Inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae is linked to actin filaments

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SUMMARY

We reported that a plasmalemmal inositol 1,4,5-trisphosphate receptor-like protein (PM InsP$_3$R-L) is localized in caveolae of various non-neuronal cells in vivo (Fujimoto et al. (1992) J. Cell Biol. 119, 1507-1513). In the present study, we investigated the distribution of PM InsP$_3$R-L in cultured cells. In mouse epidermal keratinocytes (Pam 212) cultured in standard Ca$^{2+}$ (1.8 mM), PM InsP$_3$R-L was distributed densely in the vicinity of cell-to-cell contacts. In contrast, when Pam cells were cultured in low Ca$^{2+}$ (0.06 mM) without making cell-to-cell contacts, PM InsP$_3$R-L was observed randomly; by restoring the Ca$^{2+}$ concentration, the circumferential actin filaments became obvious and the density of PM InsP$_3$R-L increased in the contact region. Treatment of Pam cells with cytochalasin D caused aggregation of caveolae where PM InsP$_3$R-L as well as F-actin and fodrin were localized. In bovine aortic endothelial cells, PM InsP$_3$R-L was aligned along actin filaments crossing the cytoplasm in various directions. PM InsP$_3$R-L of Pam cells was hardly extracted by treatment with 0.5% Triton X-100 or 60 mM octyl-glucoside in a cytoskeleton-stabilizing buffer for 15 minutes at 4°C.

The results show that the distribution of caveolae bearing PM InsP$_3$R-L changes when the actin cytoskeleton is modified. They also indicate that the association of PM InsP$_3$R-L with actin filaments may mediate the redistribution of caveolae. Since caveolae are thought to be related to signal transduction, their location defined by the actin cytoskeleton may affect the site where cellular reaction is to occur in response to various stimuli.

Key words: plasma membrane, caveola, inositol 1,4,5-trisphosphate receptor, actin, calcium, signal transduction

INTRODUCTION

Caveolae are small plasmalemmal invaginations, which are thought to exist in most cell types. On the basis of morphological observations, various functions had been ascribed to caveolae (Severs, 1988), but the discovery in recent years of several proteins that exist in caveolae (Anderson, 1993). One of the caveola-specific proteins is plasmalemmal inositol 1,4,5-trisphosphate receptor-like protein (PM InsP$_3$R-L), which we found to be localized in caveolae of vascular endothelial cells, smooth muscle cells and epidermal keratinocytes (Fujimoto et al., 1992). The protein was specifically recognized by monoclonal antibodies raised to the type I InsP$_3$R of cerebellar Purkinje cells (Furuichi et al., 1989) and showed a similar mobility to that of the latter in SDS-PAGE. Because of the probable structural similarity to the type I InsP$_3$R, we postulated that PM InsP$_3$R-L may be involved in Ca$^{2+}$ influx through the plasma membrane. In fact, several physiological studies indicated the presence of an InsP$_3$-gated Ca$^{2+}$ channel in the plasma membrane (Kuno and Gardner, 1987; Restrepo et al., 1990, Fadool and Ache, 1992). Furthermore, in T lymphocytes, a plasmalemmal protein that is reactive with a polyclonal antibody to the type I InsP$_3$R, co-caps with the T cell receptor-CD3 complex and an increase of [Ca$^{2+}$], begins immediately beneath the cap (Khan et al., 1992).

To decipher the physiological functions of PM InsP$_3$R-L, it is important to know the location of the caveolae bearing the protein within the cell and how the distribution is regulated. If PM InsP$_3$R-L mediates the Ca$^{2+}$ influx, the presence of caveolae in some portions of a cell and absence in the others should determine where the ensuing cellular responses will occur. Thus, in the present study, we used cultured cells to examine the distribution of PM InsP$_3$R-L by immunocytochemical methods and found a correlation with actin filaments. Implications of the result are discussed in terms of the physiological significance of PM InsP$_3$R-L and caveolae.

MATERIALS AND METHODS

Antibodies

Rat mAb 4C11, which recognizes the type I InsP$_3$R was obtained and
characterized as described (Maeda et al., 1988; Furushichi et al., 1989). Rabbit polyclonal antibody to rat brain fodrin was raised and affinity-purified as reported previously (Fujimoto and Ogawa, 1989). Mouse monoclonal anti-chicken caveolin (clone Z034) was purchased from Zymed Lab. Inc. (South San Francisco, CA).

Cells
Pam 212 cells, a transformed mouse keratinocyte cell line (Yuspa et al., 1980) (a gift from Dr Koji Hashimoto, Department of Dermatology, Osaka University School of Medicine, Osaka, Japan), and human fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). For bovine aortic endothelium, DMEM plus 15% FCS was used. In an experiment, Pam 212 cell was cultured in low Ca²⁺ (0.06 mM) medium plus 10% FCS deprived of Ca²⁺ by treatment with Chelex-100 resin (Bio-Rad Lab., Richmond, CA). In all the other experiments, the cells were cultured in normal DMEM at the standard Ca²⁺ concentration (1.8 mM). To examine the effect of actin disorganization, cells were treated with 1 µM cytochalasin D for 2 hours at 37°C.

Immunofluorescence microscopy
Cells cultured on glass coverslips were fixed with 3% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 5 minutes at room temperature (r.t.), permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, and treated with 2% gelatin for 10 minutes. Some cells were permeabilized with 0.5% Triton X-100 in a cytoskeleton-stabilizing buffer (70 mM KCl, 3 mM MgCl₂, 5 mM EGTA, 25 mM HEPES, pH 6.9) for 5 minutes on ice before fixation.

For single labeling of PM Ins³P/R-L, cells were incubated with either rat mAb 4C11 or non-immune rat IgG for 30 minutes, then with FITC-conjugated goat anti-rat IgG antibody (Kirkgaard & Perry Lab. Inc., Gaithersburg, MD) for 30 minutes. For double labeling of PM Ins³P/R-L and PM F-actin, rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) was mixed with the secondary antibody solution. Fodrin and PM Ins³P/R-L were doubly labeled with a mixture of rabbit anti-fodrin antibody and mAb 4C11, then with a mixture of FITC-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch Lab., Inc., West Grove, PA) and rhodamine-conjugated goat anti-rat IgG antibody (Chemicon International, Inc., Temecula, CA). The specimens were observed and photographed using a Zeiss Axioptihot fluorescence microscope.

Electron microscopy
For morphological observation, Pam 212 cells cultured on coverslips, with or without cytochalasin D treatment, were fixed with a mixture of 2% glutaraldehyde and 0.2% tannic acid in 0.1 M sodium phosphate buffer, pH 7.4, for 60 minutes at r.t., postfixed with 1% osmium tetroxide in the same buffer for 60 minutes at 4°C, and ultrathin frozen sections were prepared. Thawed sections were immunolabeled in the same manner as the permeabilized cell samples and embedded in polyvinyl alcohol (Tokuyasu, 1989).

Detergent extraction and western blotting
Cells were rinsed twice with ice-cold PBS and extracted for 15 minutes on ice with 0.5% Triton X-100 or 60 mM octyl-glucoside in cytoskeletal buffer. Solubilized proteins were centrifuged for 10 minutes at 15,000 g and the supernatant was precipitated with 5 volumes of acetone at −20°C. The precipitated proteins and the non-extracted cell residues solubilized with an equal volume of sample buffer were separated by SDS-PAGE, electrotransferred onto nitrocellulose paper and probed with mAb 4C11 and mouse anti-caveolin antibody. After the incubation with goat antibodies conjugated with horseradish peroxidase, the reaction was visualized with the ECL detection system (Amershams) as described by the manufacturer.

RESULTS

Immunofluorescence microscopy
Pam 212 cells are derived from mouse epidermal keratinocytes and retain the ability to differentiate in vitro (Yuspa et al., 1980). In DMEM of standard Ca²⁺ concentration, they make cell-to-cell contacts and show a cobblestone-like appearance. When Pam cells were fixed and labeled with mAb 4C11, immunofluorescence appeared mostly as small dots; it was most intense along the intercellular boundary (Fig. 1A). Double labeling with rhodamine-phalloidin revealed that actin filaments were concentrated at the same location (Fig. 1B).

When Pam cells were cultured in low Ca²⁺ medium, they did not make intercellular junctions, and actin filaments were not confined to the cell periphery (photograph not shown). Under this condition, the immunolabeling by mAb 4C11 still appeared in dots but they were distributed randomly (Fig. 1C). An increase in the Ca²⁺ concentration from low to standard caused a rapid distributional change in the labeling. Ten minutes after the medium change, the mAb 4C11 labeling began to accumulate in the peripheral rim of the cells (Fig. 1D) and at 2 hours the transition was complete and the labeling was seen densely near cell-to-cell contacts (photograph not shown).

The above observation showed that the distribution of PM Ins³P/R-L can change in a dynamic manner and is likely to be related to that of actin filaments. This assumption was supported by the following results. First, in Pam cells extracted with 0.5% Triton X-100 for 5 minutes before fixation with paraformaldehyde, PM Ins³P/R-L persisted and was localized along the peripheral actin filaments (Fig. 1E, F). Second, in cells treated with cytochalasin D for 2 hours, most actin filaments were disintegrated and the labeling with rhodamine-phalloidin appeared as irregularly shaped spots, many of which occurred at the cell periphery. In this specimen, double labeling showed that PM Ins³P/R-L (Fig. 2A) was codistributed in the F-actin-positive spots (Fig. 2B).

In the cytochalasin-D-treated cells, an actin-binding membrane skeletal protein, fodrin, was also colocalized with PM Ins³P/R-L in the coarse spots (Fig. 2C, D). Apparently, as actin filaments were disorganized and aggregated, both PM Ins³P/R-L and fodrin became concentrated in the same region.

The distribution of PM Ins³P/R-L and actin filaments was also correlated in the aortic endothelium (Fig. 3). It was observed most conspicuously in relatively large cells, which were occasionally seen among cultured endothelial cells. In
those cells, among numerous actin filaments criss-crossing the cytoplasm, only some showed coincident labeling of PM \( \text{InsP}_3 \text{R-L} \). On the contrary, only a fraction of mAb 4C11-positive dots were localized along actin filaments, while many others seemed to be distributed randomly and not associated with the filaments.

**Electron microscopy**

In the previous paper, we showed that PM \( \text{InsP}_3 \text{R-L} \) is localized in caveolae of various cells in vivo (Fujimoto et al., 1992). To examine whether this is also the case in Pam 212 cells, the distribution of PM \( \text{InsP}_3 \text{R-L} \) was examined by two immunoelectron microscopic methods. For pre-embedding...
immunolabeling, cells permeabilized with saponin were used. The cytoplasmic matrix was largely extracted but the membrane structure was retained fairly well. In this preparation, the labeling by mAb 4C11 was restricted to the caveolar portion of the plasma membrane (Fig. 4A-C) and uncoated vesicles in the cortical cytoplasm (Fig. 4D-F). The latter vesicles were always localized immediately beneath the plasma membrane and thus were probably caveolae that had

![Fig. 2. Pam cells treated with cytochalasin D for 2 hours. (A, B) Double labeling for PM InsP₃R-L (A) and F-actin (B). Both types of labeling accumulated as coarse spots at the cell periphery (arrows). They apparently overlap for the most part. (C, D) PM InsP₃R-L (C) and fodrin (D) were also observed to be localized in the same spots (arrows). Bar, 10 μm.](image)

![Fig. 3. Double immunolabeling of PM InsP₃R-L (A) and F-actin (B) in bovine aortic endothelial cells. Some of the PM InsP₃R-L labeling is aligned along actin filaments (arrows), but notably there are many other sites of labeling that appear to be unrelated to the filaments. Bar, 10 μm.](image)
been sectioned transversely. Undifferentiated portions of the plasma membrane and coated vesicles (arrowheads in Fig. 4D, E) were not labeled. The localization of PM InsP$_3$R-L to caveolae was confirmed by the immunolabeling of thawed frozen sections (Fig. 4G); for the preparation, cells were fixed in the PLP fixative for 60 minutes at r.t. This result denied the possibility that the localization of PM InsP$_3$R-L to caveolae was caused by cross-linking by antibodies, as observed for GPI-anchored proteins (Mayor et al., 1994).

The morphology of Pam cells incubated with cytochalasin D for 2 hours was observed by electron microscopy. In comparison with control cells, caveolae of the cytochalasin-treated cells
tended to form clusters, which exist in indentations of the cell surface like ‘bunches of grapes’ (Fig. 5A-C). Tannic acid in the primary fixative made the outer leaflet of the caveolar membrane appear denser, which indicates their continuity with the plasma membrane. Bar, 100 nm. (D) As shown by pre-embedding immunoelectron microscopy, as in Fig. 4, clustered caveolae are labeled intensely for PM InsP$_3$R-L. Bar, 100 nm.

Quantification revealed that the clustering of caveolae was significantly increased by cytochalasin D treatment (Table 1). By immunoelectron microscopy, the clusters of caveolae were shown to be heavily labeled for PM InsP$_3$R-L (Fig. 5D). Judging from the density of the labeling, the clusters are thought to correspond to coarse spots observed by immunofluorescence microscopy, which were labeled intensely by mAb 4C11 and positive for both F-actin and fodrin (Fig. 2).
Among the caveola-specific proteins, caveolin is resistant to extraction by detergents. When Pam 212 cells were treated with either 0.5% Triton X-100 or 60 mM octyl-glucoside in cytoskeleton-stabilizing buffer and examined by western blotting, the reaction with PM Ins$_3$R-L of Pam 212 cells is stronger with the residual than with the solubilized proteins for both detergents. (A) Caveolin of human fibroblasts was resistant to solubilization by Triton X-100, but was extracted effectively by octyl-glucoside seen in the present study is consistent with the assumption that this protein is responsible for the cytoskeletal association of caveolae.

The result obtained with bovine aortic endothelial cells is notable because it suggests that the association of PM Ins$_3$R-L and actin is not consistent. Some of the PM Ins$_3$R-L was aligned along actin filaments, whereas some was apparently not associated to actin; conversely, actin filaments associated with PM Ins$_3$R-L constituted only a portion of the whole population, and most were devoid of PM Ins$_3$R-L labeling. This observation suggests that PM Ins$_3$R-L and, accordingly, caveolae carrying the protein are not always anchored to actin filaments. In fact, in smooth muscle cells, although caveolae contain PM Ins$_3$R-L (Fujimoto et al., 1992), caveola-rich domains are distinct from actin-rich dense plaques. It may be reasonable to assume that the association of PM Ins$_3$R-L and actin filaments is under some regulation, but details of the molecular mechanism remain to be clarified through purification of PM Ins$_3$R-L.

It was reported that Ins$_3$R-P in the adrenal cortex and the cerebellum is only slightly dissolved by Triton X-100, suggesting an association with the cytoskeleton (Guillemente et al., 1990). Actin was recognized as a contaminant of an Ins$_3$R-P in isolating the latter from smooth muscle (Chadwick et al., 1990). Furthermore, in rat liver homogenate, Ins$_3$P-binding activity was copurified with plasma membrane markers, but when the homogenate was treated with cytochalasin B or by freeze-thawing to depolymerize actin, the activity was recovered in a separate fraction (Rossier et al., 1991). These examples suggest that an association with actin is a common feature of some Ins$_3$P-Rs. However, in the cerebellar Purkinje cell, the type I Ins$_3$P-R is mostly localized in the endoplasmic reticulum (Mignery et al., 1989; Otsu et al., 1990; Satoh et al., 1990), which does not imply a relationship with the actin cytoskeleton. One possible interpretation of the above discrepancy is that PM Ins$_3$P-Rs-Ls, but not Ins$_3$P-Rs in the intracellular organelles, associate with actin. For example, it was speculated that Ins$_3$P$_3$-binding organelles in the liver homogenate are anchored to the plasma membrane by actin filaments and detached by depolymerization of the latter (Rossier et al., 1991). At present, we do not know which protein is responsible for the Ins$_3$P-binding activity in the homogenate, but assuming that the protein is PM Ins$_3$P-R-L, the phenomenon could be ascribed to its concentration in caveolae. That is, caveolae are likely to act in combination with the plasma membrane during ordinary subcellular fractionation.

**DISCUSSION**

PM Ins$_3$P$_3$-R-L is localized in caveolae of the plasma membrane. Morphological observation has shown that caveolae tend to exist in parallel with actin filaments (Rohlich and Allison, 1976). Furthermore, myosin subfragment I was revealed to bind to caveolae, which indicates that actin is closely associated with the caveolar membrane (Izumi et al., 1988). These results suggest that the caveolar membrane contains a molecule(s) that mediates the association with actin filaments. Among the caveola-specific proteins, caveolin is resistant to Triton X-100 extraction, and was earlier thought to be anchored to the cytoskeleton (Glenney and Zokas, 1989), but a recent study showed that its insolubility is caused by association with the sphingolipid microdomain rather than by linkage to the cytoskeleton (Lisanti et al., 1993). On the other hand, the insolubility of PM Ins$_3$P$_3$R-L in both Triton X-100 and octyl-glucoside seen in the present study is consistent with the assumption that this protein is responsible for the cytoskeletal association of caveolae.

Fig. 6. Pam 212 cells and human fibroblasts were treated with either 0.5% Triton X-100 (TX) or 60 mM octyl-glucoside (OG) in cytoskeletal buffer for 15 minutes on ice. Residual (R) and solubilized (S) proteins were analyzed by western blotting. (A) The reaction with PM Ins$_3$P$_3$R-L of Pam 212 cells is stronger with the residual than with the solubilized proteins for both detergents. (B) Caveolin of human fibroblasts is resistant to extraction by Triton X-100 but is mostly solubilized by octyl-glucoside.
tion, but by disrupting the actin filaments they may be separated from the plasma membrane markers and isolated in a different fraction.

We previously found that fodrin shows a distributional change in Pam 212 cells depending on the Ca\(^{2+}\) concentration of the culture medium (Yoneda et al., 1990a,b). The dual localization of fodrin was similar to that of PM Ins\(^{3}\) P-L described here. Moreover, fodrin and PM Ins\(^{3}\) P-L were localized in the same spots in cytochalasin-D-treated cells. These results suggest that fodrin may mediate the linkage between PM Ins\(^{3}\) P-L and actin. Interestingly, ankyrin, another membrane skeletal protein that binds to fodrin in various cells, has an affinity for Ins\(^{3}\) P-Rs of the cerebellum and T lymphoma cells (Davis and Bennett, 1991; Bourguignon et al., 1993; Joseph and Samanta, 1993). On the basis of this property, it has been speculated that the Ins\(^{3}\) P-Rs are present in cytoplasmic vesicles linked to the plasma membrane by ankyrin. However, as discussed in the preceding paragraph, the Ins\(^{3}\) P-Rs recognized in the above studies may contain a PM Ins\(^{3}\) P-L that is tethered to the membrane skeleton, which is composed of actin, fodrin, ankyrin and other proteins.

Receptors and enzymes of the phosphoinositide signal transduction cascade are associated with the cytoskeleton: EGF receptor, phospholipase C-\(\gamma\), phosphatidylinositol-4 and -3 kinases, phosphatidylinositol phosphate-5 kinase, and diacylglycerol kinase (Dale, 1985; Wiegart et al., 1986; Nahas et al., 1989; Grondin et al., 1991; McBride et al., 1991; Payrastre et al., 1991). Inositol phospholipids also play an important role in regulating polymerization and depolymerization of actin through several actin-binding proteins: profilin, gelsolin and other proteins interact with phosphatidylinositol 4.5-bisphosphate (Lansing and Lindberg, 1985; Janmey and Stossel, 1989; Yonezawa et al., 1990; Yu et al., 1990; Fukami et al., 1992). Involvement of diacylglycerol in the actin nucleation process at the plasma membrane has also been demonstrated (Shariff and Luna, 1992). The cytoskeletal association of the proteins of the phosphoinositide pathway has been thought relevant in transducing cell surface events to changes in the cytoplasmic organization. Close localization of proteins acting in tandem may also be important for efficient signal transduction. PM Ins\(^{3}\) P-L may be functionally connected to the molecules listed above. It would be interesting to see whether any of the above mentioned molecules that are attached to actin filaments have a relationship with caveolae.

The discovery of caveola-specific proteins has made it possible to observe the overall distribution of caveolae by immunocytchemistry. In fibroblasts, for example, caveolae were found concentrated along the cell edges and not distributed evenly throughout the cell surface (Rothberg et al., 1992; Fujimoto, 1993). An important finding of the present study was that in an epithelial cell line, Pam 212, caveolae were densely distributed along cell-to-cell contacts and, furthermore, that the distribution changed depending upon the cellular environment. Obviously, reorganization of the actin cytoskeleton is a key factor in the distributional change. In Pam cells, an increase or decrease in the Ca\(^{2+}\) concentration of the medium caused the alteration, but any factor that affects actin filaments is likely to induce redistribution of caveolae.

The physiological significance of caveolae has been elusive (Severs, 1988), but recent reports strongly suggest that they play roles that are different from endocytosis. Firstly, it has been proposed that caveolae engage in uptake of ions and small molecules without being detached from the plasma membrane, and the process was termed potocytosis (Anderson et al., 1992). Secondly, the localization of PM Ins\(^{3}\) P-L (Fujimoto et al., 1992) and the PM Ca\(^{2+}\)-pump ATPase (Fujimoto, 1993) in caveolae is consistent with the earlier hypothesis that Ca\(^{2+}\) influx and extrusion occur through the caveolar membrane (Crone, 1986). Finally, several signaling molecules, including GTP-binding proteins and a non-receptor tyrosine kinase, c-Yes, were shown to exist in a caveolin-rich Triton X-100-insoluble complex (Sargiacomo et al., 1993). On the basis of these findings, the caveola is now regarded as an organelle connected with signal transduction (Anderson, 1993). Signals generated in caveolae are most intense in the region close to caveolae. Uneven distribution of caveolae therefore implies that those signals may be transmitted preferentially to regions where caveolae are densely distributed. The concentration of caveolae near cell-to-cell contacts of Pam cells is interesting in this context because the intercellular junction, in particular the adherence junction, is also thought to be related to signal transduction (Tsukita et al., 1992).

The linkage of PM Ins\(^{3}\) P-L to actin filaments observed in the present study is important in localizing caveolae in the correct domains of the cell surface. On the other hand, an influx of Ca\(^{2+}\) caused by some agonists may occur through PM Ins\(^{3}\) P-L in caveolae, inducing a local Ca\(^{2+}\) increase, and modulating the nearby actin cytoskeleton through Ca\(^{2+}\)-sensitive actin-binding proteins. Although speculative, modification of the cytoskeleton may even be related to the transient closing of caveolae, which is postulated to occur during potocytosis (Anderson et al., 1992). The significance of the association between PM Ins\(^{3}\) P-L and actin filaments awaits further study, but it appears to be an important interface between signal transduction and the cytoskeleton.

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