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 M_r 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.

NEUROTRANSMITTERS 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_{400}, 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 ($InsP_3R$) (Ferris et al., 1989; Maeda et al., 1990) and its cDNA was cloned (Furuichi et al., 1989). By immunocytochemical techniques using antibodies raised to the protein, $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ coded by different genes (Südhof et al., 1991) further raised the possibility that an $InsP_3R$ 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_{400}; 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_{400} 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.

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1. *Abbreviations used in this paper*: GPI, glycosylphosphatidylinositol; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, inositol 1,4,5-trisphosphate receptor.

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 $MgCl_2$, 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 P_{400} 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 Rasband, 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 electrotransferred onto nitrocellulose. Blots were incubated with mAbs to P_{400} 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 P_{400} (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 along the plasma membrane in the capillary endothelium (Fig. 1 *a*). Remarkably, most of the immunogolds were localized along the plasmalemmal caveolae and not along the other portion of the plasma membrane. Because the cryosections used in the present experiment were very thin (50–80 nm) and the continuity of the plasma membrane was clearly observed, the caveola could be recognized distinctly from the organelles in the cytoplasm, although some of the latter were often in proximity to the plasma membrane. Quantitative analysis showed that whereas the number of gold particles in the caveolar portion was 30.83/μm of the membrane, it was only 3.64/μm in the noncaveolar portion. Round cytoplasmic vesicles, most of which are likely to be caveolae as shown by serial ultrathin sectioning techniques (Bundgaard et al., 1983), were also labeled densely by mAb 4C11, but other organelles including smooth ER seemed negative (Fig. 1 *a*). In contrast, the labeling with another anti-P_{400} antibody, mAb 10A6, was seen with smooth ER, but not in the caveola or other portions of the plasma membrane (Fig. 1 *b*). The third anti-P_{400} antibody, mAb 18A10 (Fig. 1 *c*) as well as normal nonimmune rat IgG gave virtually no labeling in the endothelium processed by the same procedure.

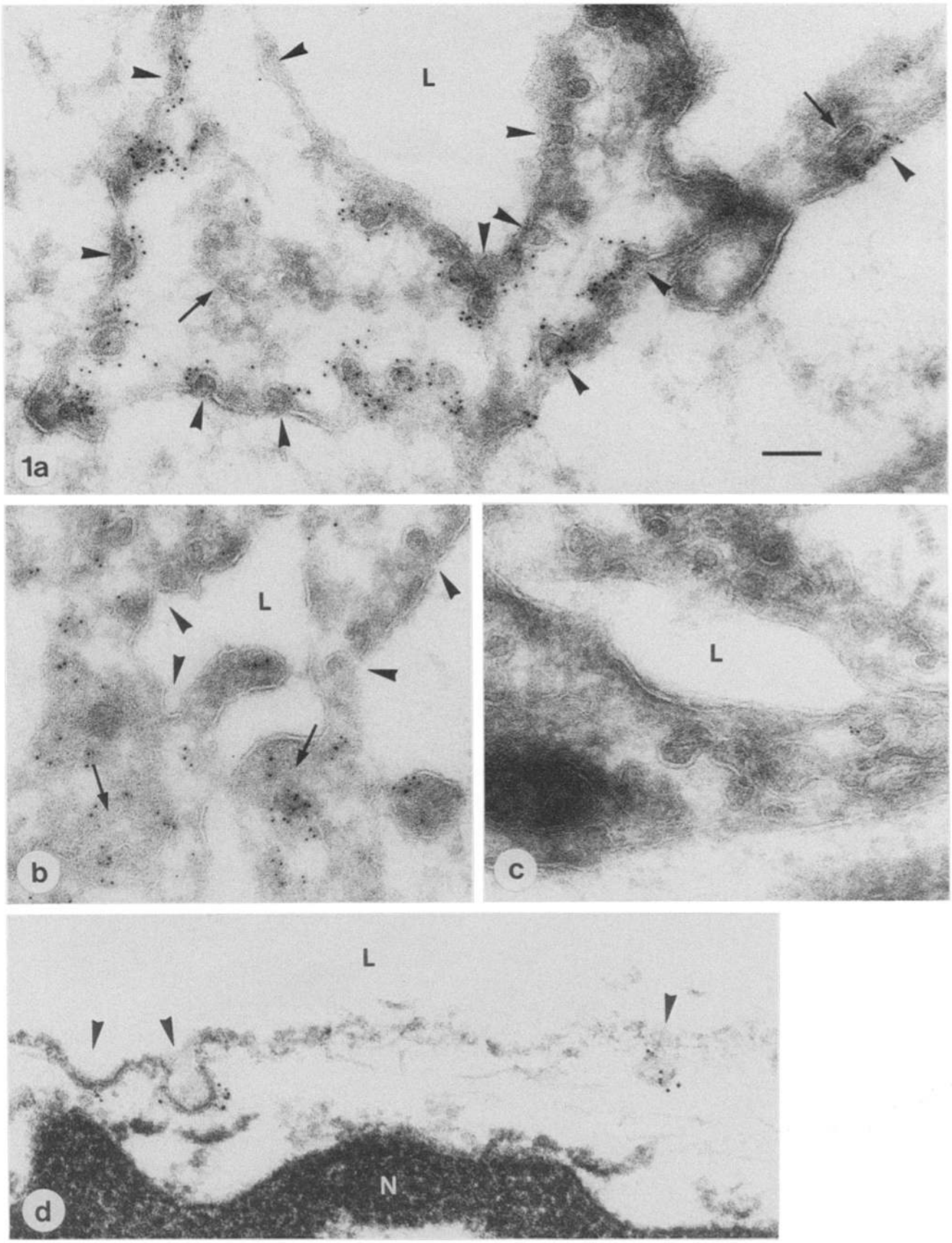
The distinction between the plasma membrane and other organelles was more definite in the preextracted cells, because only scarce organelles remained in the cytoplasm. Preembedding immunoelectron microscopy of the aortic endothelium showed that mAb 4C11 exclusively decorates invaginated portions of the plasma membrane (Fig. 1 *d*). With both immunocytochemical methods, a majority of the immunogolds along the plasma membrane were observed on the cytoplasmic surface, which disposition was the same as the cerebellar type I $InsP_3R$ in ER of the Purkinje cell (Mignery et al., 1989; Otsu et al., 1990; Satoh et al., 1990). Another characteristic shared by the cerebellar type I $InsP_3R$ and the present antigen is that most gold particles do not adhere to the membrane directly, but are separated from it by some distance (Otsu et al., 1990).

The plasmalemmal localization of the mAb 4C11 labeling occurred in other kinds of cells containing numerous caveolae as well. The visceral smooth muscle cell in the small intestine was labeled densely in the caveola (Fig. 2 *a*); in this cell, SR in the cytoplasm was also labeled by mAb 4C11 (Fig. 2 *a*). In the epidermal keratinocyte, immunogold particles for mAb 4C11 were found in the caveola along the basal (Fig. 2 *b*) as well as the lateral interdigitating (Fig. 2 *c*) cell surface, and in apparent free vesicles in the vicinity of the plasma membrane (Fig. 2 *c*). Virtually no labeling was observed in other cytoplasmic organelles.

Cell Surface Biotinylation and Analysis by Western Blotting

To verify further the presence of the protein labeled with mAb 4C11 in the plasma membrane, we selectively biotinylated the cell surface of the cultured endothelial cell

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 $InsP_3R$: (*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 labeling with mAb 4C11 is mostly localized on the cytoplasmic surface of the caveola of the endothelium. In most instances, the gold particles 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.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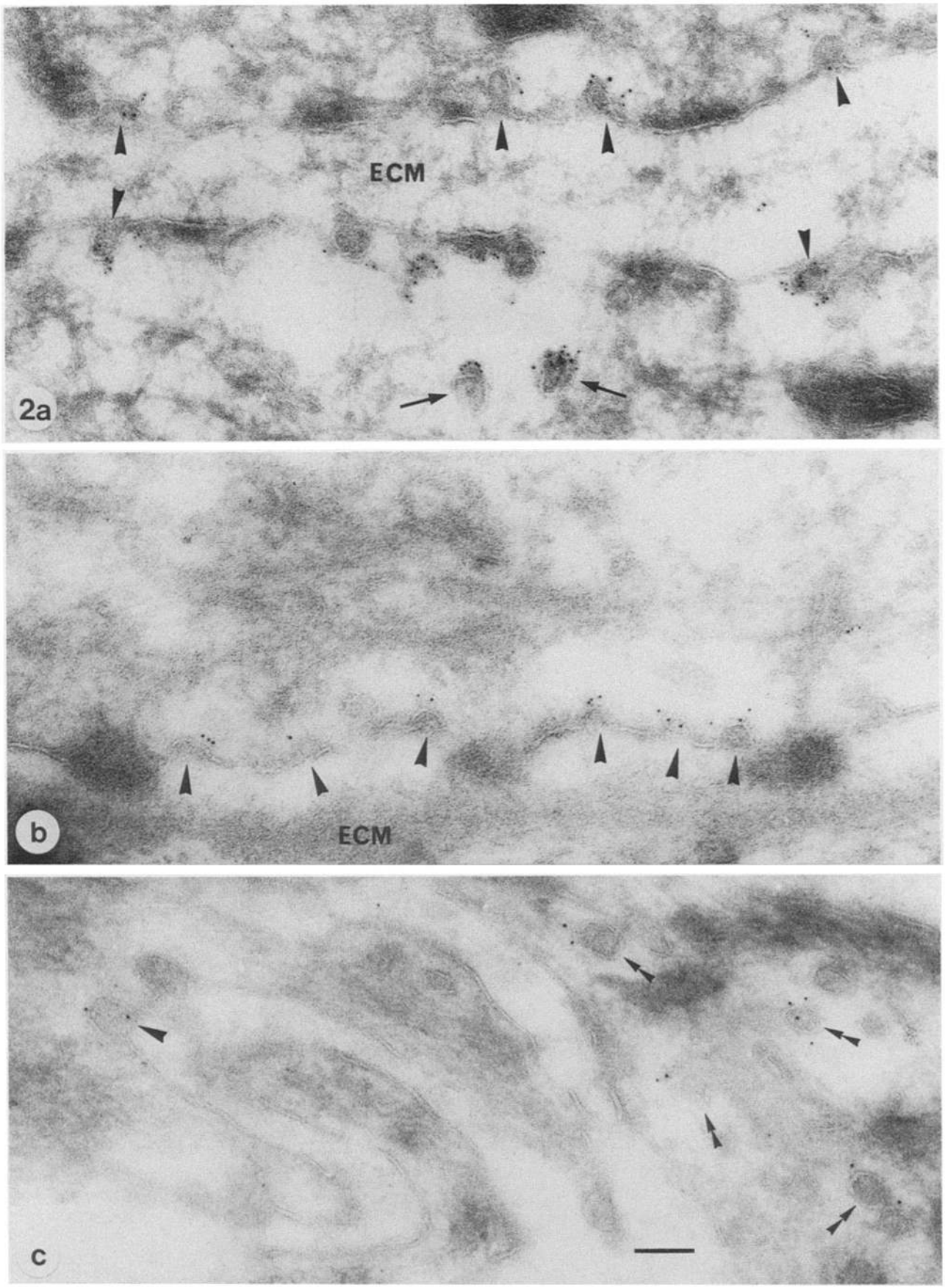
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with a membrane-impermeable reagent, sulfo-NHS-biotin (Hurley et al., 1985). Biotinylated proteins recovered with immobilized streptavidin and the residual proteins unrecovered were taken and subjected to immunoblotting with the three mAbs used for immunocytochemistry (Fig. 3). The former sample should represent only the plasmalemmal proteins exposed to the exterior surface, while the latter contains not only the cytoplasmic proteins but also plasmalemmal 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 biotinylated 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 proteins, the samples were probed with anti-ER antibody. The reaction was intense at reported mol wt (90 and 66 kD) (Louvard et al., 1982) for the residual proteins, but was negligible for the biotinylated proteins, which indicates that biotinylation of intracellular ER proteins was minimal with the present method. The reaction of mAbs 4C11 and 18A10 with the residual proteins might indicate presence of reactive proteins in cytoplasmic organelles, but considering the immunocytochemical results, it is more likely due to incomplete biotinylation 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.

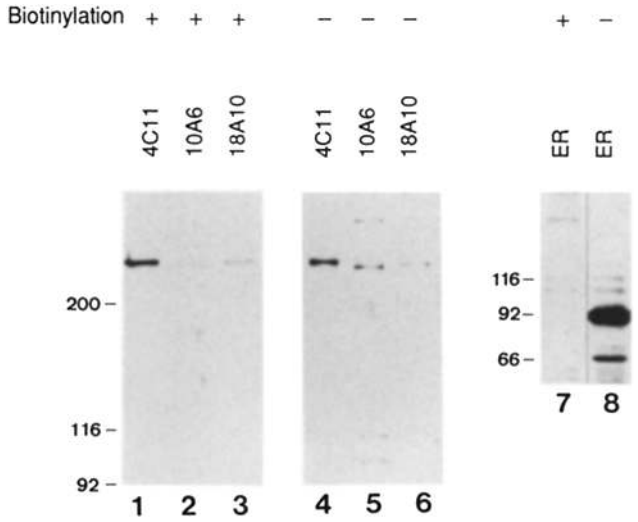

Figure 3. Immunoblot analysis of cultured endothelial cells biotinylated with a membrane impermeable reagent, sulfo-NHS-biotin. Proteins recovered by immobilized streptavidin (lanes *1–3*, *7*) and those not recovered (lanes *4–6*, *8*) were probed with the following antibodies: lanes *1* and *4*, mAb 4C11; lanes *2* and *5*, mAb 10A6; lanes *3* and *6*, mAb 18A10; lanes *7* and *8*, rabbit polyclonal antiserum to ER. The two samples were loaded to give equivalent reaction with mAb 4C11. The residual protein sample reacted positively with mAb 10A6 at 240 kD and with anti-ER antibody at 90 and 66 kD, while the proteins recovered with immobilized streptavidin did not give positive reactions with the two antibodies in the same region. The reaction with mAb 18A10 at 240 kD was weak but showed similar density in the two samples. Reaction with normal rabbit IgG and normal rat IgG was negative in both samples (photograph not shown).

Discussion

Distribution of $InsP_3R$ has been a focus of intensive research because it is an indispensable knowledge to understand 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 $InsP_3R$ (type I $InsP_3R$), antibodies were generated against the protein and several immunoelectron 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 labeling 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 immunolocalization 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 $InsP_3Rs$.

In the present study, we found that the immunocytochemical 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 biotinylation experiment, the antibody was found to recognize the plasmalemmal protein of 240 kD in mol wt. Another monoclonal anti-P_{400} antibody (mAb 18A10) did not show positive labeling in the cytochemical experiment, but in the immunochemical 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 $InsP_3R$. On the other hand, the third mAb to P_{400} (mAb 10A6) did not label the plasma membrane in immunocytochemistry nor recognized the cell surface protein in immunochemistry. Because all the spliced variants of the type I $InsP_3R$ 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 $InsP_3R$, rather than an alternatively spliced product of the same gene. Furthermore, while the Purkinje cell showed in-

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 (*arrowheads*), 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.

tense labeling with the mAbs after a potent fixation with 1% glutaraldehyde (Otsu et al., 1990), the 240-kD protein in the cells studied currently was susceptible to even a low concentration (<0.1%) of glutaraldehyde. The difference may simply reflect the abundance of the antigen in the Purkinje cell, but it may indicate divergence in the molecular architecture between the type I $InsP_3R$ and the 240-kD protein, and hence in the disposition of epitopes recognized by the mAbs.

The presence of the type I $InsP_3R$ in the plasma membrane has been thought unlikely because proteins localized both in ER and in the plasmalemma have not been known (DeCamilli et al., 1990). However, some of the newly reported $InsP_3Rs$ (Südhof et al., 1991), type II and putative type III, or other yet discovered types of $InsP_3R$ may contain different sorting signals from the type I $InsP_3R$ and may be targeted to the plasma membrane. As discussed above, the 240 kD-protein is not likely to be a product of the type I $InsP_3R$ gene. It will be interesting to see if the 240-kD protein is identical to any of the new $InsP_3Rs$. Our immunolabeling study with mAb 4C11 also showed that the endothelial ER and the smooth muscle SR are different in reactivity: the former was not labeled positively whereas the latter was decorated by the antibody. The result indicates that $InsP_3Rs$ responsible for the intracellular discharge of Ca^{2+} may be diversified in various cell types rather than restricted to the type I $InsP_3R$.

At present, we do not know to what extent the 240-kD protein is related to $InsP_3Rs$ in function, that is, whether it binds $InsP_3$, works as a Ca^{2+} channel, or both. An intriguing possibility is that the 240-kD protein identified presently in the plasma membrane is an $InsP_3R$: an $InsP_3$-gated Ca^{2+} channel. However, in the endothelium, depletion of intracellular Ca^{2+} pool (Hallam et al., 1989) and inositol 1,3,4,5-tetrakisphosphate (Lückhoff and Clapham, 1992) have been suggested to mediate the Ca^{2+} influx, while the direct action of $InsP_3$ to the plasmalemmal Ca^{2+} channel has been generally thought unlikely (Hallam et al., 1989; Lückhoff and Clapham, 1992) (but see Adams et al., 1989). Recently in the rat thymus and human Jurkat T lymphocytes, an $InsP_3R$ having lower affinity for $InsP_3$ and higher affinity for inositol 1,3,4,5-tetrakisphosphate compared to the type I $InsP_3R$ was reported to occur in the plasma membrane (Khan et al., 1992). If the 240-kD protein has similar characteristics to the lymphocyte plasmalemmal $InsP_3R$, it may be related to the inositol 1,3,4,5-tetrakisphosphate-dependent Ca^{2+} influx (Lückhoff and Clapham, 1992).

It was surprising that the 240-kD protein was localized exclusively in the caveola and not distributed in the entire plasma membrane. In addition to the receptor for 5 methyltetrahydrofolic acid (the folate receptor) (Rothberg et al., 1990*a*) and caveolin, a *v-src* tyrosine kinase substrate (Rothberg et al., 1992), the 240-kD protein is the third protein found concentrated in the caveola in normal untreated cells. The mechanism which localizes the 240-kD protein to the caveola may be different from the folate receptor, which is a glycosylphosphatidylinositol (GPI)-linked integral membrane 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 invaginations, whereas CD4 in its native transmembrane form was incorporated through the coated pit (Keller et al., 1992). Consistent with this, some other GPI-linked proteins are known to be excluded from the coated pit (Bretcher et al., 1980; Bamezai et al., 1992). Thus, GPI-linkage can be a targeting signal for caveolar localization. But the 240-kD protein probably transverses the lipid bilayer multiple times and does not have a GPI moiety; moreover, cholesterol-binding reagents, such as filipin and digitonin, which were shown to dissociate the folate receptor from the caveola (Rothberg et al., 1990*b*) did not affect the clustering of the 240-kD protein in cultured cells (data not shown). On the other hand, caveolin is exposed to the cytoplasmic surface of the plasma membrane (Rothberg et al., 1992) and hence, probably not a GPI-linked protein (Cross, 1990). It is resistant to high salt washing and Triton X-100 extraction (Rothberg et al., 1992). The property may be due to linkage to cytoskeleton, but the presence of glycosphingolipids in the surrounding lipid bilayer may cause the effect (Brown and Rose, 1992). In fact, accumulation of gangliosides in the caveolar membrane has been shown for some cells (Montesano et al., 1982; Tran et al., 1987; Parton et al., 1988). Interestingly, the 240-kD protein is also resistant to Triton X-100 extraction in some cultured cells (Fujimoto, T., S. Nakade, and K. Mikoshiba, unpublished observations). Caveolin and the 240-kD protein may be localized to the caveola by a similar mechanism.

The present finding also has important implications in elucidating the caveolar function. The caveola is a plasma 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 $InsP_3R$ in the cerebellar ER (Furuichi 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 $InsP_3R$-like protein in the caveola is consistent with the hypothesis that the plasmalemmal differentiation is involved in regulation of cytosolic Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}-related proteins are present in and around the caveola in various cell types.

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