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Localization of inositol 1,4,5-trisphosphate receptors in the rat kidney

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Localization of inositol 1,4,5-trisphosphate receptors in the rat kidney. Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) serve as intracellular calcium release channels involved in signal transduction of various hormones in the kidney. Molecular cloning studies have shown that there are three types of IP_3R, designated type 1, type 2, and type 3. To characterize their localizations in the rat kidney, we employed immunohistochemical studies using type-specific monoclonal antibodies that were raised against the 15 C-terminal amino acids of each type of IP_3R. Type 1 was detected in glomerular mesangial cells and vascular smooth muscle cells. Type 2 was expressed exclusively in intercalated cells of collecting ducts from the cortex to the inner medulla. Type 3 was expressed in vascular smooth muscle cells, glomerular mesangial cells, and some cells of cortical collecting ducts, probably principal cells. As to the subcellular distribution, type 1 and type 2 showed a homogenous distribution in the cytoplasm, whereas type 3 was present mainly in the basolateral portion of the cytoplasm. These results indicate that IP_3R isoforms were expressed in a cell-specific manner. The heterogeneous subcellular localizations among the IP_3R types suggests compartmentalization of distinct IP_3-sensitive Ca^{2+} pools.

Inositol 1,4,5-trisphosphate (IP_3) is an intracellular second messenger that is generated by hydrolysis of inositol phospholipids responding to the binding of hormones and autacoids to their receptors located on the cell surface [1]. Inositol 1,4,5-trisphosphate triggers the release of Ca^{2+} from intracellular stores by binding to an IP_3 receptor (IP_3R) [2], which constitutes an IP_3-gated Ca^{2+} channel [3–5]. The IP_3R plays an important role in regulating crosstalk between the second messengers, Ca^{2+} and IP_3. Molecular cloning studies have shown that there are three types of IP_3R derived from different genes, designated type 1 (IP_3R-1) [6–8], type 2 (IP_3R-2) [9, 10], and type 3 (IP_3R-3) [10–12]. Among the three IP_3R types, there are differences in characteristics, such as the affinity for IP_3 [9], the effect of phospholylation [13], and the binding of calmodulin [14]. In the kidney, various hormone receptors are coupled to phosphatidylinositide responses, and these include the vasopressin receptor (the V1 receptor), the endothelin receptor, and the angiotensin II receptor [15]. These IP_3-mediating hormone receptors are expressed in a cell-specific manner and play important roles in the physiological functions of the kidney. However, less is known about the properties and functions of IP_3Rs in the kidney.

Previous studies have shown the localization of mRNA for IP_3Rs in the kidney. Yang et al [16] reported the expression of mRNA for IP_3R-1 and IP_3R-2 along the microdissected nephron using reverse transcription and polymerase chain reaction (RT-PCR). Furuichi, Shiota and Mikoshiba [17] and Fujino et al [18] have demonstrated the expression of mRNA for three types of IP_3Rs in the kidney by *in situ* hybridization. However, the precise cellular and subcellular localization of IP_3Rs remains to be elucidated.

Therefore, we raised type-specific monoclonal antibodies against each type of IP_3R and examined the distribution of the IP_3R proteins at the cellular and subcellular levels in the rat kidney by immunohistochemistry.

METHODS

Antibodies

Mouse monoclonal antibodies KM1112, KM1083 and KM1082 were raised against synthetic peptides corresponding to the 15 C-terminal amino acids of IP_3R-1 (human IP_3R-1 2681-2695) [8], IP_3R-2 (human IP_3R-2 2687-2701) [10]and IP_3R-3 (human IP_3R-3 2657-2671) [10], respectively. At least 14, but perhaps all of the 15 amino acids of antigen peptides are identical to those of the rat sequence. These antibodies have been shown to recognize each type of rat IP_3R protein specifically [19, 20].

Preparation of membrane protein

Wistar rats (7 to 8 weeks, male) were anesthetized with intraperitoneal pentobarbital sodium and perfused via the left ventricle with phosphate-buffered saline (PBS). After the kidneys had been removed and minced, 9 volumes of solution containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, and 5 mM Tris-HCl, pH 7.4 were added and the specimens were homogenized in a glass-Teflon Potter homogenizer with 10 strokes at 1,000 rpm. The homogenates were

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centrifuged at 1,000 × g for five minutes at 4°C. The supernatants were centrifuged at 105,000 × g for 60 minutes at 2°C to sediment the membrane proteins. The pellets were resuspended in 1 mM EDTA, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, and 50 mM Tris-HCl, pH 7.4. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting

Ten micrograms of membrane protein from the kidney were subjected to 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Laemmli buffer system [21]. After transferring the proteins electrophoretically to nitrocellulose filters, immunodetection probing with type-specific antibodies was performed. Bound antibodies were visualized by an ECL Western blotting system (Amersham Life Sciences, Buckinghamshire, UK). To determine antigen specificity, the antibody solutions were preabsorbed with a tenfold excess of the antigen peptide.

Immunohistochemistry

Wistar rats (7 to 8 weeks, male) were anesthetized with intraperitoneal pentobarbital sodium. The kidneys were perfused via the left ventricle, first with PBS and then with 2% paraformaldehyde. The kidneys were then removed and cut into approximately 5-mm-thick sections, which were then fixed by immersion in the same fixative for two hours at 4°C. After dehydration, the tissues were embedded in paraffin.

Immunohistochemistry was performed using avidin-biotin-horseradish peroxidase complex (ABC) technique (Vectastain ABC-Elite Kit; Vector Laboratories, Burlingame, CA, USA). Three-micrometer sections of rat kidney were deparaffinized, rehydrated and then incubated for 30 minutes with 0.3% H_2O_2 to eliminate endogenous peroxidase activity. After rinsing in PBS, the sections were treated with 2% normal horse serum for one hour. The sections were incubated with anti-IP_3R antibodies at 4°C overnight. The concentrations of the first antibodies were 10 μg/ml (KM1112) or 3 μg/ml (KM1083 and KM1082). After rinsing in PBS, the sections were incubated with biotinylated horse antibody against mouse IgG (Amersham Life Sciences) for one hour. To avoid cross-reaction between the second antibody (biotinylated anti-mouse IgG) and endogenous rat IgG in the section, the second antibody was diluted with PBS containing 200 μg of normal rat IgG per ml. After rinsing, the sections were incubated for 30 minutes with Vectastain ABC reagent, followed by incubation with diaminobenzidine. The sections were then counterstained with methylgreen, examined, and photographed on a light microscope (Olympus, Tokyo, Japan). As a control, the antibody solutions were preabsorbed with a tenfold excess of the peptide.

For the double-immunofluorescense staining study, we used an affinity-purified rabbit antiserum against aquaporin-2 (AQP-2) (kindly provided by Dr. Sei Sasaki, Tokyo Medical Dental University) and a mouse monoclonal antibody against IP_3R-2 (KM1083). Sections were incubated with the two antisera, then incubated with rhodamine-conjugated anti-rabbit IgG (Cappel, West Chester, PA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Vector Laboratories).

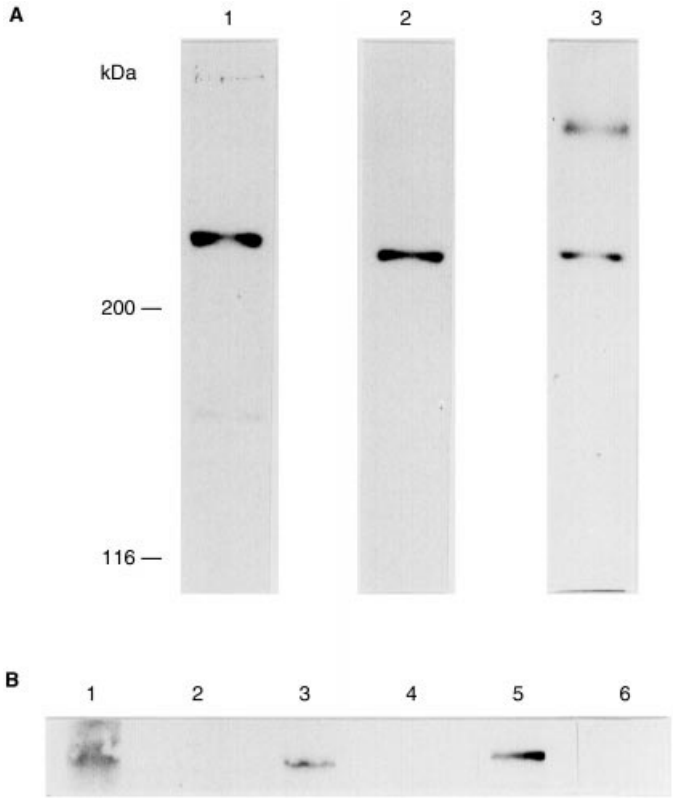

Fig. 1. (*A*) Western blots (5% SDS-PAGE) of renal membrane proteins (10 μg/lane) probed with anti-IP_3R-1 antibody (KM1112, lane 1), anti-IP_3R-2 antibody (KM1083, lane 2), or anti-IP_3R-3 antibody (KM1082, lane 3). The positions of molecular weight markers are shown on the left. (*B*) Immunoabsorption of monoclonal antibodies with the specific antigen peptide. Western blots (5% SDS-PAGE) of renal membrane proteins (10 μg/lane) focusing on the major band at ~250 kDa probed with KM1112 (lane 1), preabsorbed KM1112 (lane 2), KM1083 (lane 3), preabsorbed KM1083 (lane 4), KM1082 (lane 5), preabsorbed KM1082 (lane 6). The monoclonal antibody solutions were preabsorbed with a tenfold excess of the antigen.

RESULTS

To identify rat renal IP_3R proteins, we carried out immunoblots against membrane proteins prepared from the rat kidney. KM1112 recognized a single major band at ~250 kDa (Fig. 1A, lane 1). Similarly, KM1083 and KM1082 labeled the protein with a molecular weight of ~250 kDa (Fig. 1A, lanes 2 and 3). These results are consistent with the previous study of Sugiyama et al [19]. In immunodetection with KM1082, the upper molecular weight band was observed. This band is thought to be a dimer complex of IP_3R-3. The bands at ~250 kDa were abolished by pretreatment of the antibody with the antigen peptide (Fig. 1B). This result shows that these antibodies specifically recognize each type of IP_3R protein expressed in the kidney.

To immunolocalize IP_3Rs at the cellular and subcellular levels in the kidney, we performed immunohistochemistry on paraffin sections of paraformaldehyde-fixed rat kidney. Immunostaining of IP_3R-1 was seen in glomerular mesangial cells and in vascular smooth muscle cells of arteries and arterioles (Fig. 2A). These stainings were completely blocked by preincubation of the antibody with the peptide (Fig. 2B). No significant staining was observed in any tubular cells.

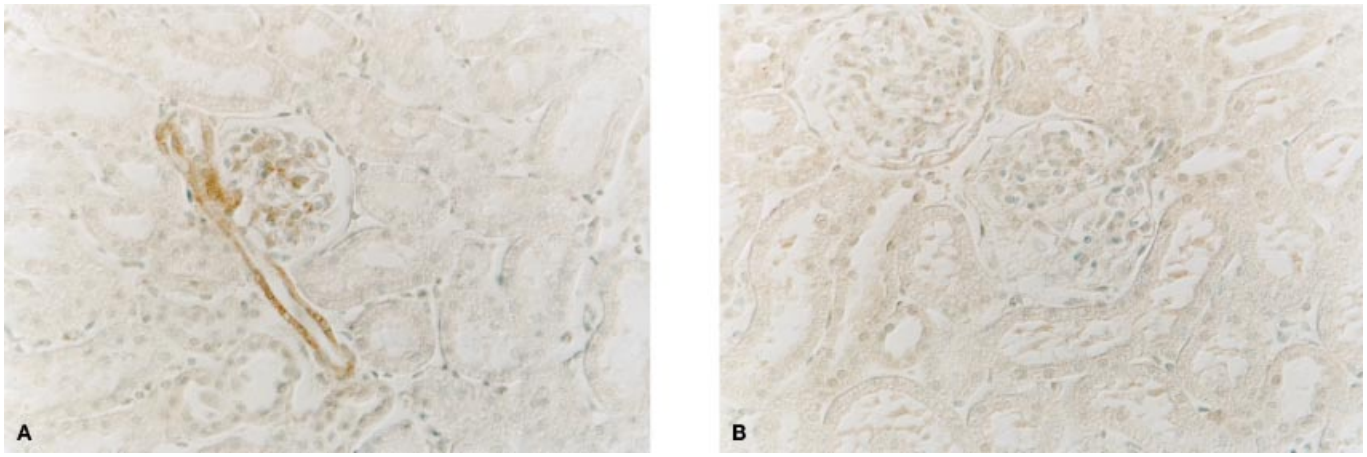

Fig. 2. Immunohistochemical localization of IP_3R-1 in rat kidney. (*A*) The sections were incubated with 10 μg/ml of KM1112 (×200). (*B*) Preabsorbed KM1112 was used instead of KM1112 (×200).

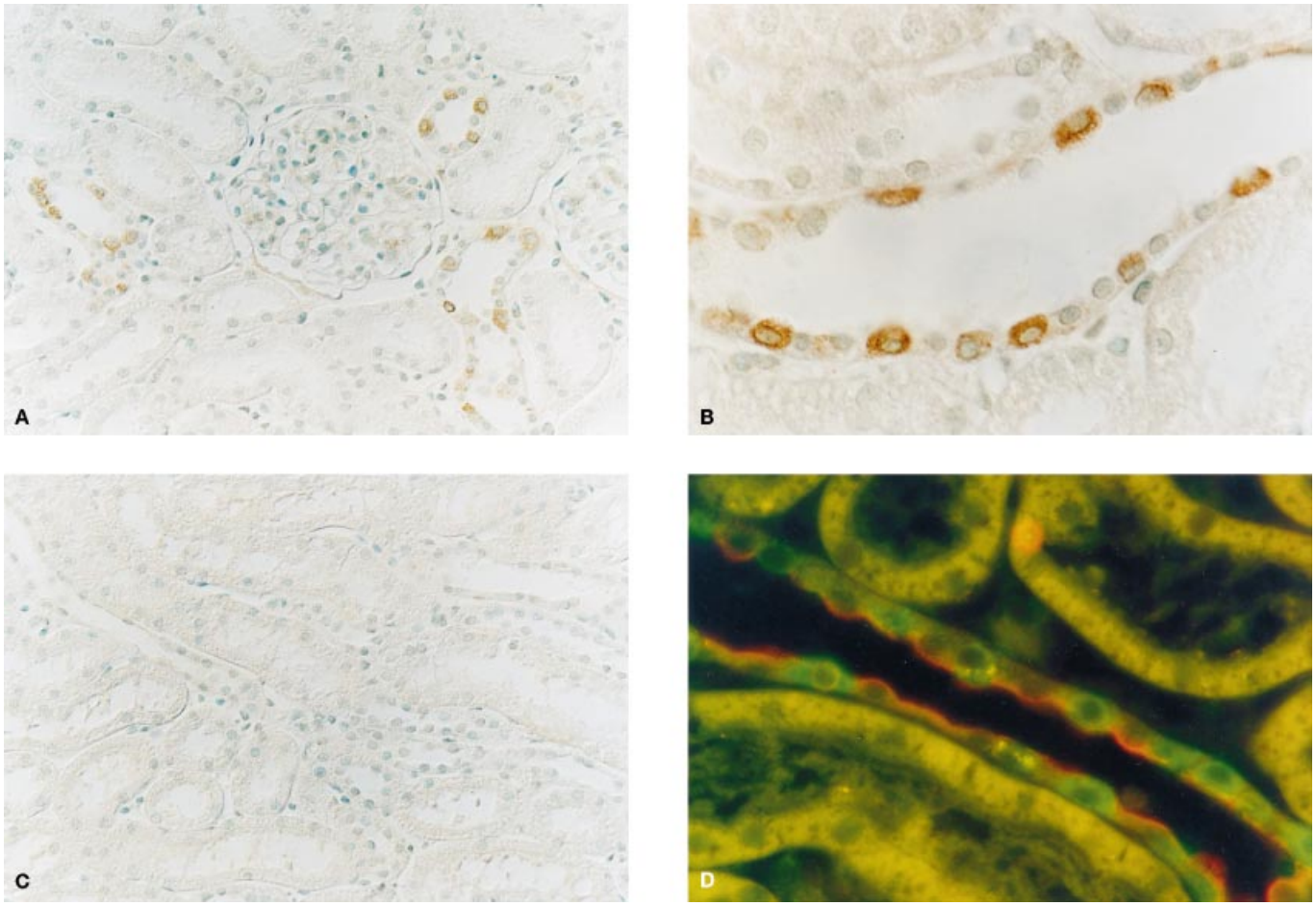

Fig. 3. Immunohistochemical localization of IP_3R-2 in rat kidney. (*A*) The sections were incubated with 3 μg/ml of KM1083 (×200). (*B*) The sections were incubated with 3 μg/ml of KM1083 (×400). (*C*) Preabsorbed KM1083 was used instead of KM1083 (×200). (*D*) Indirect immunofluorescence micrographs showing rat kidney cortex stained with IP_3R-2 antibody (yellow-green, FITC), and double labeled with anti-AQP-2 antibody (red, rhodamine) (×400).

The immunostaining of IP_3R-2 was limited in tubular cells. Cells positive for anti-IP_3R-2 antibody were localized in the cortical to the inner medullary collecting ducts, and the staining was patchy along the collecting ducts (Fig. 3 A, B). In the cortical and outer medullary collecting ducts, 30 to 40% of cells were positive for IP_3R-2. In the upper third of the inner medullary collecting duct, cells positive for IP_3R-2 accounted for approximately 10% of cells. No positive cells were observed in the

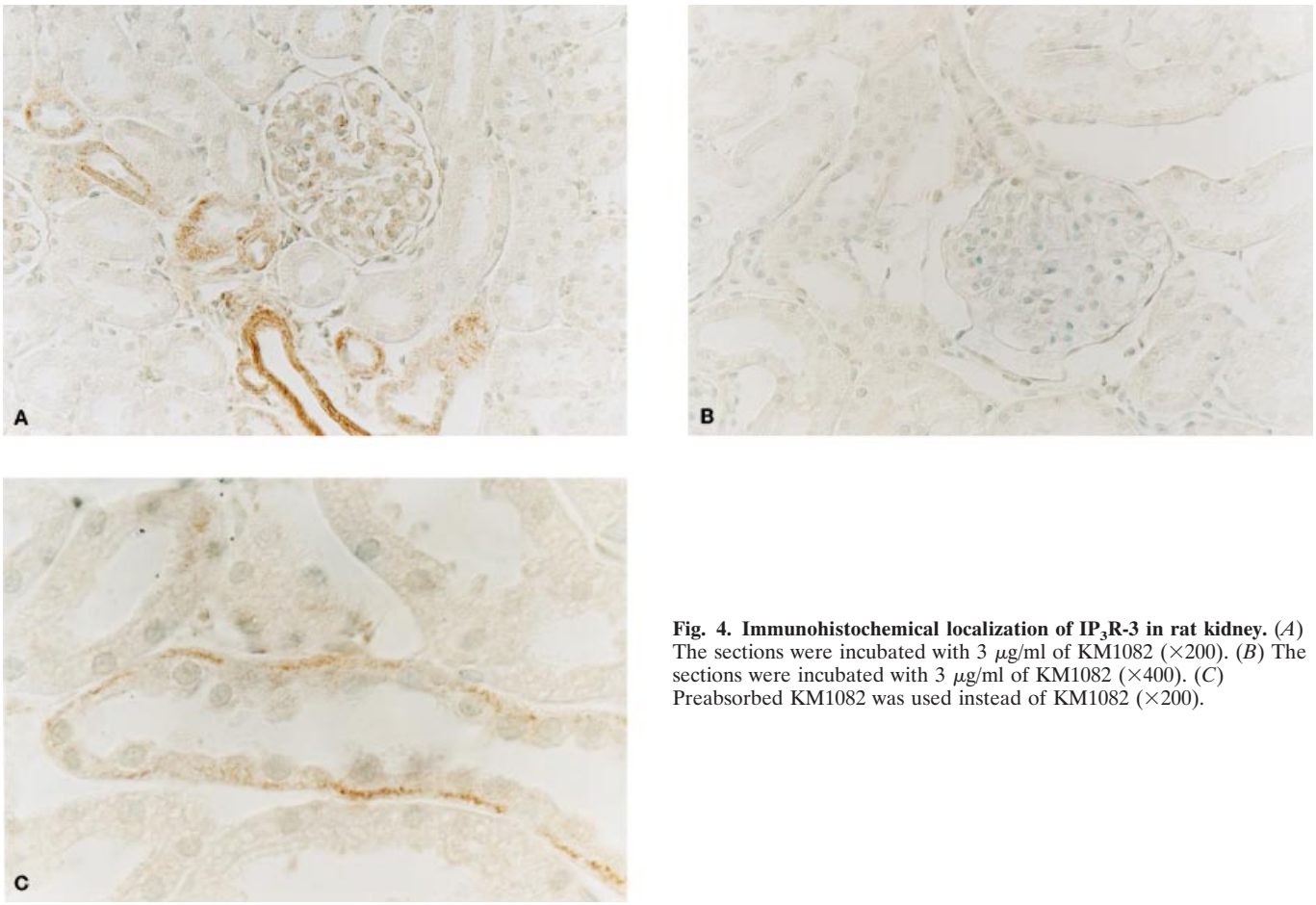

Fig. 4. Immunohistochemical localization of IP_3R-3 in rat kidney. (*A*) The sections were incubated with 3 μg/ml of KM1082 ($\times$200). (*B*) The sections were incubated with 3 μg/ml of KM1082 ($\times$400). (*C*) Preabsorbed KM1082 was used instead of KM1082 ($\times$200).

remaining part of the inner medullary collecting duct. These stainings were completely blocked by preincubation of the antibody with the peptide (Fig. 3C). To characterize the subpopulation of cells that express IP_3R-2 in these nephron segments, we used double labeling immunofluorescence with a polyclonal antibody against the aquaporin-2 (AQP-2) and the monoclonal antibody against the IP_3R-2. The collecting duct contains two distinct cell types, principal cells and intercalated cells. The AQP-2, vasopressin-stimulated water channel, has been shown to be expressed at the apical membranes of principal cells in the collecting ducts [22]. Figure 3D shows fluorescence images of rat renal cortex depicting the distributions of AQP-2 (red, rhodamine) and IP_3R-2 (yellow-green, FITC). The cells staining for AQP-2 (red) on the apical side were not stained for IP_3R-2 (green-yellow), while the cells stained for IP_3R-2 (yellow-green) in the cytoplasm were not stained for AQP-2 (red). These observations show clearly that IP_3R-2 is expressed in the intercalated cells.

The immunostaining of IP_3R-3 was observed in cortical collecting ducts, vascular smooth muscle cells, and glomerular mesangial cells (Fig. 4A). These stainings were completely blocked by preincubation of the antibody with the peptide (Fig. 4B). Although double-staining was hampered by the low efficiency of KM1082, especially in the immunofluorescence study, the positive-stained cells in the collecting ducts were thought to be principal cells based on their distribution and morphology. At a higher magnification, the polarity of IP_3R-3 protein expression was observed (Fig. 4C). In the stained cells, the basolateral aspect of the cytoplasm was intensely stained. No staining was observed in the basolateral aspect of the cell. On the other hand, in the immunohistological studies of IP_3R-1 and IP_3R-2, staining was consistently observed throughout the cytoplasm.

DISCUSSION

In the present study, we demonstrated the localization of three IP_3R types in the rat kidney using type-specific monoclonal antibodies. Immunohistochemical examination revealed that IP_3R-1 is expressed in glomerular mesangial cells and smooth muscle cells in blood vessels. Furuichi, Shiota and Mikoshiba [17] examined the localization of mRNA for IP_3R-1 by *in situ* hybridization. They reported that a strong signal was observed in vessels, while there was no signal in glomerular mesangial cells. It is likely that their technique lacked sufficient sensitivity to detect the lower levels of IP_3R-1 message present in mesangial cells. Yang et al [16] also examined the expression of mRNAs for IP_3R-1 by RT-PCR along the microdissected nephron. They found that mRNA for IP_3R-1 was widely distributed in almost every structure, with relatively high amounts in collecting ducts, while there was no signal in the proximal convoluted tubule. These apparent differences in localization between the present study and that of Yang et al [16] are most likely due to the different methodological approaches employed. Inability to detect IP_3R-1 in all tubular

cells by immunohistochemistry might be attributable to the low titer of monoclonal antibody, or a false positive signal from tubules on RT-PCR. We performed the immunohistochemistrical examination using several other antibodies, but failed to detect significant signals in any tubular cells (data not shown). Therefore, we think that the primary sites of IP_3R-1 expression are vascular smooth muscle cells and glomerular mesangial cells. These cells are believed to have a contractile function. In mesangial and vascular smooth muscle cells, angiotensin II, endothelin, and vasopressin are known to induce Ca^{2+} release via production of IP_3 and to regulate cell contractility [15]. The IP_3R-1 is thought to be a major isoform regulating the contractility of such cells.

The IP_3R-2 is expressed exclusively in the intercalated cells of collecting ducts. This observation agrees with the results of an *in situ* hybridization study [16], in which IP_3R-2 mRNA was highly concentrated in collecting ducts but nearly undetectable in the other nephron segments. The intercalated cells regulate excretion and reabsorption of acid and base [23]. However, no hormone inducing the production of IP_3 and increasing the intracellular calcium concentration in intercalated cells has as yet been identified. Moreover, little is known about the relationship between the rise in the intracellular calcium concentration and the function of intercalated cells. Physiological studies using knockout mice lacking the gene for IP_3R-2 are anticipated to reveal the function of Ca^{2+} signaling in intercalated cells.

The IP_3R-3 is expressed in vascular smooth muscle cells, glomerular mesangial cells, and some cells of the cortical collecting duct. The cells expressing IP_3R-3 in the cortical collecting ducts are thought to be principal cells. Principal cells are responsible for transepithelial sodium and water transport. Vasopressin is known to the increase cytoplasmic Ca^{2+} concentration by binding to the V_1 receptor located on both the luminal and the basolateral membrane of principal cells [24]. Ikeda et al [25] speculate that the increase in $[Ca^{2+}]_i$ mediated by the V_1 receptor may participate in negative feedback control, preventing an excessive effect of vasopressin actions mediated via the V_2 receptor. Thus, IP_3R-3 in principal cells might be involved in this negative feedback mechanism.

In terms of subcellular localization, IP_3R-1 and IP_3R-2 are expressed throughout the cytoplasm, consistent with the previously demonstrated endoplasmic reticulum localization pattern [26, 27]. On the other hand, IP_3R-3 shows a polarized distribution in the cell. On light microscopic observation, IP_3R-3 appears to be localized to the basolateral side of the cytoplasm, but not the true basolateral plasma membrane. There is a possibility that IP_3R-3 might be localized to caveolae near the basolateral plasma membrane. Fujimoto et al [28] reported that IP_3R-like protein is localized to caveolar structure of the plasma membrane. Additional immunolocalization studies at the electron microscopic level have been hampered by the apparent loss of antigenicity under fixation conditions necessary to preserve the organellar structures of the cells of interest. Immunoelectron microscopy with type-specific antibodies is necessary to resolve this problem. Kasai, Li and Miyashita [29] have proposed a model in which the heterogeneous distribution of IP_3Rs having different affinities for IP_3 plays an important role in formation of the Ca^{2+} wave. In rat pancreatic acinar cells, the Ca^{2+} concentration increase is initiated in a small trigger zone, the T zone, and then, Ca^{2+} waves propagate to reach the rest of the cell. Thus, the possibility exists that the polarized distribution of IP_3R-3 in the principal cells might serve as the molecular basis of spatiotemporal Ca^{2+} signaling.

In proximal tubule cells, endothelin [30] and parathyroid hormone (PTH) [31] are known to initiate IP_3-induced Ca^{2+} release, but we detected no isoforms of IP_3R in this segment. The affinity of the antibody might have been too low, or an IP_3R type which has not been cloned might be expressed in these tubule cells.

In summary, the present study shows that the three known IP_3R types exhibit different cellular and subcellular distributions in the rat kidney. Different IP_3Rs may have distinct functions in the rat kidney.

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APPENDIX

Abbreviations used in this article are: IP_3, inositol 1,4,5-trisphosphtate; IP_3R, IP_3 receptor; IP_3R-1, IP_3R-2 and IP_3R-3 are IP_3 receptor types 1, 2 and 3, respectively; RT-PCR, reverse transcription and polymerase chain reaction; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ABC, avidin-biotin-horseradish peroxidase complex; AQP-2, aquaporin-2; FITC, fluorescein isothiocyanate.

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