Inositol 1,4,5-trisphosphate receptor associated with focal contact cytoskeletal proteins

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Abstract The linkage between inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and cytoskeletal proteins is considered to be important in cell function. In the present study, the association of IP_3R subtypes with cytoskeletal proteins was examined using monoclonal antibodies specific to each IP_3R subtype. We found that IP_3R type 2 colocalized with talin, a focal contact cytoskeletal protein. IP_3R type 2 exhibited a patchy distribution in the peripheral cytoplasm differently from type 1 and type 3 IP_3R. Furthermore, IP_3R subtypes co-immunoprecipitated with talin, vinculin and α-actin, but not α-actinin or paxillin.

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Key words: Inositol 1,4,5-trisphosphate receptor; Cytoskeletal protein; Talin; Focal contact; Immunocytochemistry; Smooth muscle cell

1. Introduction

Phosphoinositide turnover has an important role in signal transduction, via the intracellular second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) [1]. IP_3 triggers the release of Ca^{2+} into the cytoplasm by binding to IP_3 receptors (IP_3Rs) on intracellular Ca^{2+} stores, such as the endoplasmic reticulum (ER). There are at least three distinct IP_3R subtypes [2]. It has been reported that IP_3R type 1 immunoreactivity is present in plasma membrane and closely associated with actin filaments in non-neuronal cells [3]. IP_3R type 1 is associated with ankylin, on the cytoplasmic face of the plasma membrane in T-cells [4,5]. The localization of IP_3Rs on or near the plasma membrane and linkage with cytoskeletal proteins suggest that the inositol phospholipid signal transduction pathway may have an important role in transducing cell surface events to changes in cytoplasmic organization. Thus, in the present study, we examined the cellular localization of IP_3R type 1, type 2 and type 3 and the association of these subtypes with cytoskeletal proteins in cultured smooth muscle cells using monoclonal antibodies specific to each IP_3R subtype.

2. Materials and methods

2.1. Reagents

Mouse monoclonal antibodies KM1112, KM1083 and KM1082, which specifically recognize IP_3R type 1, type 2 and type 3, respectively, were used and characterized as previously described [6]. The specificity of these antibodies was verified in our previous reports [6–8]. The monoclonal antibodies to talin, α-actinin, paxillin, vinculin and smooth muscle α-actin were purchased from Sigma (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG1b antibody, TRITC-conjugated horse anti-mouse IgG1b antibody and TRITC-conjugated horse anti-mouse IgG2a antibody were purchased from Southern Biotechnology Associates (Birmingham, AL, USA). FITC-phalloidin was purchased from Molecular Probes (Eugene, OR, USA). ECL system was from Amersham (Buckinghamshire, UK). Analytical grade reagents were from Sigma, Wako (Tokyo, Japan), Peptide Institute (Osaka, Japan), Calbiochem (San Diego, CA, USA) or Schleicher & Schuell (Dassel, Germany).

2.2. Immunocytochemistry

Rat smooth muscle cells are cultured in M199 medium supplemented with 10% (v/v) fetal bovine serum in humidified 5% CO_2-95% air atmosphere at 37°C as described previously [9]. Passage between three and five cells cultured on glass slides were fixed with 2% paraformaldehyde in 10 mM phosphate-buffered saline (PBS; pH 7.4) for 5 min at room temperature (between 20°C and 25°C). After permeabilizing cells with 0.1% Triton X-100 in PBS for 5 min, and incubation with 2% normal horse serum in PBS for 30 min at room temperature, cells were then incubated in a humidified chamber for 16 h at 4°C with KM1112, KM1083 or KM1082 diluted to 1 μg/ml, or anti-talin antibody diluted to 1:100, in PBS containing 2% normal horse serum. As controls, KM1112, KM1083 and KM1082 were preabsorbed with a 10-fold excess of each respective antigenic peptide and no specific immunoreactivity was observed for any of the antibodies (data not shown). Since antibodies used in the immunocytochemistry are all mouse monoclonal antibodies, we used isotype specific secondary antibodies for double staining. KM1083 is mouse IgG2a isotype. KM1112, KM1082 and anti-talin antibody are mouse IgG1b isotype. Cells were washed three times with PBS and incubated with a mixture of FITC-conjugated anti-mouse IgG1b antibody, for IP_3R type 1, type 3 and talin, and TRITC-conjugated anti-mouse IgG2a antibody, for IP_3R type 2, to examine double labeling of IP_3R type 2 and IP_3R type 1, type 3 or talin. As controls, KM1083 was incubated with anti-mouse IgG1b antibody. KM1112, KM1082 and anti-talin antibody were incubated with anti-mouse IgG2a antibody. No specific signal was observed in these preparations (data not shown). For double labeling of F-actin and IP_3R type 2 or type 3, FITC-phalloidin was mixed with TRITC-conjugated anti-mouse IgG1b and IgG2a antibodies. The specimens were examined using a Zeiss Axiovert microscope equipped with an epi-fluorescence apparatus and a Melidian confocal laser microscope Insight-TR (Okemos, MI, USA).

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Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; ER, endoplasmic reticulum; PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate; FITC, fluorescein isothiocyanate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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2.3. Immunochemical analysis

Immunoprecipitation was performed as described previously [6]. Briefly, cells 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 μM pepstatin A, 10 μM leupeptin and 1 mM 2-mercaptoethanol) for 30 min at 4°C. The cell lysate was centrifuged at 20 000×*g* for 30 min at 2°C. Concentration of the supernatant was 0.7 mg/ml. The supernatant was incubated with either KM1112, KM1083, KM1082 or the monoclonal antibody to talin (each at 6 μg/ml) for 1 h at 4°C, respectively, followed by the addition of anti-mouse IgG. The immune complexes were collected with pansorbin and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto nitrocellulose sheets. As a control, immunoprecipitation was also performed with denatured smooth muscle cell lysate. The sheets were blocked with skim milk and incubated with monoclonal antibodies to IP_3R type 1, type 2, type 3, talin, α-actinin, paxillin, vinculin or smooth muscle α-actin. The bound monoclonal antibodies were detected using the ECL system.

3. Results

3.1. Diffuse and patchy subcellular localization of IP_3R type 1, type 2 and type 3

Monoclonal antibodies KM1112, KM1083 and KM1082 specifically recognize each IP_3R subtype by immunoblotting (data not shown). IP_3R type 1 was distributed diffusely as a meshwork predominantly in the cytoplasm surrounding the nuclei, and to a lesser degree in the peripheral regions of the cell. A few patches of immunostaining, however, were observed near the plasma membrane (Fig. 1A). IP_3R type 2 was distributed diffusely in the cytoplasm around the nuclei and in dense patches in the peripheral regions of the cell (Fig. 1B). In at least one instance, IP_3R type 1 and type 2 colocalized in the peripheral region (Fig. 1A,B), however, IP_3R type 2 immunoreactivity near the plasma membrane was both more dense and more frequently observed than IP_3R type 1 immunoreactivity (Fig. 1B). IP_3R type 3 was diffusely distributed in the cytoplasm around the nuclei and in the peripheral regions (Fig. 1C). In some areas, IP_3R type 3 colocalized with IP_3R type 2 (Fig. 1C,D), however, IP_3R type 3 immunoreactivity was not dense and patchy like that of IP_3R type 2. There was no specific staining using non-immune antibodies (data not shown).

3.2. Colocalization of IP_3R subtypes and F-actin or talin

We investigated whether IP_3R type 2 or type 3 was associated with actin filaments using double immunofluorescence. In a few instances, colocalization was observed (Fig. 2). Interestingly, we found intense labeling of IP_3R type 2 localized to the end of the actin filaments (Fig. 2, arrow). This pattern

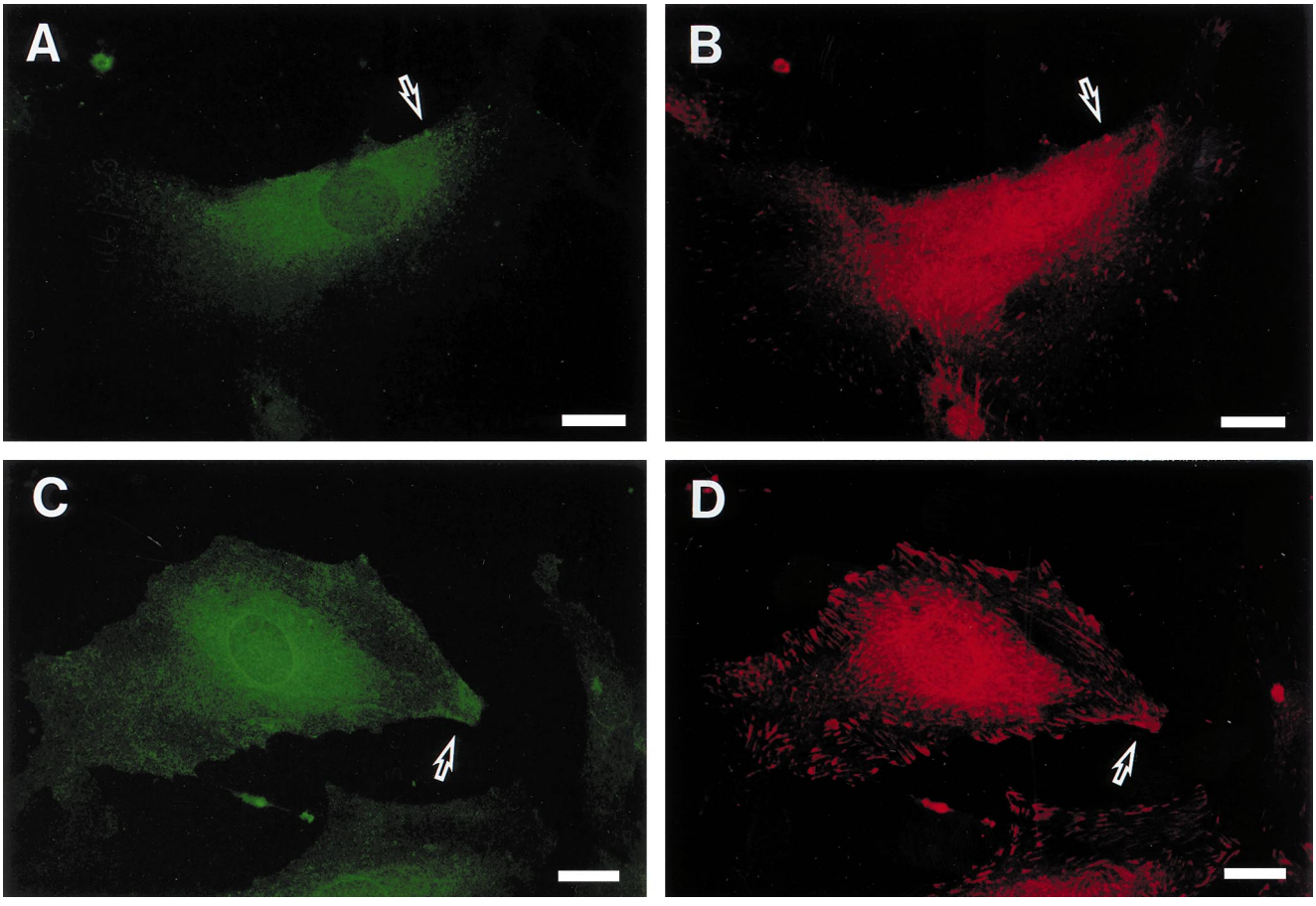

Fig. 1. Double label immunofluorescence of IP_3R subtypes in smooth muscle cells. Cultured smooth muscle cells were fixed and lysed with Triton X-100. The cells were incubated with both (A) KM1112 (anti-IP_3R type 1) and (B) KM1083 (anti-IP_3R type 2), or both (C) KM1082 (anti-IP_3R type 3) and (D) KM1083. KM1083 was visualized using TRITC-conjugated anti-IgG2a antibody (red). KM1112 and KM1082 were visualized using FITC-conjugated anti-IgG1b antibody (green). Although colocalization of IP_3R type 2 was observed with both IP_3R type 1 (A,B; arrows) and type 3 (C,D; arrows), IP_3R type 2 had a patchy immunoreactive pattern that was not observed for either IP_3R type 1 or type 3. Bar, 10 μm.

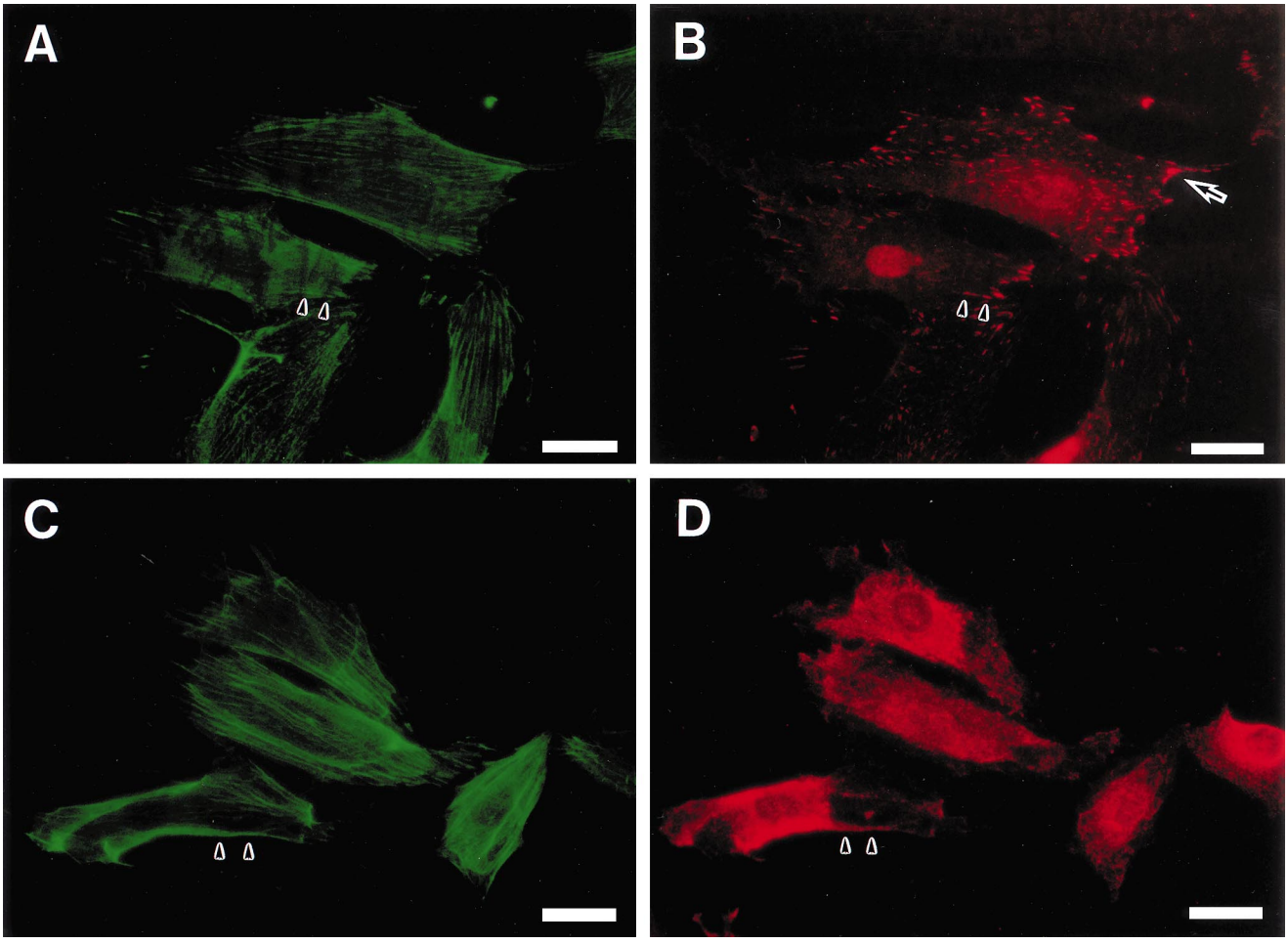

Fig. 2. Double label immunofluorescence of actin filaments and IP_3R type 2 or type 3. Cultured smooth muscle cells were fixed and incubated with FITC-conjugated phalloidin (A,C), then incubated with KM1083 (anti-IP_3R type 2) or KM1082 (anti-IP_3R type 3). KM1083 (B) and KM1082 (D) were visualized with TRITC-conjugated anti-IgG2a antibodies and anti-IgG1b antibodies, respectively. (A,B) In a few instances, colocalization of IP_3R type 2 and F-actin was observed (arrowheads). Note the dense IP_3R type 2 signal present at the end of F-actin (B, arrow). (C,D) In a few instances, colocalization of IP_3R type 3 and F-actin was also observed (arrowheads). Bar, 20 μm.

was unique to IP_3R type 2 and was not observed with IP_3R type 3.

Then we examined the double staining of IP_3R type 2 talin to examine the localization of IP_3R type 2 at focal contact. Before analyzing by confocal laser microscopy, we checked the staining pattern of the anti-talin monoclonal antibody in the cells using conventional epi-fluorescence microscopy. Secondary antibody only or normal mouse serum followed with secondary antibody did not show any staining (data not shown). The significant patchy dense signals ranging from the middle to the peripheral of cell were observed (data not shown). These staining patterns were almost identical to the previously described by Otey, C. [26], suggesting the antibody specifically stained talin. Confocal fluorescence imaging showed that the dense patches of IP_3R type 2 immunoreactivity observed at the peripheral regions of the cell colocalized with talin (Fig. 3). In these regions, fluctuation of the fluorescence across the bar was similar for both IP_3R type 2 and talin, suggesting the localization of IP_3R type 2 at focal contact.

3.3. Association of IP_3R subtypes with cytoskeletal proteins

The interaction of IP_3R subtypes with talin was examined by immunoprecipitation with monoclonal antibodies to each IP_3R subtype and subsequent immunoblotting with antibodies to each IP_3R subtype and talin. No band was detected in the immunoprecipitate using non-immune antibodies (data not shown). Talin co-immunoprecipitated with KM1112, KM1083 and KM1082, indicating an interaction between IP_3R subtypes and talin (Fig. 4A). There was no cross-reactivity between the anti-talin monoclonal antibody and any of the IP_3R subtype antibodies, indicating that the anti-talin antibody was specific to talin (Fig. 4B). When talin was immunoprecipitated, each of the IP_3Rs (type 1, 2 and 3) co-immunoprecipitated with talin, suggesting that talin may be associated with each of the subtypes (Fig. 4C). We further investigated whether the IP_3R subtypes interacted with other cytoskeletal proteins involved in the formation of focal contacts. The relative amounts of the immunoprecipitated IP_3R type 1, type 2 and type 3 were 62%, 98% and 29%, respectively. Paxillin and α-actinin did not appear to co-immunoprecipitate with any of the IP_3R subtypes (Fig. 5), while α-actin co-immunoprecipitated with IP_3R subtypes. Vinculin clearly co-immunoprecipitated with IP_3R type 3 compared with type 1 and type 2.

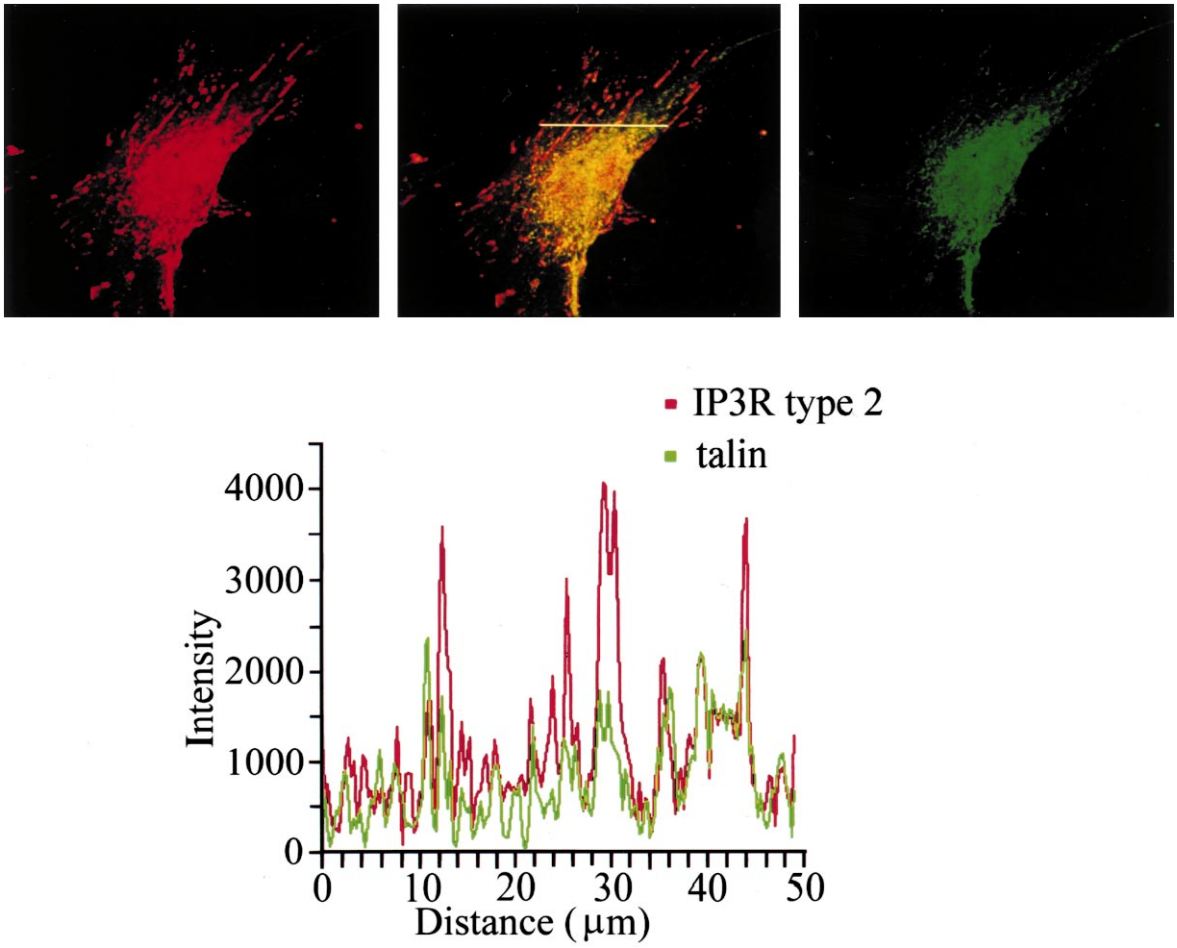

Fig. 3. Colocalization of IP_3R type 2 and talin using confocal laser microscopy. Smooth muscle cells were fixed and labeled with KM1083 (anti-IP_3R type 2) and anti-talin monoclonal antibodies. KM1083 (left; red), anti-talin (right; green), and superimposed KM1083 and anti-talin images (middle). Note, colocalized regions are yellow. The intensity of the fluorescence was measured across the bar in the middle panel. Fluctuations in the fluorescence intensity across the bar were similar for both IP_3R type 2 and talin.

4. Discussion

Smooth muscle cells express three IP_3R subtypes, and one of the subtypes, type 1, has been reported previously to be localized in the central and peripheral sarcoplasmic reticulum, as visualized using electron microscopy [10–12]. A small portion of IP_3R type 1-like protein linked to actin filaments and was localized to caveolae and the sarcoplasmic reticulum close to the plasma membrane [3,10]. In the present study, we found that IP_3R type 2 had a diffuse cytoplasmic distribution, similar to IP_3R type 1 and type 3, however, it was also present in dense patches in the peripheral cytoplasm, a pattern that IP_3R type 1 did not exhibit. IP_3R type 2 and type 3 also colocalized with actin filaments. Based on the colocalization patterns, actin filaments that linked to IP_3R type 2 were thought to be different from those linked to IP_3R type 1. Only IP_3R type 2 accumulated to the end of actin filaments and colocalized with the focal contact protein talin at focal contact. It was reported that presenilin which localized to ER was interacted with focal contact protein filamin [27,28]. IP_3R might be present on such intracellular compartment at focal contact. We also showed the first evidence that showed association of IP_3R subtypes with focal contact cytoskeletal proteins. Talin was co-immunoprecipitated with three types of IP_3R, suggesting IP_3R type 1 and type 3 were also present at focal contact. However, the amount of IP_3R type 1 and type 3 at focal contact would be small because of the relatively weak IP_3R signals at peripheral region of the cells in immunofluorescence studies. Since IP_3R channel forms heterotetramer, we could not rule out whether talin binds to each of IP_3R subtypes in the present immunoprecipitation. To address the issue, further studies are needed.

The localization raises the possibility that type 2 is involved in Ca^{2+} signaling at focal contact. Focal contacts are specialized sites consisting of assemblies of cytoskeletal proteins that have an important role in stabilizing cell adhesion, structure and mobility [14,15]. It has been reported that localized increases in intracellular [Ca^{2+}] have specific effects in smooth muscle cells [13]. An association between IP_3Rs and cytoskeletal proteins that are commonly present at focal contacts is consistent with the possibility that IP_3R may regulate proteins at focal contacts. There are many reports that inositol phospholipid [16–18] and an enzyme in the inositol signaling pathway, phosphatidylinositol kinase, phosphatidylinositol 4-phosphate kinase and phospholipase C (PLC), [19–23] have important roles in the formation of actin stress fibers. In several cases, cell adhesion and migration were regulated by an increase in intracellular [Ca^{2+}] [16,24,25]. The hydro-

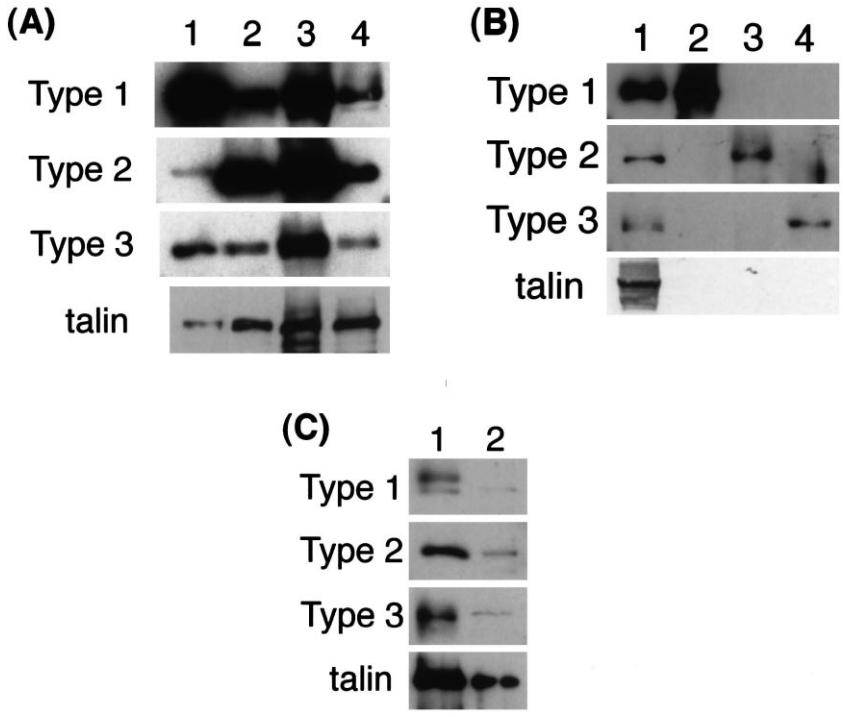

Fig. 4. Co-immunoprecipitation of IP_3Rs with talin identified using immunoblotting. Smooth muscle cell lysate (A) and denatured cell lysate (B) were immunoprecipitated with KM1112 (anti-IP_3R type 1), KM1083 (anti-IP_3R type 2) or KM1082 (anti-IP_3R type 3), and the precipitates were subjected to SDS-PAGE and blotted onto nylon membranes. The membrane was cut into strips, and each was incubated with the appropriate antibody, KM1112, KM1083, KM1082 or anti-talin. Cell lysate was used as positive control of immunoblotting. The bands indicated as type 1, 2, 3 and talin are the bands detected by the anti-IP_3R and talin antibodies, respectively. Co-immunoprecipitation of IP_3R subtypes was observed in a soluble condition in (A) but not in (B), suggesting the prevention of any protein-protein interaction in the denatured condition. (A) Columns 1, 2 and 3 correspond to the soluble cell lysate immunoprecipitated with KM1112, KM1083 and KM1082, respectively. Column 4 shows the soluble cell lysate without immunoprecipitation. (B) Column 1 shows the whole denatured protein without immunoprecipitation. Columns 2, 3 and 4 show the whole denatured protein immunoprecipitated with KM1112, KM1083 and KM1082, respectively. (C) Smooth muscle cell lysate was immunoprecipitated with anti-talin antibody and the pellet was subjected to SDS-PAGE, blotted onto nylon membranes and immunoblotted with KM1112, KM1083, KM1082 or anti-talin monoclonal antibodies. Column 1 shows immunoreactivity with KM1112, KM1083, KM1082 or anti-talin antibodies without immunoprecipitation. Column 2 shows the soluble cell lysate immunoprecipitated with talin antibody.

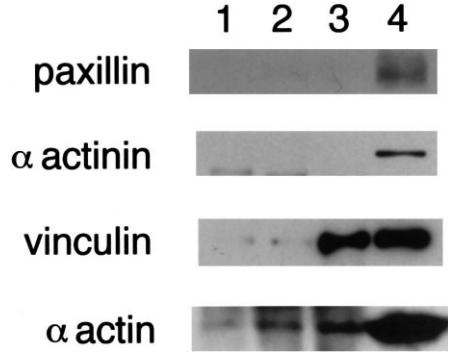

Fig. 5. Co-immunoprecipitation of cytoskeletal proteins with IP_3R. Smooth muscle cell lysate was immunoprecipitated with KM1112 (anti-IP_3R type 1), KM1083 (anti-IP_3R type 2) or KM1082 (anti-IP_3R type 3), and subjected to SDS-PAGE and blotted onto nylon membranes. The membrane was cut into strips, and each was incubated with the appropriate antibody: anti-paxillin, anti-α-actinin, anti-vinculin or anti-α-actin. As a control, cell lysate without immunoprecipitation was immunoblotted with each antibody (column 4). Note that IP_3R type 1 (column 1), type 2 (column 2) and type 3 (column 3) all bind to vinculin and α-actin, but do not bind to paxillin and α-actinin.

lysis of phosphatidylinositol bisphosphate (PIP_2) is involved in Ca^{2+} mobilization and the depolymerization of actin filaments in smooth muscle cells [16]. The association of PIP_2 with vinculin dissociates the head-tail interaction, thus exposing binding sites for talin and actin, and thereby promoting the assembly of focal contacts [17]. The hydrolysis of PIP_2 may promote the disassembly of these cytoskeletal networks via the proteolysis of talin by the Ca^{2+}-activated protease calpain II [18]. Thus, although further study is needed to elucidate the role of IP_3R, PIP_2-PLC-IP_3-IP_3R type 2 Ca^{2+} signaling may have a role in cell adhesion by regulating cytoskeletal proteins at focal contacts.

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