Localization of Inositol 1,4,5-Trisphosphate Receptor-like Protein in Plasmalemmal Caveolae

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Abstract. Activation of various receptors by extracellular ligands induces an influx of Ca\(^{2+}\) through the plasma membrane, but its molecular mechanism remains elusive and seems variable in different cell types. In the present study, we utilized mAbs generated against the cerebellar type I inositol 1,4,5-trisphosphate (InsP\(_3\)) receptor and performed immunocytochemical and immunochemical experiments to examine its localization in several non-neuronal cells. By immunogold electron microscopy of ultrathin frozen sections as well as permeabilized tissue specimens, we found that a mAb to the type I InsP\(_3\) receptor (mAb 4C11) labels the plasma membrane of the endothelium, smooth muscle cell and keratinocyte in vivo. Interestingly, the labeling with the antibody was confined to caveolae, smooth vesicular inpocketings of the plasma membrane. The reactive protein, with an Mr of 240,000 by SDS-PAGE, could be biotinylated with a membrane-impermeable reagent, sulfo-NHS-biotin, in intact cultured endothelial cells, and recovered by streptavidin-agarose beads, which result further confirmed its presence on the cell surface. The present findings indicate that a protein structurally homologous to the type I InsP\(_3\) receptor is localized in the caveolar structure of the plasma membrane and might be involved in the Ca\(^{2+}\) influx.

NEUROTTRANSMITTERS and hormones cause an increase of the cytosolic free Ca\(^{2+}\) by two independent mechanisms: by a release from intracellular stores and by an entry through the plasma membrane. Although it is established that the Ca\(^{2+}\) release is mediated by inositol 1,4,5-trisphosphate (InsP\(_3\))\(^1\), the mechanism of the Ca\(^{2+}\) influx has not been determined (Berridge and Irvine, 1989; Meldolesi et al., 1991). Besides InsP\(_3\) (Kuno and Gardner, 1987; Restrepo et al., 1990), inositol 1,3,4,5-tetrakisphosphate (Irvine, 1990), specific G proteins (Narasimhan et al., 1988; Sjölander et al., 1990), and depletion of intracellular Ca\(^{2+}\) pool (Putney, 1990) have been hypothesized to be related to the process. In the last few years, P\(_{\text{iso}}\), a 313-kD glycoprotein (with a M\(_r\) of 250,000 by SDS-PAGE) enriched in the cerebellar Purkinje cell has been identified as an InsP\(_3\) receptor (InsP\(_3\)R) (Ferris et al., 1989; Maeda et al., 1990) and its cDNA was cloned (Furuchi et al., 1989). By immunocytochemical techniques using antibodies raised to the protein, InsP\(_3\)R in the Purkinje cell has been localized in the ER, but not in the plasma membrane (Mignery et al., 1989; Otsu et al., 1990; Satoh et al., 1990). The results have been interpreted to disprove the involvement of InsP\(_3\) in the Ca\(^{2+}\) influx. However, there have been several electrophysiological experiments suggesting the presence of InsP\(_3\)R in the plasma membrane; in T lymphocytes (Kuno and Gardner, 1987) and olfactory cilia (Restrepo et al., 1990), the latter of which are virtually devoid of ER, InsP\(_3\) was shown to open a cell surface Ca\(^{2+}\) channel. The recent finding that there are diverse types of InsP\(_3\)R coded by different genes (Südhof et al., 1991) further raised the possibility that an InsP\(_3\)R may exist in the plasma membrane.

In the present study, we examined several kinds of cells by immunoelectron microscopy with a battery of mAbs raised to P\(_{\text{iso}}\); we actually found that one of the mAbs specifically decorates the plasma membrane. The existence of the reactive protein on the cell surface was verified by showing that it was biotinylated by a membrane-impermeable reagent in intact cells. Surprisingly, a majority of the immunogold labeling with the anti-P\(_{\text{iso}}\) mAb was localized in small uncoated invaginations of the plasma membrane called caveolae, whose function has not been understood unequivocally (Severs, 1988). Implications of the current findings are discussed in relation to the Ca\(^{2+}\) influx mechanism as well as the caveolar function.

Materials and Methods

Immunoelectron Microscopy

Adult DDY mice were anesthetized and perfused with 3% formaldehyde in...
0.1 M sodium phosphate buffer, pH 7.4, from the left heart ventricle. The heart, the ileum, and the back skin were removed, immersed in the same fixative for 30–40 min, rinsed, infused with 2.3 M sucrose and rapidly frozen by liquid nitrogen. Ultrathin cryosections were prepared, immunolabeled, stained with 2% neutral uranyl acetate (Tokuyasu, 1980) and embedded in 2% methyl cellulose plus 0.4% uranyl acetate (Griffiths et al., 1984).

The aorta was taken without fixation, treated with 0.01% saponin in a cytoskeletal buffer (70 mM KCl, 5 mM MgCl2, 3 mM EGTA, 0.1 mM PMSF, 25 mM Hepes, pH 6.9) for 2 min, fixed with 3% formaldehyde in the same buffer for 30 min, cryosectioned to 1 μm in thickness, immunolabeled, and embedded in Epon 812 for ultrathin sectioning.

Immunolabeling for both preparations was done with mAbs to cerebellar P400 protein (4C11, 10A6, 18A10) obtained as described previously (Maeda et al., 1988); they were used at 15 μg/ml for mAb 4C11 and at 50 μg/ml for mAb 10A6, mAb 18A10, and normal rat IgG. Colloidal gold (5 nm)-conjugated goat anti-rat IgG antibody (Amersham Corp., Buckinghamshire, UK) diluted to 1/40 was used as the secondary antibody to visualize the antigenic sites.

Quantitation of immunogold distribution was done for mAb 4C11 on micrographs enlarged to a constant magnification. Length of the caveolar and noncaveolar plasma membrane with the distinct trilaminar structure was measured using MacMeasure program, Version 1.9 (written by Dr. Wayne Rashband, National Institute of Mental Health) run on an Apple Macintosh LC computer (Apple Japan, Tokyo) equipped with Digitizer SD-510C (Wacom Co., Ltd., Tokyo) and the number of gold particles per unit length of the plasma membrane was counted. For caveolae, only the membrane profiles clearly continuous with the surface non-invaginated portion of the plasma membrane were chosen for the measurement.

**Biotinylation of Cell Surface Proteins, Their Recovery with Streptavidin-Agarose, and Western Blotting**

Endothelial cells were isolated from the bovine aorta and cultured in collagen-coated plastic dishes in DME supplemented with 15% FBS. The cells confluent in a 100-mm dish were biotinylated with 10 ml of 0.5 mg/ml sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) (Hurley et al., 1985) for 30 min at 4°C and lysed with 5 ml of 1% Triton X-100 in 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 1 μM pepstatin A, and 10 μM leupeptin for 30 min at 4°C. The extract was mixed with 300 μl of streptavidin ( Gibco BRL, Gaithersburg, MD) conjugated to Sepharose 4B (Pharmacia, Uppsala, Sweden) (at ~1 mg streptavidin/ml bead) for 8–12 h at 4°C to recover the biotinylated proteins (Sargiacomo et al., 1989). The recovered proteins as well as residual proteins in the solution precipitated with cold acetone were subjected to SDS-PAGE (gel concentration 5%) and electrophoresed on nitrocellulose. Blots were incubated with mAbs to P400 at 0.1-5 μg/ml and antiserum to ER (Louvard et al., 1982) (kindly provided by Dr. Daniel Louvard) diluted to 1/4,000. They were reacted with HRP-conjugated goat secondary antibodies and visualized by enhanced chemiluminescence (ECL) detection system (Amersham Corp.) as described by the manufacturer.

**Results**

**Immunoelectron Microscopy**

Using three mAbs specific to the cytoplasmic portion of P400 (Maeda et al., 1988; Furuichi et al., 1989), we examined various tissues with two different immunocytochemical techniques. Immunogold electron microscopy of ultrathin cryosections revealed that the labeling with mAb 4C11 exists in other cytoplasmic organelles. In most instances, the gold particles did not adhere to the membrane directly, but are separated from the caveolar membrane by amorphous material. Except for small vesicles, the smooth ER (arrows) or other organelles were not labeled with mAb 4C11. (b) mAb 10A6 marked cytoplasmic organelles (arrows), but not the caveola (arrowheads). (c) mAb 18A10 did not give specific labeling in the same preparation except for low background. L: vascular lumen; N: nucleus. Bar, 100 nm.

Figure 1. Immunogold electron microscopy of the capillary endothelium of the heart (a–c) and the aortic endothelium (d). (a–c) Ultrathin cryosections and (d) permeabilized tissue sample were labeled with rat mAbs to type I InsP3R: (a and d) mAb 4C11; (b) mAb 10A6; (c) mAb 18A10. Arrowheads show caveolae whose limiting membrane is evidently continuous with the plasma membrane. (a and d) The label-
with a membrane-impermeable reagent, sulfo-NHS-biotin (Hurley et al., 1985). Biotinylated proteins recovered with immobilized streptavidin and the residual proteins unrecov-
ered were taken and subjected to immunoblotting with the three mAbs used for immunocytochemistry (Fig. 3). The former sample should represent only the plasmalemmal pro-
teins exposed to the exterior surface, while the latter con-
tains not only the cytoplasmic proteins but also plasmalem-
mal proteins which did not react with the biotinylating reagent by the present experimental procedure. mAb 4C11
gave a positive band with both the biotinylated proteins and
the residual proteins at around 240 kD. When the two protein
samples were loaded to give a comparable reaction intensity
for mAb 4C11, the reaction with mAb 10A6 at 240 kD was
much stronger with the residual proteins than with the bi-
otinylated proteins; mAb 18A10 gave weak but equivalent
reactions with the two samples in the same mol wt range. To
show the degree of possible biotinylation of intracellular pro-
teins, the samples were probed with anti-ER antibody. The
reaction was intense at reported mol wt (90 and 66 kD) (Lou-
vard et al., 1982) for the residual proteins, but was negligible
for the biotinylated proteins, which indicates that biotinyti-

tion of intracellular ER proteins was minimal with the pres-

ent method. The reaction of mAbs 4C11 and 18A10 with the
residual proteins might indicate presence of reactive proteins
in cytoplasmic organelles, but considering the immunocyto-
chemical results, it is more likely due to incomplete biotinyli-
tion of the surface proteins. In summary, the result concludes
that the 240-kD protein reactive with mAb 4C11 and possibly
also with mAb 18A10 exists in the plasma membrane.

Discussion

Distribution of InsP$_3$R has been a focus of intensive re-
search because it is an indispensable knowledge to under-
stand the role of InsP$_3$-dependent cytosolic Ca$^{2+}$ increase in
the cellular signal transduction process. Since the cerebellar
P$_{400}$ was identified as an InsP$_3$R (type I InsP$_3$R), antibodies
were generated against the protein and several immunoelec-
tron microscopic studies have been performed with the mouse
and avian Purkinje cell (Mignery et al., 1989; Otsu
et al., 1990; Satoh et al., 1990). They showed that the label-
ing with the anti-P$_{400}$ antibody is localized in the rough and
smooth ER and in the nuclear envelope, but not in the Golgi
apparatus, plasma membrane and other organelles. The results
fit well with the generally accepted view based on
subcellular fractionation and physiological studies that ER
or related membrane compartments are the site of InsP$_3$-
sensitive Ca$^{2+}$ store. However, until today immunolocaliza-
tion study has been only successful for the Purkinje cell
where P$_{400}$ is concentrated in an extraordinary amount.
Judging from the intercellular differences concerning the
physiological role of Ca$^{2+}$, it is plausible that other cell
types, especially nonexcitable cells, may show a totally
different distribution of InsP$_3$Rs.

In the present study, we found that the immunocytochemi-

cal labeling with a mAb raised to P$_{400}$ (mAb 4C11) was

localized in the plasma membrane of the endothelium, smooth
muscle cell and keratinocyte. By the surface biotinyl-
ation experiment, the antibody was found to recognize the
plasmalemmal protein of 240 KD in mol wt. Another mono-
clonal anti-P$_{400}$ antibody (mAb 18A10) did not show posi-
tive labeling in the cytochemical experiment, but in the im-
munochernical procedure it also bound to the 240-kD
protein on the cell surface. Because of the positive reactivity
with the two mAbs and similarity in mol wt, the protein,
hereafter referred to as the 240-kD protein, is quite likely
to bear structural homology to the type I InsP$_3$R. On the
other hand, the third mAb to P$_{400}$ (mAb 10A6) did not label
the plasma membrane in immunocytochemistry nor recog-
nized the cell surface protein in immunohistochemistry. Because
all the spliced variants of the type I InsP$_3$R known so far are
recognized by the three mAbs (Nakagawa et al., 1991), the
result indicates that the plasmalemmal 240-kD protein is
possibly a product of a distinct gene from the type I
InsP$_3$R, rather than an alternatively spliced product of the
same gene. Furthermore, while the Purkinje cell showed

Figure 2. Immunogold electron microscopy of the smooth muscle cell of the ileum (a) and the epidermal keratinocyte (b and c). Ultrathin
cryosections were labeled with mAb 4C11. (a) Not only caveolae which are identified by continuity with the surface membrane (arrow-
heads), but also SR (arrows) are labeled positively in the smooth muscle cell. (b and c) The labeling with mAb 4C11 occurred exclusively
in caveolae along the basal surface of the keratinocyte in the basal epidermal layer (b, arrowheads). It was also seen in caveolae in the
lateral interdigitating plasma membrane (c, arrowhead) and in the cytoplasmic vesicles (c, double arrowheads) which might be continuous
with the plasma membrane. ECM: extracellular matrix. Bar, 100 nm.
brane protein. Recently, a recombinant protein having a GPI-linkage added to the extracellular portion of CD4 was shown to be internalized through noncoated caveola-like membrane invagination probably present in all kinds of cells. Recent studies showed that caveolae in certain cultured cells are related to the uptake of small molecules (e.g., folate) (Anderson et al., 1992), but whether caveolae in various cells have a common function is not known (Severs, 1988). For example, the caveola in the smooth muscle cell is presumed to be a static structure, but its function remains obscure (Severs, 1988); on the other hand, the caveola in the endothelium has been postulated to be pinched off to form free vesicles to transport various substances between the luminal and abluminal surfaces (Palade, 1960; Simionescu, 1988). Despite seeming differences, an analogous ridge-like structure observed on the cytoplasmic surface of the caveolae in various cells including the endothelium and smooth muscle cell (Somlyo et al., 1971; Peters et al., 1985; Izumi et al., 1989; Rothberg et al., 1992) suggests that they share some constituents. Considering the large cytoplasmic mass of the type I InsP₃R in the cerebellar ER (Furiuchi et al., 1989; Otsu et al., 1990), the 240-kD protein is likely to be a component which forms the ridge-like structure of caveolae. Additionally, although the function of the 240-kD protein is not known at present, the localization of an InsP₃R-like protein in the caveola is consistent with the hypothesis that the plasmalemmal differentiation is involved in regulation of cytosolic Ca²⁺ concentration (Popescu, 1974; Crone, 1986). As indicated from morphological studies, the caveola is closely associated with ER in the endothelium (Bundgaard, 1991) and SR in the smooth muscle (Gabella, 1981), and thus may be related to the Ca²⁺ storage function of the latter organelles. To fully understand the physiological significance of the caveola, it now seems indispensable to elucidate the function of the 240-kD protein. It will be also interesting to examine whether other Ca²⁺-related proteins are present in and around the caveola in various cell types.

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