Isolation and characterization of vascular smooth muscle inositol 1,4,5-trisphosphate receptor

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myo-Inositol 1,4,5-trisphosphate (InsP_3) receptor of porcine aorta was purified to near homogeneity and its biochemical properties were compared with those of cerebellar InsP_3 receptor of the same animal species. The aortic InsP_3 receptor consisted of equal amounts of two polypeptides with slightly differing molecular masses of around 240 kDa and was found to possess a single population of InsP_3 -binding site (K_d of 1.2 nM). The InsP_3 receptor purified from porcine cerebellum was also comprised of two polypeptides. However, the molecular mass was slightly but definitely larger, being 250 kDa, and the amounts of the two

polypeptides were not equal. The aortic InsP_3 receptor cross-reacted with polyclonal antibody specific to type 1 InsP_3 receptor as did the cerebellar InsP_3 receptor. The aortic InsP_3 receptor bound to calmodulin–Sepharose in a Ca^{2+} -dependent manner, while the cerebellar InsP_3 receptor did not. Reverse transcriptase-PCR analysis revealed two splicing variants of the type 1 InsP_3 receptor in porcine aortic smooth muscle distinct from those of the type 1 InsP_3 receptor of porcine cerebellum. The possible relevance of this difference to difference in calmodulin-binding property was discussed.

INTRODUCTION

In many cell types, including vascular smooth muscle cells, inositol 1,4,5-trisphosphate (InsP_3) mobilizes Ca^{2+} from intracellular stores in response to certain neurotransmitters, hormones and growth factors [1]. The release is initiated through binding of the compound to a specific receptor composed of four subunits constituting an InsP_3 -gated Ca^{2+} channel and localized to the endo/sarcoplasmic reticulum. Recent molecular cloning studies revealed the existence of at least four types of InsP_3 receptor subunit encoded by different genes designated as 1 [2,3], 2 [4], 3 [5] and 4 [6]. The cerebellar receptor belongs to type 1, but is further diversified by alternative splicing [7,8]. Development- and tissue-specific expression of various InsP_3 receptor isoforms has been suggested. Co-expression of different isoforms in the same cell type is also possible [7,9–11].

So far, the InsP_3 receptor has been purified from only two tissues, i.e. cerebellum [12,13] and smooth muscle [14]. In terms of partial amino acid sequences and InsP_3 binding the receptors from these two tissues resemble each other closely [15]. A 240 kDa protein isolated from porcine aortic smooth muscle as a protein kinase G substrate [16] was later found to be an InsP_3 -binding protein probably representing the major InsP_3 receptor of the aortic smooth muscle [17]. The protein shares many properties in common with InsP_3 receptor isolated from rat cerebellum, but exhibits certain important differences. While the 240 kDa protein consisted of two polypeptides with slightly differing molecular masses, the cerebellar InsP_3 receptors appeared to be single polypeptides with a slightly larger molecular mass of 250–260 kDa. The 240 kDa protein was found to bind to calmodulin in a Ca^{2+} -dependent manner while the cerebellar receptor did not.

Under the circumstances, an attempt was made to purify InsP_3 receptor from porcine aortic smooth muscle and to define its

biochemical and immunological properties in comparison with those of the InsP_3 receptor isolated from cerebellum of the same animal species and the rat. The reverse transcriptase-PCR technique was used to identify the subtypes of aortic InsP_3 receptors.

The study indicated that the aortic smooth muscle InsP_3 receptor belongs to type 1 but is structurally different from the cerebellar type 1 receptor. The possible relevance of this difference to difference in calmodulin-binding property is discussed.

MATERIALS AND METHODS

Purification of InsP_3 receptors

The InsP_3 receptor of porcine aortic smooth muscle was purified by a procedure similar to that used for purification of the cerebellar InsP_3 receptor [12], i.e. two successive affinity chromatographic runs with heparin- and concanavalin A–Sepharose columns followed by an additional affinity chromatography with calmodulin–Sepharose. Calmodulin-depleted microsomes, prepared from 400 g of tissue [16], were solubilized at 4 mg/ml protein by continuous stirring for 30 min in buffer A [0.25 M NaCl/20 mM Hepes/NaOH (pH 7.4)/1 mM EDTA/2 mM dithiothreitol/20 % (w/v) glycerol] containing 0.5 % (w/v) Triton X-100 and protease inhibitors (0.1 mM PMSF, 10 units/ml aprotinin, 4 $\mu\text{g}/\text{ml}$ leupeptin and 4 $\mu\text{g}/\text{ml}$ pepstatin A). The supernatant obtained by centrifugation at 120000 *g* for 40 min was immediately applied to a heparin–Sepharose CL-4B column (2 $\times$ 6.5 cm). The column was washed with ten column vol. of buffer A containing 0.1 % (w/v) Triton X-100 and eluted with buffer B [0.1 % (w/v) Triton X-100/0.5 M NaCl/20 mM Hepes/NaOH (pH 7.4)/2 mM dithiothreitol/20 % (w/v) glycerol]. The heparin–Sepharose pool of InsP_3 receptor was then incubated with 2 ml of concanavalin A–Sepharose for 4 h on ice with gentle shaking. The resin was washed with buffer B and eluted with

Abbreviations used: InsP_3 , *myo*-inositol 1,4,5-trisphosphate; protein kinase G, cGMP-dependent protein kinase.

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buffer B containing 1 M methyl- α -D-mannopyranoside as described previously [17]. The eluate was dialysed overnight against two changes of 200-fold excess of buffer C [0.1 % (w/v) Triton X-100/20 mM Hepes/NaOH (pH 7.4)/2 mM dithiothreitol/20 % (w/v) glycerol/0.05 mM CaCl_2] containing 0.16 M NaCl, and applied to a calmodulin-Sepharose 4B column (1.4 $\times$ 2.0 cm). The column was washed with six column vol. of buffer C containing 0.16 M NaCl and InsP_3 receptor was eluted with buffer C containing 1 M NaCl. The fractions enriched with InsP_3 receptor were combined, dialysed overnight against a 200-fold excess of buffer B, frozen in liquid N_2 and stored at -80°C .

Purification of porcine and rat cerebellar InsP_3 receptors was conducted by two successive chromatographic runs with heparin- and concanavalin A-Sepharose columns as described by Supatapone et al. [12], after preparation and solubilization with Triton X-100 of calmodulin-depleted microsomes by the method of Maeda et al. [13]. The 240 kDa protein kinase G substrate protein of porcine aortic smooth muscle was purified from calmodulin-depleted microsomes as described previously [17]. The purified proteins were frozen in liquid nitrogen and stored at -80°C until use.

Phosphorylation of InsP_3 receptors by protein kinase G

For phosphorylation of purified InsP_3 receptors, 35 μl of sample containing purified protein equivalent to 400 fmol of InsP_3 -binding site was mixed with 35 μl of 40 $\mu\text{g}/\text{ml}$ purified protein kinase G and 4 μM cGMP in TEM buffer [20 mM Tris/HCl (pH 7.4)/2 mM EDTA/25 mM 2-mercaptoethanol] and 35 μl of phosphorylation buffer [80 mM Tris/HCl (pH 7.5)/40 mM magnesium acetate/0.2 mM 3-isobutyl-1-methylxanthine]. The mixture was pre-incubated at 37°C for 5 min and the reaction was initiated by addition of 35 μl of 400 μM [γ - ^{32}P]ATP (5 Ci/mmol). At the times indicated, a 20 μl aliquot from the reaction mixture was immediately mixed with 10 μl of SDS/sample buffer [6 % (w/v) SDS/0.19 M Tris/HCl (pH 6.8)/6 % (v/v) 2-mercaptoethanol/30 % (w/v) glycerol] and heated at 37°C for 10 min. The denatured samples were separated by 6 % (w/v) SDS/polyacrylamide gel and silver-stained. The band of InsP_3 receptor was dissected and placed in 20-ml glass vials. The radioactivities of the gel pieces were counted using Soluvable (Du Pont-NEN) as a solubilizer according to the instruction manual provided by the manufacturer. The stoichiometry of phosphorylation of InsP_3 receptors was calculated on the basis of incorporated phosphates and InsP_3 -binding sites used in the experiment, assuming that each subunit of InsP_3 receptor homotetramer binds one InsP_3 molecule.

Calmodulin-binding assay

The vehicles of the InsP_3 receptors were replaced by dialysis with binding buffer [0.1 % (w/v) Triton X-100/0.16 M NaCl/20 mM Hepes/NaOH (pH 7.4)/2 mM dithiothreitol/20 % (w/v) glycerol] containing either 0.05 mM CaCl_2 or 1 mM EGTA. Then, samples containing 0.8–2.4 μg of protein (150 μl) were mixed with 70 μl of a 50 % (v/v) suspension of calmodulin-Sepharose pre-equilibrated with binding buffer containing 0.05 mM CaCl_2 or 1 mM EGTA, depending on the buffer used as a vehicle of the protein examined, vortex-mixed and incubated for 2 h at 4°C with occasional vortex mixing. After centrifugation at 100 g for 5 min to obtain the supernatant (designated as 'breakthrough'), the gel was washed four times with the same buffer. The washed gel was suspended with 70 μl of the same buffer containing 1 M NaCl, vortex-mixed and placed on ice for 30 min with occasional vortex mixing. After obtaining the supernatant ('1 M NaCl eluate') by centrifugation, the gel was further washed and

incubated with binding buffer containing 1 mM EGTA for 30 min. The supernatant ('1 mM EGTA eluate') was separated from the gel in the same way. The supernatants obtained were mixed with a half-volume of SDS/sample buffer, heated at 37°C for 10 min and analysed by SDS/PAGE.

Reverse transcriptase-PCR analysis

Total RNA was isolated from porcine aorta and cerebellum by the guanidinium thiocyanate method as described by Chomczynski and Sacchi [18]. Poly(A)⁺ RNA was isolated from the RNA preparations by oligo(dT) Latex beads (Oligotex-dT30, Takara, Kyoto, Japan). First-strand cDNA was synthesized from the poly(A)⁺ RNAs by using reverse transcriptase (Superscript II, Gibco-BRL) with random hexamer (Takara) and oligo(dT)_{12–18} (Pharmacia) as primers, and was used in each of the PCR experiments.

We first aimed to determine the partial nucleotide sequences flanking the S1 and S2 splicing segments of cDNA for porcine type 1 InsP_3 receptor. All the primers were synthesized on the basis of the rat cDNA sequence reported by Mignery et al. [3] with degenerative nucleotides introduced at the 3'-end portion of each primer. The primers used for amplification of the sequences flanking the S1 splicing segment were: IPR1-1, 5'-TTCCATGCTGA(A/G)CA(A/G)GA(A/G)AA(A/G)TT-3' (bases 1056–1078); IPR1-2, 5'-CGTTCCACCCGT(A/G/C/T)AC(A/G)AA(A/G)TA(A/G/C/T)AC-3' (bases 1770–1793). The primers used for amplification of sequences flanking the S2 splicing segment were: IPR2-1, 5'-GACATCGTGTCTGCCCT-(A/G/C/T)GA(A/G)GA-3' (bases 5145–5167); IPR2-2, 5'-CGATCGTAAAAGACCTT(A/G)AA(A/G)AA-3' (bases 5892–5914). Nucleotide numbering was based on that of the rat cDNA sequence [3]. Each reaction mixture contained 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl_2 , 0.001 % (w/v) gelatin, 0.2 mM each of dNTPs, 1 μM each of primers, 25 units/ml of Taq DNA polymerase (Perkin-Elmer/Cetus), and 4 % (v/v) first-strand cDNA products of poly(A)⁺ RNA from porcine cerebellum. After an initial denaturation at 94°C for 2 min, the reaction was cycled 35 times with 30 s at 94°C , 45 s at 48°C and 90 s at 72°C . The reaction was completed by heating for 3 min at 72°C . DNA segments of approx. 700 bp and 770 bp were amplified as predominant products by using the primer pair IPR1-1 and IPR1-2 and the primer pair IPR2-1 and IPR2-2 respectively. The amplified products were purified by electrophoresis on agarose gels and cloned to pT7Blue(R)T vectors (Novagen). Four positive clones from each of the two amplified products were sequenced for both DNA strands by using the dideoxynucleotide chain termination method and an automated DNA sequencer (Applied Biosystems, model 373A). The sequences of the four clones from the PCR product with IPR1-1 and IPR1-2 as primers were found to be identical and they showed a high homology (89.4 %) to the corresponding sequence of rat type 1 InsP_3 receptor, but a 45 bp sequence corresponding to S1 splicing segment was internally deleted in all the clones. The sequences of the four clones from the PCR product with IPR2-1 and IPR2-2 as primers were also identical and showed a high homology (89.8 %) to the rat receptor. They all contained an S2 splicing segment of 120 bp (results not shown).

PCR amplification of a fragment containing the S1 splicing segment of porcine type 1 InsP_3 receptor was performed using primers that flank the S1 splicing segment, S1-1 (5'-GAGCACAGGAAGAAGCAGCATGT-3', corresponding to rat sequence 1092–1114) and S1-2 (5'-TAGGAGTTTCTTG-GGACGAGGCT-3', corresponding to rat sequence 1467–1489). Primers that were used for amplification of a fragment

containing the S2 splicing segment were S2-1 (5'-TTCATTGCAAGTTAATAAGCAC-3', corresponding to rat sequence 5283–5306) and S2-2 (5'-AATGCTCTCATG-AAACACTCGGTC-3', corresponding to rat sequence 5781–5804). These oligonucleotide primers were synthesized on the basis of the partial nucleotide sequences of porcine type 1 InsP_3 receptor that were determined as described above (Figure 6). The reaction mixture was essentially the same as described above except that first-strand cDNA products either from porcine aorta or cerebellum poly(A)⁺ RNA were used as a template. The reaction protocol included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s, and a final extension at 72 °C for 3 min. The PCR products were purified, subcloned and sequenced as above. Quantitative PCR amplification was performed under identical conditions except that only 21–24 cycles of amplification were employed.

For quantitative analysis of splicing variants of porcine type 1 InsP_3 receptor, the PCR products amplified under the quantitative conditions were electrophoresed on 2% (w/v) agarose gels, transferred onto cationized nylon membranes (Zeta-Probe, Bio-Rad) and UV-cross-linked (Funa UV-linker, Funakoshi, Tokyo, Japan). Blots were pre-hybridized and hybridized at 37 °C in 5 × SSPE [0.9 M NaCl/50 mM NaH_2PO_4 (pH 7.4)/5 mM EDTA], 5 × Denhardt's solution (0.02% (w/v) Ficoll/0.02% (w/v) polyvinylpyrrolidone/0.002% (w/v) BSA), 0.5% (w/v) SDS and 0.1 mg/ml salmon sperm DNA. The oligonucleotide probes used for hybridizations corresponded to sequences contained commonly in splicing variants: probe 1 (5'-GTGGACCCAGACCAGGATGCC-3', corresponding to rat sequence 1326–1346) for S1-splicing variants and probe 2 (5'-GAGGCCCTCAGACAGATTTTG-3', corresponding to rat sequence 5529–5549) for S2-splicing variants. The probes were 5'-end-labelled with ³²P by using a DNA labelling kit (Megalabel, Takara, Kyoto, Japan). The blots were washed twice at 55 °C in 2 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS, then exposed to X-ray films (Fuji RX) overnight at –80 °C. Quantification of ³²P radioactivity was performed with a Fujix BAS 2000 Bio-Imaging-Analyzer (Fuji Photo Film, Tokyo, Japan).

Other analytical methods

Immunoblots of the purified InsP_3 receptors separated by SDS/PAGE were probed with T120 (a rabbit polyclonal antibody specific for type 1 InsP_3 receptor) and visualized with an enhanced chemiluminescence assay kit (Amersham) as described previously [16]. The InsP_3 receptor antibody used in this study was the one raised against a synthesized peptide corresponding to the C-terminal 19 amino acid residues of rat type 1 InsP_3 receptor [19] and was provided by Dr. T. C. Südhof (University of Texas Southwestern Medical Centre, Dallas, TX, U.S.A.).

InsP_3 -binding activity was measured at the end of each purification step for InsP_3 receptor with the polyethyleneglycol precipitation method as described previously [17]. In order to obtain saturation-binding isotherms of purified InsP_3 receptors the ion-exchange syringe column assay method developed by Hingorani and Agnew [20] with modifications as described previously [17] was used.

SDS/PAGE was conducted under denaturing conditions on a 1 mm thick, 4.5, 5 or 6% (w/v) polyacrylamide gel using the Laemmli buffer system [21]. For silver-staining of proteins separated on an SDS/polyacrylamide gel, a Silver Stain II kit (Wako Pure Chemicals, Osaka, Japan) was used. Protein con-

centration was determined by a modified Lowry method with BSA as a standard [22].

Materials

The sources of the materials used in this study were as follows: heparin–Sephacrose CL-6B, concanavalin A–Sephacrose and calmodulin–Sephacrose 4B from Pharmacia LKB Biotechnology, Uppsala, Sweden; Triton X-100, methyl- α -D-mannopyranoside, heparin sodium salt and poly(ethylene glycol)-6000 from Wako Pure Chemicals, Osaka, Japan; [γ -³²P]ATP tetra (triethylammonium) salt (10–50 Ci/mmol) from Du Pont-New England Nuclear, Boston, MA, U.S.A. All other chemicals were of the highest purity grade commercially available. Protein kinase G was purified from porcine lung as described previously [16].

RESULTS

Purification of InsP_3 receptor from porcine aorta

The InsP_3 receptor of porcine aorta was purified to near homogeneity by a procedure similar to the purification of cerebellar InsP_3 receptors combined with a final step of purification by calmodulin affinity chromatography. Table 1 illustrates the degree of purification of aortic InsP_3 receptor attained after each chromatographic step, as assessed by InsP_3 binding. Approx. 85 μg of purified InsP_3 receptor was obtained from 400 g of tissue. The purification was 375-fold that in tissue solubilized in Triton X-100. On the basis of specific activity of InsP_3 binding, the purity was comparable to that of a 240 kDa protein reported by Koga et al. [17]. All the InsP_3 -binding activity was retained by calmodulin–Sephacrose and eluted from the column with 1 M NaCl-containing buffer, a behaviour exactly the same as that reported with the 240 kDa protein [17]. Scatchard analysis of the data of saturation experiments conducted with [³H] InsP_3 revealed only a single population of binding sites with a K_d of 1.2 nM as compared with the 240 kDa protein K_d of 2.0 nM (results not shown). Porcine and rat cerebellar InsP_3 receptors also showed a single binding site with K_d values of 3.2 and 1.1 nM respectively. The InsP_3 binding was completely inhibited by heparin, a known InsP_3 receptor antagonist.

Figure 1A depicts the SDS/PAGE profile of the InsP_3 receptor purified from porcine aortic smooth muscle. The profiles of porcine aortic 240 kDa protein and InsP_3 receptors of porcine and rat cerebella are also depicted in the Figure. As is evident from the Figure the molecular sizes of the InsP_3 receptors from porcine and rat cerebella were slightly larger than those of the InsP_3 receptor and the 240 kDa protein from porcine aorta. When the main band region was enlarged by lowering the concentration of acrylamide gel, it turned out that the porcine aortic InsP_3 receptor, as well as the 240 kDa protein, was actually composed of two bands with slightly differing molecular masses containing more or less the same amount of protein (Figure 1B). The InsP_3 receptor purified from either porcine or rat cerebellum was also composed of two bands, but the band of lower molecular mass was dominant.

Phosphorylation by protein kinase G

Figure 2 depicts the time course of incorporation of phosphate into the InsP_3 receptor. For comparison, the time courses of incorporation of phosphate into the 240 kDa protein, and rat and porcine cerebellar InsP_3 receptors, are also shown in the Figure. The incorporation of phosphate was relatively rapid, becoming detectable within 2 min, and was saturated after 20 min incubation except for the InsP_3 receptor of rat cerebellum. The phosphorylation was stoichiometric with all the InsP_3 receptors

Table 1 Purification of InsP_3 receptor from porcine aorta

Fraction	Protein (mg)	InsP_3 binding* (pmol)	Specific activity (pmol/mg)	Fold purification	Yield (%)
Triton X-100 solubilizate*†	181.6	237	1.31	1	100
Heparin column					
Unbound	109.6	95.5	0.87	0.66	40.2
Bound	12.0	378	31.7	23.9	159
Concanavalin A column					
Unbound	8.83	72.0	8.27	6.31	30.3
Bound	1.68	95.2	56.7	43.3	40.1
Calmodulin column					
Unbound	0.704	0	0	0	0
Bound	0.085	42.0	491	375	17.7

* InsP_3 binding measured with 100 nM [^3H] InsP_3 by the poly(ethylene glycol) precipitation method.

† Triton X-100 solubilizate of calmodulin-depleted microsomes prepared from 400 g of porcine aorta as starting material.

Figure 1 SDS/PAGE profiles of purified InsP_3 receptor and the 240 kDa protein kinase G substrate protein of porcine aorta and purified cerebellar InsP_3 receptors

Silver-stained gels are shown. Electrophoresis calibration kits (Pharmacia) were used for molecular mass estimation. Lanes: 1, InsP_3 receptor of porcine aorta (0.24 μg); 2, 240 kDa protein of porcine aorta (0.24 μg); 3, InsP_3 receptor of porcine cerebellum (0.35 μg); 4, InsP_3 receptor of rat cerebellum (0.28 μg). In (A) proteins were separated on a 6% polyacrylamide gel. In (B) proteins were separated on a 4.5% polyacrylamide gel to improve resolution of the two closely arrayed bands of porcine aortic InsP_3 receptor and the 240 kDa protein. An enlargement of the main band region is shown.

as well as with the 240 kDa protein: 1 mol of phosphate was incorporated per mole of InsP_3 receptor or 240 kDa protein at maximum incorporation.

Calmodulin binding

In the presence of 50 μM Ca^{2+} , porcine aortic InsP_3 receptor as well as the 240 kDa protein was retained by calmodulin-Sepharose affinity gel and eluted with 1 M NaCl. Further incubation with EGTA resulted in elution neither of the InsP_3 receptor nor of the 240 kDa protein (Figure 3A). The binding to calmodulin was Ca^{2+} dependent; in the absence of Ca^{2+} all the InsP_3 receptor as well as the 240 kDa protein applied to

Figure 2 Time course of phosphorylation by protein kinase G of purified InsP_3 receptor and the 240 kDa protein of porcine aorta and cerebellar InsP_3 receptors

Phosphorylation was performed with 10 $\mu\text{g}/\text{ml}$ protein kinase G as described in the Materials and methods section. The incorporation of phosphate into the respective proteins was calculated on the basis of incorporated phosphate and InsP_3 -binding sites used in the experiment assuming that each subunit of InsP_3 receptor homotetramer binds one InsP_3 molecule. Symbols: ●, 240 kDa protein; ○, porcine aortic InsP_3 receptor; △, porcine cerebellar InsP_3 receptor; ▲, rat cerebellar InsP_3 receptor. Each value represents the mean of two experiments.

calmodulin-Sepharose failed to adhere to the gel and was found in the breakthrough (Figure 3B).

In contrast, almost all the porcine and rat cerebellar InsP_3 receptors appeared in the breakthrough even in the presence of Ca^{2+} , suggesting no, or very weak, binding of these proteins to calmodulin. The minute amount detected in 1 M NaCl eluate (Figure 3A) may not be an experimental artifact since it was not detected under Ca^{2+} -free conditions (Figure 3B).

Immunoreactivity

Like the 240 kDa protein, the InsP_3 receptor purified from porcine aorta, cross-reacted well with the polyclonal antibody specific to type 1 InsP_3 receptor (T120) (Figure 4). Some minor immuno-reactive bands with lower molecular masses that were detected may represent degradation products of proteolysis

Figure 3 Binding to calmodulin-Sepharose of purified InsP_3 receptor and the 240 kDa protein of porcine aorta and cerebellar InsP_3 receptors

The binding to calmodulin-Sepharose was examined by the method described in the Materials and methods section. All the protein samples were subjected to calmodulin-Sepharose chromatography, fractionated into the 'breakthrough' fraction (lane 2), '1 M NaCl eluate' fraction (lane 3) and '1 mM EGTA eluate' fraction (lane 4) and separated on SDS/6%-polyacrylamide gels. Lane 1 shows the samples applied. Binding of the proteins to calmodulin-Sepharose was conducted in the presence of 50 μM Ca^{2+} (A) or 1 mM EGTA in place of Ca^{2+} (B).

Figure 4 Immunoblotting of purified InsP_3 receptor and the 240 kDa protein of porcine aorta and cerebellar InsP_3 receptors

Purified protein samples, each containing 0.2 μg of protein, were separated on an SDS/6%-polyacrylamide gel and the gel was processed for immunoblotting with a polyclonal antibody specific to type 1 InsP_3 receptor as described in the Materials and methods section. (A) Silver-stained gel and (B) an immunoblot. Lanes: 1, InsP_3 receptor of porcine aorta; 2, 240 kDa protein of porcine aorta; 3, InsP_3 receptor of porcine cerebellum; 4, InsP_3 receptor of rat cerebellum.

during purification. The minor bands with similar molecular masses were also found in the purified 240 kDa protein, and the amounts of these proteins relative to the major bands were similar both with InsP_3 receptors and the 240 kDa protein, suggesting that they were derived from the same origin. Thus, the porcine InsP_3 receptor is immunologically identical with the 240 kDa protein, and both proteins belong to the type 1 InsP_3 receptors.

Figure 5 PCR analysis of the type 1 InsP_3 receptor splicing variants in porcine aorta and cerebellum

First-strand cDNAs synthesized from poly(A)⁺ RNAs from porcine aorta (lane 1) and cerebellum (lane 2) were used as templates with primers flanking the S1 splicing segment (A) or primers flanking the S2 splicing segment (B). Primer pair S1-1 and S1-2 was used for amplification of fragments containing the S1 splicing segment and primer pair S2-1 and S2-2 for amplification of fragments containing S2 splicing segment. PCR products were separated on a 3%-agarose gel (A) or a 2%-agarose gel (B). No PCR products were amplified with poly(A)⁺ RNA as templates under similar conditions. The sequences and positions of these primers are indicated in Figure 6. Further experimental details are described in the Materials and methods section.

Splicing variants of type 1 InsP_3 receptor

Although the InsP_3 receptors purified from porcine aorta and cerebellum were found to be of type 1, there were differences between these two receptors as regards molecular composition, molecular mass and binding to calmodulin, differences that may be best explained by the presence of differing splicing variants. Two alternative splicing sites are known in the type 1 InsP_3 receptor: the one termed S1 is located in the N-terminal ligand-binding domain and the other site termed S2 lies in the coupling or regulatory domain [7]. We synthesized oligonucleotide primers flanking the area of S1 and S2 and employed them in PCR amplification using first-strand cDNAs derived from poly(A)⁺ RNAs of porcine aorta and cerebellum as templates.

Figure 5 depicts PCR products from porcine aortic and cerebellar cDNAs. With primers flanking the S1 splicing segment (Figure 6), two DNA fragments were amplified from both aorta and cerebellum (Figure 5A). The length of each of the two products appeared to be the same in the two tissues and was as predicted from the positions of the primers used. The long product was approximately 45 bp longer than the short, suggesting that the former contains the S1 splicing segment while the S1 segment is deleted in the latter. With primers flanking the S2 splicing segment (Figure 6), only one DNA fragment was amplified from either aorta or cerebellum (Figure 5B). The product of cerebellar cDNA was 120 bp longer than that of aortic cDNA, a length compatible with that of the S2 splicing segment.

The amplified DNA fragments were separately subcloned and sequenced (Figure 6). The longer DNA fragments from aorta and cerebellum cDNA obtained with the primers flanking the S1 splicing segment were identical in length (398 bp) and in sequence, and contained the S1 splicing segment (45 bp). On the other hand, the shorter DNA fragments produced from the same tissues were also identical in length (353 bp) and sequence, but lacked the S1 splicing segment. The PCR product from cerebellar cDNA with primers flanking the S2 splicing segment was 522 bp in length and contained the S2 splicing segment (120 bp), which was absent in the corresponding PCR product (402 bp) from

Figure 6 Nucleotide sequences of PCR-amplified DNA fragments of type 1 InsP_3 receptor splicing variants

(A) The sequence of a DNA fragment (398 bases) containing the S1 splicing segment and (B) the sequence of a DNA fragment (522 bases) containing the S2 splicing segment. The positions of primers for PCR are underlined. The S1 and S2 splicing segments are in boxes. Deduced amino acids are also shown (using the single letter code) below the nucleotide sequences.

aortic cDNA. Besides the two splicing variants, Nakagawa et al. [7] demonstrated two intermediate forms with partial regions of the full S2 splicing segment. We sequenced five clones from the PCR product of cerebellar cDNA with primers flanking the S2 splicing segment, all of which contained the full-length S2 segment.

The reverse transcriptase-PCR analyses suggested the expression in porcine aorta of two splicing variants of type 1 InsP_3 receptor, S1-inserted or S1-lacking form, both of which lacked S2 (S1+/S2- and S1-/S2-). In contrast, it was suggested that porcine cerebellum expresses the two S1-splicing variants that contain the full-length S2 (S1+/S2+ and S1-/S2+). Southern blotting analyses of PCR products amplified under quantitative conditions enabled us to estimate the relative levels of the different splicing variants. By using oligonucleotide probe (probe 1) corresponding to the sequence commonly found in the S1-splicing variants, the relative levels for S1- form and S1+ form were estimated to be almost equal in aorta (46.0% and 53.9% respectively), while the S1- form was more abundant in the cerebellum than S1+ form (61.3% and 38.6% respectively). The relative ratio of expression of type 1 InsP_3 receptor in cerebellum and aorta estimated by using a probe (probe 2) corresponding to the sequence commonly found in the S2 splicing variants was 8.5:1 (results not shown).

DISCUSSION

In the present study, InsP_3 receptor was isolated from porcine aortic smooth muscle and its biochemical properties were com-

pared with those of the porcine aortic 240 kDa protein and porcine and rat cerebellar InsP_3 receptors. The 240 kDa protein is a protein originally isolated as a protein kinase G substrate [16], which was later found to represent an InsP_3 -binding protein [17].

The method used for isolation of InsP_3 receptor from aortic smooth muscle was essentially similar to those used by previous investigators [12,13] for isolation of cerebellar InsP_3 receptors except that calmodulin-Sepharose chromatography was added as a final step. By adding calmodulin-Sepharose chromatography it was feasible to purify the receptor to near homogeneity with a high yield: about 85 μg of purified receptor was obtained from 400 g of porcine aortic smooth muscle, a yield which was seven times higher than that of Chadwick et al. [14] who, using additional sucrose density-gradient steps, obtained 53 μg of purified receptor from 1.6 kg of bovine aortic smooth muscle.

The receptor obtained was shown to share many properties in common with the 240 kDa protein and cerebellar InsP_3 receptors: like them it is a heparin-binding glycoprotein. InsP_3 binds to the protein with a similar high affinity to those found with the 240 kDa protein and cerebellar InsP_3 receptors, and the binding was heparin inhibitable. It is a good substrate for protein kinase G. The extent and kinetics of phosphorylation were not different from those of the 240 kDa protein and porcine cerebellar InsP_3 receptor. The kinetics of phosphorylation of rat cerebellar InsP_3 receptor was significantly slower than those of other proteins, confirming our previous observation [17]. Phosphorylation of all the InsP_3 receptors, including the 240 kDa protein, was stoichiometric: at maximum incorporation, approx. 1 mol of phosphate was incorporated per mole of the InsP_3 receptor subunit.

However, the InsP_3 receptor isolated from porcine aorta differed from cerebellar InsP_3 receptors in two important points. First, like the 240 kDa protein, the InsP_3 receptor from porcine aorta was found to bind to calmodulin in the presence of Ca^{2+} , while the InsP_3 receptors purified from porcine and rat cerebellum did not bind either in the presence or absence of Ca^{2+} . The lack of binding of rat cerebellar receptor to calmodulin was also demonstrated by Supattapone et al. [23]. In our previous study, in which calmodulin-binding activity was tested with calmodulin overlay technique [16], the binding was not detected with the 240 kDa protein. However, in our previous work, samples were treated with SDS, electrophoresed and Western blotted. Thus, denaturation of the protein that might have occurred may have been the cause of a loss of calmodulin-binding activity. The concentration of calmodulin used in the calmodulin overlay experiment being too low may have also been the cause of the failure.

Secondly, there is a difference in apparent molecular mass. In SDS/PAGE analysis, the porcine aortic InsP_3 receptor, as well as the 240 kDa protein, showed a similar molecular mass of 240 kDa as compared with 250 kDa for porcine and rat cerebellar InsP_3 receptors. Furthermore, although all the InsP_3 receptors and 240 kDa protein presented itself on SDS/PAGE as two closely arrayed bands (doublet) with slightly differing molecular masses, there are differences in the relative amounts of these two bands. While the amount of the two bands is almost equal in the InsP_3 receptor as well as in the 240 kDa protein from porcine aorta, the band of lower molecular mass was dominant in InsP_3 receptors from either porcine or rat cerebellum. The two bands comprising the InsP_3 receptor and the 240 kDa protein of porcine aorta and the two bands comprising the cerebellar InsP_3 receptors were phosphorylated equally well by protein kinase G [17].

In the present study both the aortic and cerebellar InsP_3 receptors and the aortic 240 kDa protein cross-reacted with a polyclonal antibody specific to type 1 InsP_3 receptor. Type 1 or

Figure 7 Amino acid sequence of the putative calmodulin-binding site of the aortic InsP_3 receptor

Identified calmodulin-binding sites of chicken gizzard myosin light-chain kinase [25], rat olfactory cyclic nucleotide-gated cation channel [26] and human erythrocyte plasma membrane Ca^{2+} -pump ATPase [27] are also shown for comparison. Symbols: ●, basic, positively charged amino acids; ○, hydrophobic, aliphatic amino acids; □, hydrophobic, aromatic amino acids. The site for insertion of the S2 splicing segment in porcine aortic type 1 InsP_3 receptor is indicated by an arrow and the S2 splicing segment in porcine cerebellum type 1 InsP_3 receptor is underlined.

the cerebellar form of the InsP_3 receptor has two splicing segments designated S1 and S2 [7–9]. The S1 splicing segment lies near the N-terminal InsP_3 -binding domain and codes for 15 amino acids and S1 splicing variants show distinct distribution in brain and peripheral tissues. The S2 splicing segment is located between two putative phosphorylation sites for cAMP-dependent protein kinase and codes for 40 amino acids. In the nervous system, the longer form with S2 predominates, while the shorter form without S2 predominates in the peripheral tissues [7,9,10].

To demonstrate the presence or absence of these splicing segments in *InsP₃* receptors isolated from the porcine aorta, reverse transcriptase-PCR analysis was conducted. The analysis clearly indicated the absence of S2 in the aortic *InsP₃* receptor. Full-length S2 was found in all the cerebellar receptors. Thus, the fact that the molecular mass of the aortic *InsP₃* receptor is lower than those of the cerebellar receptors may be explained by the lack of an S2 splicing segment in the aortic smooth muscle *InsP₃* receptor.

As regards the S1 splicing segment, both variants were found in almost equal quantity in the aortic InsP₃ receptor. Thus, the two closely arrayed bands observed on SDS/PAGE of porcine aortic InsP₃ receptor may represent the presence of both variants. In porcine cerebellum, the S1-lacking variant was the predominant form; this is in harmony with the observations made with SDS/PAGE.

Komalavilas and Lincoln [24] recently identified Ser-1755 of the rat cerebellar InsP_3 receptor as a site for phosphorylation by protein kinase G. The corresponding Ser residue in the porcine type 1 InsP_3 receptor sequence may serve as a phosphorylation site for protein kinase G. Although the S2 splicing segment lies near the putative phosphorylation site for protein kinase G, the lack of an S2 splicing segment in porcine aortic type 1 InsP_3 receptor appears not to have affected the phosphorylation by protein kinase G. The lack may, however, have a profound influence on the calmodulin-binding property of the type 1 InsP_3 receptor. In Figure 7, calmodulin-binding sites of well-known calmodulin-regulated proteins, such as myosin light-chain kinase,

olfactory cyclic nucleotide-gated cation channel and plasma membrane Ca^{2+} -pump ATPase, are depicted. The binding sites are characterized by (1) the presence of a basic amino acid every 3–6 amino acid residues; and (2) a strong predominance of aromatic or aliphatic hydrophobic amino acids in clusters between the basic amino acids. In Figure 7 the amino acid sequence of the putative calmodulin-binding site of the porcine type 1 InsP_3 receptor is also shown. As is evident from the Figure, a sequence that characterizes the representative calmodulin-binding proteins is present in the type 1 splicing variants expressed in porcine aorta also. However, through insertion of the S2 splicing segment, the putative calmodulin-binding site is separated at the mid-point and half of the characteristic sequence is lost in the splicing variants found in porcine cerebellum. We therefore propose that the calmodulin-binding property of the porcine aortic InsP_3 receptor is introduced through deletion of S2. Further study is obviously required to disclose the physiological significance of the presence of a calmodulin-binding site in a splicing variant of the type 1 InsP_3 receptor.

In conclusion, the 240 kDa protein, previously found in porcine aorta as a protein kinase G substrate, is identical with a major InsP_3 receptor in that tissue. The porcine aortic smooth muscle InsP_3 receptors represent splicing variants of the type 1 InsP_3 receptor that are structurally distinct from those of the type 1 InsP_3 receptors expressed in the cerebellum. It shares many properties in common with the cerebellar InsP_3 receptor but differs in one important point, i.e. calmodulin binding, which may be physiologically relevant and can be a useful index for subclassification of InsP_3 receptors.

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