

# Monoclonal antibodies distinctively recognizing the subtypes of inositol 1,4,5-trisphosphate receptor: Application to the studies on inflammatory cells

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**Abstract** Monoclonal antibodies were raised that specifically recognize the COOH-terminal sequences and the loop sequences between the fifth and the sixth transmembrane spanning regions of human inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) type 1, 2 and 3. Western blot analysis using Jurkat cells, mouse cerebellum, COS-7 expressing IP<sub>3</sub>R type 3 cDNA showed that those monoclonal antibodies reacted specifically with each of these three IP<sub>3</sub>R subtypes and that they do not cross-react. These antibodies could be used for the specific immunoprecipitation of IP<sub>3</sub>Rs. Using these monoclonal antibodies, the expression profiles of IP<sub>3</sub>R-subtype proteins were found to be different among inflammatory cells such as macrophages, polymorphonuclear cells, mast cells, eosinophils, splenocytes, thymocytes and megakaryocytic cells. Usually, more than one type of IP<sub>3</sub>R were expressed in a cell simultaneously. The observation of CMK cells under immunofluorescence confocal microscopy revealed that IP<sub>3</sub>R type 1 and type 2 are located at different subcellular fractions.

**Key words:** Monoclonal antibody; Intracellular calcium; Inositol 1,4,5-trisphosphate receptor; Mast cell; PMN; Macrophage; Thymocyte; Splenocyte; Megakaryocyte

## 1. Introduction

Phosphatidyl inositol 4,5-bisphosphate is hydrolyzed in response to receptor stimulation on the plasma membrane to produce inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol. IP<sub>3</sub> is a physiological ligand known to mediate internal Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> storage sites by binding to its specific receptor named the IP<sub>3</sub> receptor (IP<sub>3</sub>R) [1]. The IP<sub>3</sub>R in cerebellar Purkinje cells forms a tetramer in order to function as a Ca<sup>2+</sup> channel [2,3]. This signal transduction system is used in cells of diverse types and is an essential process leading to specific cellular responses [4]. Recently, proteins immunoreactive to anti-IP<sub>3</sub>R antibodies or IP<sub>3</sub>-gated Ca<sup>2+</sup> channels have been reported to exist on the plasma membranes of T cells, B cells, olfactory neurons and endothelial cells [5–9].

The IP<sub>3</sub>R encoded in the first cloned mouse IP<sub>3</sub>R cDNA from cerebellar Purkinje cells is now termed IP<sub>3</sub>R type 1 [10], since recent molecular cloning studies, including our own studies on human receptors, have revealed that there is a family of IP<sub>3</sub>Rs. The full-length cDNAs for IP<sub>3</sub>R subtypes, termed IP<sub>3</sub>R type 2 and type 3, have been isolated [11–15]. The presence of another type of IP<sub>3</sub>R, type 4, is also suggested [16]. Our North-

ern and dot blot analyses of human hematopoietic cell lines using the subtype-specific cDNA regions of these human IP<sub>3</sub>Rs as probes revealed that each IP<sub>3</sub>R subtype is differently expressed in these cells and that most of cells studied express at least two distinct types of IP<sub>3</sub>R simultaneously. Moreover, it was proved that the expression profile of these subtypes in each cell changed dynamically in response to stimuli which induce cell differentiation [17]. These findings prompted us to investigate the difference in functions, and tissue/cellular and subcellular localization of these IP<sub>3</sub>R subtypes. Immunohistochemical studies using the monoclonal antibodies raised against IP<sub>3</sub>R type 1 have shown that whilst the subtype is distributed in many areas of the brain, its expression is especially high in the Purkinje cells [18]. However, to date, such studies for IP<sub>3</sub>R type 2 and type 3 are rather limited [11,12,14] essentially because of the lack of monoclonal antibodies specific to each of IP<sub>3</sub>R subtypes, especially those to IP<sub>3</sub>R type 2 and type 3. Such a set of monoclonal antibodies could also be a great help for the analysis of the subunit composition of the Ca<sup>2+</sup> channels to examine if they are homotetramer or heterotetramer.

In spite of their high level identities [13–15], the cloning and the sequencing analysis of the three subtypes of human IP<sub>3</sub>R cDNA enabled us to find several subtype-specific regions [13,15]. In this study, we have established the monoclonal antibodies specific to each of the three IP<sub>3</sub>R subtypes using the synthetic peptides corresponding to the loop region between the fifth and the sixth transmembrane regions and to the COOH-terminal regions. Employing these monoclonal antibodies, we have analyzed the expression of the IP<sub>3</sub>R subtypes in inflammation-related blood cells, and have found that each member of the IP<sub>3</sub>R family is expressed differently among those

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**Abbreviations:** IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IP<sub>3</sub>R, IP<sub>3</sub> receptor; ELISA, enzyme linked immunosorbent assay; PMN, polymorphonuclear cell; SD rat, Sprague–Dawley rat; FITC, fluorescein isothiocyanate.

cell types as well as being localized at different subcellular compartments.

## 2. Materials and methods

### 2.1. Cells and cell culture

A human T cell line (Jurkat) and a megakaryocytic CMK cell line were maintained as described [17]. COS-7 and COS-7 expressing human IP<sub>3</sub>R type 3 were grown according to Hino et al [13]. Cerebellum was obtained from ICR mouse. Macrophages and polymorphonuclear cells (PMN) were harvested from the peritoneal cavities of Sprague–Dawley (SD) rats after intraperitoneal injection of thioglycollate broth [19] and casein [20], respectively. Mast cells from the same cavity of SD rat were purified using Ficoll 400 (Pharmacia LKB Biotechnology, Sweden) [21]. Splenocyte and thymocyte suspensions from SD rats were prepared as described [22,23]. Eosinophils were harvested from the peritoneal cavities of Hartley guinea pig after intraperitoneal injection of normal horse serum [24]. The purity of these cells prepared from rats and guinea pigs were more than 90%.

### 2.2. Preparation of peptide antigens

Peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) method on a peptide synthesizer PSSM8 (Simadzu, Japan). The synthesized peptides corresponding to the IP<sub>3</sub>R COOH-terminal regions were CLGHPHNMNVNPPQQA (peptide-1C, amino acid residues 2,681–2,695), CLGSNTPHVNHMMPPH (peptide-2C, 2,687–2,701) and CRQLGFVDVQNS\*ISR (peptide-3C, 2,657–2,671) for IP<sub>3</sub>R type 1, type 2 and type 3, respectively. The peptides corresponding to the IP<sub>3</sub>R loop region between the fifth and the sixth transmembrane regions were CGESLANDFLYSDVS\*R (peptide-1L, 2,483–2,497), CGSHQVPTMTLTMMME (peptide-2L, 2,436–2,450) and CSPLGMPHGAAAFVDT (peptide-3L, 2,410–2,424) for IP<sub>3</sub>R type 1, type 2 and type 3, respectively. Serines designated as S\* were introduced instead of the original cysteines to avoid disulfide bond formation. Cysteins were attached to all of the peptides as the N-terminal spacer amino acids used as the coupling sites for the preparation of keyhole limpet hemocyanin (KLH; Carbiochem, USA)-peptide conjugates.

### 2.3. Immunization and preparation of hybridoma

Procedures for immunization of BALB/c mice with the peptide-KLH conjugates, cell fusion and hybridoma selection were as described [25]. Aliquots of the culture supernatants from each of the hybridomas were tested in ELISA for the presence of mouse immunoglobulins with specificity directed against the original immunizing antigens. These supernatants were also tested for antibody activity against the other types of IP<sub>3</sub>R peptides to examine their specificity.

### 2.4. Screening of candidate monoclonal antibodies by ELISA

The antigens used in the ELISA screening were the thyroglobulin-peptide conjugates. The conjugates were coated on 96-well ELISA PLATTE (Greiner, Germany) by passive absorption for 16 h at 4°C. The plates were washed with phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), and blocked by addition of 1% BSA in PBS followed by incubation for 1 h at room temperature. After washing, the culture supernatant of each hybridoma was added and incubated for 2 h at room temperature. The plates were washed with 0.05% Tween 20 in PBS followed by addition of the peroxidase-conjugated rabbit anti-mouse immunoglobulin solution (Dako, Japan), and incubated for 1 h at room temperature. After washing, the plates were incubated at room temperature with peroxidase substrate (2,2'-azino bis-(3-ethylbenzothiazoline-6-sulfonic acid). The color was quantified by measuring the absorption at 415 nm on a micro-plate reader NJ2001 (Japan Intermed).

### 2.5. Determination of immunoglobulin isotypes

The supernatants of the hybridoma cultures were tested as described above with the exception of the second antibody. For this purpose, peroxidase-conjugated rabbit anti-mouse IgG1, IgG2a, IgG2b and IgG3 monoclonal antibodies (Zymed, USA) were used.

### 2.6. Membrane preparation and Western blotting

Cells were homogenized in ice-cold 5 mM Tris-HCl (pH 7.4) contain-

ing 1 mM EDTA, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Wako Pure Chemical Industries, Ltd., Japan), 10  $\mu$ M Pepstatin A (Peptide Institute, Inc., Japan) and 10  $\mu$ M Leupeptin (Peptide Institute Inc., Japan), and centrifuged at 2,000  $\times$  g for 10 min at 4°C. The supernatants were further centrifuged at 100,000  $\times$  g for 30 min at 4°C. The resultant pellets were resuspended in a solution containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 mM PMSF, 10  $\mu$ M Pepstatin A and 10  $\mu$ M Leupeptin. The membrane fraction and the cells dissolved in the sample buffer (0.1% SDS, 125 mM Tris-HCl, pH 6.8) were subjected to 6% SDS-PAGE, followed by electroblotting onto nitrocellulose sheets (Schleicher and Schuell, Germany). The sheets were blocked with skim milk, and further incubated with monoclonal antibodies. The monoclonal antibodies tested were those to the COOH-terminal peptide of IP<sub>3</sub>R type 1, KM1112, to the COOH-terminal peptide of IP<sub>3</sub>R type 2, KM1083, KM1084, KM1085, KM1086, KM1087 and KM1089, to the COOH-terminal peptide of IP<sub>3</sub>R type 3, KM1076, KM1077, KM1078, KM1079, KM1080, KM1081 and KM1082, and to the loop peptide of IP<sub>3</sub>R type 3, KM1106, KM1107, KM1108, KM1109 and KM1110. As a check for specificity, monoclonal antibodies preincubated with 10  $\mu$ g/ml of their immunized peptides were used. The bound monoclonal antibodies were detected using the ECL system (Amersham, UK).

### 2.7. Immunoprecipitation

Immunoprecipitation was performed as described [26]. Briefly, membrane were solubilized in the lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 5 mM EDTA, 0.1 mM PMSF, 10  $\mu$ M Pepstatin A, 10  $\mu$ M Leupeptin and 1 mM 2-mercaptoethanol) for 30 min at 4°C. After centrifugation at 20,000  $\times$  g for 30 min at 2°C, the supernatants were boiled for 5 min in the lysis buffer containing 6 M urea, 20 mM dithiothreitol, 1% SDS and 1% Triton X-100, and dialyzed against 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 10  $\mu$ M Pepstatin A and 10  $\mu$ M Leupeptin. The solution was incubated with 6  $\mu$ g/ml KM1112, KM1083 and KM1082 for 1 h at 4°C, respectively, followed by addition of anti-mouse IgG. The immune complexes were collected with Pansorbin (Calbiochem, USA) and subjected to SDS-PAGE.

### 2.8. [<sup>3</sup>H]IP<sub>3</sub> binding assay

Solubilized membranes in the lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1 mM PMSF) were incubated with 1/20 vol. of normal mouse serum for 1 h at 4°C and then pre-cleared with protein G agarose beads (Vivax Biotechnology, USA) for 1 h at 4°C. The supernatant was incubated with 6  $\mu$ g/ml KM1083 for 1 h at 4°C followed by addition of protein G agarose beads and incubated further for 1 h at 4°C. The beads were washed with the lysis buffer and incubated with 10 nM [<sup>3</sup>H]IP<sub>3</sub> (777 GBq/mmol, NEN/DuPont, USA) for 10 min on ice. After centrifugation at 10,000  $\times$  g for 5 min, the beads were suspended in AQUASOL 2 (NEN/DuPont, USA) and radioactivity was analyzed by liquid scintillation counting.

### 2.9. Fluorescence confocal microscopy

Suspensions of CMK cells in PBS were plated onto poly-L-lysine coated glass slides and incubated at 37°C for 15 min. The adherent CMK cells were fixed in 2% paraformaldehyde, 0.025 M L-lysine and 0.01 M of periodate in sodium phosphate buffer (pH 6.2) for 15 min on ice. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature followed by blocking with 2% normal horse serum for 30 min at room temperature. The cells were incubated with 2  $\mu$ g/ml KM1112 or KM1083 for 16 h at 4°C. Normal mouse serum and the monoclonal antibodies preincubated with 10  $\mu$ g/ml of its immunized peptides were used as controls. The cells washed with PBS were incubated with biotinylated horse anti-mouse immunoglobulin (Vector, USA) for 1 h at room temperature. Then the cells were rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector, USA). The stained cells were examined under fluorescence confocal microscopy using In-SIGHT-IQ (Meridian Instruments Far East K.K., Japan).

## 3. Results

### 3.1. Characterization of monoclonal antibodies

Mice were immunized with the conjugates containing the

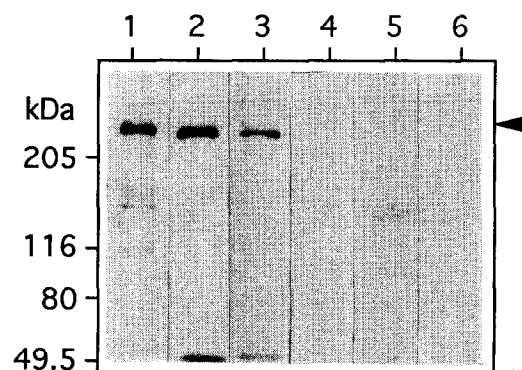


Fig. 1. The specificity of the monoclonal antibodies raised against the peptides corresponding to the IP<sub>3</sub>R COOH-terminal regions. Membrane fraction of Jurkat were subjected to SDS-PAGE (10  $\mu$ g protein/lane) and Western blotting. The blots were incubated with the monoclonal antibody KM1112 against peptide-1C for IP<sub>3</sub>R type 1 (lane 1), KM1083 against peptide-2C for IP<sub>3</sub>R type 2 (lane 2), and KM1082 against peptide-3C for IP<sub>3</sub>R type 3 (lane 3). The reactions of the monoclonal antibodies preincubated with each immunizing peptide-1C (lane 4), -2C (lane 5) and -3C (lane 6) were also shown. Arrowhead indicates the position of IP<sub>3</sub>Rs.

COOH-terminal or the loop amino acid sequences of human IP<sub>3</sub>Rs. Preliminary screening by ELISA of the specificities of the obtained hybridoma supernatants identified a clone denoted KM1112 recognizing the COOH-terminal peptide of IP<sub>3</sub>R type 1, six clones (denoted KM1083 to KM1087 and KM1089, respectively) recognizing the COOH-terminal peptide of IP<sub>3</sub>R type 2, seven clones (denoted KM1076 to KM1082, respectively) recognizing the COOH-terminal peptide of IP<sub>3</sub>R type 3 and five clones (denoted KM1106 to KM1110, respectively) recognizing the loop peptide of IP<sub>3</sub>R type 3. Hybridomas producing antibodies to the loop peptides of IP<sub>3</sub>R type 1 and type 2 were not obtained. These antibodies showed no cross-reactivity in ELISA to the peptides other than the immunizing antigens (data not shown). The isotype of these antibodies was determined to be IgG1 with the exception of KM1081 and KM1086 (IgG2b) and KM1078, KM1080, KM1083 and KM1089 (IgG2a).

The specificity of those monoclonal antibodies to each IP<sub>3</sub>R subtype was demonstrated by Western blot analysis using Jurkat cells, mouse cerebellum that expresses IP<sub>3</sub>R type 1 dominantly, COS-7 and a recombinant COS-7 expressing IP<sub>3</sub>R type 3 cDNA [13]. As shown in Fig. 1, KM1112, KM1083 and KM1082 specifically reacted with the Jurkat proteins of about 250 kDa. Staining of the bands was blocked by the immunizing peptide-1C, -2C and -3C, respectively. Moreover, the monoclonal antibodies KM1112, KM1083 and KM1082 reacted only with the immunoprecipitated proteins of about 250 kDa with KM1112, KM1083 and KM1082, respectively. No cross-reactivity was demonstrated (Fig. 2). It was reported that IP<sub>3</sub>R type 1 and IP<sub>3</sub>R type 3 gave specific bands of about 250 kDa on SDS-PAGE using an anti-mouse IP<sub>3</sub>R type 1 monoclonal antibody 4C11 [18] and an anti-IP<sub>3</sub>R type 3 polyclonal antibody [13], respectively. The antibody 4C11 was shown to react with the protein with the same migration in this study (data not shown). As indicated in Fig. 3, KM1112 specifically reacted with a 250 kDa protein of cerebellum and did not react with that of COS-7 expressing IP<sub>3</sub>R type 3. Staining of the band was blocked by the immunizing peptide-1C (data not shown). While the monoclonal antibodies KM1082 and KM1109 reacted with the 250 kDa protein of COS-7 expressing human IP<sub>3</sub>R type 3, they did not react with that of cerebella. Staining of the band was blocked by the immunizing peptide-3C (data not shown). These results indicated that our monoclonal antibodies specifically recognize IP<sub>3</sub>R type 1 and type 3.

As shown in Fig. 3B, the monoclonal antibody KM1083 recognizes proteins of about 250 kDa which do not react with KM1112, KM1082 and KM1109. Since we had no recombinant cells expressing IP<sub>3</sub>R type 2 cDNA, we examined if the protein recognized by KM1083 bound IP<sub>3</sub>. The membrane of Jurkat which is known to express IP<sub>3</sub>R type 2 [17] were solubilized with Triton X-100 and immunoprecipitated with KM1083. As shown in Fig. 4, the [<sup>3</sup>H]IP<sub>3</sub> binding activity was detected in KM1083 immunoprecipitates. The result in combination with the specific reactivity with the peptide-2C (Fig. 1) and with one of the three Jurkat IP<sub>3</sub>R subtypes other than those of type 1 and type 3 (Fig. 2) clearly show that KM1083 recognizes IP<sub>3</sub>R type 2 specifically.

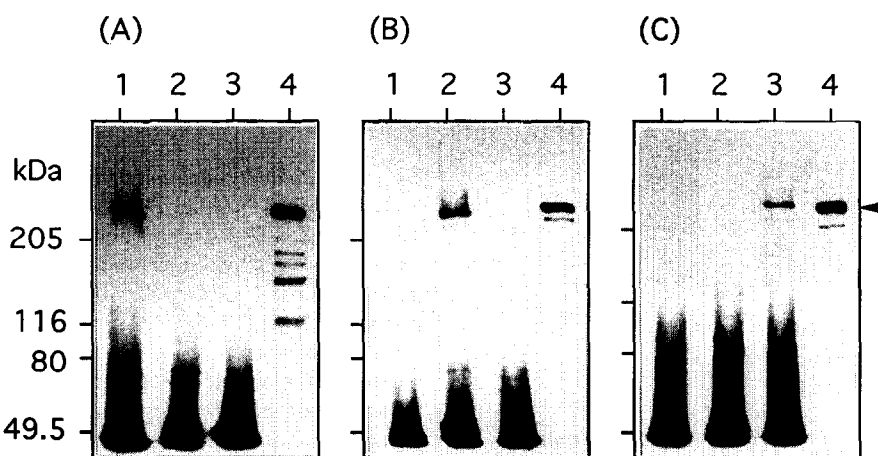


Fig. 2. The specificity of the monoclonal antibodies against each of IP<sub>3</sub>R subtypes. Solubilized Jurkat membrane proteins were immunoprecipitated with the monoclonal antibodies KM1112 (lane 1), KM1083 (lane 2) and KM1082 (lane 3). The immunoprecipitates and membrane fraction (lane 4, 10  $\mu$ g protein/lane) of Jurkat were subjected to SDS-PAGE and Western blotting using KM1112 (panel A), KM1083 (panel B), and KM1082 (panel C). Arrowhead indicates the position of IP<sub>3</sub>Rs.

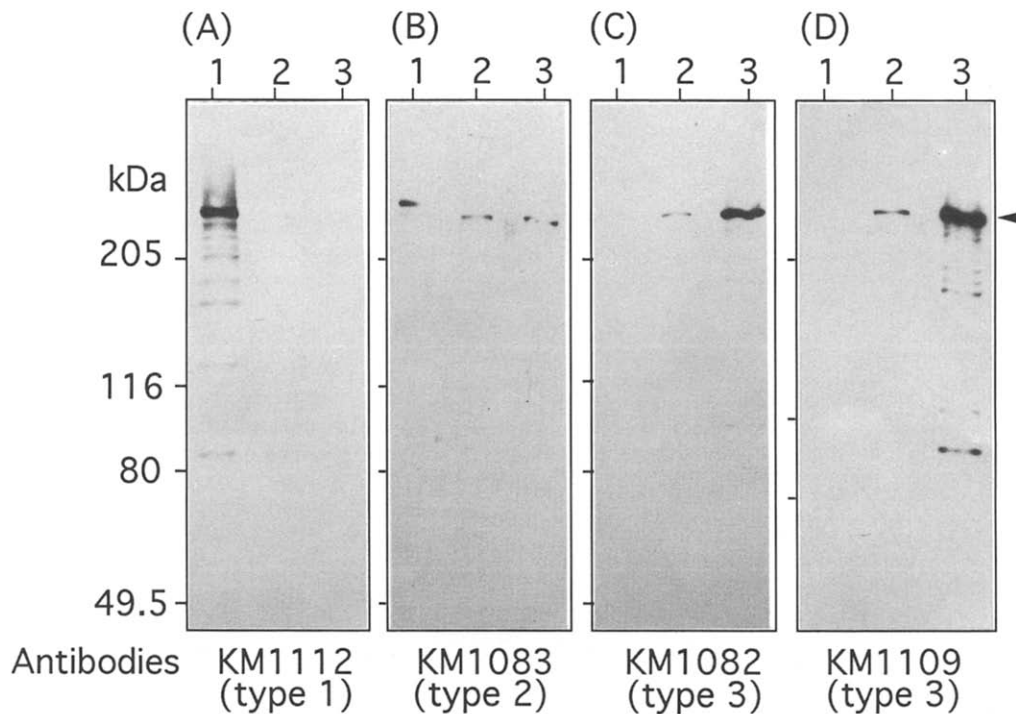


Fig. 3. Western blot analysis showing the specificity of the monoclonal antibodies raised against the peptides corresponding to the IP<sub>3</sub>R COOH-terminal regions and the loop regions between the fifth and the sixth transmembrane regions of IP<sub>3</sub>Rs. Membrane fractions (2 µg protein/lane) of mouse cerebellum (lane 1), COS-7 (lane 2) and COS-7 expressing human IP<sub>3</sub>R type 3 cDNA (lane 3) were subjected to SDS-PAGE and Western blotting using the monoclonal antibodies KM1112 (panel A), KM1083 (panel B), KM1082 (panel C) and KM1109 against peptide-3L for IP<sub>3</sub>R type 3 (panel D). Arrowhead indicates the position of IP<sub>3</sub>Rs.

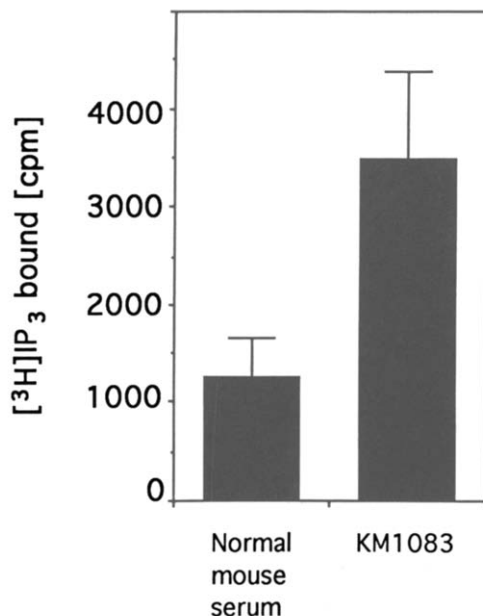


Fig. 4. Specific [<sup>3</sup>H]IP<sub>3</sub> binding of the protein of Jurkat recognized by the monoclonal antibody KM1083 raised against peptide-2C for IP<sub>3</sub>R type 2. Jurkat membranes were solubilized and immunoprecipitated with KM1083. For control, normal mouse serum was used instead of KM1083. Specific [<sup>3</sup>H]IP<sub>3</sub> binding was measured by liquid scintillation counting.

All other monoclonal antibodies established also gave bands of about 250 kDa on Western blot analysis (data not shown).

### 3.2. Expression of IP<sub>3</sub>R subtypes in inflammatory cells

The expression of IP<sub>3</sub>R subtype proteins in macrophages, PMN, mast cells, splenocytes and thymocytes of rats, and eosinophils of guinea pigs was examined by Western blot analysis using the monoclonal antibodies KM1112, KM1083 and KM1082. Those cells were found to express IP<sub>3</sub>R subtypes of about 250 kDa in cell type-specific manner (Fig. 5). Macrophages and PMN predominantly express IP<sub>3</sub>R type 2. Mast cells and eosinophils express IP<sub>3</sub>R type 1 and type 2. IP<sub>3</sub>R type 2 and type 3 are extensively expressed in thymocytes and splenocytes.

### 3.3. Subcellular localization of IP<sub>3</sub>R subtypes in CMK

Our previous Northern blot analysis had shown that CMK cells expressed IP<sub>3</sub>Rs type 1 and type 2 [17]. The same expression profile of IP<sub>3</sub>Rs were obtained by Western blot analysis (data not shown). CMK cells were stained with the monoclonal antibodies KM1112 and KM1083, and observed under fluorescence confocal microscopy (Fig. 6). Under the same conditions of staining and microscopic observation, KM1083 stained rather homogeneously the cytoplasmic space with many strongly stained small domains (Fig. 6B), whilst KM1112 stained intensively a few areas of the cytoplasm (Fig. 6A). Very interestingly, KM1083 stained positively the regions near the plasma membrane in a patchy manner. Even when the signals from the cytoplasm were roughly normalized (Fig. 6, insets), no staining

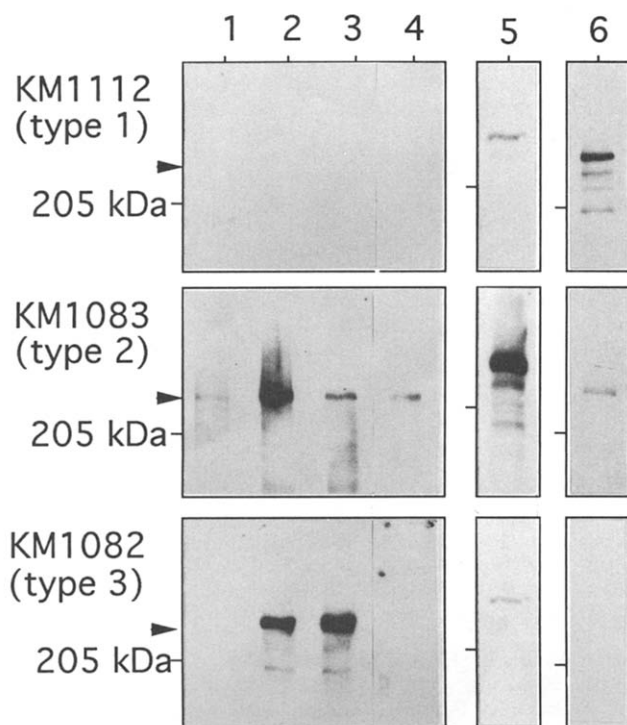


Fig. 5. Western blot analysis of IP<sub>3</sub>R subtypes in inflammatory cells. Macrophages (lane 1), thymocytes (lane 2), splenocytes (lane 3), PMN (lane 4) and mast cells (lane 5) of rat, and eosinophils of guinea pig (lane 6) were solubilized in the sample buffer and subjected to SDS-PAGE and Western blotting (50  $\mu$ g protein/lane). IP<sub>3</sub>R subtypes were detected with the monoclonal antibody KM1112 to IP<sub>3</sub>R type 1 (upper panels), KM1083 to type 2 (middle panels) and KM1082 to type 3 (bottom panels), respectively. Arrowheads indicate the position of IP<sub>3</sub>Rs.

of the regions of plasma membrane was observed for KM1112. These staining patterns were blocked by the immunizing peptide-1C and peptide-2C, respectively (data not shown). The result indicates that the subcellular localization of these two IP<sub>3</sub>R subtypes in CMK is different.

#### 4. Discussion

Monoclonal antibodies are inevitably the tool for studying the structures, functions and tissue/subcellular localization of IP<sub>3</sub>Rs. In practice, using monoclonal antibodies against IP<sub>3</sub>R type 1, tissue and cellular localization, phosphorylation and tetramer formation of the IP<sub>3</sub>R subtype have been elucidated [2,3,18,27]. It was shown by cDNA cloning, Northern blot and in situ hybridization analysis that there are at least three subtypes in the IP<sub>3</sub>R family and that these subtypes are differently expressed in tissues [11–16] and in many specific hematopoietic cells [17].

Using the synthetic peptides corresponding to the COOH-termini and the loops between the fifth and the sixth transmembrane regions of human IP<sub>3</sub>R subtypes [13], we succeeded in establishing the hybridomas producing anti-IP<sub>3</sub>R type 1, 2 and 3 monoclonal antibodies with high specificity. Employing these antibodies, we revealed that IP<sub>3</sub>R type 2 and 3 are expressed in thymocytes. It has been reported that, though specific [<sup>3</sup>H]IP<sub>3</sub> binding activities of thymus and Jurkat plasma membranes are similar, it is quite different from that of cerebellum, and that thymus IP<sub>3</sub>R protein possesses glycans of higher sialic acid content than cerebellum IP<sub>3</sub>R (IP<sub>3</sub>R type 1) [28]. We found that the possible N-glycosylation site of IP<sub>3</sub>R type 3 is different from that of IP<sub>3</sub>R type 1 and 2 with respect to the number of sites and the nearby amino acid sequences [13]. Such differences may lead to different sugar contents among these IP<sub>3</sub>R subtypes. Thus, the previously characterized IP<sub>3</sub>R in thymus might be the IP<sub>3</sub>R type 3 detected in this study. It has been reported that IP<sub>3</sub>R of the T-lymphoma cell line forms complexes with cytoskeletal proteins such as ankyrin well known to bind a number of plasma membrane-associated proteins [29]. Thus, it is very interesting to examine IP<sub>3</sub>R subtypes associated with such cytoskeletal proteins with respect to the regulation of their channel activities in cytoskeleton-dependent manner.

The expression profiles of IP<sub>3</sub>R subtypes protein were quite different among inflammatory cells tested. Interestingly, plural types of IP<sub>3</sub>R were expressed in the same cells simultaneously. These results agree with our previous report analyzing IP<sub>3</sub>R

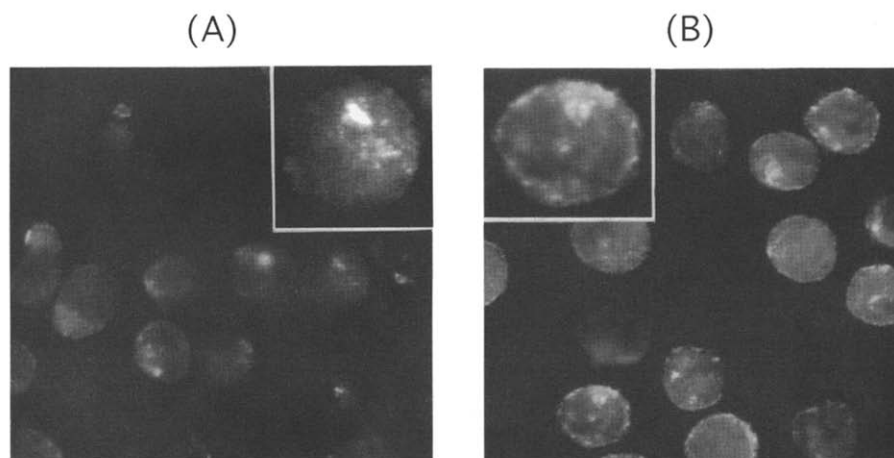


Fig. 6. Immunofluorescence staining of IP<sub>3</sub>R type 1 and 2 in CMK cells. Fixed and permeabilized CMK cells were immunostained with the monoclonal antibody KM1112 to IP<sub>3</sub>R type 1 or KM1083 to IP<sub>3</sub>R type 2. FITC positive cells stained with KM1112 (A) and KM1083 (B) were examined under fluorescence confocal microscopy. Insets show cells stained with KM1112 and KM1083, respectively, with rough normalization of signals from their cytoplasmic regions.

subtype expression by Northern blot analysis [17]. Such cell-type specific expression of IP<sub>3</sub>R subtypes may be tightly related to specific cellular functions such as phagocytosis, secretion and chemotaxis. This finding also raises a question whether the Ca<sup>2+</sup> channels formed by IP<sub>3</sub>R are homomeric or heteromeric tetramers [17]. Since our monoclonal antibodies immunoprecipitate each of the three IP<sub>3</sub>R subtypes, these antibodies will be a useful tool to analyze the subunit composition of the channels.

Immunohistochemical studies using antibodies raised against type 1 protein or unidentified IP<sub>3</sub>R protein from a T cell line have revealed that IP<sub>3</sub>R localized also to plasma membrane [8,9] and perinuclear membrane [30,31]. To date, no data are available for cells expressing plural types of IP<sub>3</sub>R about the subcellular localization of each of these IP<sub>3</sub>R subtypes. Surprisingly, the immunocytochemical examination of CMK cells revealed that the subcellular localization is distinctively regulated for each of IP<sub>3</sub>Rs. Platelets are produced at the surface of megakaryocytes presumably being accompanied with the reconstruction of cytoskeletons. The IP<sub>3</sub>R type 2 located near the plasma membrane may contribute to the process by releasing Ca<sup>2+</sup> at appropriate times during the reconstruction. Immunocytochemical observation by electron microscope using these monoclonal antibodies will give more detailed subcellular localization.

The COOH-terminal regions of each IP<sub>3</sub>R subtype are well conserved among human, mouse and rat [10–15]. This study has shown that our monoclonal antibodies raised against the synthetic peptides corresponding to human IP<sub>3</sub>R subtypes react with the counterparts of rodent IP<sub>3</sub>R subtypes. Thus, our monoclonal antibodies have provided a route for the immunohistochemical and immunocytochemical study of the normal and human disease model-animals established in these species. Such studies are now in progress and will be reported.

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