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Immunohistochemical Localization of Type 2 Inositol 1,4,5-Trisphosphate Receptor to the Nucleus of Different Mammalian Cells

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Abstract The inositol 1,4,5-trisphosphate receptor ($InsP_3R$) is a ligand-gated Ca^{2+} channel responsible for the release of Ca^{2+} from intracellular stores in the response of a wide variety of cells to external stimuli. Molecular cloning studies have revealed the existence of three types of $InsP_3R$ encoded by distinct genes. In the study presented here, we used selective anti-$InsP_3R$ antibodies to determine the intracellular location of each $InsP_3R$ subtype in bovine aortic endothelial cells, bovine adrenal glomerulosa cells, and COS-7 cells. $InsP_3R1$ was found to be widely distributed throughout the cytosol and most abundantly in the perinuclear region identified as the endoplasmic reticulum (co-localization with protein disulfide isomerase). The intracellular location of $InsP_3R3$ was similar to that of $InsP_3R1$. Surprisingly, $InsP_3R2$ was found mostly associated to the cell nucleus. This observation was made with two antibodies recognizing different epitopes on $InsP_3R2$. Binding studies revealed the presence of a high affinity-binding site for [^3H] $InsP_3$ on purified nuclei from bovine adrenal cortex. Confocal images showed that $InsP_3R2$ was not confined to the nuclear envelope but was distributed relatively uniformly within the nucleus. Our results demonstrate that the three types of $InsP_3R$ are not similarly distributed within a specific cell type. Our results also suggest the existence of an intranuclear membrane network on which $InsP_3R2$ is abundantly expressed. J. Cell. Biochem. 85: 219–228, 2002. © 2002 Wiley-Liss, Inc.

Key words: inositol trisphosphate receptor; nucleus; immunohistochemical localization; calcium signaling

Many hormones, growth factors, and neurotransmitters mediate their action by elevating the intracellular Ca^{2+} concentration through the phosphoinositides hydrolysis pathway [Berridge, 1995; Clapham, 1995]. These hormones activate G-protein-coupled receptors or tyrosine kinase receptors that activate phospholipase C isoenzymes, which hydrolyze phosphatidylinositol 4,5-bisphosphate and generate the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ binds a receptor/channel responsible for the release of Ca^{2+} from intracellular stores [Yoshida and Imai, 1997].

The purification and characterization of $InsP_3$ receptor ($InsP_3R$) from different tissues revealed that it is a glycosylated protein with an apparent molecular weight ranging from 220 to 260 kDa [Maeda et al., 1988; Supattapone et al., 1988; Chadwick et al., 1990]. Molecular cloning studies revealed the existence of three types of $InsP_3R$ encoded by distinct genes and designated $InsP_3R1$ [Furuichi et al., 1989; Mignery et al., 1990], $InsP_3R2$ [Sudhof et al., 1991; Yamamoto-Hino et al., 1994], and $InsP_3R3$ [Blondel et al., 1994; Maranto, 1994; Yamamoto-Hino et al., 1994]. $InsP_3R$ is structurally and functionally divided into three major domains: a ligand binding domain, a modulatory domain, and a Ca^{2+} channel domain [Mignery and Sudhof, 1990]. The percent identity among the three receptor subtypes (68% in their binding domain, 53% in their modulatory domain, and 59% in their pore-forming domain) predicts that they share an overall similar structural organization but may have distinct regulatory mechanisms [Blondel et al., 1993]. The Ca^{2+} channel is formed by the non-covalent association of four $InsP_3Rs$ of the same type (homotetramer) or of

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different types (heterotetramer) [Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz and He, 1995].

Studies on $InsP_3R$ distribution have shown that the three receptor subtypes can be co-expressed in many tissues [Newton et al., 1994; Wojcikiewicz, 1995]. $InsP_3R1$ is expressed in most, if not all, cell types and is particularly abundant in cerebellar Purkinje cells [Furuichi and Mikoshiba, 1995; Joseph, 1996]. In contrast, $InsP_3R2$ and $InsP_3R3$ are more sparingly distributed among tissues, although they are prominent in certain cell types [De Smedt et al., 1994]. When they are expressed within the same cell, it is not clear that the three types of $InsP_3R$ have the same intracellular localization. In the study presented here, we used selective antibodies recognizing each one of the three types of $InsP_3R$ [Poitras et al., 2000] to evaluate by immunofluorescence the intracellular localization of $InsP_3Rs$. Our results show that the three types of $InsP_3R$ co-localize at the endoplasmic reticulum and that $InsP_3R2$ is abundant at the cell nucleus. These results suggest that $InsP_3R$ subtypes may have distinctive roles within a cell.

MATERIALS AND METHODS

Materials

$InsP_3$ (trilithium salt) was obtained from LC Services Corporation (Woburn, MA). $[^3H]InsP_3$ (40 Ci/mmol) and ECL immunodetection kit were from Amersham Canada Ltd. (Oakville, ON). Propidium iodide and Texas red-conjugated goat antibody against mouse IgG were obtained from Molecular Probes (Hornby, ON). Mouse anti-protein disulfide isomerase antibody was from Stressgen Biotechnologies Corp. (Victoria, BC). Fluorescein isothiocyanate (FITC)-conjugated sheep antibody against rabbit IgG was from Roche Molecular Biochemicals (Laval, QC). NADPH was obtained from Fluka (Oakville, ON). Rabbit polyclonal antibodies used for immunofluorescence and Western blot studies (anti-$InsP_3R1$, anti-$InsP_3R2$, and anti-$InsP_3R3$) were produced in our laboratory by immunizing rabbits against C-terminal epitopes selective to each $InsP_3R$ subtype. The antibodies were purified with Protein-A agarose beads and their selectivity was established as previously described [Poitras et al., 2000]. An anti-$InsP_3R2$ antibody raised against an N-terminal epitope of $InsP_3R2$ was obtained from Affinity Bioreagents, Inc. (Golden, CO). All other reagents were from Sigma (St. Louis, MO) or from Fisher Scientific, Inc. (Fairlawn, NJ).

Electrophoresis and Immunoblotting

Endothelial cells were subjected to one freeze-thaw cycle and scraped gently in PBS (3.5 mM NaH_2PO_4, 17.4 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 3.5 mM KCl, 0.9 mM $MgCl_2$, 137 mM NaCl). After centrifugation at 13,000*g* for 15 min at 4°C, broken cells were solubilized for 1 h at 0°C in a medium containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100 and the proteases inhibitors cocktail Complete™ (Boehringer-Mannheim Canada, Laval, QC). Insoluble material was precipitated by centrifugation at 13,000*g* for 30 min at 4°C and 500 µl of the supernatant was mixed with 500 µl of 2 × Laemmli's buffer (60 mM Tris/HCl pH 6.8, 10% glycerol, 2% SDS, 125 mM dithiothreitol, 0.3% Bromophenol Blue), boiled for 5 min, and loaded onto a 1 mm thick, 5% acrylamide gel that was subjected to a constant voltage of 200 V for 85 min at 4°C. Proteins were electrotransferred to a PVDF membrane at a constant current of 0.5 A for 4 h at 4°C. Blotted membranes were incubated for 1 h at room temperature in 5% (w/v) non-fat dried milk in PBST (PBS containing 0.1% Tween-20). The blots were then incubated for 2 h at room temperature with different anti-$InsP_3R$ antibodies (1: 1,000 dilution) in the blocking buffer described above. After extensive washing with PBST, the blots were incubated for 1 h at room temperature with a peroxidase-conjugated donkey antibody against rabbit IgG (1: 1,000 dilution). After extensive washing with PBST, the immunoreactivity was detected with ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ) on a Bio-Max ML film (Eastman Kodak Co., Rochester, NY).

Isolation of Nuclei

To obtain a large amount of working material, intact nuclei were isolated from bovine adrenal cortex glands that were obtained at a nearby slaughterhouse. Bovine adrenal cortexes (dissected free of medullary tissue) were homogenized with a polytron in a medium containing 1.3 M sucrose, 1 mM $MgCl_2$, and 10 mM potassium phosphate pH 6.8. The homogenate was centrifuged for 15 min at 1,000*g* and the resulting pellet was suspended in a minimal volume of homogenization medium. This suspension

was mixed with a medium containing 2.4 M sucrose, 1 mM $MgCl_2$, and 10 mM potassium phosphate pH 6.8 to reach a final sucrose concentration of 2.2 M. After a centrifugation for 1 h at 100,000*g*, the resulting nuclear pellet was resuspended in a medium containing 0.25 M sucrose, 4 mM $MgCl_2$, and 20 mM Tris/HCl pH 7.5 and washed by centrifugation for 15 min at 1,000*g*. The final nuclear pellet was stored at $-80°C$. The purity of the nuclei preparation was evaluated with the NADPH cytochrome C reductase assay as described by Crane and Low [1976]. Briefly, NADPH (0.1 mg/ml), cytochrome C (0.3 mg/ml), and KCN (1 mM) were pre-incubated for 2 min in potassium phosphate (0.1 M) pH 7.2. The reaction was started by adding the cellular fraction in a final volume of 1 ml. Absorbency was recorded at 550 nm.

$InsP_3$ Binding Assay

Nuclei (5 mg of protein/assay) were incubated for 30 min at $0°C$ in a medium containing 25 mM Tris/HCl pH 8.5, 110 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4, 1 mM EDTA, in a final volume of 500 µl with appropriate concentrations of $[^3H]InsP_3$ and non-radioactive $InsP_3$. Non-specific binding was determined in the presence of 1 µM $InsP_3$. Incubations were terminated by centrifugation at 15,000*g* for 15 min and the supernatants were removed. Bound $[^3H]InsP_3$ was recovered in the pellet and evaluated by liquid scintillation spectrometry.

Immunofluorescence Staining

Endothelial cells were seeded on micro cover glasses (22 mm square) in 6-wells plates and maintained in culture until they reached 50% confluence. Cells were washed with PBS and fixed with 100% methanol for 10 min at $-20°C$. Non-specific sites were blocked with 1% BSA in PBS for 1 h at room temperature. After being washed, cells were incubated for 1 h at room temperature with primary anti-$InsP_3R$ antibodies (1 µg/ml) or with primary anti-protein disulfide isomerase antibodies (1: 50 dilution) prepared in PBS. After three washes with PBS, cells were incubated for 1 h at room temperature with secondary FITC-conjugated sheep antibodies against rabbit IgG (1: 50 dilution) or with Texas red-conjugated goat antibodies against mouse IgG (1:500 dilution). To label the nuclei, fixed cells were incubated with propidium iodide (0.13 µg/ml) for 30 min at room temperature. In control experiments performed in parallel, no specific immunofluorescent staining was observed when primary antibodies were omitted. After extensive washing with PBS, cover glasses were mounted on microscope slides and examined on a Leica Leitz microscope equipped for epifluorescence. Pictures were obtained with a Leica camera (Wild MPS48).

Confocal Microscopy

Immunofluorescence staining was performed as described above. Slides were examined with a scanning confocal microscope (NORAN Instruments, Inc., Middleton, WI) equipped with a krypton/argon laser and coupled to an inverted microscope with a $40 \times$ oil immersion objective (Nikon). Specimens were excited at 488 nm. Emitted FITC fluorescence was measured at wavelengths 525–550 nm. Optical sections were collected at 1 µm intervals with a 10 µm pinhole aperture. Digitized images were obtained with 256 times line averaging and enhanced with the Intervision software (NORAN Instruments, Inc.) on a Silicon Graphics 02-workstation.

RESULTS

Presence of the Three Types of $InsP_3R$ in Bovine Aortic Endothelial Cells

To determine which types of $InsP_3R$ are expressed in our population of bovine aortic endothelial cells, Western blot analyses were performed using selective antibodies raised against specific epitopes in the C-terminus of each $InsP_3R$ subtype. Cells were solubilized in 1% Triton X-100, subjected to SDS–PAGE electrophoresis (5% acrylamide) and electrotransferred to a PVDF membrane. Immunodetection of the $InsP_3Rs$ was done using the anti-$InsP_3R1$ antibody (Fig. 1A), the anti-$InsP_3R2$ antibody (Fig. 1B), or the anti-$InsP_3R3$ antibody (Fig. 1C). Proteins migrating with relative molecular masses of about 250 kDa were revealed with all three antibodies. These results indicate that all three types of $InsP_3R$ are expressed in bovine aortic endothelial cells.

Intracellular Location of $InsP_3Rs$ in Bovine Aortic Endothelial Cells

We investigated the location of $InsP_3Rs$ in bovine aortic endothelial cells by immunocytochemistry with the selective polyclonal rabbit antibodies described above. The reaction of these antibodies was revealed with an FITC-conjugated anti-rabbit IgG secondary antibody,

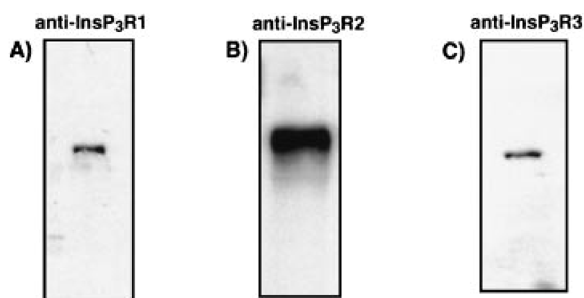

Fig. 1. Bovine aortic endothelial cells express the three types of $InsP_3Rs$. Bovine aortic endothelial cells were solubilized in Laemmli's buffer, loaded on a 5% acrylamide gel (50 µg/lane), subjected to electrophoresis and electrotransferred to PVDF membranes which were then probed with anti-$InsP_3R1$ (**A**), anti-$InsP_3R2$ (**B**), or anti-$InsP_3R3$ (**C**) antibodies. Staining was obtained with a peroxidase-conjugated secondary antibody (1/1,000). The immunoreactivity was detected with ECL (Amersham). This typical experiment is representative of three experiments with different cell preparations.

and the cells were examined with a Leica fluorescence microscope. Figure 2A shows the immunostaining obtained with the anti-$InsP_3R1$ antibody. The fluorescence is widely distributed throughout the cell with a higher intensity in the perinuclear region corresponding to the endoplasmic reticulum. The outer limits of the cell are not clearly defined, which indicate that the plasma membrane is not immunostained. The nucleus appearing as a somber region within the cell in Figure 2A as a red circular structure in Figure 2B (propidium iodide staining) is relatively poorly stained with the anti-$InsP_3R1$ antibody. Superimposition of the two images (Fig. 2C) demonstrates that $InsP_3R1$ is predominantly localized to the endoplasmic reticulum and that it colocalizes relatively poorly with a marker of the cell nucleus.

Figure 2D shows the immunostaining obtained with the anti-$InsP_3R2$ antibody. The fluorescence is confined to the cell nucleus that is strongly stained and to the perinuclear region that is moderately stained. Figure 2E shows the immunostaining obtained with the anti-protein disulfide isomerase antibody (a specific marker of the endoplasmic reticulum) which labels the perinuclear region. The superimposition of images 2D and 2E demonstrates that a small proportion of $InsP_3R2$ colocalizes with the marker of the endoplasmic reticulum whereas the largest proportion of $InsP_3R2$ localizes to the cell nucleus where no protein disulfide isomerase can be found (Fig. 2F).

Figure 2G shows the immunostaining obtained with the anti-$InsP_3R3$ antibody. The fluorescence is widely distributed within the cell, with a proeminent location at the perinuclear region. $InsP_3R3$ is nearly absent from the cell nucleus which appears as a dark round space in the middle of the cell. These results suggest that the three subtypes of $InsP_3R$ have distinct intracellular locations in bovine aortic endothelial cells. It must be mentioned that in control studies where the primary anti-$InsP_3R$ antibodies were omitted and where only the FITC-conjugated secondary antibody was used, no immunostaining could be detected under our experimental conditions (data not shown).

The presence of $InsP_3Rs$ on the endoplasmic reticulum was presumed since this organelle is known to be the intracellular Ca^{2+} store from which $InsP_3$ releases Ca^{2+}. However, the abundance of $InsP_3R2$ associated with the cell nucleus was rather unexpected and surprising. To substantiate this observation, we used another anti-$InsP_3R2$ antibody that recognizes an epitope located on the N-terminus of $InsP_3R2$. Figure 2H shows that this antibody intensely stained the nucleus of bovine aortic endothelial cells. When this antibody was pre-incubated with an excess of neutralizing peptide (which corresponds to the epitope against which the antibody was raised), no cellular staining was observed (data not shown). This control experiment further demonstrates the specificity of our immunocytochemical approach to localize the $InsP_3R2$.

Intracellular Location of $InsP_3Rs$ in Bovine Adrenal Glomerulosa Cells and in COS-7 Cells

To verify whether the intracellular location of $InsP_3Rs$ found in bovine aortic endothelial cells was a unique property of these cells or was common to other cell types, we repeated our immunocytochemical approach with COS-7 cells and bovine adrenal glomerulosa cells, also known to express the three types of $InsP_3R$. Figure 3A–C shows the immunostaining of bovine adrenal glomerulosa cells with anti-$InsP_3R1$, anti-$InsP_3R2$, and anti-$InsP_3R3$ antibodies, respectively. As previously observed with endothelial cells, $InsP_3R1$ and $InsP_3R3$ are predominantly located to the endoplasmic reticulum whereas $InsP_3R2$ is predominantly located to the cell nucleus. Very similar results were obtained with COS-7 cells (Fig. 3D–F). Identical results were also obtained with human aortic endothelial cells (data not shown).

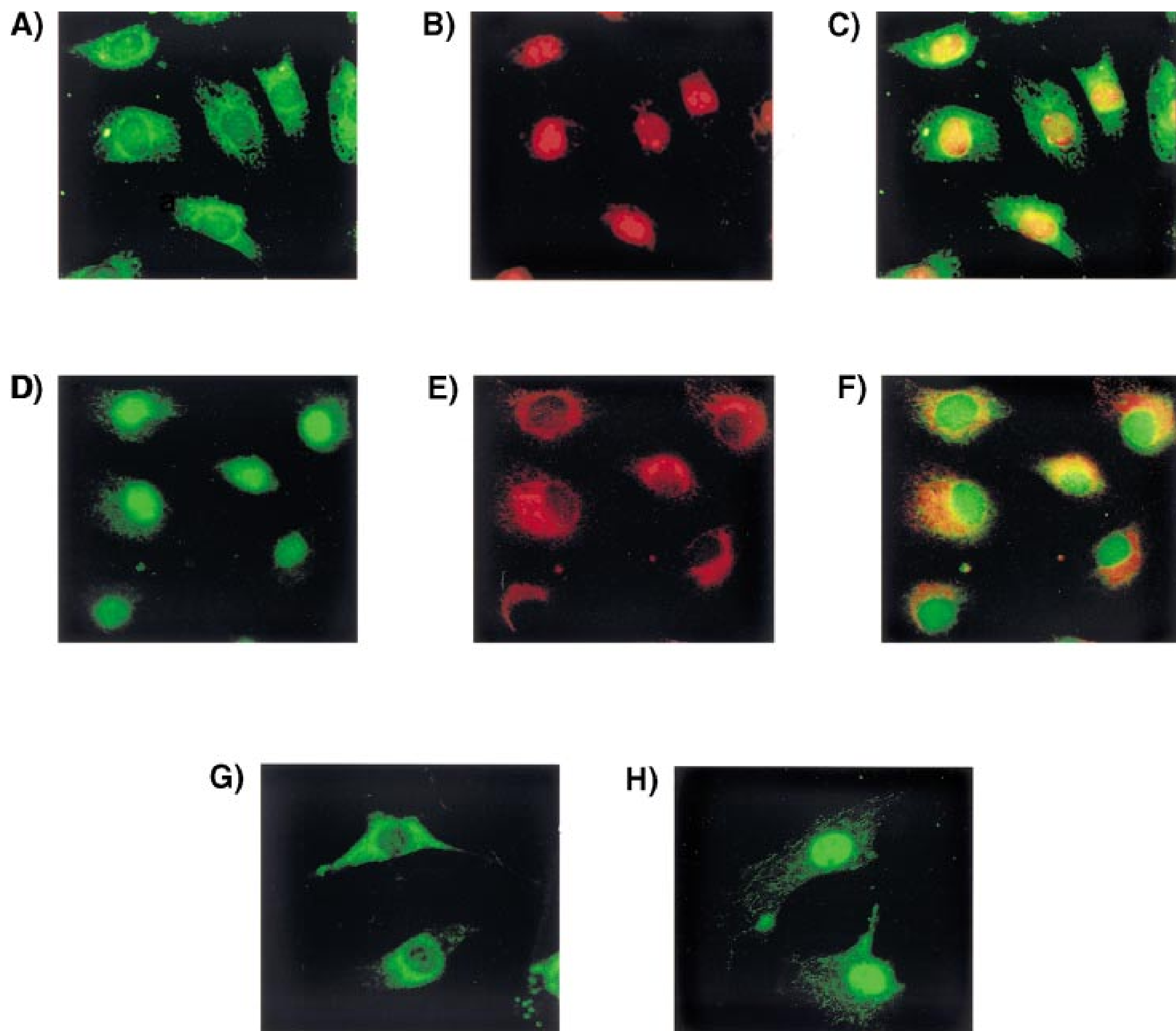

Fig. 2. Intracellular localization of InsP3Rs in bovine aortic endothelial cells. Cells were grown on cover glasses, fixed with methanol, and incubated with our anti-$InsP_3R1$ (**A**), anti-$InsP_3R2$, (**D**) or anti-$InsP_3R3$ (**G**) antibodies. In **H**, fixed cells were incubated with an anti-$InsP_3R2$ antibody recognizing an epitope located on the N-terminus of $InsP_3R2$. Fluorescent staining (green) was obtained with an FITC-conjugated secondary antibody. Cell nuclei were also stained (red) with propidium iodide (**B**). To identify the endoplasmic reticulum, cells were incubated with an anti-protein disulfide isomerase antibody (**E**) and stained (red) with a Texas red-conjugated secondary antibody. Panel **C** shows an overlay of images A and B. Panel **F** shows an overlay of images D and E. These results are representative of similar observations obtained in at least three independent experiments.

$InsP_3$ Binding Sites on Purified Nuclei From Bovine Adrenal Cortex

To further substantiate the observation that $InsP_3R2$ appears to be abundant in the cell nucleus, we directly assessed the $InsP_3$ binding properties of purified bovine adrenal cortex nuclei. The purified nuclei were first evaluated for their content of NADPH cytochrome C reductase, a specific marker of the microsomes. The specific activity of NADPH cytochrome C reductase in the nuclei fraction was as low as 0.16 ± 0.05 nmol/min/mg, a value which is about two orders of magnitude lower than the activity found in the microsomal fraction (16 ± 3.4 nmol/min/mg). $[^3H]$-$InsP_3$ binding studies were performed with this purified nuclei fraction. Figure 4 shows a typical dose-displacement curve where the specific binding of $[^3H]$-$InsP_3$ was progressively inhibited by increasing concentrations of non-radioactive $InsP_3$. Tracer binding was significantly inhibited with 0.1 nM $InsP_3$ and was completely abolished with 1 μM $InsP_3$. The IC50 (concentration inhibiting 50% of tracer binding) was 1.02 ± 0.23 nM $InsP_3$ (mean ± SD of three independent experiments).

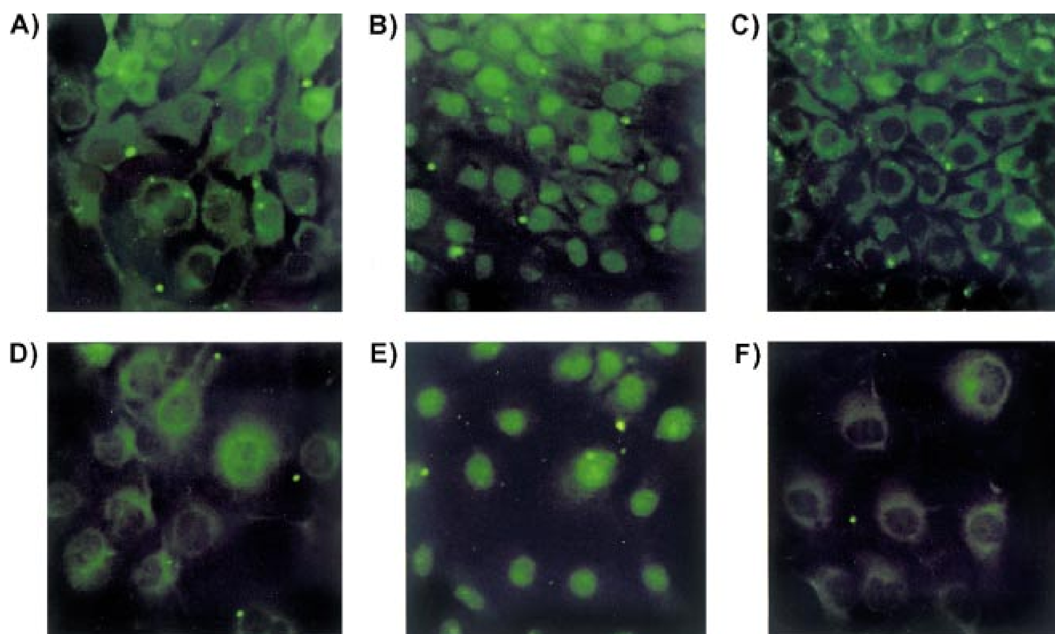

Fig. 3. Intracellular localization of $InsP_3Rs$ in bovine adrenal glomerulosa cells and COS-7 cells. Bovine adrenal glomerulosa cells (**A**,**B**,**C**) and COS-7 cells (**D**,**E**,**F**) were cultured on cover glasses, fixed with methanol and incubated with anti-$InsP_3R1$ (A,D), anti-$InsP_3R2$ (B,E), or anti-$InsP_3R3$ (C,F) antibodies. Fluorescent staining was obtained with an FITC-conjugated secondary antibody. Similar results were obtained with at least three different cell preparations.

Evaluation by Confocal Microscopy of the Cellular Location of $InsP_3R2$

The nuclear envelope is known to be a double membrane composed of two lipid bilayers in continuity with the endoplasmic reticulum membrane. It can thus be regarded as a specialized region of the endoplasmic reticulum. To better evaluate the precise location of $InsP_3R2$ associated with the nucleus, confocal microscopy was performed on bovine aortic endothelial cells immunocytochemically labeled with the anti-$InsP_3R2$ antibody. Figure 5 shows typical successive confocal images (0.25 μm width) of a single fixed cell. Starting from underneath the cell where no fluorescence can be detected, up to the superior edge of the cell, the laser scan shows that $InsP_3R2$ is highly enriched in each of the different sections of the nucleus. These results indicate that $InsP_3R2$ is not only confined to the nuclear envelope but is also present deeper within the nuclear structure.

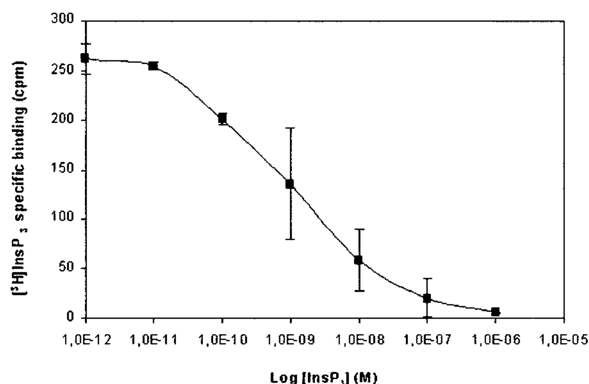

Fig. 4. $InsP_3$ binding sites on cell nuclei. Purified nuclei from bovine adrenal cortex (2.5 mg of protein) were incubated at 0°C for 30 min with 1 nM $[^3H]InsP_3$ and increasing concentrations of unlabeled $InsP_3$ in a final volume of 500 μl. Non-specific binding was determined in the presence of 1 μM $InsP_3$. Incubations were terminated by centrifugation at 15,000*g* for 15 min at 4°C. Each point is the mean ± SD of triplicate values.

DISCUSSION

Endothelial cells are known to respond to several Ca^{2+} mobilizing hormones and to express all the components of the Ca^{2+} signaling pathway. In the study presented here, we have used a Western blot approach to demonstrate that bovine aortic endothelial cells express the three types of $InsP_3R$. These results are in accordance with those of Mountian et al. [1999] who used a RT-PCR approach to demonstrate that the three types of $InsP_3R$ are expressed in

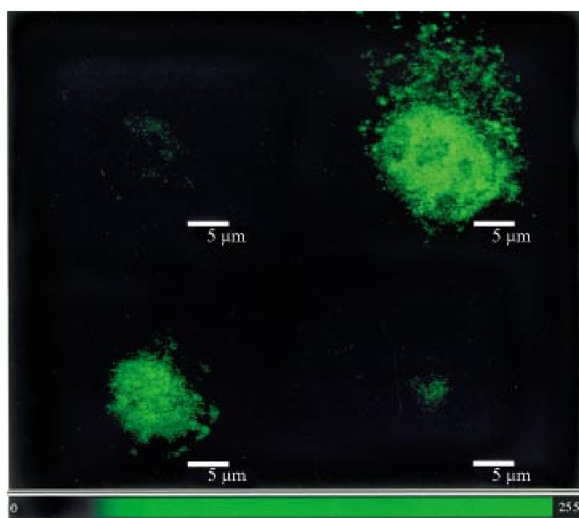

Fig. 5. Intranuclear localization of $InsP_3R2$. Bovine aortic endothelial cells were grown on cover glasses, fixed with methanol, incubated with our anti-$InsP_3R2$ antibody, stained with an FITC-conjugated secondary antibody, and observed by confocal laser microscopy. Successive images (0.25 μm depth) were taken starting from underneath the cell (top left) up to the superior edge of the cell (bottom right). Four representative images selected at varying depth are shown. These results are representative of similar observations obtained in five independent experiments.

rat and human endothelial cells. The main purpose of the present study was to determine the subcellular localization of $InsP_3R$ in a cell type expressing all three types of $InsP_3R$. With rabbit polyclonal antibodies recognizing selective epitopes on the C-termini of $InsP_3R1$, $InsP_3R2$, and $InsP_3R3$, our immunofluorescence studies revealed that the three types of $InsP_3R$ co-localize in certain regions of the cell, which correspond to the endoplasmic reticulum. $InsP_3R1$ is widely distributed within the cell and co-localizes in the perinuclear region with a specific marker of the endoplasmic reticulum. $InsP_3R1$ is also found throughout the cytosol, probably on certain portions of the endoplasmic reticulum that extend from the perinuclear region to the outer limits of the cell. Because the outer limits of the cell were never clearly delineated with our immunofluorescence approach, it is unlikely that $InsP_3R1$ is present in significant amounts at the plasma membrane. We observed that the intracellular localization of $InsP_3R3$ is similar to that of $InsP_3R1$. The endoplasmic reticulum constitutes the intracellular Ca^{2+} pool from which Ca^{2+} is released during agonist-induced cell activation. The integration of $InsP_3Rs$ into the endoplasmic reticulum membrane is thus fully compatible with a role of intracellular Ca^{2+} channel.

The most unexpected observation made with our immunofluorescence approach was the strong staining of the cell nucleus with the anti-$InsP_3R2$ antibody. The nucleus was stained with an antibody recognizing an epitope on the C-terminus of $InsP_3R2$, and it was equally stained with another antibody recognizing an epitope on the N-terminus of $InsP_3R2$. The staining of the cell nucleus was relatively weak with the anti-$InsP_3R1$ antibody, and it was virtually null with the anti-$InsP_3R3$ antibody. These results suggest that, in bovine aortic endothelial cells, $InsP_3R2$ is associated with the cell nucleus. We made the same observation with our immunofluorescence approach in COS-7 cells and in bovine adrenal glomerulosa cells. We also showed that isolated nuclei bind $[^3H]InsP_3$ with a high affinity (1.02 nM) comparable to that observed in preparations enriched in $InsP_3R2$ [Sudhof et al., 1991; Parys et al., 1995] and comparable to that of immunoprecipitated $InsP_3R2$ from bovine adrenal cortex [Poitras et al., 2000]. To our knowledge, this is the first study showing the localization of $InsP_3R2$ to the cell nucleus. A few previous studies suggested the presence of $InsP_3Rs$ on the nuclear membrane. Sullivan et al. [1993] demonstrated that $InsP_3$ stimulates the fusion of nuclear vesicles bound to chromatin in Xenopus egg extracts. Humbert et al. [1996] demonstrated the presence of $InsP_3$ binding sites in a rat liver membrane preparation enriched in inner nuclear membrane. Functional studies monitoring Ca^{2+} changes in the nucleoplasm and electrophysiological studies on isolated nuclei suggested that $InsP_3Rs$ are present on nuclear membranes [Mak and Foskett, 1994; Hennager et al., 1995; Stehno-Bittel et al., 1995]. These studies however did not clearly identify the $InsP_3R$ subtype present in their preparations. In cultured skeletal muscle, Jaimovich et al. [2000] observed an important staining of the nuclear envelope with an anti-$InsP_3R1$ antibody. They did not observe any specific staining within the nucleus.

Relatively few studies have addressed the question of intracellular localization of $InsP_3Rs$. With an immunogold electron microscopy approach, Fujimoto et al. [1992] demonstrated that an anti-$InsP_3R1$ antibody labels the caveolae of keratinocytes, endothelial and smooth muscle cells. They suggested that $InsP_3R1$

might be involved in Ca^{2+} influx. Fujimoto et al. [1995] further showed that the association of $InsP_3R1$ with actin filaments might mediate the redistribution of caveolae. In WB rat liver epithelial cells, Joseph et al. [1995] showed with an immunofluorescent approach that $InsP_3R1$ and $InsP_3R3$ have very similar subcellular localizations, being excluded from the nucleus, widely distributed throughout the cell and most abundantly in the perinuclear region. In different secretory cells, $InsP_3R3$ has been found at the secretory pole and associated with secreting granules [Blondel et al., 1994; Maranto, 1994; Nathanson et al., 1994; but see Ravazzola et al., 1996]. In rat airway epithelial cells, Sugiyama et al. [1996] showed different subcellular distributions for each type of $InsP_3R$. $InsP_3R1$ was found in the apical thin cytoplasmic area, $InsP_3R3$ was expressed mainly in the supranuclear cytoplasm whereas $InsP_3R2$ was found in the entire cytoplasm. The study also demonstrated that the expression of $InsP_3R$ subtypes varied within the different cell types composing the airway epithelium. A very recent study by Sugiyama et al. [2000] demonstrated that in rat smooth muscle cells, $InsP_3R2$ has a diffuse cytoplasmic distribution, similar to that of $InsP_3R1$ and $InsP_3R3$ but is also present in dense patches in the peripheral cytoplasm, a pattern that $InsP_3R1$ does not exhibit. Interestingly, the study also showed that the anti-$InsP_3R2$ antibody strongly stains the nuclear region (a result that was not emphasized by the authors).

The nuclear envelope is a double membrane system in direct continuity with the endoplasmic reticulum membranes. The presence of $InsP_3Rs$ on the nuclear envelope is therefore not unexpected. Our results obtained with the confocal microscopy approach suggest however that $InsP_3R2$ is not only confined to the nuclear envelope but is also distributed deeper within the structure of the nucleus. Since $InsP_3R2$ is an intrinsic membrane protein, these results imply the existence of an intra-nuclear membrane network. Tubular membrane structures have been described by Fricker et al. [1997] in the nuclei of many mammalian cell types. They suggested that these membranes derived from the nuclear envelope, form long, dynamic tubular channels that extend deep into the nucleoplasm. These nuclear channels show specific morphologies ranging from single short stubs to multiple, complex, branched structures. With a confocal microscopy approach, Lui et al. [1998] further showed that the nuclei of HeLa cells contain tubular structures that can store and release Ca^{2+}. These studies suggesting the presence of membrane structures within the cell nucleus are compatible with the intranuclear immunoreactivity of anti-$InsP_3R2$ antibodies. The diffuse distribution of anti-$InsP_3R2$ staining shown in our confocal images does not fit perfectly with the specific distribution of the tubular membrane structures described by Fricker et al. [1997]; therefore $InsP_3R2$ may reside on a presently uncharacterized membrane network that may or may not be in direct contact with the tubular membrane structures. Many components of the Ca^{2+} signaling pathway, including Ca^{2+} ATPase, phosphoinositides and phospholipase C, have been shown to be localized at the cell nucleus [for review see Malviya and Rogue, 1998]. The $InsP_3R2$ may be an important component of the intranuclear Ca^{2+} signaling machinery.

In conclusion, we have shown that the three types of $InsP_3R$ are expressed in bovine aortic endothelial cells. $InsP_3R1$ and $InsP_3R3$ are localized to the endoplasmic reticulum whereas $InsP_3R2$ is abundant at the cell nucleus where it may play a role in the regulation of Ca^{2+}-dependent nuclear processes such as protein import and gene transcription.

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