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Identification of free coated pinocytic vesicles in Swiss 3T3 cells

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Whether or not free coated vesicles are involved during internalization of ligands bound to the receptors of coated pits is controversial. Free coated vesicles cannot be identified with certainty in random individual thin sections – reconstructions based on consecutive thin sections are required. The thickness of the sections determines the reliability of such reconstructions. In the present study, serial section electron microscopy was applied to Swiss 3T3 cells and the topographical resolution yielded by 80 nm and 20 nm sections was compared. Swiss 3T3 cells in monolayer at 37°C were exposed for 5 min to cationized ferritin (CF) which is a marker of pinocytic vesicles. Subsequently the cells were fixed, pelleted and further processed for electron microscopy. The results showed that reconstructions of coated CF-labeled structures based on consecutive sections of an average thickness of ~80 nm could not be performed with certainty. A substantial fraction (25%) of the examined profiles appeared to be free vesicles, but narrow surface connections could easily have been missed in these thick sections. The series of the much thinner 20 nm sections provided a better resolution allowing the narrowest surface connections to be identified. Accordingly, the number of truly free, coated vesicles was much lower than the number of apparently free vesicles in the thick sections. However, free coated vesicles labeled with CF were identified in the consecutive 20 nm sections (4% of the examined profiles).

Key words: serial sections/coated pits and vesicles/Swiss 3T3 cells

Introduction

Coated pits at the cell surface are involved in receptor-mediated internalization of a variety of ligands (Goldstein *et al.*, 1979; Helenius *et al.*, 1980, 1983; Pearse and Bretscher, 1981; Pastan and Willingham, 1981b, 1983; Anderson and Kaplan, 1983; Salisbury *et al.*, 1983; Steinman *et al.*, 1983). The internalized molecules rapidly reach an acidic, prelysosomal compartment (Maxfield, 1982), the endosome or receptosome, which appears to be the site for uncoupling of ligand and receptor prior to receptor-recycling (Geuze *et al.*, 1983). This compartment may also be the site for translocation of bacterial and plant toxin A moieties (Olsnes and Sandvig, 1983) and for the entry of nucleocapsids (of membrane-enveloped virus) into the cytosol (Helenius *et al.*, 1980; Marsh and Helenius, 1980). Moreover, coated pits are also involved in fluid-phase endocytosis and may, at least in certain cell types, be the only vehicle for endocytosis (Marsh and Helenius, 1980; van Deurs *et al.*, 1981, 1982).

Divergent viewpoints on the events which lead to the internalization of ligands from the surface-connected coated pits have been put forward. Some advocate that coated pits give rise to free coated vesicles (truly free pinosomes) which – presumably after the coat material has been shed – deliver their load to a prelysosomal vacuole, the endosome (Helenius *et al.*, 1983). However, coated pits are considered by others to be permanently surface-associated structures from which an independent organelle, the receptosome, buds off and carries ligands into the cell, where the receptosome constitutes the acidic prelysosomal compartment (Willingham and Pastan, 1980, 1983a, 1983b; Willingham *et al.*, 1981; Pastan and Willingham, 1981a, 1981b, 1983). The latter hypothesis is partly based on a series of ultrastructural studies, including serial section analysis, which failed to demonstrate free coated pinocytic vesicles in Swiss 3T3 cells. Surface connections were reported to be present in all examined cases.

Recently, serial section electron microscopy was applied to L-cells and human skin fibroblasts (Petersen and van Deurs, 1983). Consecutive sections of an average thickness of ~20 nm were used for reconstructions of the organization of coated pinocytic structures. Free coated pinocytic vesicles were identified in both cell types. These results were, however, questioned by Willingham and Pastan (1983b) on the basis of their results with the Swiss 3T3 cells. In consecutive sections (80–100 nm thick) of these cells they did not identify any free coated pinocytic vesicles and they suggested that results obtained with much thinner consecutive sections overestimate the number of free vesicles due to low membrane contrast in the individual sections. A theoretical analysis of these technical problems led us to the conclusion that appropriate section thickness rather than the contrast level determines the reliability of the reconstruction (van Deurs *et al.*, 1983). The thinner the sections are, the more precisely the reconstruction can be performed.

With the present report we intend to settle the discussion on the existence of free coated pinocytic vesicles. We have extended our serial section studies to Swiss 3T3 cells, the cell type used by Willingham and Pastan. To emphasize the importance of section thickness, the organization of coated structures in these cells were analyzed in consecutive sections of average thicknesses of about either 20 nm or 80 nm (the thickness used by Willingham and Pastan). A comparison of the results obtained from the two classes of serial sections has led to the following: (i) the 80 nm consecutive sections are far too thick to allow reconstruction of the organization of coated profiles and their relation to the surface membrane; (ii) the ultrathin consecutive sections yielded the required topographical resolution and free, coated pinocytic vesicles were found.

Results and Discussion

An exogenous marker of pinocytosis is needed in the study of coated pits and vesicles, because coated vesicles are also formed from cytoplasmic organelles (Petersen and van Deurs, 1983). Cationized ferritin (CF) is a distinct and easily identifiable marker of coated pits and apparently free, coated

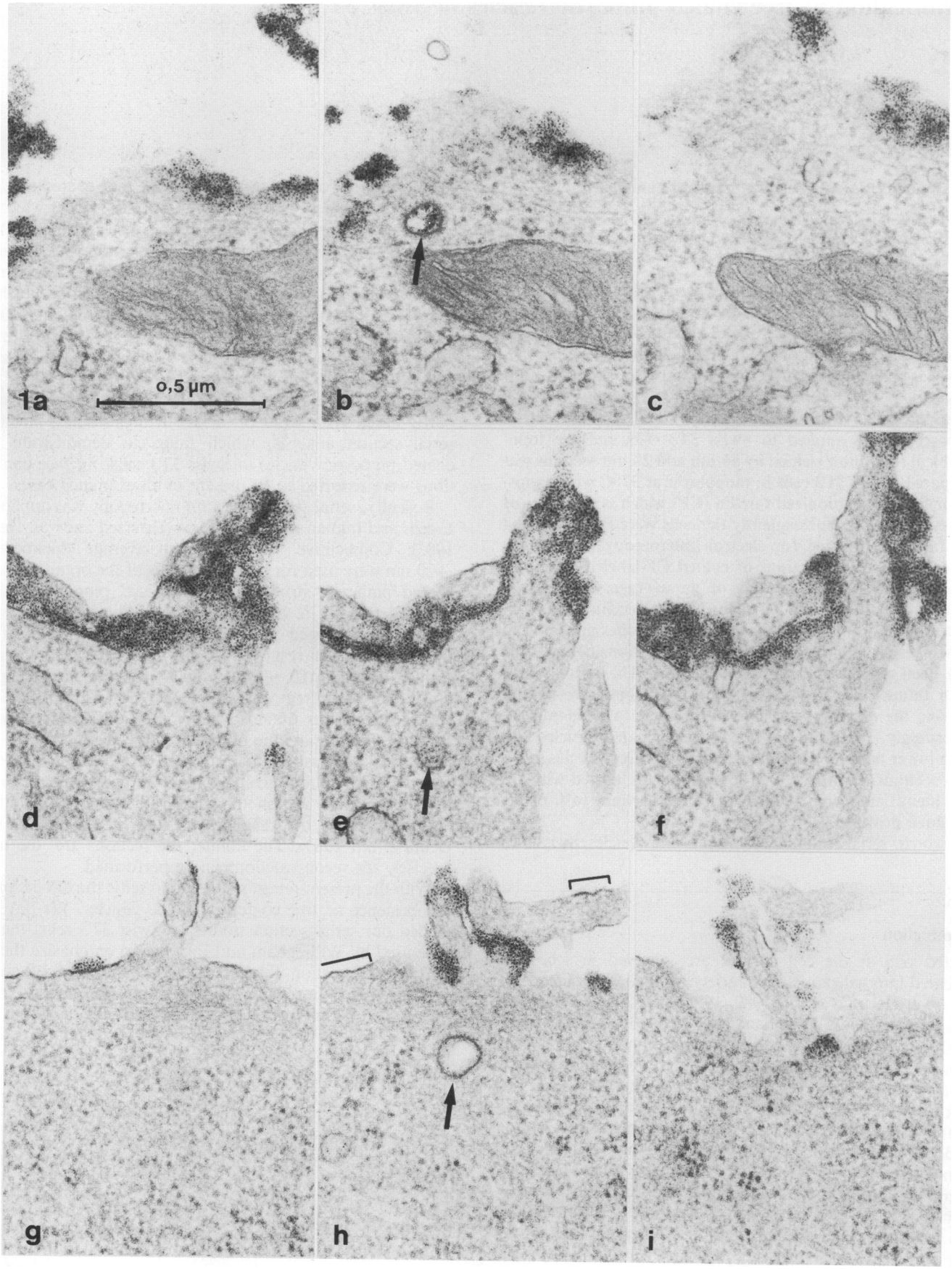

Fig. 1. Portions of three series of 80 nm sections (**a**–**c**, **d**–**f** and **g**–**i**), each of which includes a coated vesicular profile in the mid-section (arrows). The profiles in **b** and **e** are distinctly CF-labeled, while the profile in **h** contains very little, or no CF. It is impossible to draw any serious conclusion about the possible surface relations of these coated profiles; they cannot be judged surface-connected. The bars in **h** show that the 'unit membrane' becomes distinct only in short segments (i.e., when cut perpendicular because of the extreme section thickness used). Microscope operated at 80 kV.

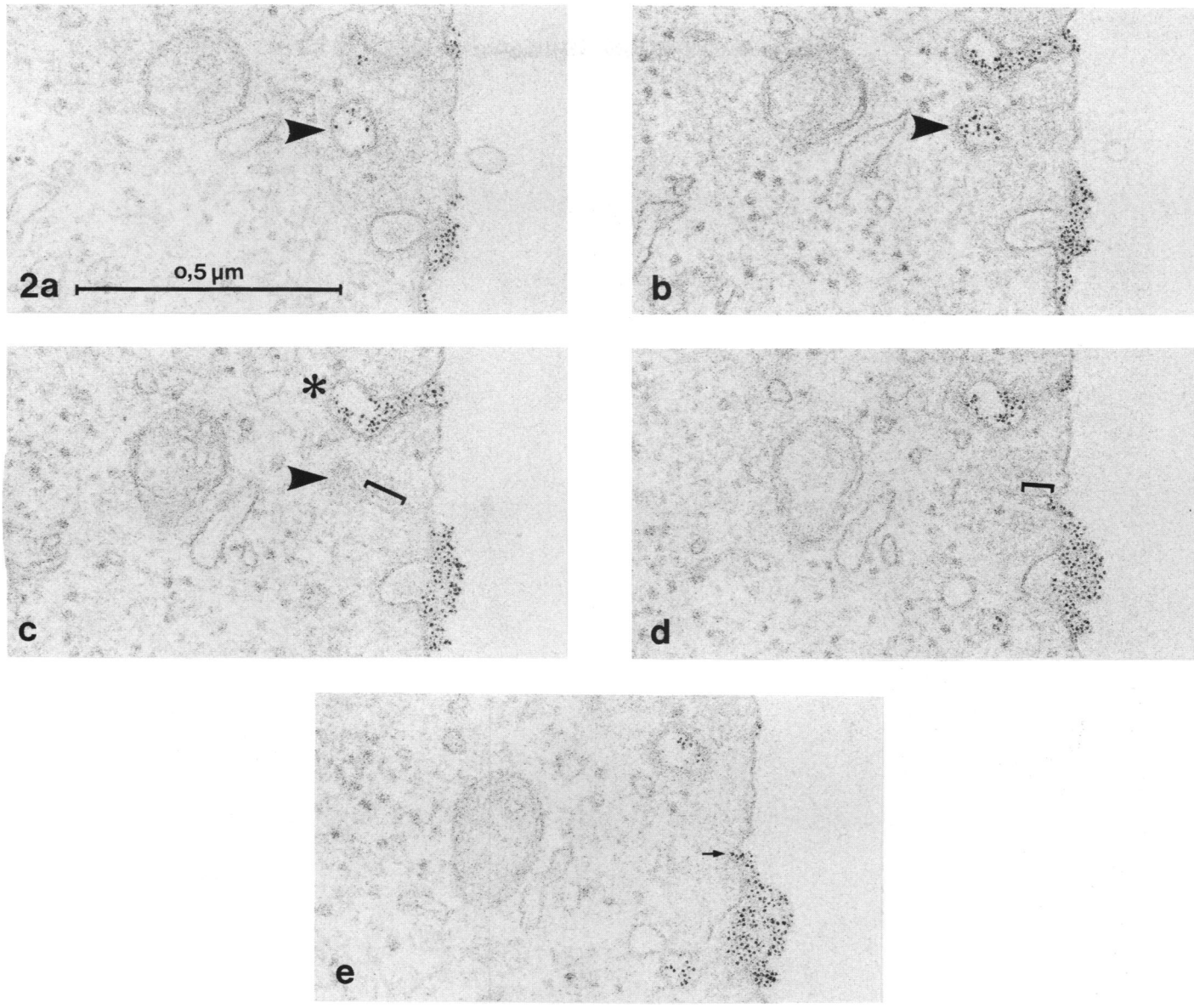

Fig. 2. Series of five 15–20 nm conecutive sections showing some surface-connected coated pits with CF. Note that the profile labeled with arrowheads is distinct in **a** and **b**; in **c** only a 'shadow' remains, but a segment of a very narrow neck is present (bar), and in **d** this neck communicates with the cell surface (bar). In **e**, a small surface invagination is the last sign of the neck in the series of sections. Note that at the coated pit labeled by an asterisk, the 'unit membrane' can be distinctly seen for a relatively long distance (because of the small section thickness). EM operated at 80 kV.

vesicles (van Deurs *et al.*, 1981, 1982; Petersen and van Deurs, 1983). With an incubation time of only 5 min, it is reasonable to assume that coated vesicular profiles containing CF derive from the plasma membrane and thus are pinocytic.

In the present study, CF was seen – often in clusters – on the cell surface of Swiss 3T3 cells, in coated pits, in coated vesicular profiles (preferentially localized close to the cell surface), in numerous uncoated vesicular profiles, and in large (endocytic) vacuoles.

In the 80 nm sections, membrane contrast was very high (Figure 1). However, it was difficult to analyze the membranes in detail. The plasma membrane is an undulating structure implying that only small membrane segments extend perpendicularly to the plane of sectioning all the way through very thick sections. Consequently, in projected images of the 80 nm sections, plasma membranes appeared blurred in most of their outline (Figure 1h), an appearance which complicates reconstructions of membranous structures.

Reconstructions of discrete structures contained in a series of 80 nm sections were also complicated by the fact that the picture of the tissue changes abruptly from one section to the next. Distances between structures under reconstruction and the selected 'landmarks' or 'reference points' varied so much that the tracing of a given structure throughout the series became very uncertain. It was often difficult or impossible to decide whether discrete structures present in neighbouring sections were part of one structure or represented two different structures in close proximity. For example, if there is an apparently free vesicular profile in one section and a surface-connected vesicular profile approximately at the same location in the following section, then the first vesicular profile can represent either a truly free vesicle or an extension of the surface-connected vesicle.

Coated vesicular profiles with CF could be followed in up

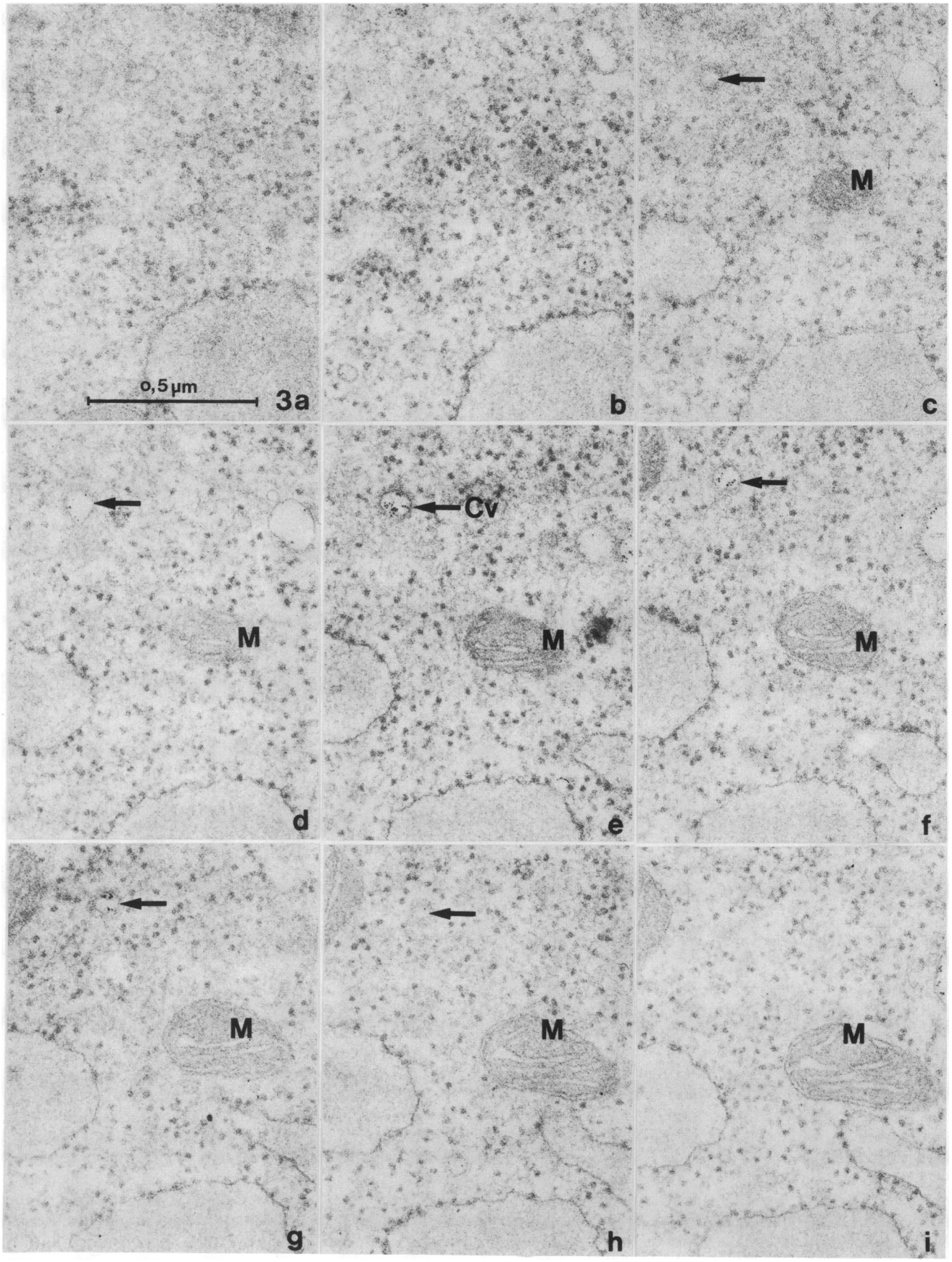

Fig. 3. Series of nine consecutive ~20 nm sections showing a coated vesicular profile with CF (arrows; in **e** labeled Cv). The brief (~5 min) labeling with CF ensures that the structure is pinocytic ('incoming'). No surface connections (or connections to any internal membrane compartment) can be demonstrated; the profile 'starts' as a 'ring-shaped shadow' in **c**, and ends as a similar shadow in **h**. M, mitochondrion (used as a 'landmark' during the preparation of the plate). EM operated at 60 kV.

to three consecutive 80 nm sections. Surface connections could not be identified for 26 out of 103 coated profiles with CF included in the analysis (Figure 1). This observation is inconsistent with the results of Willingham and Pastan (1983a, 1983b). They found 263 out of 263 vesicular profiles to be surface-connected in 70–100 nm consecutive sections of Swiss 3T3 cells.

Coated pits may be connected to the cell surface by very long (up to 1000 nm), narrow (~17 nm) and often very tortuous necks. Such structures were recently illustrated by micrographs of 120 nm thick sections (Willingham and Pastan, 1984). It is noteworthy, however, that the 'unit membrane' of the apparent necks was well defined in these very thick sections. We consider it unlikely that a tubular neck of ~20 nm diameter can present distinct membranes when it is contained in a 120 nm thick section. Alternatively, these apparent necks may represent 'foldings' of the surface membrane extending throughout the entire section. Such foldings which appear as a neck in random individual thin sections were demonstrated during the reconstructions of coated profiles in skin fibroblasts and L-cells (Petersen and van Deurs, 1983). Moreover, it is difficult to imagine that very convoluted necks up to 1000 nm long will aways be contained in one and the same section, as reported by Willingham and Pastan (1984).

In the 20 nm sections, the contrast was indeed significantly lower, but membranous structures could be analysed and traced with better accuracy. Even very narrow necks connecting vesicular profiles with the cell surface could be detected (Figure 2). Obviously, such a neck could easily be missed if it was shared by two neighbouring 80 nm sections. During the analysis of the series of 20 nm sections, we traced the organization of 152 coated vesicular profiles labeled with CF. By far the majority (146) turned out to be coated pits provided with more or less narrow surface connections (Figure 2). The truly free profiles (Figure 3) were located several vesicle diameters from the surface membrane or deeper in the cytoplasm, in some cases close to the Golgi apparatus or between the Golgi and the nucleus.

The very different outcome of serial sectioning of the two different thicknesses is in accordance with observations on vesicular profiles in capillary endothelium (Bundgaard, 1983). The number of free vesicles is overestimated if the thickness of the consecutive sections approaches the diameter of the vesicular structures. It is therefore surprising that Willingham and Pastan (1983a, 1983b) did not observe apparently free coated vesicles in their 70–100 nm section study.

The number of free coated pinocytotic vesicles in Swiss 3T3 cells is low compared with our previous findings on L-cells and human skin fibroblasts (Petersen and van Deurs, 1983). This may be related to variations in the lifetime of a coated vesicle, i.e., the period from its formation, by pinching off from the cell surface, to the time when the vesicle's coat is shed. This aspect has been discussed in some detail previously (Petersen and van Deurs, 1983). Other things being equal, the shorter the lifetime, the fewer the number of free coated vesicles in the cytoplasm.

Furthermore, the fixation procedure used for electron microscopy presumably prevents further formation of coated vesicles from the cell surface while some of those already formed will shed their coat before being 'stabilized' by the fixative. This implies that the number of free coated vesicles will be underestimated (see also McGuire and Twietmeyer, 1983).

Finally, it is noteworthy that the reconstructions did not reveal 'receptosome-like' structures in relation to the coated pits. Such structures were observed in individual sections, but the reconstructions always demonstrated that they were foldings of the plasma membrane (see Figure 2 in Petersen and van Deurs, 1983). If 'receptosomes' were contained in the series of 20 nm sections, they would be identified unequivocally.

Conclusion

The present experiments on Swiss 3T3 cells, which extend our previous results on L-cells and human skin fibroblasts, show: (i) that an analysis of 80 nm consecutive sections often fails to demonstrate connections between coated vesicular profiles and the plasma membrane, which leads to an overestimation of the number of free vesicles; (ii) that consecutive sections, ~20 nm thick, provide a topographical resolution which allows identification of the smallest necks between coated vesicles and the plasma membrane; (iii) that free coated pinocytic vesicles occur in Swiss 3T3 cells; and (iv) that receptosome-like structures budding off from coated pits were not contained in the 20 nm consecutive sections.

The findings support the concept that free coated pinosomes ferry internalized ligands into the cell and fuse (presumably after the coat had been shed) with a prelysosomal vacuolar compartment, the endosome (Helenius *et al.*, 1983).

Materials and methods

Swiss 3T3 cells were grown in monolayers under standard conditions in Dulbecco's modified medium supplemented with 5% NCS.

Cells were rinsed twice with pre-warmed phosphate-buffered saline (PBS) (37°C), and CF (Miles Yeda, Miles Laboratories, Inc., Elkhart, IN), 0.1 mg/ml PBS, was added. After 5 min of incubation at 37°C, cells were rinsed four times with ice-cold PBS and fixed overnight with 1% formaldehyde, 1.25% glutaraldehyde and 0.1 M sodium cadodylate buffer, pH 7.2.

After fixation the cells were scraped off the plates and centrifuged at 1600 *g*. Pellets were post-fixed for 1 h at 4°C with 2% OsO_4 in cacodylate buffer, pH 7.2. Subsequently, the cells were block-stained for 1 h at 20°C with 1% uranyl acetate in distilled H_2O, dehydrated in a graded series of ethanol and embedded in Epon.

Ribbons of thin sections were cut with a diamond-knife and collected on Formvar-covered single slot grids. The results are based on series containing 5–15 consecutive sections of an average thickness of ~80 nm and on series containing 15–20 sections of an average thickness of ~20 nm. Section thickness was estimated by counting the number of sections which contained spherical structures of known diameter. The sections were not post-stained because this procedure inevitably gives some contamination which may lead to breakage of the supporting film or to blurring of crucial details.

The consecutive sections were examined in a Jeol 100 CX electron microscope and structures of interest were photographed at 60 or 80 kV throughout the series.

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