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Different modes of internalization of proteins associated with *adhaerens* junctions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material

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The distribution and fate of two junctional complexes, *zonula adhaerens* and desmosomes, after dissociation of cell-cell contacts is described in MDBK cells. Junctions were split between adjacent cells by treatment with EGTA and proteins associated with the plaques of *zonulae adhaerentes* and desmosomes were localized by immunological methods. Splitting of these junctions is accompanied by the dislocation of desmosomal plaque protein from the cell periphery and its distribution in punctate arrays over the whole cytoplasm. By contrast, vinculin associated with *zonulae adhaerentes* is still seen at early times (0.5 – 1 h) in a conspicuous belt-like structure which, however, is displaced from the plasma membrane. Strong vinculin staining is maintained on leading edges of free cell surfaces. Electron microscopy of EGTA-treated cells exposed to colloidal gold particles reveals the disappearance of junctional structures from the cell periphery and the concomitant appearance of a distinct class of gold particle-containing vesicles which are coated by dense plaques. These vesicle plaques react with antibodies to desmosomal plaque proteins and are associated with filaments of the cytokeratin type. In the same cells, extended dense aggregates are seen which are most probably the membrane-detached vinculin-rich material from the *zonula adhaerens*. The experiments show that, upon release from their junction-mediated connections with adjacent cells, major proteins associated with the cytoplasmic side of the junctions remain, for several hours, clustered within plaques displaced from the cell surface. While plaque material of *adhaerens* junctions containing vinculin is recovered in large belt-like aggregates, desmosomal plaque protein remains attached to membrane structures and appears on distinct vesicles endocytotically formed from half-desmosomal equivalents. Observations of similar desmosome-derived vesicles in various tissues suggest that this form of endocytotic resumption of desmosomal material may also take place in living organisms.

Key words: epithelial cells/desmosomes/endocytosis/junctional complexes/cytoskeleton

Introduction

In epithelial tissues, and also in layers of epithelial cells growing *in vitro*, cell-to-cell communication and coherence appears to involve four types of junctions: tight junctions, gap junctions, junctions of the *adhaerens* type, and desmosomes. Each of these junctions is characterized by its special ultrastructural organization (Farquhar and Palade, 1963; Staehelin, 1974) and probably also protein composition (e.g., Colaco and Evans, 1981; Nicholson *et al.*, 1981; Bok *et al.*, 1982; Hertzberg *et al.*, 1982; for desmosomes see below). Such junctions represent localized domains in which certain membrane proteins are clustered and restricted from dispersion by lateral diffusion. This also seems to hold for the corresponding asymmetric junction-like specializations, i.e., "focal adhesions" related to *adhaerens*-type junctions and "hemi-desmosomes", which have been described in associations with the basal lamina or, in cell cultures, with the artificial substratum. Establishment and maintenance of local clusters of integral membrane molecules has often been discussed in relation to possible constraint exerted through interactions with membrane-associated components, including parts of the cytoskeleton (Singer, 1974; Nicolson *et al.*, 1977). Restriction of plasma membrane components to certain parts of the cell surface has been demonstrated for apical *versus* basolateral regions of polarized epithelial cells (e.g., Quaroni *et al.*, 1979; Hauri *et al.*, 1980; Louvard, 1980; Franke *et al.*, 1981a; Green *et al.*, 1981), and it has been shown that such proteins may be released from such constraints when epithelial cells are dissociated (Pisam and Ripoche, 1976; Ziomek *et al.*, 1980).

In the present study we report on the fate of plasma membrane specializations associated with two types of junctions, the *adhaerens* junctions and the desmosomes, upon cell dissociation induced by removal of calcium ions with EGTA. This procedure is very effective in separating epithelial cells by splitting their junctional complexes in a symmetric fashion, leaving initially "half-junction" structures on the surfaces of the dissociated cells (e.g., Sedar and Forte, 1964; Muir, 1967; Borysenko and Revel, 1973; Overton, 1974; Cereijido *et al.*, 1978; Meldolesi *et al.*, 1978; Hoi Sang *et al.*, 1979). For this study we have chosen cultured kidney epithelial cells of the bovine Madin-Darby line MDBK which, like the corresponding canine MDCK cells, have maintained the typical polar architecture of tubular kidney epithelial cells and show the subapical sequence of all four types of junctions, transepithelial electrical resistance, active ion and fluid transport and apical *versus* basolateral segregation of normal and viral membrane components (Misfeldt *et al.*, 1976; Rindle *et al.*, 1979; Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan and Pendergast, 1980; Green *et al.*, 1981). Specifically, we have studied the *adhaerens*-type junctions forming a typical subapical belt (*zonula adhaerens*) and the desmosomes. Both types of junctions are intimately associated, on their cytoskeletal aspects, with dense plaques which in turn serve as specific anchorage structures for different cytoskeletal filaments. *Adhaerens*-type junctions are covered by a dense, finely filamentous web at which actin microfilaments insert and which is enriched in a protein of mol. wt. 130 000 termed vinculin; antibodies to vinculin allow specific visualization of this type of junctional complex (Geiger, 1979; Geiger *et al.*, 1980, 1981). Correspondingly, the cytoplasmic side of the desmosomal membrane is characterized by a dense plaque enriched in a group of cytoskeletal proteins which are insoluble in various buffers and detergents (Skerrow and Matoltsy, 1974; Drochmans *et al.*, 1978; Franke *et al.*, 1981b;

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Gorbsky and Steinberg, 1981). Antibodies to such high mol. wt. (164 000–250 000) polypeptides of the desmosomal plaque have been shown to be valuable in visualizing the distribution of desmosomal material (Franke *et al.*, 1981b, 1982a). We have used antibodies to these junction marker proteins in order to follow the fate of junctional plaque material upon cell separation.

Results

Immunofluorescence microscopy

In densely grown monolayers of MDBK cells immunofluorescence microscopy using antibodies against desmosomal plaque proteins cell-to-cell boundaries appeared as linear arrays of fluorescent "dots", each dot representing a desmosome (Figure 1a, b). Occasionally, some dots were also seen in cytoplasmic regions seemingly away from the lateral plasma membrane (Figure 1a, c) but, as discussed elsewhere (Franke *et al.*, 1981b), such dots could reflect diverse situations including desmosomes located at partially overlapping cell margins and hemi-desmosomes at the bottom surface. The typical dotted line staining was restricted to cell-to-cell boundaries. Sparsely grown cell colonies showed positive reaction only in regions of cell-to-cell attachment but not on free cell edges (Figure 1c). Using staining with antibodies to vinculin we observed a continuous belt delineating cell-to-cell boundaries and additional staining of focal adhesions located toward the substratum (Figure 1d). Free cell edges were also stained by the vinculin antibodies but here the staining often was more patchy (Figure 1e).

Removal of calcium ions from the cell medium by addition of EGTA resulted in obvious changes of these staining patterns. Already 30 min after application of EGTA a dislocation of desmosomal plaque proteins from the cell periphery was recognized, often still showing linear arrays of dots (Figure 2a). Regions of close apposition of cell membranes were generally devoid of desmosomal plaque staining (Figure 2b, c). Upon prolonged exposure (1–3 h) of cells to EGTA, antibody label appeared in punctate arrays randomly distributed throughout the cytoplasm (Figure 2d), in many cells showing juxtanuclear enrichment (Figure 2e). Such punctate staining was noted, in many cells, at even longer periods of exposure to EGTA (3–4 h; at 4 h, of course, most cells had detached from the substratum).

By contrast, antibody staining for vinculin showed, at early times (30–60 min) of EGTA treatment, the formation of a conspicuous belt-like structure displaced from the plasma membrane (Figure 3a, b). This band of vinculin staining, probably derived from the subapical *zonula adhaerens*, seemed to fade more rapidly upon further exposure to the chelating agent and was lost, in most cells, after 3 h. In addition, strong vinculin staining was also observed at focal adhesions and on free cell edges throughout the whole period of EGTA treatment (Figure 3c, d).

Essentially identical results were obtained with the various EGTA concentrations examined. Staining of EGTA-treated cells with antibodies to cytokeratins showed no gross change of the meshwork of cytokeratin filaments in these cells (not shown; for cytokeratin staining of normal MDBK cells see Franke *et al.*, 1982a, 1982b). Using double immunofluorescence microscopy with guinea pig antibodies to desmosomal plaque proteins and a monoclonal murine antibody to cytokeratin, we were able to detect numerous sites of attachment of cytokeratin fibrils to the intracellular dots of desmosomal plaque material, at least at 30 and 60 min of EGTA treatment (not shown).

Electron microscopy

When examined by electron microscopy (Franke *et al.*, 1981b), cell-to-cell contacts of MDBK cells showed, like in MDCK cells, frequent desmosomes and *adhaerens* junctions (Figure 4a, b) most of which seemed to be integrated into the *zonula adhaerens* belt. MDBK cells exposed to EGTA and colloidal gold, as a general marker for unspecific endocytosis, were devoid of junctional structures (for MDCK cells see Cereijido *et al.*, 1978; Hoi Sang *et al.*, 1979). Instead, we found extended belt-like or diverticulate aggregates of dense material (Figure 4c) in the subapical cytoplasm which resembled in structure the *zonula adhaerens* mats of untreated cells, were stained with vinculin antibodies (not shown), and seemed to represent membrane-detached *zonula adhaerens* plaque material. Concomitantly, numerous vesicles were seen which were partly covered by a "cap" of an electron-dense coat associated with tufts of intermediate-sized filaments (Figure 4d–g). Many of these vesicles, which were recognized throughout the whole cytoplasm including the juxtanuclear region, contained gold particles (Figure 5). Besides spheroidal vesicles, some profiles of tennis racket-shaped or disk-like vesicles, with asymmetric or symmetric desmosome-like morphology, were noted, similar to the intracellular desmosome-like structures described in various tissues and cell cultures (e.g., Cook and Stevens, 1970; von Bülow and Klingmüller, 1971; Caputo and Brandi, 1972; Schenk, 1975; Petry, 1980). Electron microscopic immunolocalization revealed intense staining of the dense "caps" on the vesicle surfaces with antibodies to desmosomal protein (Figure 4h), indicating the origin of these vesicles from "half-desmosomes" (single plaques) formed early during EGTA treatment.

We further examined the true vesicle nature of such gold-containing membrane-structures associated with desmosomal plaque protein in serial sections. Figure 5 presents an example of such a series of consecutive thin sections which includes several spheroidal dense plaque bearing vesicles that contain gold particles, thus proving their endocytotic derivation from desmosomal membrane domains.

Discussion

Immunolocalization using antibodies to desmosomal plaque proteins allows the study of the distribution of desmosomal components in whole cells, independent of their morphological appearance. When junctional complexes are split by removal of calcium ions most of the desmosomal plaque domains do not remain for long periods of time at the plasma membrane but are selectively taken up and translocated deeper into the cytoplasm. As shown previously in other cells, half-desmosomal plaques ("single plaques") can still be seen shortly after splitting junctions by treatment of tissues or cell cultures with proteolytic enzymes and/or agents chelating divalent cations (Overton, 1968, 1974; Borysenko and Revel, 1973; Pisam and Ripoche, 1976; Cereijido *et al.*, 1978; Overton and DeSalle, 1980). Even after several hours of exposure to EGTA, occasional single plaque structures can still be seen on lateral and apical surfaces of dissociated cells (not shown), but the vast majority of these plaques is rapidly internalized by an endocytotic process. After 30 min of treatment most of the plaque protein, and probably also desmosome-specific integral membrane components, is located in the cell interior. These plaque residues are associated with a distinct class of

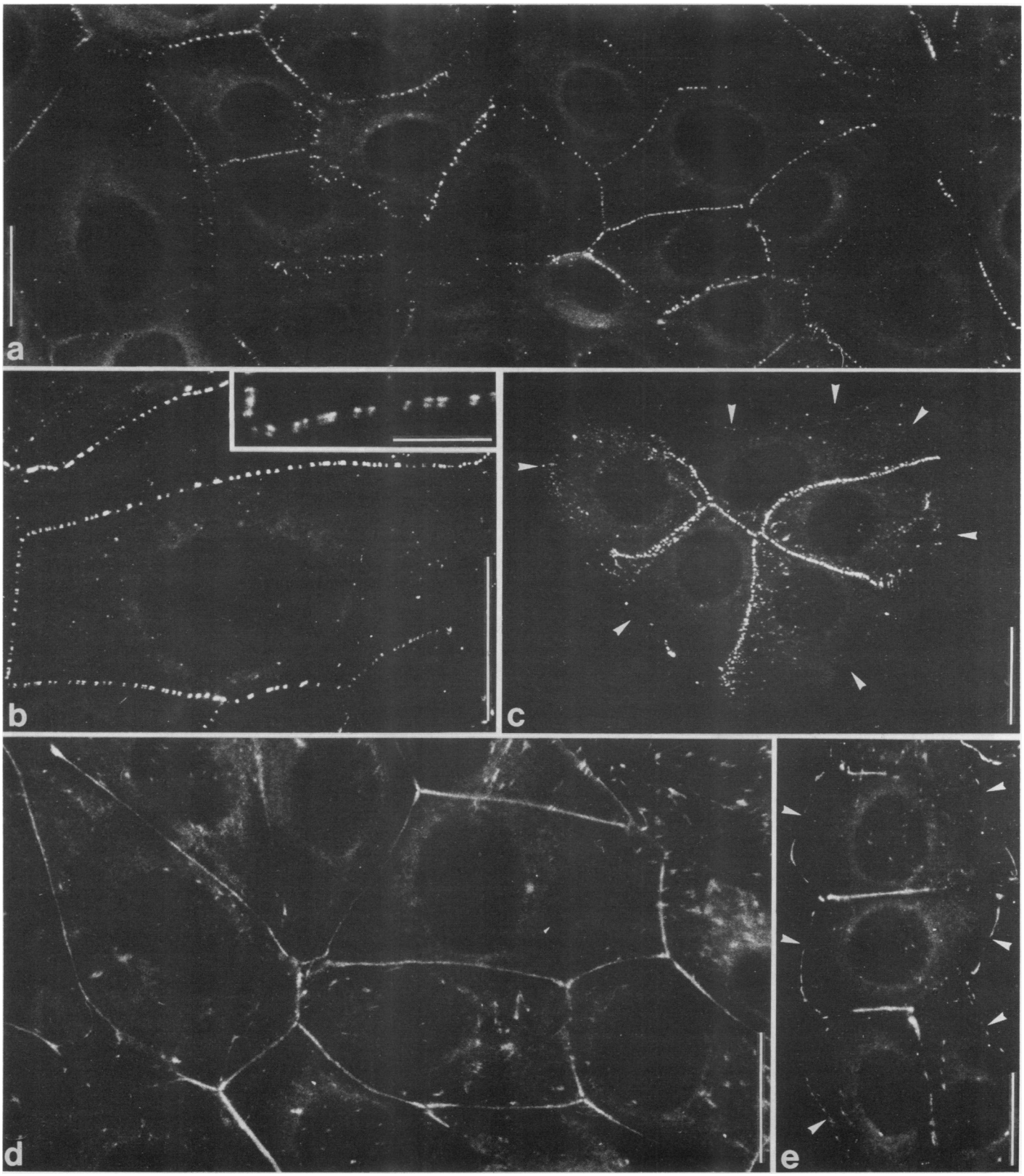

Fig. 1. Monolayer culture of MDBK cells stained for immunofluorescence microscopy with antibodies against desmosomal plaque proteins (**a**–**c**) and to vinculin (**d**, **e**). Antibodies to desmosomal plaque proteins stain cell-to-cell boundaries which appear as series of distinct fluorescent dots (**a**). Higher magnification (**b**) allows identification of individual desmosomes and, occasionally (insert in **b**), resolution of both plaques of a single desmosome separated by the intercellular gap. No fluorescence in typical desmosomal dots is seen on free cell surface edges of cell colonies (**c**, arrowheads). Staining with antibodies to vinculin also demarcates cell-to-cell boundaries but appears in the form of continuous lines, representing the belts of the *zonula adhaerens* (**d**, **e**). Vinculin is also present at focal adhesions close to the substratum (small comma-shaped structures in **d** and **e**) and on free cell surface edges (demarcated by arrowheads in **e**). Bars represent 20 μm (**a**–**f**) and 5 μm (insert in **b**).

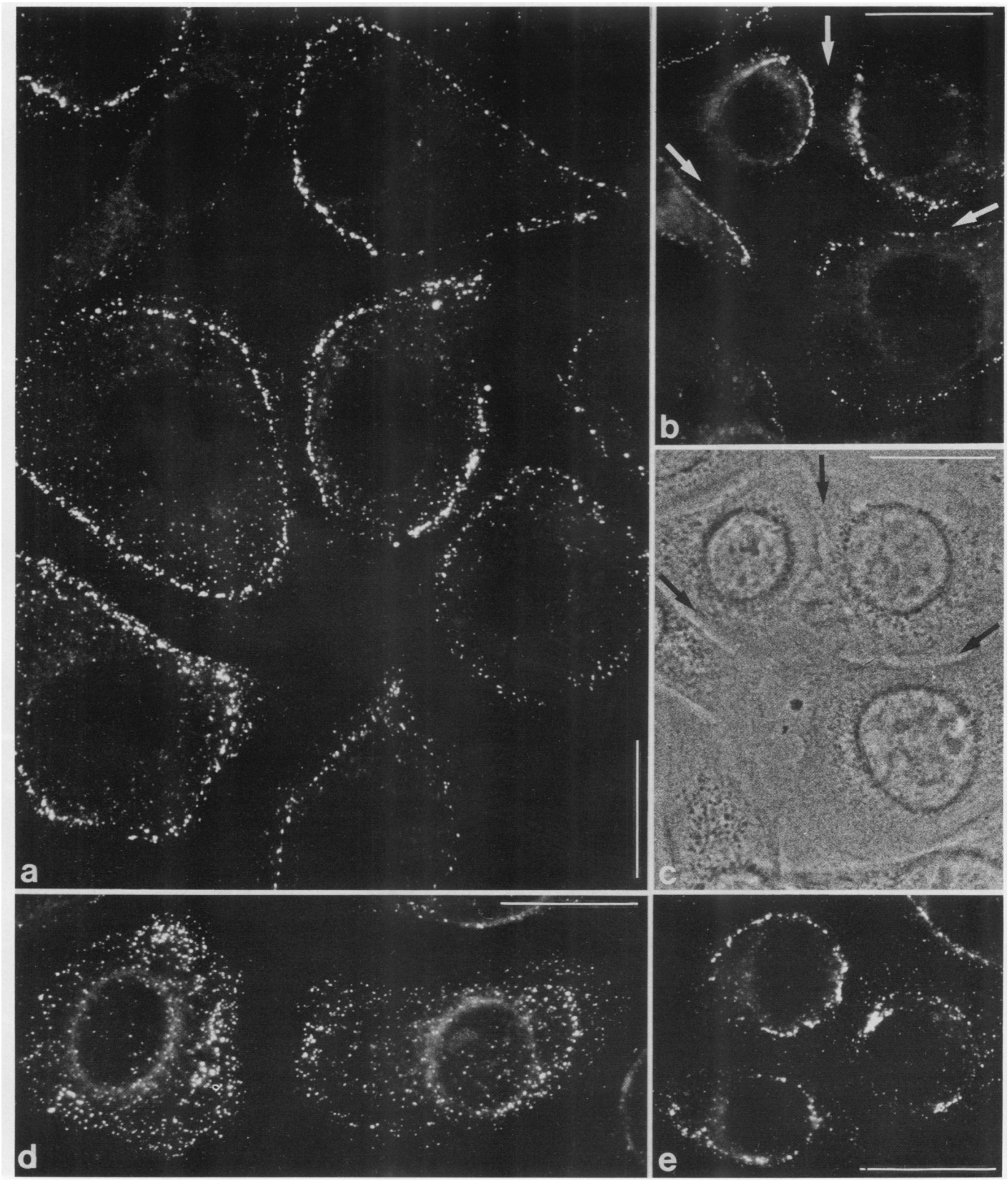

Fig. 2. Immunofluorescence microscopy using antibodies to desmosomal plaque protein on a culture of MDBK cells similar to that shown in Figure 1 which has been incubated in medium containing 2 mM EGTA. After 30 min of EGTA treatment most fluorescent dots appear in the cell interior, often still showing curvilinear arrays somewhat retracted from the cell periphery (**a, b**). This displacement of desmosomal plaque antigen is especially clear from comparison with phase contrast microscopy (**c**, arrows in **b** and **c**). 1 h after addition of EGTA the fluorescent dots are randomly distributed throughout the cytoplasm (**d**). At later time points (3 h) of EGTA treatment a certain proportion of the cells shows enrichment of desmosomal plaque protein in the juxtanuclear cytoplasm (**e**). Bars, 20 μm.

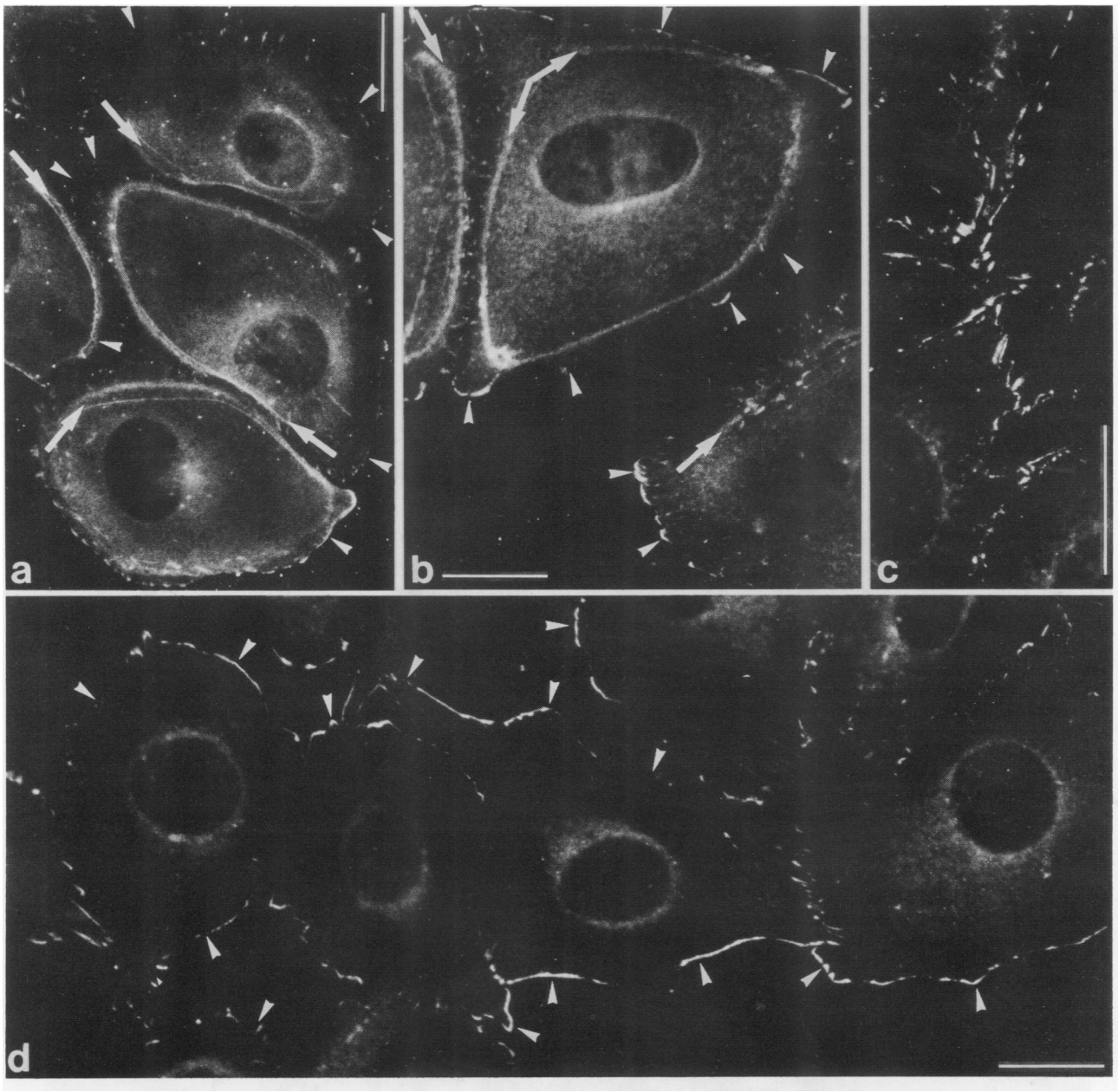

Fig. 3. Immunofluorescence microscopy using antibodies to vinculin on MDBK cells after incubation in medium containing 2 mM EGTA. After 30–60 min of EGTA treatment, vinculin staining appears in conspicuous belt-like structures away from the cell periphery (arrows in **a** and **b**). Upon prolonged treatment (3 h) this belt-like fluorescence has disappeared whereas vinculin antibody staining on cell edges is still seen (**c** and **d**; demarcated by arrowheads in **a**, **b**, **d**). Bars, 20 μm.

vesicles and often are attached to tufts of tonofilaments. Similar structures have previously been described in cells dissociated by proteolytic enzymes and have been interpreted as invaginated or vesiculated parts of half-desmosomes (Overton, 1968, 1974, 1977; Berry and Friend, 1969; Borysenko and Revel, 1973; Fukuyama *et al.*, 1974). Our present study, involving only EGTA for cell dissociation and using a molecular marker for identification of desmosomal material, confirms and extends these observations and further allows the conclusion that most of the desmosomal plaque protein remains in polymeric aggregates attached to a membrane. The question of whether the intracellular membrane profiles bearing plaque material are endocytotic vesicles or merely deeply invaginated surface membrane areas (for discussion, see Berry and Friend, 1969; Petry, 1980) has been answered by our electron microscopic examination of serial sections through gold particle-containing regions, showing that these structures are indeed endocytotic vesicles. During the vesicular internalization of desmosomal plaque proteins, the attachment of tonofilaments apparently can be maintained, indicating that the forces of endocytotic internalization are also sufficient to derange and re-arrange, at least in some places, the cytokeratin filament system (see also Overton and DeSalle, 1980) which forms a structural continuum with the desmosomal plaques (Franke *et al.*, 1978; Sun and Green, 1978).

We conclude that mechanisms exist which allow the desmosomal plaque proteins, and probably also the associated desmosomal membrane domains, to escape the dissipative forces leading to lateral redistribution which for various membrane proteins and external surface coat materials occurs

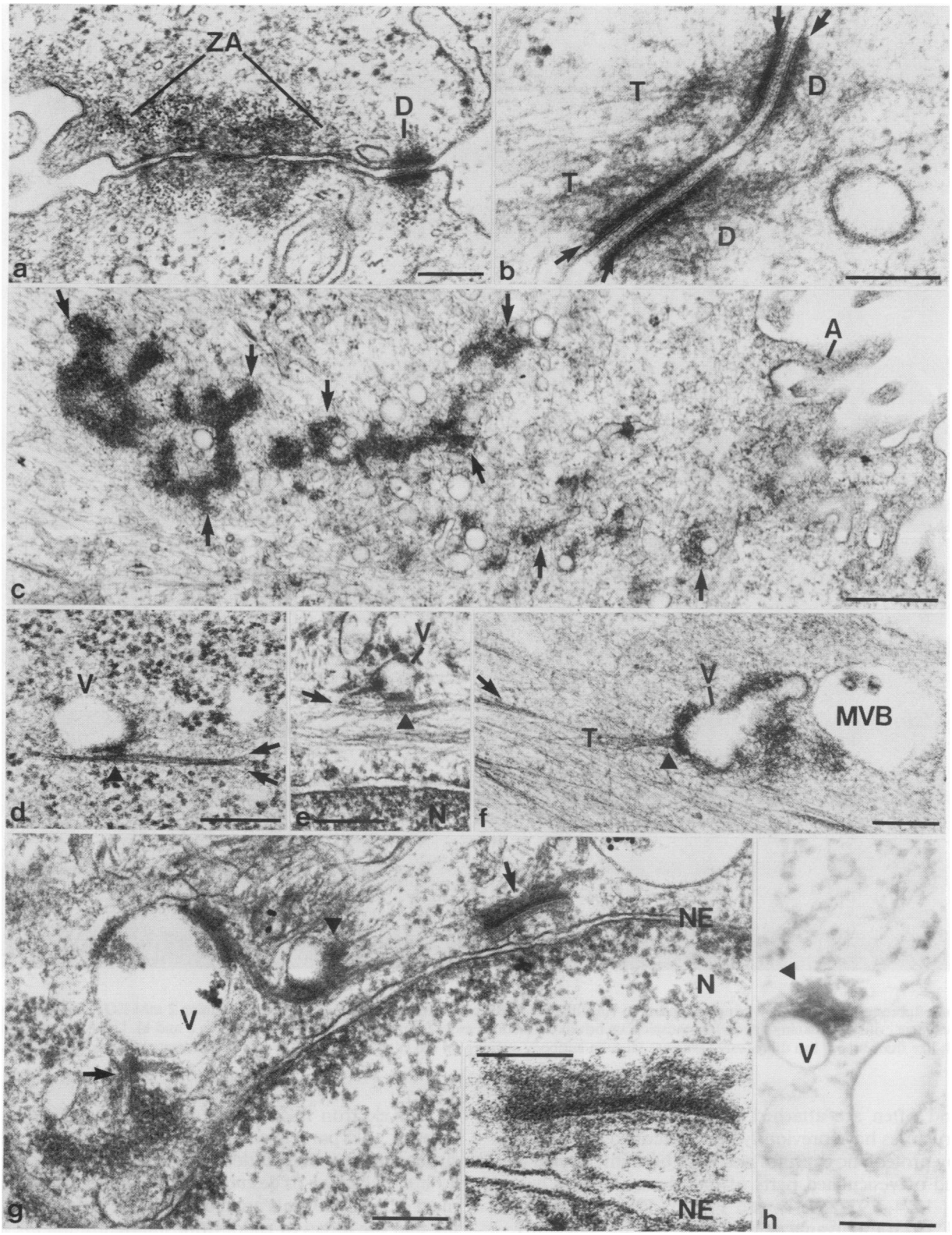

Fig. 4. Electron micrographs of MDBK cells showing normal junction morphology of *zonula adhaerens* (ZA in **a**) and desmosomes (D in **a** and **b**; arrows in **b** denote desmosomal plaques; T, tonofilaments). Cells treated with 2 mM EGTA for 2 h (**c**–**h**) show dense aggregates of finely filamentous material, probably derived from the *zonula adhaerens*, located away from the plasma membrane (arrows in **c**; A, apical surface). Desmosomes are no longer visible. A certain class of vesicles associated with "caps" of densely stained material, probably derived from desmosomal plaques (arrowheads), and tonofilaments (arrows in **d**–**f**) is now prevalent. A typical assembly of various kinds of vesicles in juxtanuclear position is shown in (**g**), including "tennis racket"- and disc-shaped vesicle formations with ultrastructural features typical of desmosomal organization (arrows and insert). Electron microscopic localization, using the immunoperoxidase technique, of antibodies to desmosomal plaque protein results in staining of the dense "caps" on these vesicles (**h**, arrowhead). MVB, multivesicular body; V, vesicles; N, nucleus; NE, nuclear envelope. Bars represent 0.5 μm (**c**), 0.2 μm (**a**, **b**, **d**–**h**), and 0.1 μm (insert in **g**). Magnifications: **a**, **d**, **f** x 57 000; **b**, **h** x 82 000; **c** x 31 000; **e** x 62 000; **g** x 64 000.

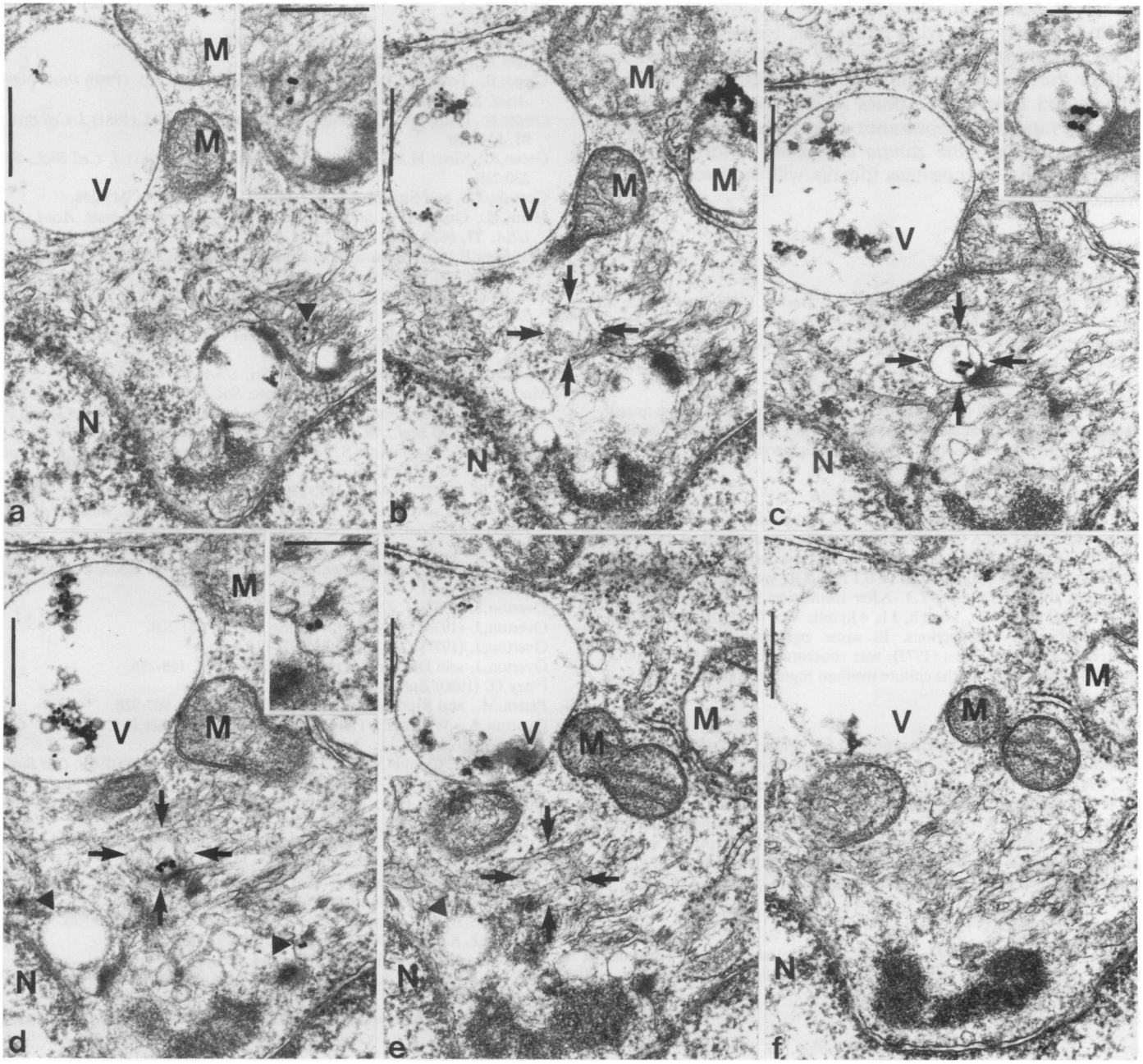

Fig. 5. Electron micrographs of consecutive serial sections through the juxtanuclear region of a MDBK cell 2 h after exposure to 2 mM EGTA applied together with colloidal gold particles, showing numerous vesicles associated with dense plaque-like structures and tufts of tonofilaments. Many of these vesicles contain gold particles (arrowheads in **a**, **d**, and **e**; some are shown at higher magnification in the inserts in **a**, **c**, and **d**). The arrows in **b**–**e** point to the same vesicle seen in grazing section (**b**), cross-section (**c**), in another grazing section (**d**), and to a corresponding region in the subsequent section (**e**). V, vacuole; M, mitochondria; N, nucleus. Bars denote 0.2 μm (**a**–**f**) and 0.1 μm (inserts in **a**, **c**, and **d**). x 72 000 and x 144 000 (inserts in **a**, **c**, and **d**).

rapidly upon cell dissociation (e.g., Pisam and Ripoche, 1976; Ziomek *et al.*, 1980). Vesicle-like structures associated with caps resembling desmosomal plaques are not restricted to cell cultures periodically exposed to trypsin or EDTA but are also found in various epithelial tissues (Schenk, 1975; Petry, 1980). This may indicate that the process of endocytotic resumption of desmosomal material also occurs in living organisms, perhaps to meet requirements of reversible cell-cell-interactions and changes in cell shape, including those taking place during mitosis as well as during epithelial cell detachment and locomotion such as in embryogenesis and metastasis.

Our study also illustrates that the processes for internalization of junctional plaque material are different for the two types of plaque-associated junctions of the cell apex: the *zonula adhaerens* releases its plaque in large aggregates detached from the membrane, whereas the desmosomal plaque material maintains its membrane association and is endocytosed in vesicles.

Upon prolonged exposure to EGTA (3–4 h) not only the

vinculin staining of the belt-structure fades but also the number of desmosome-derived vesicles positive for plaque protein is greatly reduced in many cells. However, when the EGTA treatment is stopped by shift to normal cell culture medium the cells are capable of re-establishing cell-to-cell contacts and form desmosomes as well as *adhaerens* junctions. The rates of re-appearance differ, however, for the two types of junctions: the *zonula adhaerens* is formed much earlier than the desmosomes (details will be described in a forthcoming paper).

Materials and methods

Cell culture

MDBK cells (ATCC CCL 22; Madin and Darby, 1958) were grown in minimal essential medium with non-essential amino acids and Earle's basal salt solution containing reduced bicarbonate concentration (0.85%) and 10% fetal calf serum.

Antibodies

Guinea pig antibodies to desmosomal plaque protein from bovine muzzle (Franke *et al.*, 1981b), rabbit antibodies to chicken gizzard vinculin (Geiger, 1979), and guinea pig antibodies to epidermal prekeratin from bovine snout (Franke *et al.*, 1978, 1980) have been described.

Dissociation of cells and desmosomal junctions

EGTA was used for chelating calcium ions in the culture medium. A stock solution of 0.15 M EGTA was added to the medium to obtain final concentrations of 2 and 4 mM. As this procedure resulted in a decrease of the pH value of the culture medium, some drops of 0.1 N NaOH were immediately added to re-adjust the pH value to 7.3. After incubation at 37°C for different periods of time (0, 30 min, 1 h, 2 h, 3 h, 4 h) cells were fixed for light and electron microscopical examinations. In some experiments, colloidal gold, prepared according to Frens (1973), was concentrated by centrifugation (3 min, 8000 *g*) and added to the culture medium together with the EGTA (~8 OD_{520}).

Microscopy

Cells grown on cover slips were processed for indirect immunofluorescence microscopy and electron microscopy of ultrathin sections as described (Franke *et al.*, 1981b). For electron microscopic immunolocalization, cells were fixed with 4% formaldehyde in phosphate buffer saline (pH 7.2) for 30 min and incubated in 1% Triton X-100 (same buffer) for 1 min, prior to incubation with antibodies (for details see Kartenbeck *et al.*, 1981). Light microscopy was performed with a Zeiss microscope (Photomikroskop III), and electron micrographs were taken with a Siemens Elmiskop 101.

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