Calcium Pump of the Plasma Membrane Is Localized in Caveolae

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Abstract. The Ca^{2+} pump in the plasma membrane plays a key role in the fine control of the cytoplasmic free Ca^{2+} concentration. In the present study, its subcellular localization was examined with immunocytochemical techniques using a specific antibody generated against the erythrocyte membrane Ca^{2+} pump ATPase. By immunofluorescence microscopy of cultured cells, the labeling with the antibody was seen as numerous small dots, often distributed in linear arrays or along cell edges. Immunogold EM of cryosections revealed that the dots correspond to caveolae, or smooth invaginations of the plasma membrane. The same technique applied to mouse tissues in vivo showed that the Ca^{2+} pump is similarly localized in caveolae of endothelial cells, smooth muscle cells, cardiac muscle cells, epidermal keratinocytes and mesothelial cells. By quantitative analysis of the immunogold labeling, the Ca^{2+} pump in capillary endothelial cells and visceral smooth muscle cells was found to be concentrated 18–25-fold in the caveolar membrane compared with the noncaveolar portion of the plasma membrane. In renal tubular and small intestinal epithelial cells, which have been known to contain the Ca^{2+} pump but do not have many caveolae, most of the labeling was randomly distributed in the basolateral plasma membrane, although caveolae were also positively labeled.

The results demonstrate that the caveola in various cells has the plasmalemmal Ca^{2+} pump as a common constituent. In conjunction with our recent finding that an inositol 1,4,5-trisphosphate receptor-like protein exists in the caveola (Fujimoto, T., S. Nakade, A. Miyawaki, K. Mikoshiba, and K. Ogawa. 1992. *J. Cell Biol.* 119:1507–1513), it is inferred that the smooth plasmalemmal invagination is an apparatus specialized for Ca^{2+} intake and extrusion from the cytoplasm.

THE plasmalemmal (PM)[1] Ca^{2+} pump extrudes Ca^{2+} from the cytoplasm against a steep concentration gradient and is essential in maintaining intracellular Ca^{2+} homeostasis (Carafoli, 1987). The pump is thought to be ubiquitous in eukaryotic cells as a single transmembrane protein of 70–150 kD (Carafoli, 1991). Four isoforms (PMCA1-4) encoded by a multigene family have up to 85–90% similarity to each other in amino acid sequences; in addition, several alternatively spliced products are known to be expressed differentially in various cells (Strehler, 1991; Wang et al., 1992). Because of recent cloning experiments, the structure and function relationship of the PM Ca^{2+} pump is rapidly unfolding. On the other hand, our knowledge concerning localization of the PM Ca^{2+} pump is limited to a few calcium-transporting tissues (Kumar and Penniston, 1991) and only light microscopic techniques have been applied for its immunocytochemical detection. Examination of the ultrastructural localization of the PM Ca^{2+} pump, particularly in cells not specialized in Ca^{2+} transport, is indispensable to clarify its general physiological significance.

Recently we found that a 240-kD protein recognized by mAbs to the cerebellar type I inositol 1,4,5-trisphosphate ($InsP_3$) receptor is enriched in the caveola of vascular endothelial cells, smooth muscle cells, and epidermal keratinocytes (Fujimoto et al., 1992). Although its function has yet to be analyzed, the protein may mediate Ca^{2+} entry through the plasma membrane. This assumption is consistent with the hypothesis that the physiological role of the caveola is to regulate cytosolic Ca^{2+} concentration through a combination of channel-mediated influx and energy-requiring extrusion (Crone, 1986). Now that a molecule possibly responsible for the influx has been localized in the caveola, it is intriguing to ask whether it is at this site that the PM Ca^{2+} pump is engaged in active Ca^{2+} extrusion. Enzyme histochemical studies have reported the presence of Ca^{2+}-dependent ATP hydrolyzing activity in caveolae of endothelial cells and smooth muscle cells, but the technique used is not specific to the PM Ca^{2+} pump ATPase, and is likely to have detected nonspecific ecto-ATPases as well (Ogawa et al., 1986; Nasu and Inomata, 1990).

In the present study, we generated polyclonal antisera to the Ca^{2+} pump isolated from erythrocyte membrane, and conducted immunofluorescence and immunoelectron microscopic studies by the affinity-purified antibody. As a result, labeling for the PM Ca^{2+} pump was found to exist exclu-

1. *Abbreviations used in this paper*: $InsP_3$, inositol 1,4,5-trisphosphate; PM, plasmalemmal.

sively in the plasma membrane and showed marked concentration in the caveolar portion of fibroblasts, vascular endothelial cells, smooth muscle cells, cardiac muscle cells, epidermal keratinocytes, and peritoneal mesothelial cells. Combined with the presence of $InsP_3$ receptor-like protein, this result infers that the caveola is a structure involved in the regulation of the intracellular free Ca^{2+} concentration.

Materials and Methods

Antibody

The Ca^{2+} pump ATPase of the plasma membrane was purified from the fresh pig erythrocyte as described (Niggli et al., 1979). Briefly erythrocyte ghosts were treated with Triton X-100 and the solubilized proteins were applied to a calmodulin column in the presence of Ca^{2+}. The fractions eluted from the column with EDTA were applied to preparative SDS-PAGE (Laemmli, 1970) and a band of ~140 kD was cut out, minced, and mixed with complete and incomplete Freund adjuvants for primary and booster injections, respectively. Sera obtained from immunized rabbits were affinity-purified with the 140-kD protein electrotransferred to Immobilon membrane (Millipore Corp., Bedford, MA) (Olmsted, 1981).

To examine the specificity of the obtained antibody, human erythrocyte ghosts, and the total lysate of human fibroblasts and bovine aortic endothelial cells in culture were subjected to SDS-PAGE (6% acrylamide gel), transferred to nitrocellulose, and incubated with either the affinity-purified rabbit anti-PM Ca^{2+} pump antibody or normal rabbit IgG at 1 μg/ml. For comparison, the blots were also treated with the same concentration of mouse monoclonal anti-human erythrocyte Ca^{2+}-ATPase antibody (KY-1) (Ogurusu et al., 1990) (kindly provided by Dr. M. Shigekawa, Department of Molecular Physiology, National Cardiovascular Center Research Institute, Osaka, Japan). They were further incubated with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (KPL Inc., Gaithersburg, MD) and the reaction was visualized by the ECL detection system (Amersham, Buckinghamshire, UK) according to the manufacturer's instruction.

To test if the rabbit and mouse antibodies recognize the same protein, solubilized human erythrocyte membrane proteins were reacted with either the mouse KY-1 antibody or normal mouse IgG, and then with goat anti-mouse IgG antibody conjugated to agarose beads. Immunoprecipitated samples were treated in a sample buffer, subjected to Western blotting, and probed with the rabbit anti-PM Ca^{2+} pump antibody by the same procedure as described above.

Immunofluorescence Microscopy of Fibroblasts in Culture

Human fibroblasts explanted from the biopsied normal human skin (gift of Dr. Yutaka Nagano, Department of Geriatric Medicine, Kyoto University, Kyoto, Japan), bovine aortic endothelial cells, and mouse BALB/c 3T3 cells (obtained from Japanese Cancer Research Resources Bank, Tokyo, Japan) were cultured on glass coverslips in DME (Nissui Pharmac. Co., Tokyo) supplemented with 10 or 15% FBS. The cells were fixed with 3% formaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. In some experiments, the dorsal plasma membrane was removed by adhering and then pulling apart nitrocellulose filter (Fujimoto et al., 1991). The obtained unroofed samples, consisting of the ventral plasma membrane and some residual cytoskeleton, were fixed with a mixture of 3% formaldehyde and 0.01% glutaraldehyde and treated with 1 mg/ml sodium borohydride. Both specimens were incubated with 2% gelatin for 10 min before immunolabeling.

The affinity-purified rabbit anti-PM Ca^{2+} pump antibody was used at 10–20 μg/ml. As controls, the antibody preabsorbed with the antigen blot on nitrocellulose filter as well as preimmune rabbit IgG obtained from the same animals were used. The secondary antibody was FITC-conjugated goat anti-rabbit IgG antibody (10–20 μg/ml) (Cappel Laboratories, Durham, NC). Some specimens were incubated with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) to visualize F-actin. All the fluorescent specimens were mounted with a glycerol/Mowiol 4-88 (Calbiochem-Novabiochem Corp., La Jolla, CA) mixture containing 1,4-diazobicyclo-[2,2,2]-octane (Eastman Kodak Co., Rochester, NY) (Harlow and Lane, 1988) and observed by a Zeiss Axiophoto fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

Immunoelectron Microscopy

Adult DDY mice were anesthetized with pentobarbital and perfused from the left heart ventricle first with saline containing heparin and then with 5% formaldehyde (freshly depolymerized from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.4. Tissues were excised, trimmed into small pieces, and kept in the same fixative for up to 60 min. Cultured human fibroblasts were fixed similarly on culture dishes, scraped with a rubber policeman, and embedded in 10% gelatin. Both samples were infused with buffered 2.3 M sucrose, rapidly frozen, and kept in liquid nitrogen. Ultrathin (50–100 nm) cryosections were prepared as described (Tokuyasu, 1980) and subjected to indirect immunogold labeling. The primary antibodies were used at the same concentration as above, and 5 nm colloidal gold-conjugated goat anti-rabbit IgG antibody (Amersham) diluted to 1/40 was used for the secondary antibody. The specimens were adsorption stained with neutral uranyl acetate (Tokuyasu, 1980) and embedded in a mixture of 2% methylcellulose (Nakalai Tesque, Kyoto, Japan) and 0.4% uranyl acetate (Griffiths et al., 1984).

Quantitation of the immunogold labeling in ultrathin cryosections of capillary endothelial cells and smooth muscle cells was done using MacMeasure program, version 1.9 (written by Dr. Wayne Rasband, National Institute of Mental Health). The caveolar and noncaveolar plasma membrane with the definite trilaminar structure was selected, and the number of gold particles per unit length of membrane was counted. The ratio of positively labeled caveolae was also calculated for the two cell types.

Results

Specificity of the Antibody

Pig erythrocyte Ca^{2+}-ATPase obtained by the calmodulin-affinity column appeared as a single band of 140 kD (Fig. 1 *A*); the band was excised from the gel and used as the anti-

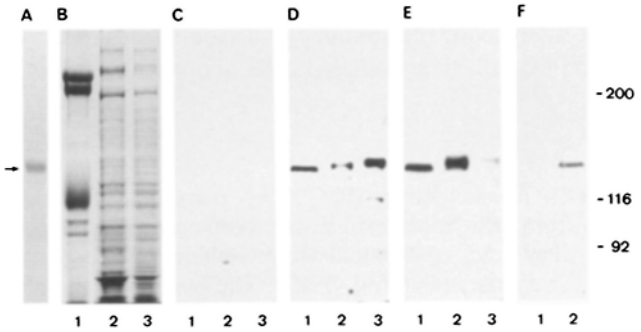


Figure 1. (*A*) Ca^{2+}-ATPase obtained from the pig erythrocyte membrane by the calmodulin-affinity column is shown. The dense band of 140 kD (*arrow*) was cut out and used to generate the rabbit antibody. (*B–E*) Human erythrocyte ghosts, human fibroblasts, and bovine aortic endothelial cells were electrophoresed using a 6% acrylamide gel, transferred to nitrocellulose filter, and treated with normal nonimmune rabbit IgG, the affinity-purified rabbit anti-pig erythrocyte Ca^{2+}-ATPase antibody, and the mouse monoclonal anti-human erythrocyte Ca^{2+}-ATPase antibody (KY-1). Coomassie blue staining of the gel (*B*), immunoblotting results with normal rabbit IgG (*C*), the rabbit anti-Ca^{2+}-ATPase antibody (*D*), and the mouse KY-1 antibody (*E*) are shown. (Lane *1*) Human erythrocyte; (lane *2*) human fibroblast; and (lane *3*) bovine aortic endothelium. Control lanes showed only background staining, while both the rabbit and mouse anti-Ca^{2+}-ATPase antibodies recognized a band of 140 (human erythrocyte), 142 (human fibroblast), and 145 (bovine aortic endothelium) kD. (*F*) Immunoprecipitates obtained either with normal mouse IgG (lane *1*) or with the mouse KY-1 antibody (lane 2) were probed with the rabbit anti-Ca^{2+}-ATPase antibody. The rabbit antibody recognized a single band at 140 kD only in the sample precipitated with the mouse KY-1 antibody.

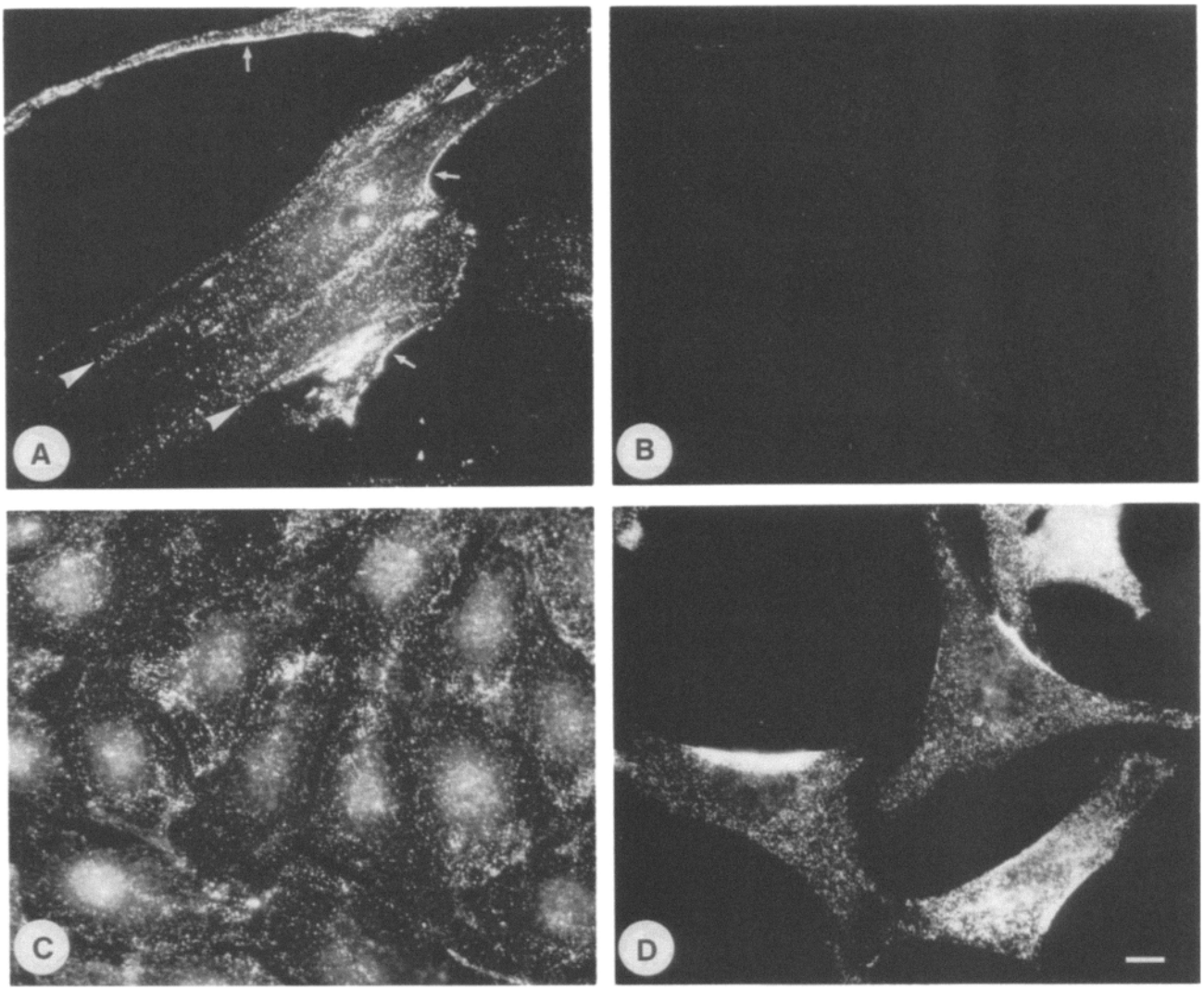


Figure 2. Immunofluorescence microscopy of cultured cells. Human fibroblasts (*A* and *B*), bovine aortic endothelia (*C*), and mouse BALB/c 3T3 cells were incubated with the anti-erythrocyte Ca^{2+}-ATPase antibody (*A*, *C*, and *D*) or the antibody preabsorbed with the isolated Ca^{2+}-ATPase (*B*). The reaction with the anti-Ca^{2+}-ATPase was seen as small dots in all three samples. They were often observed in linear arrays (*arrowheads*) and along cell edges (*arrows*). The preabsorbed antibody caused only faint background staining. Bar, 10 μm.

gen to raise antisera in rabbits. Immunoblotting was done to test the specificity of the obtained antibody. Human erythrocyte ghosts, human fibroblasts, and bovine aortic endothelial cells were electrophoresed in a 6% polyacrylamide gel (Fig. 1 *B*), transferred to nitrocellulose filter, and probed with the affinity-purified rabbit antibody. All the samples showed a positive band, but with slightly different mobility: 140 kD for the erythrocyte, 142 kD for the fibroblast, and 145 kD for the endothelium (Fig. 1 *D*). Normal rabbit IgG did not react markedly with any of the three samples (Fig. 1 *C*).

The same blot samples were reacted with a mouse monoclonal anti-human erythrocyte Ca^{2+}-ATPase antibody (KY-1), which was previously shown to precipitate the enzyme activity from solubilized membrane proteins (Ogurusu et al., 1990). In all of the three cell specimens, the mouse antibody labeled a band at the same molecular weight region as the rabbit antibody (Fig. 1 *E*). Furthermore, the rabbit anti-PM Ca^{2+} pump antibody reacted positively with a band of 140 kD which was immunoprecipitated by the mouse antibody (Fig. 1 *F*). These results show that the rabbit antibody raised to the 140-kD protein of the pig erythrocyte membrane specifically recognizes the PM Ca^{2+} pump in various specimens.

Immunofluorescence and Immunoelectron Microscopy of Fibroblasts in Culture

Immunofluorescence labeling with the anti-PM Ca^{2+} pump antibody was observed as small dots in various cultured cells. In human fibroblasts, many dots were scattered in the outspread portion of the cell; an intense accumulation of the dots was frequently seen as linear arrays in a longitudinal direction and along the cell edges (Fig. 2 *A*). Staining was abolished when the antibody was preabsorbed with the anti-

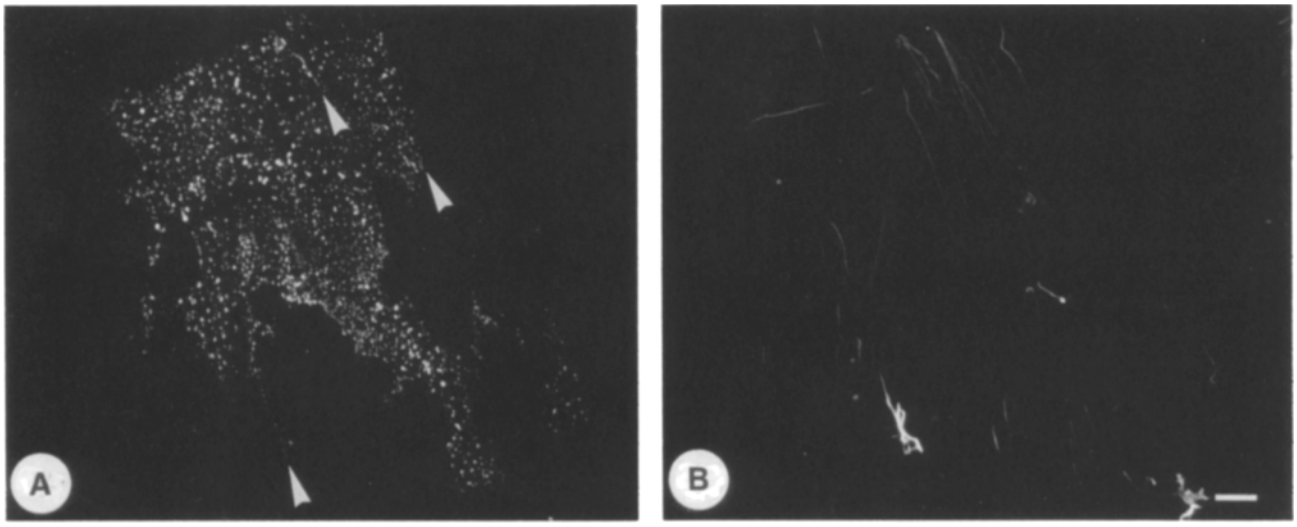


Figure 3. Immunofluorescence microscopy of unroofed human fibroblasts. The dorsal plasma membrane and most of the cytoplasm were removed and the remaining basal half of the cell was doubly labeled for the PM Ca^{2+} pump (*A*) and F-actin (*B*). Scarcity of F-actin indicates that the unroofing procedure was done successfully. The labeling for the PM Ca^{2+} pump is clearly observed as numerous small dots; some of them seem to be aligned along the remaining F-actin (*arrowheads*). Bar, 10 μm.

gen blot on nitrocellulose filter (Fig. 2 *B*), or when preimmune IgG was used (photograph not shown). The punctate labeling for the PM Ca^{2+} pump was also seen in bovine aortic endothelial cells (Fig. 2 *C*) and mouse BALB/c 3T3 cells (Fig. 2 *D*), but the labeling in linear arrays as observed in human fibroblasts was less frequent.

Localization of labeling for the PM Ca^{2+} pump in the plasma membrane was shown by using an unroofed specimen of human fibroblasts. A nitrocellulose filter was adhered to the dorsal surface of cultured cells and then pulled apart. When performed successfully, the dorsal plasma membrane and most of the cytoplasm were removed, with only the basal

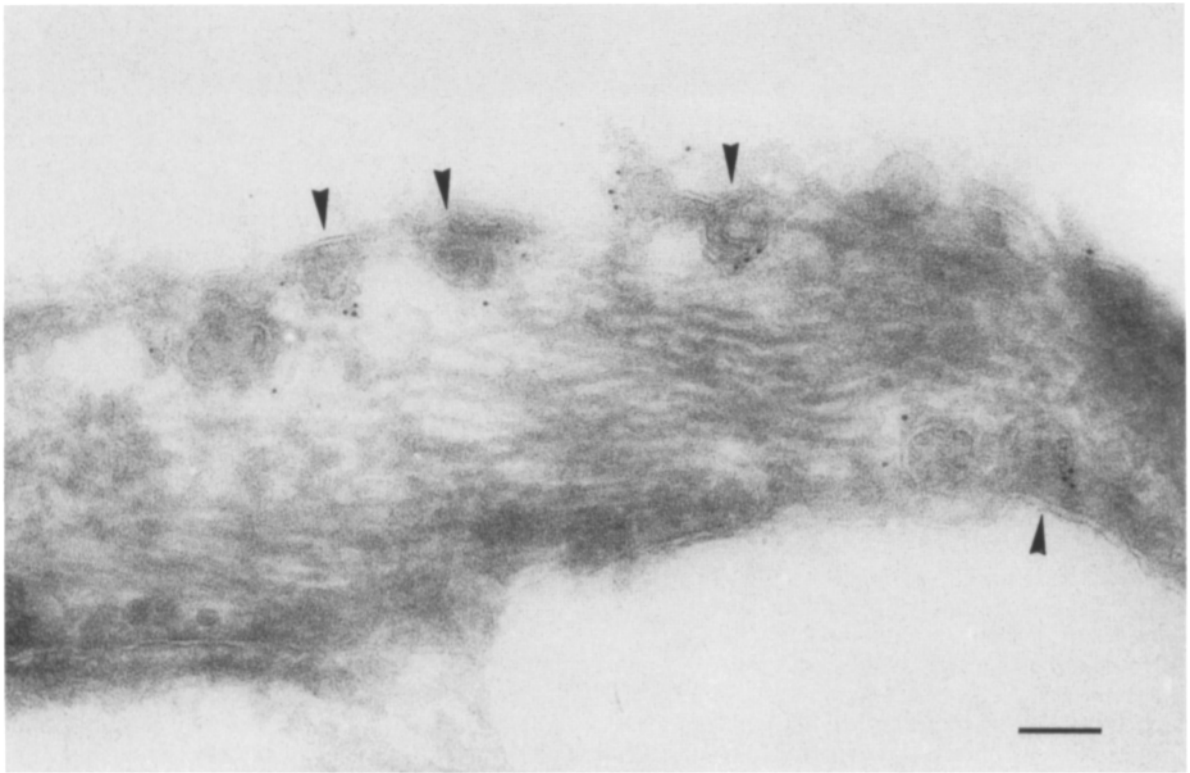


Figure 4. Immunogold EM of human fibroblasts in culture by ultrathin cryosectioning. Immunogolds are found to be concentrated around caveolae of the plasma membrane (*arrowheads*). Bar, 100 nm.

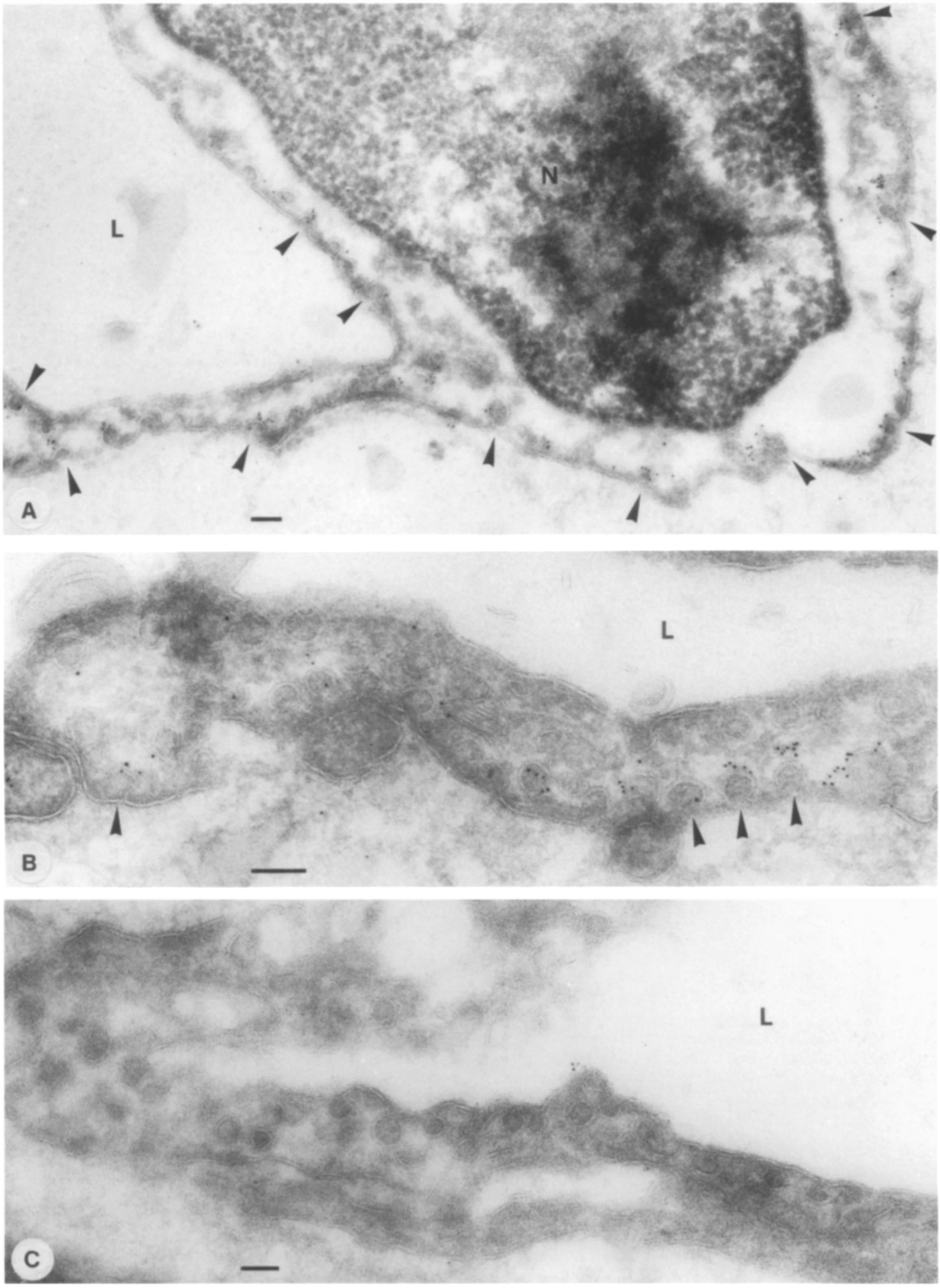


Figure 5. Immunogold EM of ultrathin cryosections. Mouse capillary endothelial cells of the heart. Labeling was done with the anti-Ca^{2+}-ATPase antibody (*A* and *B*) and normal rabbit IgG (*C*). In the endothelium, immunogolds were mostly localized in the caveola of the plasma membrane (*arrowheads*) and in the cytoplasmic vesicles. The luminal and the abluminal caveolae were both labeled (*A*), but in some cells labeling occurred more intensely either in the luminal (photograph not shown) or the abluminal caveolae (*B*) than those of the other surface. Normal rabbit IgG gave virtually no labeling (*C*). *L*, capillary lumen; *N*, nucleus. Bars, 100 nm.

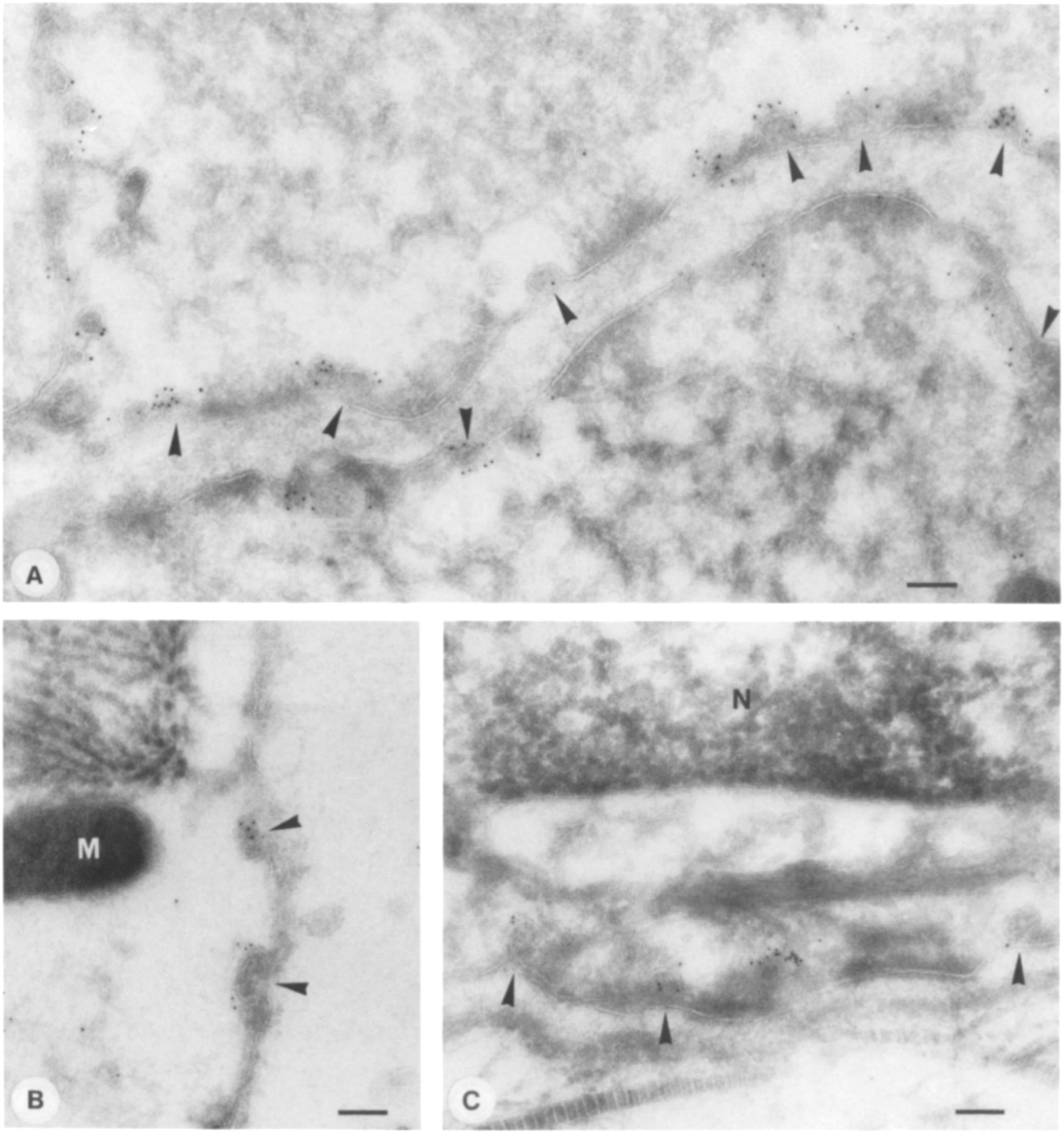
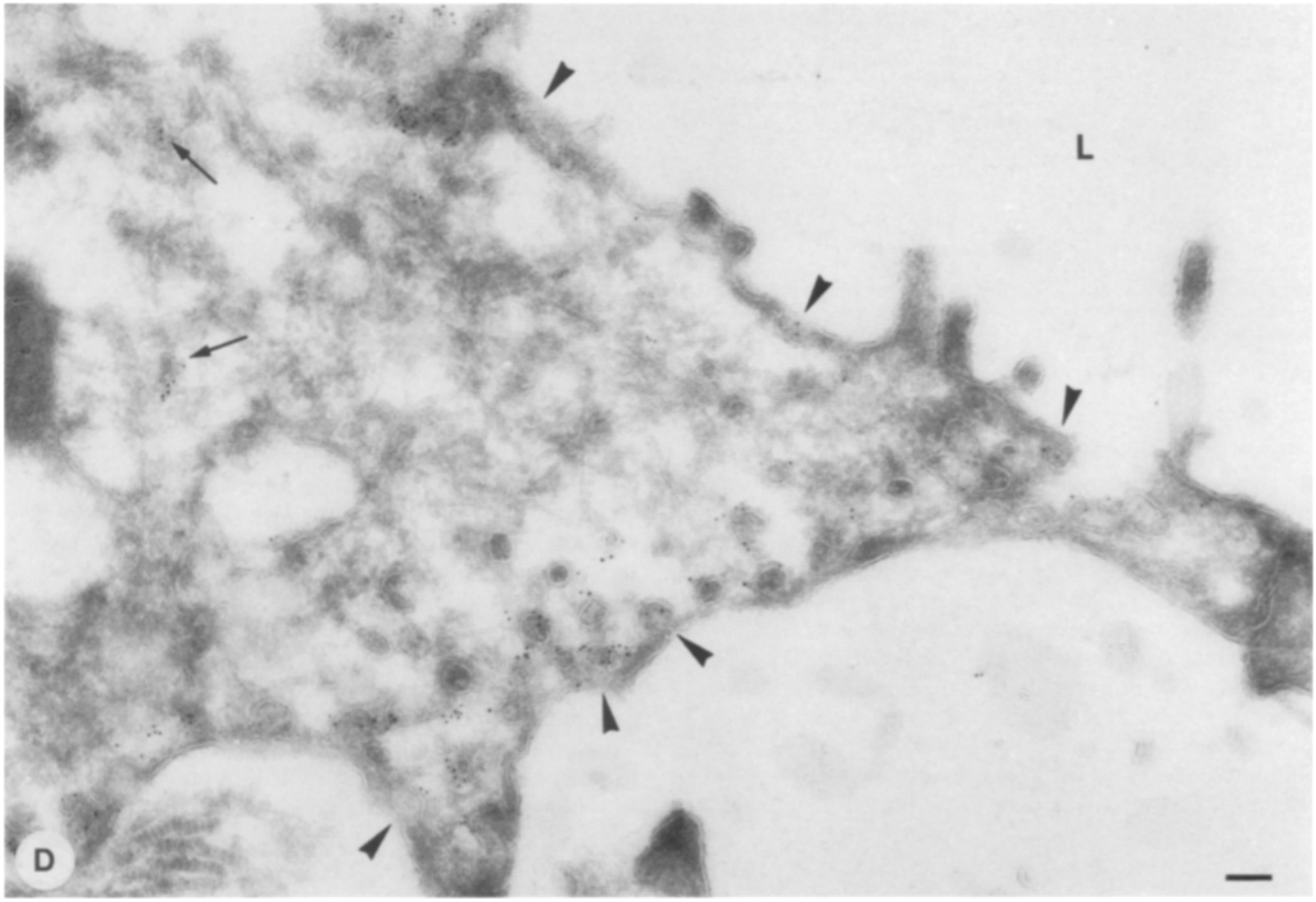


Figure 6. Immunoelectron microscopy of various caveola-rich cells of the mouse. (*A*) Smooth muscle cell of the small intestine; (*B*) cardiac muscle cells; (*C*) epidermal keratinocytes; and (*D*) peritoneal mesothelial cells. In all the specimens, the caveola (*arrowheads*) was labeled for the PM Ca^{2+} pump, whereas the noncaveolar region of the plasma membrane was virtually devoid of the labeling. In mesothelial cells, small vesicles in the deep cytoplasm were also labeled (*arrows*). *M*, mitochondrion; *N*, nucleus; *L*, peritoneal lumen. Bars, 100 nm.

plasma membrane and a small number of cytoskeletal components remaining on the substrate (Fujimoto et al., 1991). Marked reduction of F-actin visualized by rhodamine–phalloidin indicated that the cytoplasm was largely eliminated by the unroofing procedure (Fig. 3 *B*). In such specimens, the labeling for the PM Ca^{2+} pump was observed as dots in a single focal plane, which most likely represents the adhering basal plasma membrane (Fig. 3 *A*). Some of the labeled dots were apparently aligned along the remaining actin filaments.

In ultrathin cryosections of the human fibroblast, immunolabeling was concentrated beneath the plasma membrane, and observed around caveolae, or smooth uncoated invagina-



tion of the plasma membrane (Fig. 4). The results indicated that dots seen by immunofluorescence microscopy most likely correspond to caveolae.

Immunogold EM of Cells In Vivo

Fine localization of the PM Ca^{2+} pump in mouse tissues in vivo was also examined by immunogold EM of ultrathin cryosections. In several tissues studied, vascular endothelial cells showed intense labeling with the antibody. In capillary endothelial cells, the labeling was mostly localized in the caveola of the luminal and abluminal surfaces as well as in apparent free cytoplasmic vesicles, whereas the remaining portion of the plasma membrane was scarcely labeled (Fig. 5 *A*). (Most of the vesicles probably adhere to the cell surface in reality and thus are the same structure as caveola (Frokjaer-Jensen, 1980; Bundgaard et al., 1983), but in this paper, only those membrane profiles clearly continuous with the plasma membrane will be described as caveolae.) In some specimens, the luminal or abluminal caveolae were labeled more densely than the other group (Fig. 5 *B*), which may imply heterogeneity of caveolae and a preferential distribution of caveolae bearing the PM Ca^{2+} pump along one surface of the endothelium under some as of yet unknown condition. Endothelial cells of larger vessels were also labeled almost exclusively in the caveolae and vesicles in the cytoplasm (photograph not shown). Control specimens treated with normal nonimmune rabbit IgG instead of the anti-PM Ca^{2+} pump antibody showed only a low background level of gold particles (Fig. 5 *C*).

Smooth muscle cells of the small intestinal wall were also labeled specifically in the caveolar portion (Fig. 6 *A*), as were those of the uterus and the blood vessels (photograph not shown). In this cell type as well, the continuity of the membrane was important in the identification of caveolae, because the sarcoplasmic reticulum may exist closely apposed to the plasma membrane (Gabella, 1981). Quantitative analysis of the immunolabeling result showed that in capillary endothelial cells and intestinal smooth muscle cells, 70–90% of caveolae are positive for the PM Ca^{2+} pump and, moreover, that the labeling is 18–25 times more concentrated in the caveolar portion than in the noncaveolar portion of the plasma membrane (Table I). The labeling in apparent vesicles of endothelial cells was not counted in the above quantification, and the concentration of the PM Ca^{2+} pump in the caveola should be more than the above figure if the vesicles are actually connected to the plasma membrane.

Similar caveolar accumulation of the PM Ca^{2+} pump immunolabeling was seen in other kinds of cells which exhibit many caveolae in the plasma membrane; cells examined include cardiac muscle cells (Fig. 6 *B*), epidermal keratinocytes (Fig. 6 *C*), peritoneal mesothelial cells (Fig. 6 *D*), and fibroblasts in the dermal connective tissue (photograph not shown). In mesothelial cells, in addition to caveolae attached

Table I. Quantitative Analysis of Immunogold Labeling for PM Ca^{2+} Pump

	Number of gold particles per μm of the plasma membrane*			Percentage of caveola labeled§	Total number of gold particles counted	Total length of membrane measured	Number of micrographs used
	Caveola‡ (A)	Noncaveola‡ (B)	A/B				
						μm	
Capillary endothelium	13.82	0.54	25.59	70.7	862	115.4	10
Smooth muscle cell	26.58	1.45	18.33	90.9	1431	122.6	10

* Length of the plasma membrane with distinct trilaminar profile was measured for the caveolar and noncaveolar portion of the plasma membrane and the number of immunogold particles per unit length of the respective membrane was calculated.
‡ Results significantly different at the 0.005% level by *t* test.
§ Percentage of caveolae labeled with more than one gold particle.

to the apical and basal plasma membranes, vesicles deep in the cytoplasm were labeled for the PM Ca^{2+} pump, but it is not known whether the cytoplasmic vesicles in this type of cell are connected to the surface membrane as in endothelial cells (Frokjaer-Jensen, 1980; Bundgaard et al., 1983).

Epithelial cells of the renal distal convoluted tubule and the small intestine have been known to possess the PM Ca^{2+} pump in the basolateral plasma membrane (Nellans and Popovitch, 1981; Borke et al., 1989). The cells were examined by the same procedure as the caveola-rich cells described above. As a result, the labeling for the PM Ca^{2+} pump was found to be distributed mostly in a random manner along the basolateral plasma membrane of the epithelial cells (Fig. 7). Caveolae were observed only rarely in these cells, but when present they too were labeled positively.

In all the cells examined, a majority of the labeling in the caveola was observed along the cytoplasmic surface of the plasma membrane, and relatively few gold particles were seen on the exoplasmic side (Figs. 4–6). This distribution is consistent with the molecular structure of the PM Ca^{2+} pump; it traverses the lipid bilayer multiple times but most of the molecular mass is thought to exist in the cytoplasm (Carafoli, 1991; Strehler, 1991; Wang et al., 1992). By contrast, the immunogolds distributed along the basolateral plasma membrane of the renal tubular epithelium (Fig. 7) and the intestinal epithelium (photograph not shown) were

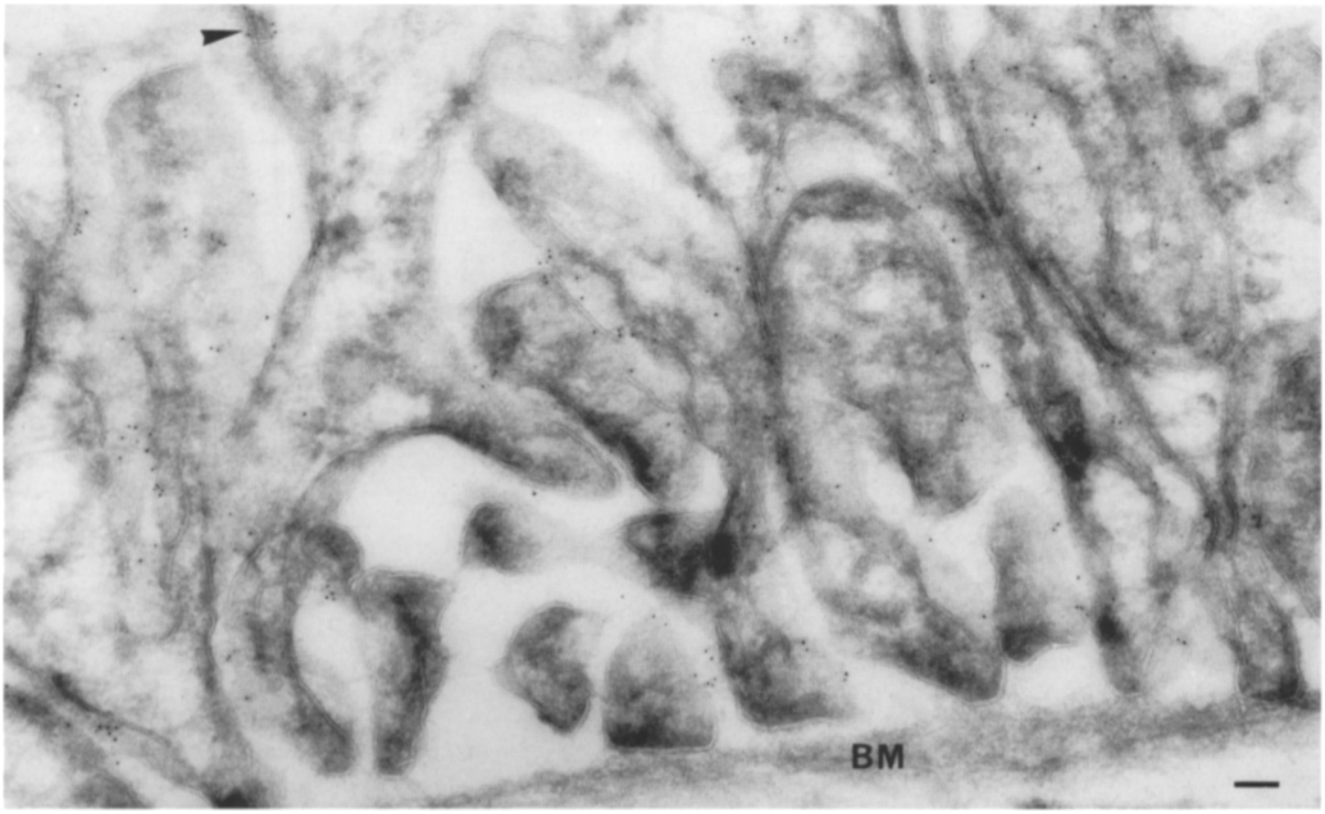


Figure 7. Immunoelectron microscopy of the distal convoluted tubular epithelium of the mouse kidney. The plasma membrane of the basal infolding was intensely labeled for the PM Ca^{2+} pump. Most of the immunogold labeling was distributed randomly along the membrane, but caveolae (*arrowhead*) seen only infrequently in this cell were also labeled. *BM*, basement membrane. Bar, 100 nm.

seen frequently on the exoplasmic surface. The implication of this discrepancy is not clear at present, but it might reflect some molecular difference in the PM Ca^{2+} pump in the caveolar vs. noncaveolar plasma membrane.

Discussion

Biochemical and immunohistochemical studies at the light microscopic level have shown that the plasma membrane of several Ca^{2+} transporting epithelia is not homogeneous in the content of PM Ca^{2+} pump; some domains of the plasma membrane seem to have a much greater amount of the protein than other domains. The domains hitherto reported to contain abundant amounts of PM Ca^{2+} pump are the basolateral surface of small intestinal (Nellans and Popovitch, 1981) and renal distal convoluted tubular (Borke et al., 1989) epithelial cells, the sinusoidal surface of hepatocytes (Kessler et al., 1990), and the apical surface of the oviduct epithelial cells of the laying hen (Wasserman et al., 1991). Considering the physiological function of these epithelia, the polarized distribution of the PM Ca^{2+} pump most likely reflects a voluminous transport of Ca^{2+} toward the PM Ca^{2+} pump-rich surface. It is especially evident in oviduct epithelial cells of the laying hen which needs a large amount of Ca^{2+} for eggshell formation, but the PM Ca^{2+} pump exists in virtually every kind of cell. In cells not related to the extensive Ca^{2+} transport, the amount of extruded Ca^{2+} should be far smaller than in the Ca^{2+} transporting epithelium, but the PM Ca^{2+} pump is nevertheless thought to be critical in the maintenance of the low intracellular Ca^{2+} concentration (Carafoli, 1987).

In the present cytochemical study, we found that in fibroblasts, endothelial cells, smooth muscle cells, cardiac muscle cells, keratinocytes, and mesothelial cells, the PM Ca^{2+} pump is preferentially localized in the caveola, or uncoated plasma membrane invagination. It did not show the polarized distribution as observed in the transporting epithelium. The existence of caveolae has been known for many years (Yamada, 1955), but their physiological significance has remained elusive to date (Severs, 1988; Anderson, 1991). Several different functions, such as a membrane reservoir for stretch, pinocytosis, a part of the T system, and Ca^{2+} extrusion, have been ascribed to caveolae (Masson-Pevet et al., 1980; Severs, 1988). These presumptive functions seem to fit well with caveolae in some kinds of cells but not in others. For example, caveolae in smooth muscle cells appear immobile and not involved in pinocytosis (Gabella, 1981), while in other kinds of cells, it is more than likely that some substances are endocytosed through uncoated invaginations (Goldberg et al., 1987; Rothberg et al., 1990). Hence it is generally thought that, although appearing similar in profile in thin section electron micrographs, caveolar function may be variable across different kinds of cells.

Concerning the caveola in capillary endothelial cells, there have been two conflicting views about its nature. A prevailing theory is that caveolae, often called plasmalemmal vesicles in this cell, are engaged in transcytosis (Simionescu, 1983; Palade, 1988). Numerous tracer studies have shown that blood-borne molecules appear first in caveolae of the luminal surface, then in the cytoplasmic vesicles, and finally in caveolae of the abluminal surface as well as in the subendothelial space. Presence of specific binding sites for albumin indicates that endothelial caveolae are related to receptor-mediated endocytosis (Ghitescu et al., 1986; Milici et al., 1987). Conversely, serial ultrathin sectioning studies have shown that almost all apparent free vesicles in capillary endothelial cells are actually caveolae with a limiting membrane that is continuous with the plasma membrane (Frokjaer-Jensen, 1980; Bundgaard et al., 1983). Close apposition of the caveola and ER has also been observed (Bundgaard, 1991). Based on these findings, it has been argued that endothelial caveolae are very similar to those in smooth muscle cells and are not likely engaged in pinocytosis; it has been proposed alternatively that the caveola is involved in the regulation of the cytosolic free Ca^{2+} concentration (Crone, 1986; Bundgaard, 1991). The presence of the two proteins related to transmembrane Ca^{2+} transport, the PM Ca^{2+} pump and a 240-kD protein recognized by a mAb to the cerebellar $InsP_3$ receptor (Fujimoto et al., 1992), evidently supports the latter contention in the above controversy. On the other hand, it does not refute the possibility that endothelial caveolae, or plasmalemmal vesicles, also perform other important functions. For example, Ca^{2+} influx and extrusion mediated by the two molecules may be induced through activation of receptors localized in the caveolar membrane, and may be related to transcytotic movement of their ligands.

In capillary endothelial cells, more than 70% of the caveolae were positive for the PM Ca^{2+} pump, and labeling for the 240-kD $InsP_3$ receptor-like protein was also found in a majority of them (Fujimoto et al., 1992). Thus it can be safely said that at least some caveolae express both proteins simultaneously. This also applies to smooth muscle cells in which the percentage of positively labeled caveolae is even higher for the two proteins than it is in endothelial cells. The coexistence of a Ca^{2+} channel for discharge (or influx) and a Ca^{2+} pump for removal (or extrusion) is reminiscent of the sarcoplasmic reticulum and ER, which are thought to be the intracellular Ca^{2+} stores (Meldolesi et al., 1990). If the analogy with the sarcoplasmic reticulum goes further, the caveola may also contain a functional analogue of calsequestrin, a low affinity/high capacity Ca^{2+}-binding protein (MacLennan and Wong, 1971), since it is logical in terms of physiological efficiency to have a Ca^{2+} buffer in its lumen. Consistent with this assumption is the report that Ca^{2+} exists abundantly in the caveola of smooth muscle cells (Popescu, 1974; Suzuki and Sugi, 1989). However, the molecular mechanism responsible for the accumulation has not been elucidated. It will be interesting to see if the distribution of plasmalemmal Ca^{2+}-binding proteins, such as glycoproteins isolated from the cardiac sarcolemma (Michalak et al., 1990), is related to the caveola.

Coexistence of the PM Ca^{2+} pump and $InsP_3$ receptor-like protein may be important in coordinating their activity through the inositol phospholipid signaling pathway. After receptor activation, $InsP_3$ and diacylglycerol are generated through hydrolysis of phosphatidylinositol 1,4-bisphosphate by phospholipase C. $InsP_3$ and/or its metabolites are likely to open the plasmalemmal Ca^{2+} channel (Kuno and Gardner, 1987; Irvine, 1990; Restrepo et al., 1990; Luckhoff and Clapham, 1992). On the other hand, the breakdown of phosphatidylinositol 1,4-bisphosphate (Ronner et al., 1977) and the increase of $InsP_3$ (Kuo and Tsang, 1988; Davis et al., 1991) are both inhibitory to the PM Ca^{2+} pump activity. Due to the copresence of the PM Ca^{2+} pump and the $InsP_3$

receptor-like protein, inositol phosphates can stimulate Ca^{2+} entry and inhibit its extrusion simultaneously to effectively induce a rise of intracellular Ca^{2+}. Another important consequence likely caused by caveolar accumulation of the two Ca^{2+} transporting proteins is that the Ca^{2+} concentration in its vicinity may change more drastically than other regions of the cytoplasm. Other plasmalemmal Ca^{2+} transporting proteins may have a different distribution (Frank et al., 1992) and thus it is hard to predict the magnitude of regional differences in the Ca^{2+} concentration. But if it occurs, as postulated for synaptic vesicles (Kelly, 1992), it might be related to the fission/fusion process between the caveola and the plasma membrane in potocytosis (Anderson et al., 1992).

A question remaining to be answered is whether all the caveolae in a cell are of the same kind. Although there were always some caveolae which were not labeled positively for the PM Ca^{2+} pump or the 240-kD $InsP_3$ receptor-like protein by immunoelectron microscopy, uneven penetration of the antibodies leading to false negative reaction cannot be ruled out. The possible diversity of caveolae, however, may be recognizable as a variation in the characteristic ridge or stripe-like ultrastructure seen on its cytoplasmic surface (Somlyo et al., 1971; Peters et al., 1985; Izumi et al., 1989; Anderson, 1991; Rothberg et al., 1992). Based on the cloned cDNA structure, not only the PM Ca^{2+} pump (Carafoli, 1991; Strehler, 1991; Wang et al., 1992) but also the 240-kD $InsP_3$ receptor-like protein, if the latter is structurally similar to the cerebellar type I $InsP_3$ receptor (Furuichi et al., 1989), should have the large cytoplasmic mass, which could explain the ridge-like structure of the caveolar membrane. Moreover, because the two proteins differ considerably in size (140 vs. 240 kD), some visible differences in the macromolecular structure may exist between them. Variation of patterns made by ridges in the caveolar membrane (Rothberg et al., 1992) is interesting in this context.

The present findings may be also relevant to pathological conditions in which changes have been known to occur in the caveola. In spontaneously hypertensive rats, an increased density of caveolae has been observed in capillary endothelial cells (Hazama et al., 1979) and cardiac muscle cells (Goto et al., 1990). The phenomenon has been generally taken to indicate that pinocytosis is enhanced in those cells. But if the caveola is an organelle related to regulation of the intracellular Ca^{2+} concentration, it is probably related to abnormalities of Ca^{2+} metabolism in the disease; in fact, an increase of the intracellular Ca^{2+} concentration and defective plasmalemmal Ca^{2+} transport have been reported in various cells of hypertensive animals (Postnov and Orlov, 1980; Orlov et al., 1983; Resink et al., 1986). It is not clear whether the increase of caveolae is a result or a cause of the diseased state, but it certainly influences the Ca^{2+} homeostasis in affected cells and might be related to progression and/or clinical manifestations of the illness.

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