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ORIGINAL PAPER

Ocadaic Acid Retains Caveolae in Multicaveolar Clusters

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Abstract Caveola-mediated endocytosis exists parallel to other forms of endocytosis. Being ligand-triggered, caveolar endocytosis provides a more selective and highly regulated way for uptake of specified substances. Internalized caveolae accumulate in intermediate organelles called caveosomes. It is still debated whether caveosomes are independent organelles or the downstream caveosomes interact with the classical endocytotic compartments. In our work caveola internalization was stimulated with a serine/threonine phosphatase (PP1 and PP2A) inhibitor (ocadaic acid—OA). To find out whether caveolar clusters are really independent organelles or they are still connected to the cell surface we used an electron dense surface marker, ruthenium red (Ru red). Since we were especially interested in the fate of caveolar clusters, the cells were treated with OA for longer time. Stimulating caveola-mediated endocytosis, OA treatment resulted in a significant increase in the number of caveolar cluster. Most of these clusters were found Ru red positive indicating that they were still conneted to the cell surface. Our double labeling experiments on ultrathin frozen sections clearly showed that in OA-treated cells caveolae are not transported to late endosomes instead they are accumulted in large multicaveolar clusters. We think that PP2A can be one of the key components to regulate the fusion of various endocytotic compartments and /or the trafficking along the microtubules.

Keywords Caveola · Alternative endocytosis · Caveosomes · Ocadaic acid

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Introduction

Caveolae are detergent resistant, highly hydrophobic membrane domains composed of mainly cholesterol and sphingolipids. [1–4]. The main structural proteins of caveolae are members of the caveolin gene family, caveolin-1, caveolin-2 and caveolin-3. Morphologically defined caveolae can be formed by the expression of caveolin-1, caveolin-2 is present mainly in the Golgi network, but it can also create huge hetero-oligomers with caveolin-1, and is thought to play an accessory role in caveolae formation [5, 6]. Caveolin-3 (M caveolin) is a muscle specific isoform [7], but it was also detected in glial cells [8] and chondrocytes [9].

Although caveolae are not normally involved in endocytosis, interaction of caveolae or caveolin with specific ligands can trigger the rapid internalization of caveolae. [10–22].

Caveolar budding is regulated by kinases and phosphatases. Caveolin-1 was described to be phosphorylated [23] on tyrosine residue 14 by Src family kinase [13, 24] and tyrosine phosphorylation of caveolin-1 resulted in caveolar internalization [25]. Caveolin-2 also undergoes Src-induced phosphorylation on tyrosine 19 [26]. Phosphocaveolin-2 (TyrP19) was stricktly co-localized with phosphocaveolin-1 (TyrP14) indicating that the simultaneous phosphorylation of caveolin-1 and caveolin-2 might equally be important in regulation of caveolae pinching off from the plasma membrane. We also showed [27] that the phophorylation of caveolin-2 induced internalization of cavolae in peritoneal macrophages. Treatment with a serine/threonine phosphatase inhibitor (ocadaic acid) causes massive mobilization of caveolae [13, 17, 27–30]. Ocadaic acid inhibits protein phosphatases, especially PP1 and PP2A. [31]. PP1 and PP2A are two major classes of serine/threonine protein

phosphatases that dephosphorylate a broad spectrum of protein kinases [32]. The exact mechanism by which a serine/threonine phosphatase (PP1 and PP2A) inhibitors can stimulate cavolar endocytosis is not known.

Stimulated caveolar internalization is always accompanied with the appearance of grape-like multicaveolar complexes called caveosomes [18]. These multicaveolar complexes distinct from classical endocytotic compartments they never fused with lysosomes thus viruses could escape lysosomal degradation [18, 33]. The further fate of caveosomes is not entirely known. It is also debated whether caveosomes are independent organelles or ligands entering caveosomes can be transported along the classical endocytotic pathway.

In our work we were interested in the ocadaic acid (OA) stimulated internalization of caveolae. We found that after OA treatment large amount of caveolar clusters, caveosome-like structures appeared in the cytoplasm of HepG2 cells. Using a special surface marker (ruthenium red) we tried to answer the question whether these caveosome-like structures were really independent organelles. Our electron microscopic results showed that most of these caveolar clusters were labeled with ruthenium red indicating that they were obviously connected to the cell surface. Using double labeling immunocytochemistry on ultrathin frozen section we found that in OA-treated cells caveolae were not transported to CD63 labeled multivesicular bodies. Our morphological and morphometrical results suggest that OA treatment stimulates the formation of multicaveolar complexes and arrests the caveolar endocytosis by inhibiting the maturation and sorting of the early endocytotic organelles.

Materials and Methods

Cells HepG2 cell line (ATCC: HB8065) was used for all experiments. For electon-microscopic studies the cells were grown on tissue culture flasks in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin-G, 0,1 mg/ml streptomycin, 1 mg/ml ciprofloxacin.

Reagents Ocadaic acid and ruthenium red (Ru red)were obtained from Sigma (Sigma Chemical Co., Sigma-Aldrich Kft)

Antibodies Polyclonal anti-caveolin-1 (Transduction Laboratories), mouse-anti-CD63 antibodies (PeliCluster), polyclonal anti-mouse IgG (obtained from Dako Laboratories) and monoclonal anti-dynaminII (from Transduction Laboratories) were used for immunogold labelling on ultrathin frozen sections. For electron microscopic localization of the primary antibodies protein-A gold (d:10 nm and/or 15 nm) was used (Cell Microscopy Center, Utrecht, The Netherlands).

Experimental Procedures The serine/threonin phosphatase inhibitor (ocadaic acid) was used in 4 nM for 30 min, 60 min and 180 min in 37°C. Each experimental group was divided into two groups: one was processed for common electron microscopy, while the other was prepared for cryo-ultramicrotomy and immunocytochemistry on ultrathin frozen sections.

Electron Microscopy Cells were fixed with 2% glutaraldehyde, for 2 h at room temperature. After fixation cells were scraped by rubber scraper in PBS contained 5% gelatine. Cells were washed with Milloning's phosphate buffer (pH: 7.4), then pelleted with 10% gelatine, dehydrated and embedded in Polybed epoxi resin. Ultrathin sections were contrast stained with uranyl acetate and lead citrate, respectively.

To visualize the surface-connected structures in some experiments, ruthenium red (Ru red) was used. In this case, the cells were fixed in 0.5 mg/ml Ru red containing freshly prepared glutaraldehyde for 1 h at room temperature. This was followed by washing in 0.1 M cacodylate buffer (pH: 7.4) and post-fixation in 0.5 mg/mlRu red containing OsO_4 for 30 min at room temperature, in dark. Then the cells were washed, scraped by rubber scraper and processing for classical EM embedding procedure. Ultrathin sections were contrast stained only with lead citrate for 5 min.

Immunoelectron Microscopy Cells were fixed by adding 4% freshly prepared formaldehyde in 0.1 M phosphate buffer (pH 7.4) to and equal volume of culture medium for 5 min., followed by post-fixation in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) without medium for 24 h at 4°C. Cells were stored until further processing in 1% formaldehyde at 4°C. Processing of cells for ultrathin cryosectioning and immunolabelling according to the protein A-gold method was done as described by Slot (et al. 1991). In brief, fixed cells were washed with 0.05 M glycin in PBS, scraped gently from the dish in PBS containing 1% gelatine and pelletted in 12% gelatine in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose at 4°C and afterwards mounted on aluminium pins and frozen in liquid nitrogen. To pick up ultrathin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used.

Morphometry and Statistical Analysis Twenty to 22 electron micrographs were randomly taken from each experimental group. The number of morphologically identified caveolae connected to the plasma membrane and caveolar

clusters were counted The surface volume ratio was calculated according to Weibel et al [34]. Results were expressed in number of caveola and caveola cluster per surface area of the cell. To quantitate the caveolin-1 containing multivesicular bodies/late endosomes, anti-CD63 and/or anti-caveolin-1 labelled organelles were counted in 10–15 randomly chosen individual cells. Values were expressed as percentage of the total number of gold particles detected above various compartments (plasma membrane versus caveolae versus caveola clusters). StatSoft Statistica 6.1 software was used for statistical analysis.

Results

Electron Microscopical Studies In control HepG2 cells single caveolae are abundantly present on the plasma membrane (Fig. 1a). When 4 nM ocadaic acid was used to stimulate caveolae pinching off from the plasma membrane, grape-like multicaveolar complexes, caveola clusters were formed (Fig 1b,c). By the time of the ocadaic acid treatment the number of caveolar clusters gradually and significantly increased (Table 1). Our electron microscopical results showed that many of these caveolar clusters were present deeper in the cytoplasm, at a distance from the plasma membrane suggesting that they were detached from the plasma membrane (Fig. 1c), while others were connected to the plasma membrane through narrow tubular invaginations (Fig. 1b). Using a special surface marker (ruthenium red) we could label all the structures that were connected to the cell surface eventhough they seem to be independent or detached from the plasma membrane (Figs. 2, 3, and 4). We have done a detailed ultrastructural and morphometrical analysis on the number and the morphology of Ru red positive organelles. In contol cells (Fig. 2a) numerous Ru red positive caveola were found close to or at the cell surface. Many of them seemed to be single, already pinched off vesicles, the Ru red positivity, however, clearly showed that they were still surface-connected.

In OA treated cells caveolar clusters started to appear. These clusters showed either tube-like (Fig. 2b) or grape-like appearance (Fig. 2c), sometimes they were present deeper in the cytoplasm as irregular shaped caveola clusters (Fig. 2d). After 60 min OA treatment similar structures were found in the cytoplasm (Fig. 3a,b, and c). The number of surface-connected single caveolae has significantly decreased (Table 1). Irregular-shaped caveola clusters were present close to the cell surface, part of them was labeled with Ru red indicating that they were still in connection with the cell surface (Fig. 3c: see Ru red positive vesicle cluster). 3 h OA treatment has not increased significantly the number of caveola clusters (Table 1), Ru red positive structures were found deeper in the cytoplasm, but they became more complex (Fig. 4a,b) Some of them were strongly labeled while others contained only a few Ru red cristals (Fig. 4b) suggesting that the plasma membrane invagination connecting them with the cell surface became very narrow.

Immunocytochemistry on Ultrathin Frozen Section In control cells caveola clusters connected to the cell surface

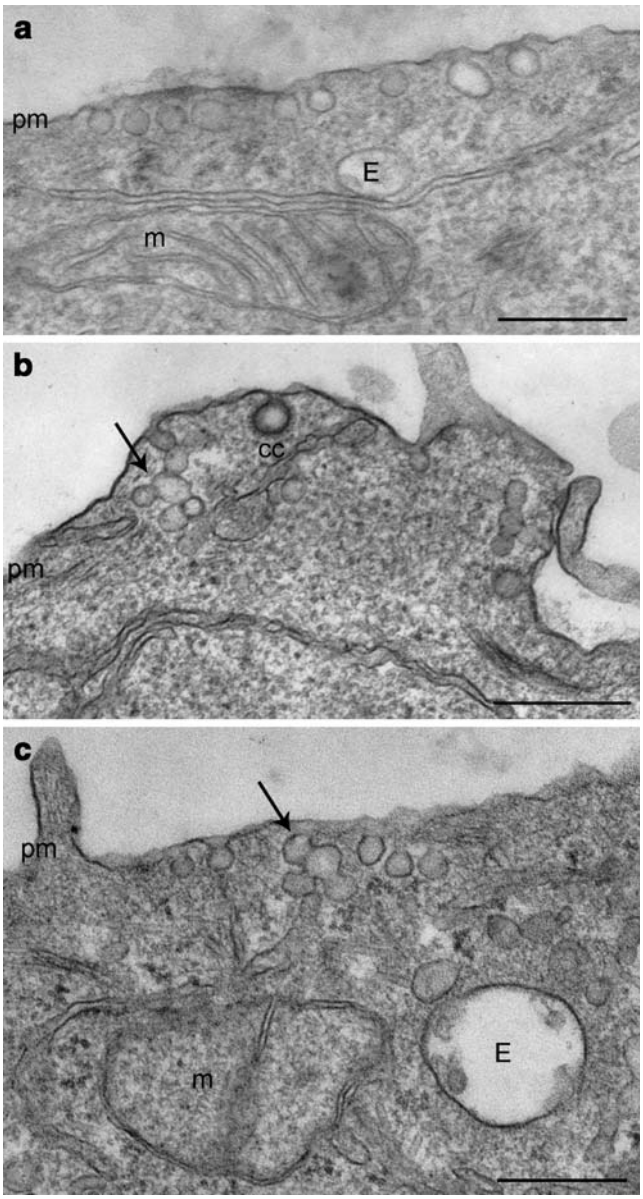

Fig. 1 Effect of ocadaic acid (OA) treatment on HepG2 cells. **a** Control cells: numerous single caveolae are present on the plasma membrane. **b**, **c** Ocadaic acid treatment results in the appearance of caveolar clusters. Some of them are obviously connected to the cell surface (*arrow* in **b**) while other seems to be independent structures (*arrow* in **c**). Cc: clathrin coated pit. *E* endosomes, *m* mitochondria, *pm* plasma membrane. Bars:200 nm

Table 1 Effect of OA treatment ont he number of surface connected caveolae and caveola clusters

treatment	N of caveolae on the plasma membrane	N of caveola cluster
CTR	1.24	0.027
4 nM OA 30 min	0.13	0.08
4 nM OA 60 min	0.09	0.12
4 nM OA 180 min	0.08	0.21

(Values are expressed as number of structures/plasma membrane unit.)

showed caveolin-1 labeling (Fig. 5a). Caveolin-1 and CD63- labeled multivesiclar bodies were present deeper in the cytoplasm (not showed). After 3 h OA treatment caveolin-1 labeled caveola clusters were still present close to the plasma membrane, the size of them has significantly increased (Fig. 5b). When anti-dynamin antibody was used on ultrathin frozen sections we often found labeling around the narrow tubular membrane invaginations by which the caveolar clusters were connected to the cell surface (Fig. 5c) indicating that they might pinch off.

Most of the multivesicular bodies were labeled only anti-CD63 antibody. Our morphometrical data (Table 2) clearly showed that OA treatment resulted in an increased caveolar cluster formation, but the number of CD63 and caveolin-1 positive multivesicular bodies has not increased, indicating that OA treatment arrests the caveolar internalization at the level of cavolar cluster formation.

Discussion

Although caveolae are highly immobile lipid domains of the plasma membrane, under special conditions (like binding specific ligands to their receptors) they can pinch off from the plasma membrane. Recently it is generally accepted that caveola-mediated endocytosis functions as a true alternative uptake mechanism paralelly to the clathrin-mediated pathway. Caveolar endocytosis is regulated by kinases and phosphatases. Tyrosin phosphorylation of caveolin-1 (and maybe caveolin-2) can initiate budding and internalization of caveolae. The GTP-binding protein dynamin, that is temporally associated to caveolae triggers fission of caveolae by constriction its neck subsequent to the hydrolysis of GTP. Treatment with a serine/threonine phosphatase inhibitor (ocadaic acid) causes massive mobilization of caveolae [13, 17, 24, 28–30]. Since ocadaic acid inhibits protein phosphatases, especially PP1 and PP2A. [31] it seems likely that these phosphatases play important role in caveola-mediated endocytosis. The exact mechanism by which serine/threonine phosphatase (PP1 and PP2A) inhibitors can stimulate cavolar endocytosis is not known.

After internalization stimulus grape-like multicaveolar complexes called caveosomes are appearing in the cyto-

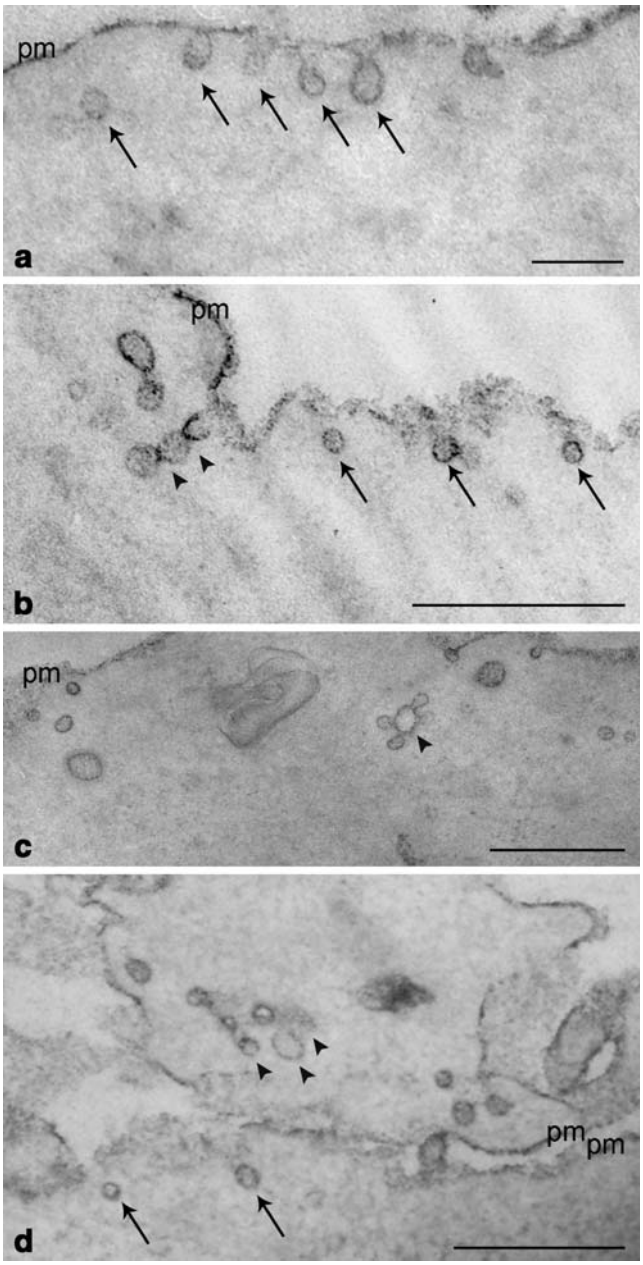

Fig. 2 Ruthenium red (Ru red) labeling of the surface connected structures. **a** In control cells many Ru red positive single caveolae are found on or close to the plasma membrane (*arrows*). **b**, **c**, and **d** After 30 min OA treatment caveolar clusters started to appear in the cytoplasm. The morphology of these clusters were very much variable: tube-like composed of fused vesicles (*arrowheads* in **b**), grape-like (*arrowhead* in **c**) or irregular shaped (*arrowheads* in *d*). Although some of them were present deeper in the cytoplasm, the Ru red labeling indicates their connection with the cell surface. Arrows show Ru red positive single caveolae close to the plasma membrane (pm). Bars: **a**: 200 nm, **b**, **c**, and **d**: 500 nm

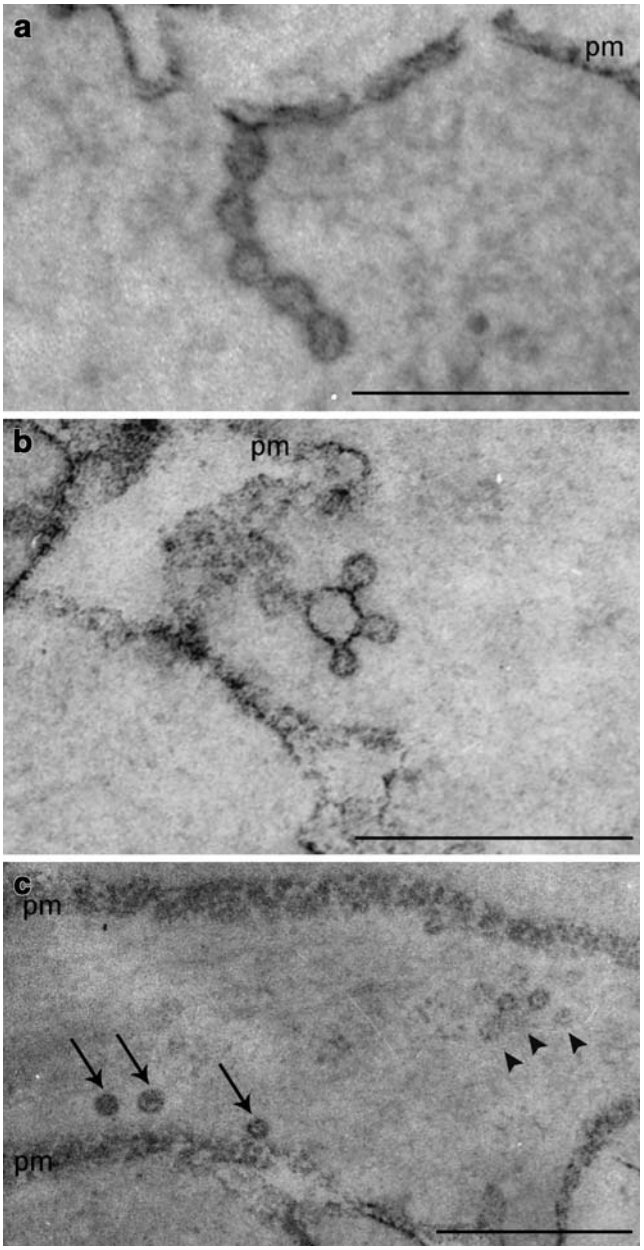

Fig. 3 Surface connected structures labeled with Ru red after 60 min OA treatment. Long tubular structures composed of fused vesicles, grape-like or irregular shaped caveola clusters are present in the cytoplasm. The tubular structure (**a**) is obviously connected to the cell surface but the grape-like (**b**) and the irregular shaped clusters (**c**) are present deeper in the cytoplasm. Ru red labeling indicates their connection with the cell surface. *Arrows* show Ru red labeled single caveolae. Bars: **a**: 400 nm; **b**, **c**: 500 nm

plasm [18, 33]. Until now only a few electron microscopical pictures were published about the morphology of these organelles. It is also debated whether these cavolar clusters are independent entities since many of them are connected to the cell surface by very narrow tubular plama membrane invaginations. According to recent knowledge caveosomes are multicaveolar structures of heterogeneous morphology, they are supposed to be early endosome-equivalent intermediate organelles in caveolar endocytosis. The further fate of caveosomes is not entirely known. It is also questionable whether caveosomes are the only intermediate organelles for ligands internalized by caveolae or caveola-mediated and the classical endocytotic pathways could communicate with each other.

There are data showing that ligands internalized by cavolae can be driven to the classical endocytotic organelles [18, 35, 36]. The existence of two caveolar trafficking routes involving caveosomes and early endosomes raises the questions wether caveosomes are independent structures or the downstream caveosomes interact with the classical endocytotic compartments. Studying caveolar internalization in HepG2 cells we found that ocadaic acid treatment increased significantly the number of caveola clusters having similar morphology as caveosomes. Many of these caveosome-like, multicaveolar complexes were connected to cell surface by a narrow tubular plasma membrane invaginations but some of them seemed to be independent

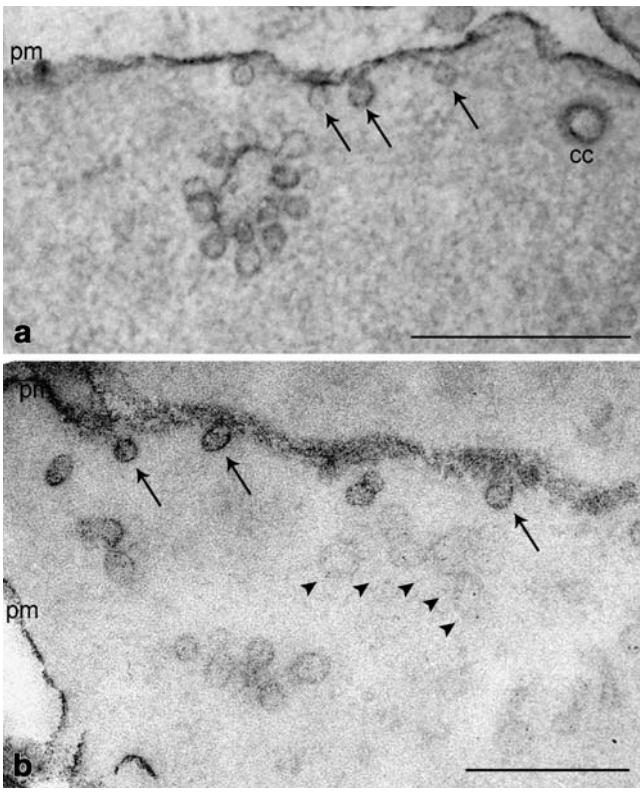

Fig. 4 Ru red labeled caveolar clusters after 3 h OA treatment. Ru red positive caveolar clusters are present deeper in the cytoplasm. Some of them are strongly labeled with Ru red while others (*arrowheads*) contain only a few Ru red cristals (**b**) indicating that the membrane invagination by which they are connected to the cell surface is very narrow. *Arrows* show single Ru red positive caveolae. cc: clathrin coated "vesicle" labeled with Ru red. The Ru red labeling indicates that this coated "vesicle" is still in connection with the plasma membrane; pm: plasma membrane. Bars: **a**: 500 nm; **c**: 400 nm

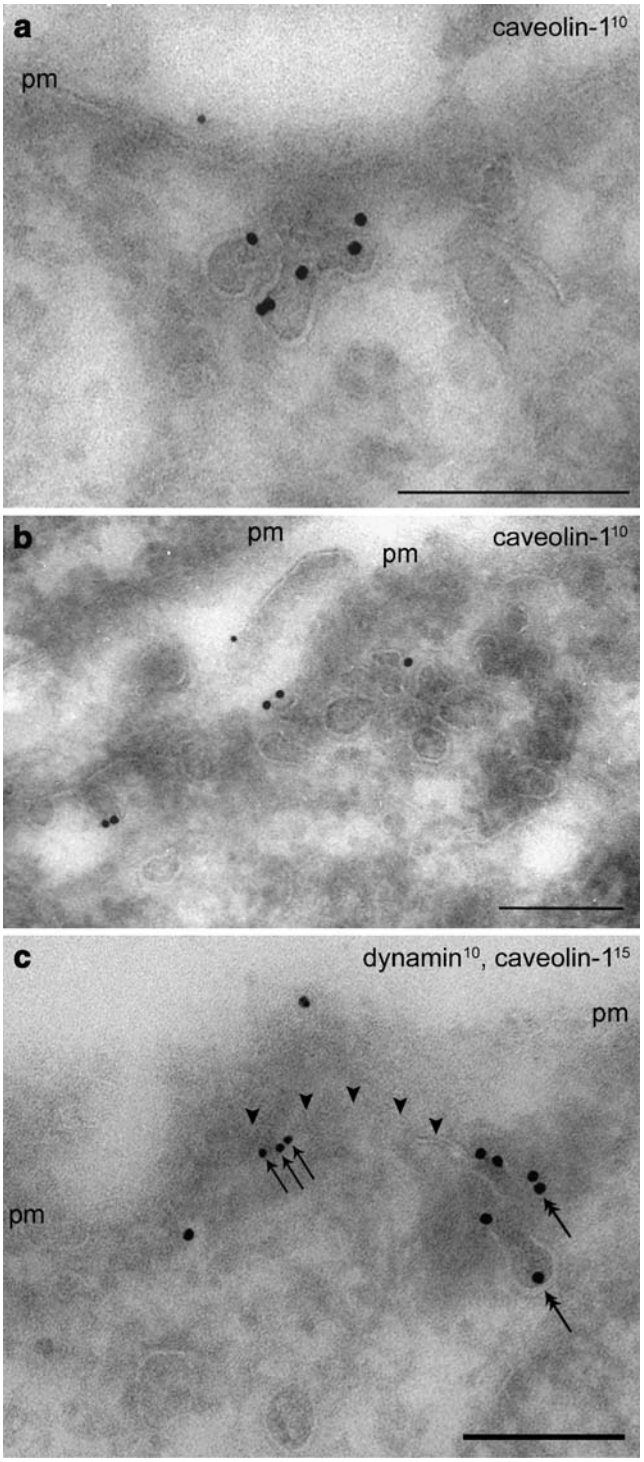

Fig. 5 Detectation of caveolin-1 on ultrathin frozen section. **a** In control cells caveola cluster connected to the cell surface shows caveolin-1 labeling. **b** After 3 h OA treatment caveolin-1 labeled caveolar clusters are still present close to the cell surface, the size of them has significantly increased. pm: plasma membrane. **c** Caveola-cluster labeled with anti-caveolin-1 antibody (*double arrows*) has a long tubular connection (*arrowheads*) with the plasma membrane. Dynamin (*smaller gold particles*, *arrows*) are found along this tubular invagination. Bars: 200 nm

structures in the cytoplasm. When ruthenium red (Ru red)—an electron dense surface marker—was used to label the cell surface, many of these structures were Ru red positive indicating that they were still connected with the cell surface. These results strongly support that a significant portion of these multicaveolar complexes described as caveosomes are not independent structures.

Caveolin-1 in many cells is evident on the cell surface and within the Golgi complex, and only partial colocalization can be detected with endosomal markers such as EEA1, a marker of the early sorting endosome [37] or CD63, late endosomal marker [38]. When caveolar endocytosis was stimulated by albumin in HepG2 cells, albumin was found to accumulate in large, caveolin-1 positive caveosome-like caveola clusters. On the same time the number of caveolin-1 and CD63 containing multivesicular bodies significantly increased indicating that caveolae-mediated endocytosis of albumin resulted in an increased caveolar trafficking along the classical endosomal degradative pathway [38]. The morphological entities by which the communication can occur are not known. Caveolin-1 containing subdomains pinching off from caveosomes as vesicles can function as mediators between the caveolar and the classical endocytotic pathways. It can not be excluded, however, that caveolar clusters "en mass" can pinch off and fuse with endosomes. Using anti-dynamin antibody we found that dynamin is accumulating around the narrow tubular invaginations by which caveolar clusters are connected to the cell surface indicating that the "en mass" fission of caveolar clusters might occur.

When caveolar internalization was stimulated with OA treatment in HepG2 cells, numerous caveola cluster appeared in the cytoplasm but there was no increase in the number of CD63 and caveolin-1 double labeled multivesicular bodies. These results strongly suggest that OA treatment arrests caveolar internalization at the level of caveolar cluster. Since OA specifically inhibits serine/threonine phosphatases it seems likely that these phosphatases, especially PP2A play important role in cargo transfer,

Table 2 Distribution of caveolin-1 in different cellular compartments after various time OA treatment

Cellular compartment	Non-treated cells (control)	4 n OA 30 min	4n OA 60 min	4 nM OA 180 min
Plasma membrane	42.9%	30.7%	20.3%	15.3%
Pinched off caveolae	9.2%	28.2%	25.2%	23.4%
Pinched off caveolae in caveola-clusters	6.1%	8.5%	21.9%	28.5%
CD63 labeled late endosomes	25.8%	8.5%	8.3%	7.2%

(Values are expressed as percentage of the total number of gold particles detected above varioous compartments of the cytoplasm)

most probably at the level of endosomal sorting [39–41]. By dephosphorylation of proteins present in the endosomal membrane, PP2A can regulate maturation of endosomes, fusion of endosomes with lysosomes [39, 42, 43]. If PP2A is inhibited the classical endocytotic sorting is blocked and the cargo can stuck in one of the intermediate compartments. This might be the explanation why SV40 virus is retained in caveosomes resulting in an escape from lysosomal degradation The small T-antigen of SV40 virus is known to bind and inhibit PP2A [44], by which it can interfere the maturation of virus and caveolin containing endosomes (caveosomes) and endosome/ lysosome fusion. It seems likely that the interaction of the cargo with caveolar components, caveolin itself or any of the regulatory kinases and/or phosphatases can be an important determinant for the final destination of the cargo.

PP2A can also regulate the movement of endocytotic compartments along the microtubules [39–41, 45]. It can not be excluded that inhibiting PP2A, ocadaic acid can block the microtubules directed transport that can result in an increased number of caveolar cluster.

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