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Hepatic stellate cells express Ca^{2+} pump-ATPase and Ca^{2+}-Mg^{2+}-ATPase in plasma membrane of caveolae

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Abstract: Intracytoplasmic free calcium ions (Ca^{2+}) are maintained at a very low concentration in mammalian tissue by the extrusion of Ca^{2+} across a steep extracellular Ca^{2+} gradient, mainly through the activity of plasma membrane Ca^{2+} pump-ATPase. The present study aimed to identify, by electron cytochemical and electron immunogold methods, the ultrastructural localizations of two types of plasma membrane Ca^{2+}-ATPase; Ca^{2+}-Mg^{2+}-ATPase and Ca^{2+} pump-ATPase, in hepatic stellate cells. Liver tissues and isolated hepatic stellate cells (HSCs) were studied. The ultrastructural localization of Ca^{2+}-Mg^{2+}-ATPase activity was examined by the electron cytochemical method of Ando. The localization of Ca^{2+} pump-ATPase was identified by immunofluorescence. The ultrastructural localization of Ca^{2+} pump-ATPase was identified by the electron immunogold method. The cytochemical reaction products of Ca^{2+}-Mg^{2+}-ATPase activity were localized on the outer (cavity) side of the plasma membrane of caveolae. Immunofluorescence of Ca^{2+} pump-ATPase was seen as small dots along the cell edge in HSCs. Immunogold particles indicating the presence of Ca^{2+} pump-ATPase were identified on the inner (cytoplasmic) side of the plasma membrane of caveolae. We localized Ca^{2+} pump-ATPase on the inner side of the plasma membrane caveolae and Ca^{2+}-Mg^{2+}-ATPase on the outer leaflet of the caveolar plasma membrane in stellate cells, suggesting that Ca^{2+} pump-ATPase may play a key role in the Ca^{2+} reflux.

Key words: hepatic stellate cell, Ca^{2+}-Mg^{2+}-ATPase, Ca^{2+} pump-ATPase, immunofluorescence, ultrastructural localization

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Introduction

The morphology of the hepatic sinusoidal wall resembles that of capillaries, in that the endothelial cells lining the sinusoidal wall correspond to capillary endothelial cells. Hepatic stellate cells (also called Ito cells, and fat-storing cells), which correspond to pericytes around capillaries, are present in the Disse's spaces around the hepatic sinusoids, where a large number of cytoplasmic projections surround the sinusoidal endothelial cells.[1]

The calcium ion (Ca^{2+}) pump located in the plasma membrane extrudes Ca^{2+} from the cytoplasm against a steep concentration gradient and is essential in maintaining intracellular Ca^{2+} homeostasis.[2] Four isoforms of the plasma membrane Ca^{2+} pump (1-4) encoded by a multigene family share up to 85%–90% similarity in amino acid sequence. In addition, several alternatively spliced products are known to be expressed differentially in various cells.[3,4] In immunofluorescence and immunoelectron microscopic studies, the Ca^{2+} pump in capillary endothelial cells and visceral smooth muscle cells has been found to be 18- to 25-fold more con-centrated in the invaginated caveolar membrane compared with the noncaveolar portion of the plasma membrane.[4,5] However, little is known about the ultrastructural localization of the plasma membrane Ca^{2+} pump in hepatic stellate cells.

The present study was designed to elucidate the electron cytochemical expressions of two different types of plasma membrane calcium ion-ATPase; Ca^{2+} pump-ATPase and Ca^{2+}-Mg^{2+}-ATPase, in hepatic stellate cells.

Materials and methods

Experimental animals

Male Wistar rats, weighing 350–400 g, were used in this study. The animals were housed in individual cages and

allowed free access to chow and water until the start of the study.

In-vivo study

Immunohistochemistry for localization of Ca^{2+} pump-ATPase in liver tissue

After they had fasted for 24 h, the rats were anesthetized with sodium pentobarbital (30 mg/kg body weight, i.p.). The liver was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) via a polyethylene catheter placed into the thoracic aorta, at 100 mmHg perfusion pressure. Immediately after the start of perfusion with the fixative, the vena cava was cut above the entry of the hepatic vein in order to maintain a free flow of the fixative throughout the liver. The thoroughly perfused areas were cut into approximately $10 \times 10 \times 10$-mm blocks and further fixed in periodate-lysine-paraformaldehyde (PLP) solution for 6 h, rinsed in 15% sucrose and then in 30% sucrose in phosphate buffer at 4°C for 6 h. Small liver tissue blocks were mounted in OCT compound (Miles, Elkhart, IN, USA), quenched in isopentane, cooled with liquid nitrogen, and stored at −80°C until use. Five-μm frozen sections were cut and mounted on poly-L-lysine-coated glass slides (SuperFrost+; Matsunami Glass, Tokyo, Japan). Cryosections were air-dried for 30 min and rinsed in phosphate buffered saline (PBS) for 5 min and then reacted overnight with a monoclonal antibody against human erythrocyte Ca^{2+} pump-ATPase (Sigma Biosciences, St. Louis, MO, USA) at 4°C and then incubated with 1:100 dilution of horseradish peroxidase-conjugated anti-mouse IgG goat antibody (Cosmo Bio, Tokyo, Japan) at 4°C. After repeated washes with PBS, the sections were reacted with diaminobenzidine solution containing 0.01% H_2O_2. The sections were then counterstained with hematoxylin for light microscopic study.

In-vitro study

Isolation of hepatic stellate cells

Hepatic stellate cells were isolated by the method of Knook et al.[6] After perfusion of the liver with 0.05% collagenase (type 1, Worthington Biochemical, Freefold, NJ, USA) and 0.1% pronase E (Kaken Pharmaceutical, Urayasu, Japan) in Hanks' balanced salt solution (HBSS; Nissui Pharmaceutical, Tokyo, Japan) at 37°C, at a flow rate of 10 ml/min for 15 min, the liver was minced and incubated with HBSS containing both enzymes at 37°C for 30 min. The resultant cells were collected after passage through a nylon mesh. The recovered cells were overlaid on a double-layer of 13% and 18% metrizamide (Sigma Chemical, St. Louis, MO, USA) solutions, and centrifuged at 1400 *g* for 17 min at room temperature. Hepatic stellate cells were collected from the top layer of the 13% metrizamide solution. The isolated hepatic stellate cells were suspended in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical) containing 10% fetal calf serum (FCS; GIBCO Laboratories, Life Technologies, Grand Island, NY, USA), seeded on plastic dishes at a density of 1.25×10^5 cells/cm^2, and incubated for 24 h.

Electron cytochemistry for Ca^{2+}-Mg^{2+}-ATPase in isolated hepatic stellate cells

For electron cytochemistry of plasma membrane Ca^{2+}-Mg^{2+}-ATPase,[7] cells cultured on a glass coverslip were fixed in a mixture of 1% paraformaldehyde and 0.5% glutaraldehyde in 0.1% cacodylate buffer, pH 7.2, for 10 min at 4°C, and were then washed overnight in 0.25 M sucrose with 0.1% cacodylate buffer (pH 7.2) for 10 min at 4°C. Samples were incubated in a basic reaction mixture (250 mM glycine buffer [pH 9.0], 3 mM ATP (Sigma Chemical) as substrate, $CaCl_2$, 2.5 mM levamisole [KW-2-LE-T; Kyowa, Tokyo, Japan], and 2 mM lead citrate) for 30 min at 37°C, rinsed in 0.1 M cacodylate buffer (pH 7.2) containing 0.25 M sucrose, and postfixed with 1% osmic acid solution for 30 to 60 min at 4°C. The sections were then dehydrated through a graded series of ethanol and embedded in Epon. These embedded sections were cut with an LKB ultratome, post-stained with uranyl acetate, and examined under a JEM 1200 EX electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

Immunofluorescence microscopy for Ca^{2+} pump-ATPase in hepatic stellate cells

The cells were fixed in PLP fixative for 30 min and then incubated overnight at 4°C with a monoclonal antibody against human erythrocyte membrane Ca^{2+} pump-ATPase (Sigma Biosciences) in PBS containing 1% normal goat serum (NGS). After being washed by transfer through five successive drops of PBS, the secondary antibody; fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Cosmo Bio) diluted 1/50 in PBS, was transferred through five drops of PBS. The specimens were mounted in a glycerol/mowiol 4-88 (Calbiochem-Novabiochem, La Jolla, CA, USA) mixture in PBS and observed under a Zeiss Axiophoto fluorescence microscope (Carl Zeiss, Thornwood, NY, USA).

Immunoelectron microscopy for Ca^{2+} pump-ATPase in hepatic stellate cells

The procedures were a modification of those described by Berryman and Rodewalde[8] and Ogi et al.[9] Some isolated cultured cells were fixed in PLP for 1 h. After the cells were washed with several changes of cold 3.5% sucrose in 0.1 M phosphate buffer containing 0.5 mM calcium chloride for 2 h, free aldehyde was quenched with 50 mM ammonium chloride in sucrose-phosphate

buffer containing 0.5 mM calcium chloride, pH 7.4, for 1 h at 0°C. To remove phosphate ions, the tissue was rinsed four times (4 × 15 min) with cold 0.1 M maleate buffer (final pH, 6.0) containing 3.5% sucrose, pH 6.5, and was then post-stained for 60 min at 0°C with 2% uranyl acetate in sucrose-maleate buffer (final pH, 6.0). Additional vehicles that were used with 2% uranyl acetate for 30 min at 0°C included distilled water (final pH, 4.0), 0.05 M maleate buffer (final pH, 4.2), and veronal acetate buffer (final pH, 4.2). The tissue was dehydrated for 45 min in 50% acetone, followed by sequential 45-min incubations at −20°C in 70% and 90% acetone, a 60-min incubation in a mixture of LR Gold (London Resin, England): acetone (1:1) at −20°C, 60-min incubation in LR Gold: acetone (7:3) at −20°C, two changes (60 min, then overnight) of 100% LR Gold at −20°C, and finally two changes (60 min, then overnight) of 100% LR Gold plus 0.5% initiator

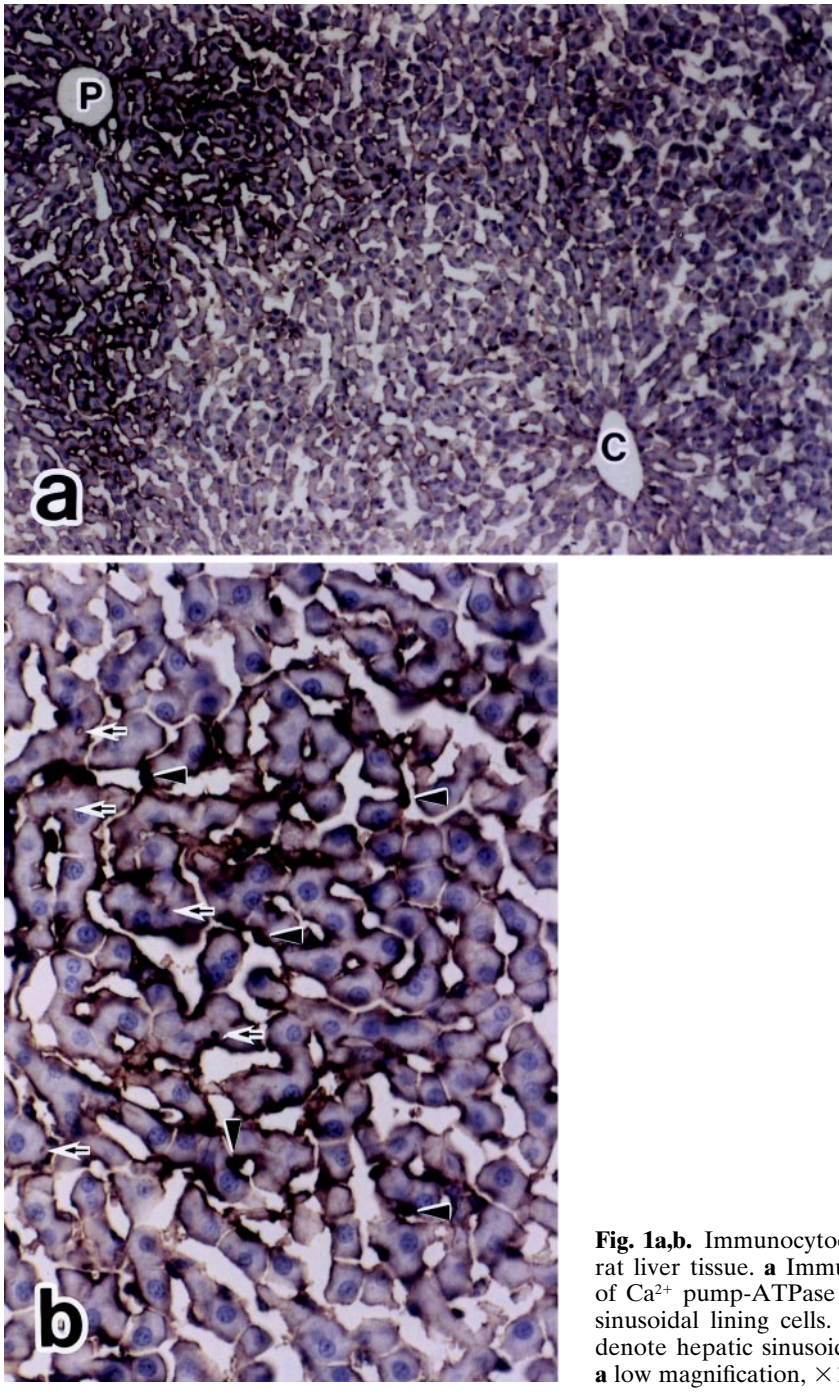

Fig. 1a,b. Immunocytochemical localization of Ca^{2+} pump-ATPase in rat liver tissue. **a** Immunopositive substances indicating the presence of Ca^{2+} pump-ATPase are located on the bile canaliculi and hepatic sinusoidal lining cells. **b** *Arrowheads* denote bile canaliculi. *Arrows* denote hepatic sinusoidal lining cells. *P*, portal vein; *C*, central vein. **a** low magnification, ×100; **b** high magnification, ×400

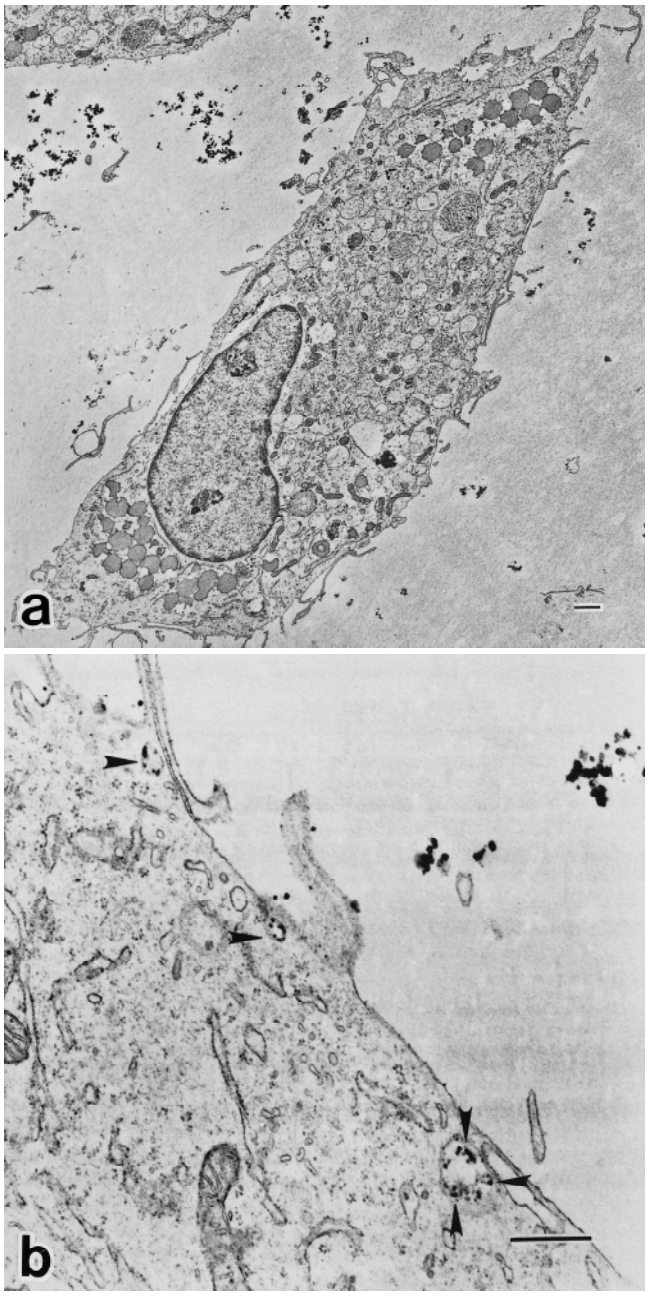

Fig. 2a,b. Electron cytochemical expressions of Ca^{2+}-Mg^{2+}-ATPase in isolated hepatic stellate cells. **a** The cytochemical reaction products of Ca^{2+}-Mg^{2+}-ATPase activity are localized on the outer surface (cavity side) of the plasma membrane of caveolae. **b** *Arrowheads* denote reaction products of Ca^{2+}-Mg^{2+}-ATPase. Counterstained with lead citrate. **a** low magnification; **b** high magnification. Bars denote 1 μm

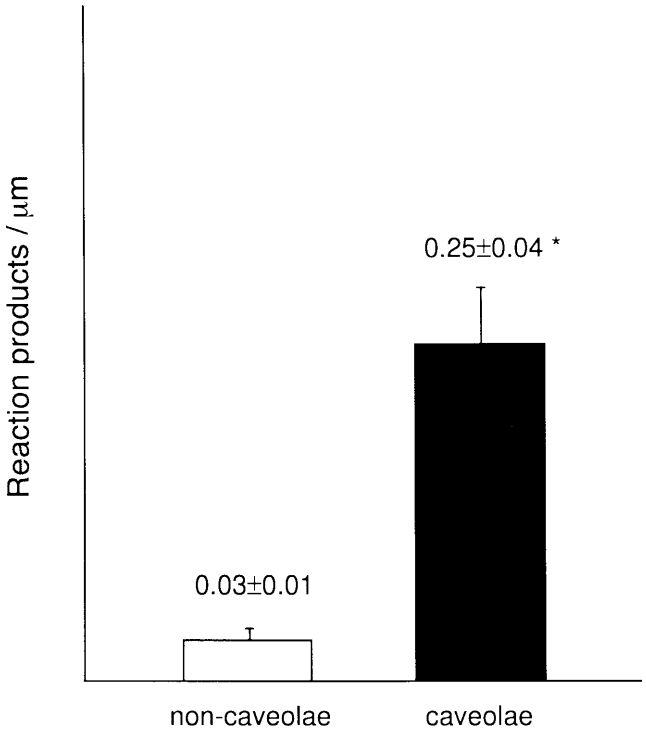

Fig. 3. Quantitative analysis of Ca^{2+}-ATPase. The reaction product is mainly localized in caveolae. $n = 7$; $*P < 0.001$

(benzoin ether or benzoin methyl ether) at −20°C. Samples were embedded in fresh LR Gold resin plus initiator in BEEM capsules and were polymerized at a wavelength of 366 nm (DSK ultraviolet polymerizer, TUV-200; Dosaka EM, Osaka, Japan) for 24 to 30 h at −20°C. Specimen blocks were stored at room temperature. Silver sections were mounted on 400-mesh nickel grids and were treated for 5 min with Tris-buffered saline (TBS; 50 mM Tris, 150 mM sodium chloride, pH 7.4). Sections were then blocked for 10 min with 5% normal goat serum (NGS) diluted in TBS. The grids were incubated overnight at 4°C with the primary antibody (monoclonal antibody against human erythrocyte membrane Ca^{2+} pump-ATPase; Sigma Biosciences) containing 1% NGS and then washed by transfer through five successive drops of TBS containing 1% NGS (3 min per drop). For the secondary antibody, 5-nm colloidal gold-conjugated anti-mouse IgG antibody (Cosmo Bio) diluted 1/50 in TBS was transferred to the grid through five successive drops of TBS (3 min per drop), and then rinsed with water.[9] Antibody complexes were stabilized with 2% aqueous glutaraldehyde for 5 min. After being rinsed with water, the specimens were stained for 15 min in 2% aqueous osmium tetroxide, rinsed with water again, and then examined under the JEM 1200 EX electron microscope at an accelerating voltage of 80 kV.

Quantitative analysis

Ca^{2+}-ATPase

Caveolae-associated and non-caveolae-associated Ca^{2+}-ATPase activities on each electron micrograph were quantitated using an image analyzer (Olympus high

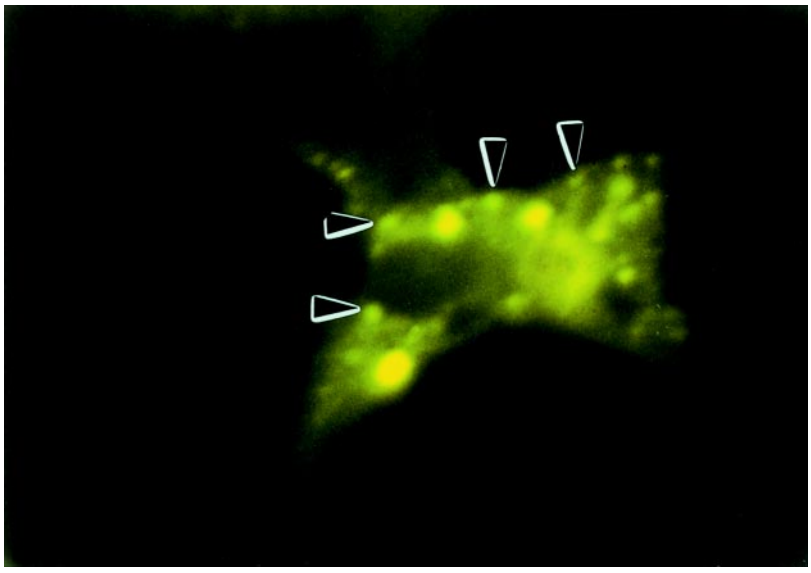

Fig. 4. Immunofluorescent detection of Ca^{2+} pump-ATPase in isolated hepatic stellate cells. **a** Reaction with the anti-Ca^{2+} pump-ATPase is seen as small dots (*green fluorescence*; *arrowleads*) around hepatic stellate cells. **b** *Yellow fluorescence* (*arrowheads*) denotes vitamin A autofluorescence. $\times 600$

speed color image analyzer SP 500; Olympus, Tokyo, Japan) by tracing the total length (A) of caveolar or non-caveolar plasma membranes and the total area (B) of the cytochemical reaction products of Ca^{2+}-ATPase activity inside or outside the caveolae. Ca^{2+}-ATPase activity was expressed as a percentage (B/A).

Ca^{2+} pump-ATPase

Immunogold labeling in ultrathin sections of hepatic stellate cells was quantitated using a Mac measure program, version 1.61. The caveolar or non-caveolar plasma membrane was selected, and the number of gold particles per unit length of the membrane was counted.

The statistical significance of differences between two groups was assessed with Student's *t*-test, and $P < 0.05$ was regarded as indicating a significant difference. Data values are expressed as means ± SEM.

Results

In-vivo study (liver tissue)

Immunocytochemical expressions of Ca^{2+} pump-ATPase protein. Immunoperoxidase-positive substances showing the presence of Ca^{2+} pump-ATPase were located on the hepatic sinusoidal lining cells in the periportal area (zone 1) in rat liver (Fig. 1a,b).

In-vitro study (isolated hepatic stellate cells)

Electron cytochemical expressions of $Ca^{2+}Mg^{2+}$-ATPase. By electron cytochemistry, electron-dense cytochemical reaction products showing the presence of Ca^{2+}-Mg^{2+}-ATPase activity were clearly demonstrated on the outer (cavityside) surface of the plasma membranes of caveolae in isolated stellate cells (Fig. 2a,b). Quantitative analysis showed that Ca^{2+}-ATPase was mainly localized in caveolae (Fig. 3).

Immunofluorescence microscopy. Immunofluorescent reaction with the anti-Ca^{2+} pump-ATPase antibody was seen as small fluorescent dots around the hepatic stellate cells (Fig. 4).

Immunogold electron microscopic localizations of Ca^{2+} pump-ATPase. By immunogold electron microscopy, electron-dense particles showing the presence of Ca^{2+} pump-ATPase were mainly located on the inner (cytoplasmic) side of the plasma membrane of caveolae (Fig. 5a,b). Morphometric analysis of immunogold particle labeling for plasma membrane showed that Ca^{2+} pump ATPase was localized in caveolae (Fig. 6).

Discussion

Caveolae have been proposed to be involved in the regulation of intracytoplasmic free Ca^{2+} concentration.[4] As shown in the present study, not only Ca^{2+} pump-ATPase but also Ca^{2+}-Mg^{2+}-ATPase is localized on the membrane of caveolae in hepatic stellate cells. Previous enzyme histochemical studies demonstrated the presence of Ca^{2+}-dependent ATP-hydrolyzing activity in the caveolae of endothelial cells and smooth muscle cells,[10] but the technique used was not specific to plasma membrane Ca^{2+}-Mg^{2+}-ATPase, and likely also detected nonspecific ecto-ATPase.[11–13]

Canalicular membrane-associated liver ecto-ATPase has been implicated in bile secretion.[14] Demonstration of canalicular $Ca^{2+}Mg^{2+}$-ATPase was reported to be unaffected by the presence of glutaraldehyde.[7]

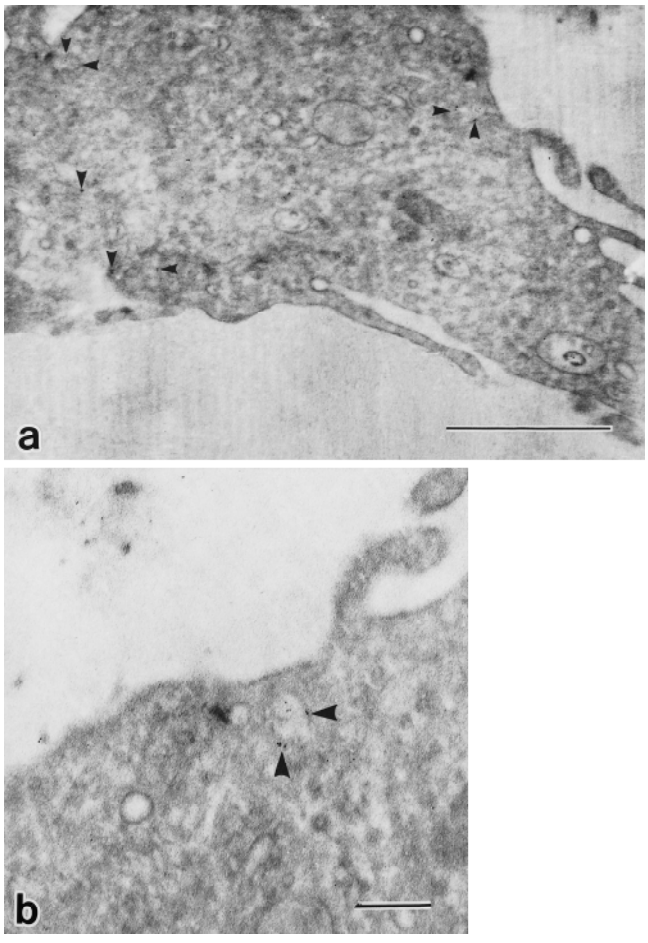

Fig. 5a,b. Immunogold electron micrographs showing the ultrastructual localizations of Ca^{2+} pump-ATPase in isolated hepatic stellate cells. Ultrastructual localization of Ca^{2+} pump-ATPase showing that Ca^{2+} pump-ATPase is mainly localized on the inner side of the plasma membrane of caveolae (cytoplasmic side). *Arrowheads* denote the presence of Ca^{2+} pump-ATPase. Uranyl acetate en-bloc-stained. **a** Low magnification, the *bar* denotes 1 μm; **b** high magnification, *bar* denotes 200 nm

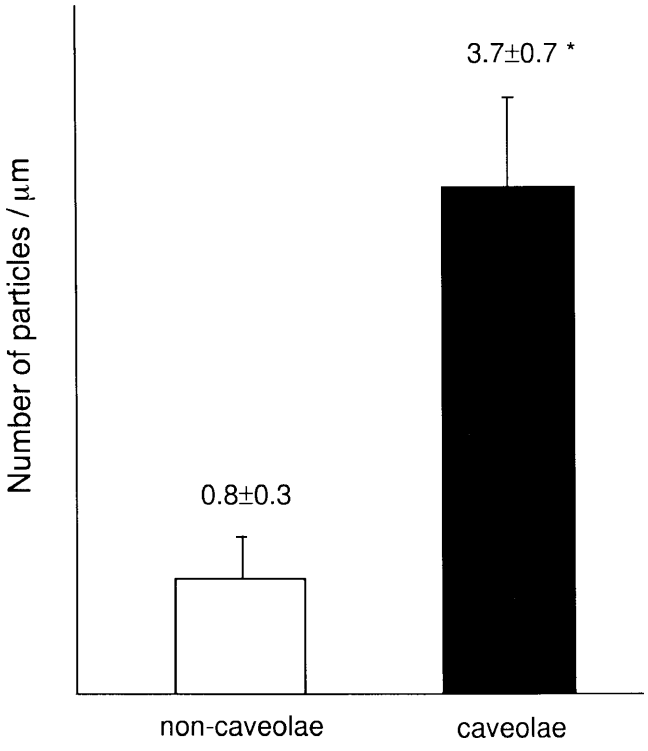

Fig. 6. Morphometric analysis of immunogold particle labeling for plasma membrane. Ca^{2+} pump-ATPase is localized in caveolae. $n = 6$; $*P < 0.01$

We used a fixative containing 0.5% glutaraldehyde. In our study, the reaction product of $Ca^{2+}Mg^{2+}$-ATPase formed by the Ando one-step method becomes invisible in the presence of large amounts of glutaraldehyde. By shortening the incubation time of isolated stellate cell sections in the substrate, we were able to prove conclusively the precise location of this enzyme activity on the caveolar membrane. We suggest that this $Ca^{2+}Mg^{2+}$-ATPase is different in function from the ecto-ATPase.

In the present study, immunogold electron microscopy of cryosections, using monoclonal antibody against Ca^{2+} pump-ATPase of erythrocyte plasma membrane revealed that Ca^{2+} pump-ATPase was present on the inner (cytoplasmic) side of the caveolae of the plasma membrane. We employed a modification[9] of the immunogold method of Berryman and Rodewalde,[8] because it is superior to most previous methods in providing good preservation of membrane ultrastructure and retention of membrane phospholipid. In the present study, using a modification of this method[8] with periodate-lysine-paraformaldehyde, we achieved good preservation of the ultrastructure of the plasma membrane, providing evidence that Ca^{2+} pump-ATPase is located on the cytoplasmic side of the caveolar plasma membrane. It is noteworthy that the present electron cytochemical study (TEM using a modification of the Ando one-step method) not only localized Ca^{2+}-Mg^{2+}-ATPase on the caveolar membrane of hepatic stellate cells but also clealy demonstrated its presence on the outer (cavity) side of the plasma membrane. There are some possible reasons why electron cytochemical studies so far have not demonstrated Ca^{2+}-Mg^{2+}-ATPase activity on the membrane of the caveolar membrane. As shown in this study, Ca^{2+}-Mg^{2+}-ATPase activity in the stellate cell plasma membrane is much lower than that in the bile canalicular membrane.[13] Because the fixative contains 0.5% glutaraldehyde, the reaction product of $Ca^{2+}Mg^{2+}$-ATPase formed by the Ando one-step method becomes invisible in the presence of large amounts of glutaraldehyde. By shortening the incubation time of isolated

hepatic stellate sections in the substrate, we were able to prove conclusively the precise location of this enzyme activity on the caveolar membrane, whereas Ca^{2+} pump-ATPase was demonstrated on the inner side of the caveolar membrane (cytoplasmic side). It is well documented that plasma membrane Ca^{2+} pump-ATPase plays a crucial role in maintaining a very low intracellular free Ca^{2+} concentration against a high concentration of extracellular free Ca,$^{2+}$ through the extrusion of intracellular free Ca^{2+}.[4] It is possible that cytochemical Ca^{2+}-Mg^{2+}-ATPase activities may function in parallel with the Ca^{2+} pump-ATPase activity related to the active extrusion of Ca^{2+} from the cytoplasm, because the structure of Ca^{2+} pump-ATPase is of the transmembraneous type.[15] The structural and functional relation between the two enzymes remain to be further clarified.

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