marker enrichment factors

Marker	I	II	III	IV	V	VI	VII
<i>p</i> -NPPase	0.5	0.9	1.0	1.0	6.8	0.6	0.0
NADPH-cytochrome c reductase	1.0	0.8	1.0	0.7	3.1	1.2	1.2
InsP_3	0.2	1.2	0.8	0.7	4.4	1.4	0.6
InsP_4	0.1	0.7	1.5	1.2	6.4	0.1	1.0

Activity enrichments resulting from equilibrium density gradient separation and high-speed differential sedimentation of membrane vesicles from colonic smooth muscle. Enrichments were calculated as the ratio of percentage recovered enzyme or receptor activity to that of protein contained within the same density fraction, and reflect the concentrations of specific markers within membrane populations.

Figure 6 Distribution of ATP-dependent ^{45}Ca uptake in circular smooth muscle membrane density fractions. ^{45}Ca uptake was measured as described in Methods. The ratio of ^{45}Ca uptake in the presence of ATP to that in the absence of ATP was calculated for each fraction. SR: sarcoplasmic reticulum; SL: sarcolemmal. Values represent means of four experiments. Standard deviations were less than 15%.

activities present in this region (Figure 5). Significant InsP_3 binding activity was also found in the region of density fractions IV–VI, albeit much less localized than the InsP_3 binding activity seen in fraction II (Figure 7a). InsP_4 binding displayed a well-defined bimodal distribution (Figure 7b) which resembled closely that of *p*-NPPase, with distribution modes centered in density fractions II and V. The highest enrichments for both InsP_3 and InsP_4 binding were found in fraction V; enrichment for InsP_4 binding in this fraction was $1.5\times$ that of InsP_3 binding (Table 1), and reflects the relatively large peak of InsP_4 binding found in this region of the gradient. Indeed, assuming a bimodal distribution in fractions II and V, one can see that the overlap in these two patterns sums in fractions III and IV. InsP_4 binding is therefore equally distributed between regions of the density gradient which appear to contain intracellular membranes (e.g. fraction II) and regions containing membranes of plasma membrane origin (e.g. fraction V).

Figure 7 Distribution of [³H]-InsP₃ and [³H]-InsP₄ specific binding in subcellular fractions prepared from canine colonic circular smooth muscle. The subcellular fractions of colon smooth muscle were prepared from discontinuous sucrose density gradients as described in Methods. The binding of [³H]-InsP₃ (15 nM; a) and [³H]-InsP₄ (15 nM; b) to the fractions was performed at pH 8.5 ([³H]-InsP₃) and pH 5.0 ([³H]-InsP₄). Nonspecific binding was obtained in the presence of 12 μ M unlabelled InsP₃ or InsP₄. The highest of [³H]-InsP₃ specific binding was in the light-density fraction II, whereas [³H]-InsP₄ specific binding was equally distributed between II and V fractions. SR: sarcoplasmic reticulum; SL: sarcolemmal. Values are means of two separate experiments performed in triplicate. Standard deviations were less than 15%.

Discussion

This study demonstrates for the first time the presence of high affinity InsP₄ binding sites in smooth muscle and their localization to plasma as well as intracellular membranes. Both InsP₃ and InsP₄ binding sites have been demonstrated in gradient-fractionated native membranes and in solubilized membranes. Detergent solubilization of InsP₃ and InsP₄ binding sites in smooth muscle increased radioligand binding with affinities comparable to those of the receptors in native membranes measured by others (Donie *et al.*, 1990; Hingorani & Agnew, 1991). The distinct pH-dependencies of the InsP₃ and InsP₄ receptor binding are in broad agreement with previous findings by other groups studying brain (Challiss *et al.*, 1991; Donie & Reiser, 1991; Theibert *et al.*, 1990) and is likely to relate to the required ionization state of the phosphate substituents of the ligand. By using ¹H- and ³¹P-n.m.r. spectroscopy, White *et al.* (1991) could demonstrate that InsP₃ had five equatorial substituents and one axial substituent. No detectable change in conformation was observed over the pH range 4–11. The increase in affinity of InsP₃ for

its receptor with increasing pH was explained by ionization of the 4- and 5-phosphate groups. On the other hand, the high affinity conformation of the InsP₄ binding sites was stabilized at acidic pH, facilitating their detection by radioligand binding studies. Such cellular pH changes occurring during Ca²⁺ regulation following addition of agonists (Danthuluri *et al.*, 1990; Kohmoto *et al.*, 1990) are likely to play an important role in modifying the binding properties of the inositol polyphosphates and may be the critical factor in understanding the action of InsP₄ in excitable cells.

InsP₃ receptors have been identified in a number of tissues, including smooth muscle (Chadwick *et al.*, 1990). The cerebellar membrane contains a high density of apparently homogeneous InsP₃ receptors which have been cloned and shown to have a molecular weight of 313,000 (Furuichi *et al.*, 1989; Maeda *et al.*, 1990). InsP₃ receptors isolated from smooth muscle are structurally and functionally similar to those from cerebellum (Marks *et al.*, 1990). Our data demonstrate a relatively high density of InsP₃ receptors in detergent-solubilized membranes of canine colon smooth muscle. The affinity of InsP₃ receptors defined in the present study is similar to that found in other smooth muscle preparations (Chadwick *et al.*, 1990; Varney *et al.*, 1990). In accordance with previous reports by a number of groups (Worley *et al.*, 1987; Chadwick *et al.*, 1990; Varney *et al.*, 1990; Challiss *et al.*, 1991), InsP₃ receptors characterized in colon smooth muscle are apparently homogeneous. Although there are slight differences in the affinities of the InsP₃ receptor for InsP₃ among tissues, there is no consistent pattern to suggest distinct subtypes of the InsP₃ receptor (Varney *et al.*, 1990).

The initial phase of receptor-mediated Ca²⁺ signalling, involving Ca²⁺ release from intracellular stores by InsP₃ is well characterized. It has been demonstrated that InsP₃ induces Ca²⁺ release from reconstituted vesicles containing the brain InsP₃ receptor (Ferris *et al.*, 1989). More recently, the purified InsP₃ receptor from smooth muscle has been reconstituted into planar lipid bilayers and demonstrated to be an InsP₃ activated Ca²⁺ channel (Mayrleitner *et al.*, 1991). The intracellular localization of the InsP₃-sensitive Ca²⁺ pool is thought to be in endoplasmic reticulum (so called sarcoplasmic reticulum in smooth muscle). Subcellular fractionation studies did in fact localize the InsP₃-sensitive Ca²⁺ pool to the microsomal fraction most enriched in endoplasmic reticulum (Joseph *et al.*, 1984; Prenki *et al.*, 1984; Ross *et al.*, 1989). In the present study, the subcellular fraction with the largest peak of InsP₃ binding was found to contain membrane vesicles of predominantly intracellular origin on the basis of its content of the endoplasmic reticulum marker NADPH-cytochrome c reductase as well as large amounts of membrane-associated protein. In addition to enzyme markers, ATP-dependent calcium accumulation was also found to be highest in these membrane vesicles. The same result has been demonstrated in uterine smooth muscle where ATP-dependent calcium accumulation is a property of sarcoplasmic reticulum (Carsten & Miller, 1980). Although it is possible for the cell membrane to have intrinsic ATP-dependent calcium accumulation, this activity is at best weak, both in colon and myometrium. The loss of inhibition of calcium uptake by thapsigargin in density fractions may be due to the loss of its binding sites during sucrose density gradient separation. Interestingly, InsP₃ binding was also present in detectable quantities in regions of the density gradient thought to contain vesicles derived from the plasma membrane (Figure 5). Similar results were obtained in subcellular fractions prepared from rat hepatocytes (Guillemette *et al.*, 1988; Rossier *et al.*, 1991). There is considerable evidence that at least a portion of the InsP₃-sensitive pool, so called subplasmalemmal cisternae, might be localized near the plasma membrane. Using polyclonal antibodies, Ross *et al.* (1989) localized the InsP₃ receptor not only to the endoplasmic reticulum, but also to the subplasmalemmal cisternae. It is possible that the enrichment of InsP₃ binding activity in plasma membrane fractions in the present study is due to

co-equilibration of subplasmalemmal cisternae with the plasma membranes. On the other hand, the InsP₃ receptor may also localize directly to the plasma membrane. In fact, electron microscopy studies using three different monoclonal antibodies indicated the presence of InsP₃ receptors on the plasma membranes in cerebellar Purkinje cells (Furuichi *et al.*, 1989). The localization of InsP₃ receptor on the plasma membrane or on the cell organelle near plasma membrane may suggest an important role for the InsP₃ receptor in regulation of Ca²⁺ homeostasis. It has been demonstrated that muscarinic stimulation of parotid cells induces the opening of Ca²⁺-sensitive ion channels in the plasma membrane prior to increases in cytosolic Ca²⁺ concentrations (Foskett *et al.*, 1989). More directly, InsP₃ has been found to activate Ca²⁺ channels in the plasma membrane of T lymphocytes (Kuno & Gardner, 1987).

Although the InsP₄ receptor has been described in cerebellum, parathyroid glands, adrenal cortex, and HL-60 cells (Bradford & Irvine, 1987; Enyedi & Williams, 1988; Enyedi *et al.*, 1989; Donie *et al.*, 1990), it has not yet been characterized in smooth muscle. In the present study, we have identified binding sites for InsP₄ with an affinity of approximately 3 nM at a concentration of 127 fmol mg⁻¹ protein in detergent-solubilized membranes prepared from canine colonic circular smooth muscle. Similar affinities for InsP₄ were reported in cerebellum (Doine *et al.*, 1990; Theibert *et al.*, 1991). The finding that competition for [³H]-InsP₄ by unlabelled InsP₃ is incomplete raises the possibility of heterogeneity of [³H]-InsP₄ binding sites. In fact, heterogeneous [³H]-InsP₄ binding sites have been reported in cerebellum, adrenal cortex, and parathyroid glands (Enyedi & Williams, 1988; Enyedi *et al.*, 1989; Challiss *et al.*, 1991). However, both saturation and competition analysis of InsP₄ receptor binding in the present study reveal a single class of binding sites for InsP₄ with an affinity similar to that of the high affinity binding sites described in other tissues. The low affinity binding sites may not have been observed in the present study simply because the concentrations of [³H]-InsP₄ used in the present study were not high enough. Alternatively, it is possible that the heterogeneity of InsP₄ binding sites only exists with respect to an action of InsP₃. That is, perhaps InsP₃ acts as an endogenous competitor at some but not all InsP₄ receptors. This possibility is intriguing in light of the distribution of smooth muscle InsP₄ binding sites to two distinct regions of the muscle cell.

Our data provide evidence that the InsP₄ receptor is present in significant quantities on the plasma membrane, as well as on membranes of intracellular origin. High enrichment factors for InsP₄ binding in density fractions thought to contain vesicles of plasma membrane origin may suggest a significant function for InsP₄ in the plasma membrane (Table 1). It has been proposed that InsP₄ acts with InsP₃ as a

second messenger by promoting extracellular Ca²⁺ entry (Gawler *et al.*, 1991). Using single cell voltage clamping methods, Morris *et al.* (1987) have demonstrated that InsP₄, in the presence of InsP₃, evokes a sustained increase in Ca²⁺-activated K⁺ current which is dependent on external Ca²⁺. While it is intriguing to consider a potential role for InsP₃/InsP₄ action in regulation of colonic smooth muscle Ca²⁺-activated K⁺ channels in light of the presence of this channel in circular muscle cells (Carl *et al.*, 1991), it is unlikely that this is the site of action of InsP₄ in smooth muscle since our repeated attempts to regulate this channel with InsP₄ in the presence and absence of InsP₃ failed (data not shown). The localization of InsP₄ binding to the sarcoplasmic reticulum in the present study lends support to the proposed role of InsP₄ in the modulation of an InsP₃-sensitive Ca²⁺ pool as well as possible direct effect upon intracellular Ca²⁺ mobilization. The presence of an additional [³H]-InsP₄ binding to fraction VII is intriguing but not readily explained. What is clear, however, is that [³H]-InsP₄ binds to both plasma and intracellular membranes and may enjoy a physiological function in the regulation of intracellular calcium in smooth muscle.

The demonstration of high affinity InsP₃ and InsP₄ receptors in canine colon circular smooth muscle provides evidence for the roles of InsP₃ and InsP₄ as second messengers in this tissue. In our recent study, we have demonstrated that activation of muscarinic receptors in colon smooth muscle produces a rapid accumulation of InsP₃ (Zhang & Buxton, 1991). InsP₄ accumulation was relatively delayed, which might be due to the similar activities of InsP₃ 3-kinase and the 5-phosphatase enzymes responsible for the formation and removal of InsP₄, respectively. Alternatively, it is possible that the radioactive method we have employed does not permit detection of labelled InsP₄ until late times due to the low abundance of this inositol isomer expected in the smooth muscle cells.

The rapid accumulation of InsP₃ is likely to play a direct role in initiation of the early tonic contraction of colon smooth muscle following activation of muscarinic receptors since removal of extracellular Ca²⁺ does not abolish the contraction, while subsequent phasic contractions are dependent on extracellular Ca²⁺. Recently, Murthy *et al.* (1991) have demonstrated the existence of high affinity InsP₃ receptors in circular smooth muscle of guinea-pig intestine, in which InsP₃ elicited concentration-dependent contractions and increases in intracellular Ca²⁺. The role of InsP₄ in regulating intracellular Ca²⁺ oscillations, which correlate well with phasic contractions in colon smooth muscle, is not entirely clear. Given the fact that InsP₄ is generated in response to M₃ muscarinic receptor stimulation and InsP₄ receptors are present on plasma membranes, InsP₄ may play an important role in regulating Ca²⁺ homeostasis based on the model proposed by Irvine (1990; 1992).

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(Received November 3, 1992

Revised March 4, 1993

Accepted March 12, 1993)