EVIDENCE FOR THE PARTICIPATION OF ACTIN MICROFILAMENTS AND BRISTLE COATS IN THE INTERNALIZATION OF GAP JUNCTION MEMBRANE

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ABSTRACT

Thin sections of rabbit granulosa, human SW-13 adrenal cortical adenocarcinoma, and mouse B-16 melanoma cells revealed an apparent single-layered basket of 4- to 7-nm filaments surrounding cytoplasmic gap junction vesicles. This interpretation was based upon apparent longitudinal, cross, and *en face* sections of structures surrounding the vesicle profiles in tissue treated with tannic acid-glutaraldehyde. In granulosa cells incubated with the S-1 fragment of heavy meromyosin, arrowhead-decorated filaments were observed at the periphery of the gap junction vesicles, suggesting that these filaments contained actin. In addition, we found that small gap junctional blebs and vesicles at the cell surface were coated with short electron-dense bristles similar in appearance to the clathrin-containing coat of coated vesicles of nonjunctional membrane. The role of these and other cytoskeletal elements in the possible endocytosis of gap junction membrane is discussed.

KEY WORDS actin · microfilaments · bristle coat · gap junction membrane · endocytosis

Recent studies have documented the association of actin microfilaments with cell membranes involved in a variety of cell processes including cell locomotion, cytokinesis, and contraction of the intestinal brush border (16, 17, 35). Other elements of the contractile apparatus of skeletal muscle, including myosin and α-actinin, have also been localized in proximity to these microfilaments, which supports the hypothesis that an actomyosin system may bring about the observed movements of these membranes (6, 7, 10, 12, 13, 36, 39, 40, 43). Although it now seems possible that an actomyosin system may be involved in the process of membrane endocytosis, actin filaments, myosin, or α-actinin have not been documented in such functionally implicit relationships with identifiable endocytotic membrane.

The present study provides ultrastructural evidence for the endocytosis of a specialized membrane region, the gap junction, in three cell types, thus confirming the work of several investigators (2, 5, 11, 26, 28). In addition, we describe an apparent single-layered basket of microfilaments surrounding cytoplasmic gap junction vesicles and a bristle coat on small invaginations of gap junction membrane at the cell surface identical to that associated with single membrane pits or vesicles described in a variety of tissues. The possible role of these and other cytoskeletal and cytomuscular structures in gap junction endocytosis is discussed.

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MATERIALS AND METHODS

Tissues

Mature Graafian follicles were removed from untreated virgin New Zealand white rabbits or from rabbits stimulated 5–12 h previously with an ovulating dose (150 IU) of human chorionic gonadotrophin (hCG; Sigma Chemical Co., St. Louis, Mo.). Human adenocarcinoma cells of the adrenal cortex (SW-13) were obtained from Leibovitz (Scott and White Clinic, Temple, Texas) in their 61st passage and cultured in L-15 medium (Grand Island Biological Co. [GIBCO], Grand Island, New York) with cortisone (10 mg/ml), insulin (0.02 U/ml), streptomycin (0.1 mg/ml), penicillin (0.06 mg/ml), and fungizone (0.01 mg/ml, GIBCO). Cells in the 159th passage were used in this study. Cell cultures were maintained in a Napco automatic CO_2 incubator (National Appliance Co., Portland, Oregon) in a 5% CO_2 atmosphere at 37°C. The medium was buffered with L-arginine at pH 7.1–7.2. B-16 melanoma cells were obtained from Dr. Rebecca Fidler (Dept. of Surgery, University of Iowa, Iowa City) and were maintained in M-199 medium with 10% fetal calf serum buffered to pH 7.2–7.4 with bicarbonate-CO_2 (GIBCO) in a 5% CO_2 atmosphere at 37°C. Single SW-13 and B-16 cells, detaching from the monolayer during culture, were pelleted by light centrifugation (400 *g*) before further processing.

Electron Microscopy

For thin-section electron microscopy, follicles were cut into quarters in a fixative solution containing 2% neutralized tannic acid and 2.5% glutaraldehyde buffered to pH 7.2 with 0.05 M cacodylate at room temperature (47). SW-13 monolayers were fixed in their dishes and pelleted foating SW-13 and B-16 cells were fixed in polypropylene centrifuge tubes with the same fixative or in 2.5% glutaraldehyde buffered with 0.05 M cacodylate. All tissues were subsequently fixed in 1% osmium in 0.05 M cacodylate for 20 min, dehydrated in ethanol, and embedded in Araldite (Cargille, R. P., Laboratories, Inc., Cedar Grove, N. J.).

Tissues for freeze fracture were fixed in 2.5% glutaraldehyde with 2% sucrose in 0.05 M cacodylate, buffered to pH 7.4 at room temperature for 10 min. These were stored in 30% glycerol on ice for 3 h to 2 d before freezing and fracturing in a Balzers BAE 121 freeze-fracture apparatus (Balzers Corp., Nashua, N. H.). Thin sections and replicas were viewed in a Philips 300 electron microscope.

S-1 Decoration

Fresh heavy meromyosin (HMM) subfragment-1 (S-1) was prepared according to the procedure of Weeds and Taylor (49) and concentrated by pressure dialysis to a final concentration of 8.8 mg/ml in a pH 7.0 phosphate buffer which contained 20 mM KCl. The specific activities, measured under the conditions of Kielley and Bradley (20) and Kielley et al. (21), were 5.2 and 2.5 μmol min^{-1} mg^{-1} protein for the EDTA and the calcium ATPase assays, respectively. Actin-binding capability of the S-1 was confirmed by the observation of a large endothermic enthalpy when S-1 and rabbit skeletal muscle F-actin were mixed in a heat conduction calorimeter (P. Ritchie and C. Swenson, unpublished results). The purified S-1 was dialyzed against standard salt solution (SSS) (38) overnight at 4°C and was used full strength or diluted to 4.4 mg/ml in SSS.

Mature rabbit ovarian follicles were removed from ovaries 5 h after i.v. injection of an ovulating dose (150 IU) of hCG and quartered in 50% glycerol (vol/vol) in a modified standard salt solution (MSS) (38). After 20 min the tissue was transferred to 25% glycerol in MSS and washed for 10 min (38). After the glycerol solution was drained as completely as possible, the tissues were rinsed twice with MSS and then incubated with 8.8 mg/ml or 4.4 mg/ml S-1 in SSS for 12 h at 4°C. These tissues were then washed in SSS and fixed in tannic acid-glutaraldehyde for 1 h at room temperature as described and processed for electron microscopy.

RESULTS

Structure of Gap Junctions in Granulosa, SW-13, and B-16 Cells

Gap junctions in thin sections of all three cell types were characterized by the intimate apposition of dense, slowly curving membranes of adjoining cells. The total width of these profiles including dense cytoplasmic leaflets was ~18 nm. These structures were further characterized by a dense continuous intercellular lamella or a series of intercellular densities with a periodicity of 4–10 nm (Figs. 1 and 5). In glycerinated granulosa cells, where the plane of the junction was variable, *en face* sections of the vesicle membrane also demonstrated a hexagonal lattice work of electron-dense spots 2.5–5.0 nm in diameter (Fig. 1). Their center-to-center spacing was ~11 nm while the lattice constant measured ~9.6 nm. Freeze-fracture replicas revealed characteristic gap junction particles and pits in all three cell types (Fig. 2). In granulosa cells, these aggregates varied in size from a few particle diameters to 23 μm^2, while in SW-13 cells, the largest aggregates were ~2 μm^2. Gap junction plaques observed in B-16 cells were usually ~0.05 μm^2 and were further characterized by dense particle packing. These junctions are similar to those observed in normal melanocytes (unpublished results of K. Joos and W. Larsen) and in cultured mouse S-91 melanoma cells (23). Thin sections also revealed numerous cytoplasmic annular gap junction profiles and highly irregular junctional profiles, still connected to nonjunctional membrane in all three cell types (Figs. 1, 3–8, 11 and 12). Infrequently, small desmosomes and intermediate junctions were observed in granulosa and SW-13 cells. Neither thin sections nor freeze-fracture replicas contained evidence of the existence of tight junctions in granulosa, SW-13, or B-16 cells.

Cytoplasmic Gap Junction Vesicles in a Filamentous Basket

Sections through the cytoplasm of rabbit gran-

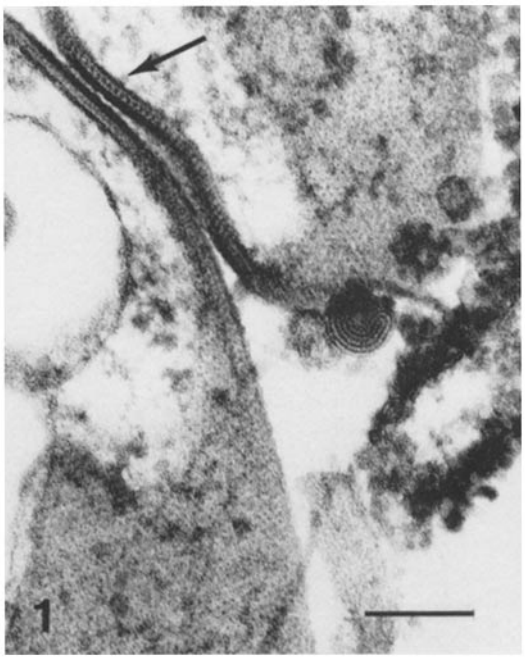


FIGURE 1 Sectioned gap junction vesicles in rabbit granulosa cell after glycerination. Periodic intercellular densities observed in cross-sectioned membrane (arrow) are organized in typical gap junction hexagonal lattice in *en face* views. Bar, 0.1 μm. × 142,000.

ulosa cells revealed numerous bimembranous annular gap junctional profiles surrounded by apparent longitudinal, cross, and *en face* sections of filaments measuring between 4 and 7 nm in diameter. In some sections a thin continuous line ~4–7 nm thick was observed ~10 nm from and precisely parallel to the gap junction membrane (Fig. 3 *a* and *b*). Other micrographs revealed a single row of electron-dense spots incompletely circumscribing the annular junctional membrane profile (Fig. 3 *c* and *d*). The spacing between these periodic densities varied from 10 to 40 nm. In areas where the junctional vesicle was sectioned tangentially, parallel electron-dense filaments 4–7 nm thick and spaced 10–40 nm apart were observed (Figs. 3 *c* and 4). Apparent sections of thin filaments were also observed at the periphery of SW-13 and B-16 cell annular junctional profiles (Fig. 5 *a* and *b*).

The filaments surrounding the junctional vesicle always appeared thinner and less dense than groups of larger 8- to 12-nm filaments which were rarely observed in proximity to gap junction membrane (Fig. 6 *a* and *b*). For this reason we considered the possibility that the filamentous basket was composed of actin microfilaments. To further characterize these filaments then, we attempted to decorate them with S-1 of HMM, which is a diagnostic test for the presence of actin.

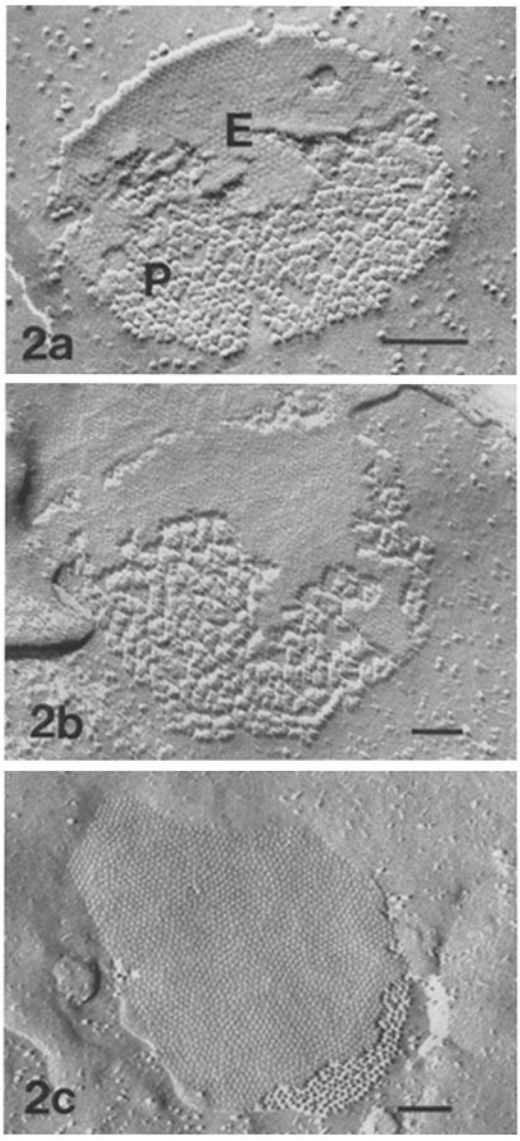


FIGURE 2 Freeze-fracture replicas of gap junctions of (*a*) rabbit granulosa cell, × 10,000; (*b*) SW-13 adenocarcinoma cell in the monolayer, × 68,000; and (*c*) cultured B-16 melanoma cell, × 70,000. Characteristic P-fracture face (*P*) particles and E-fracture face (*E*) pits are evident. Bars, 0.1 μm.

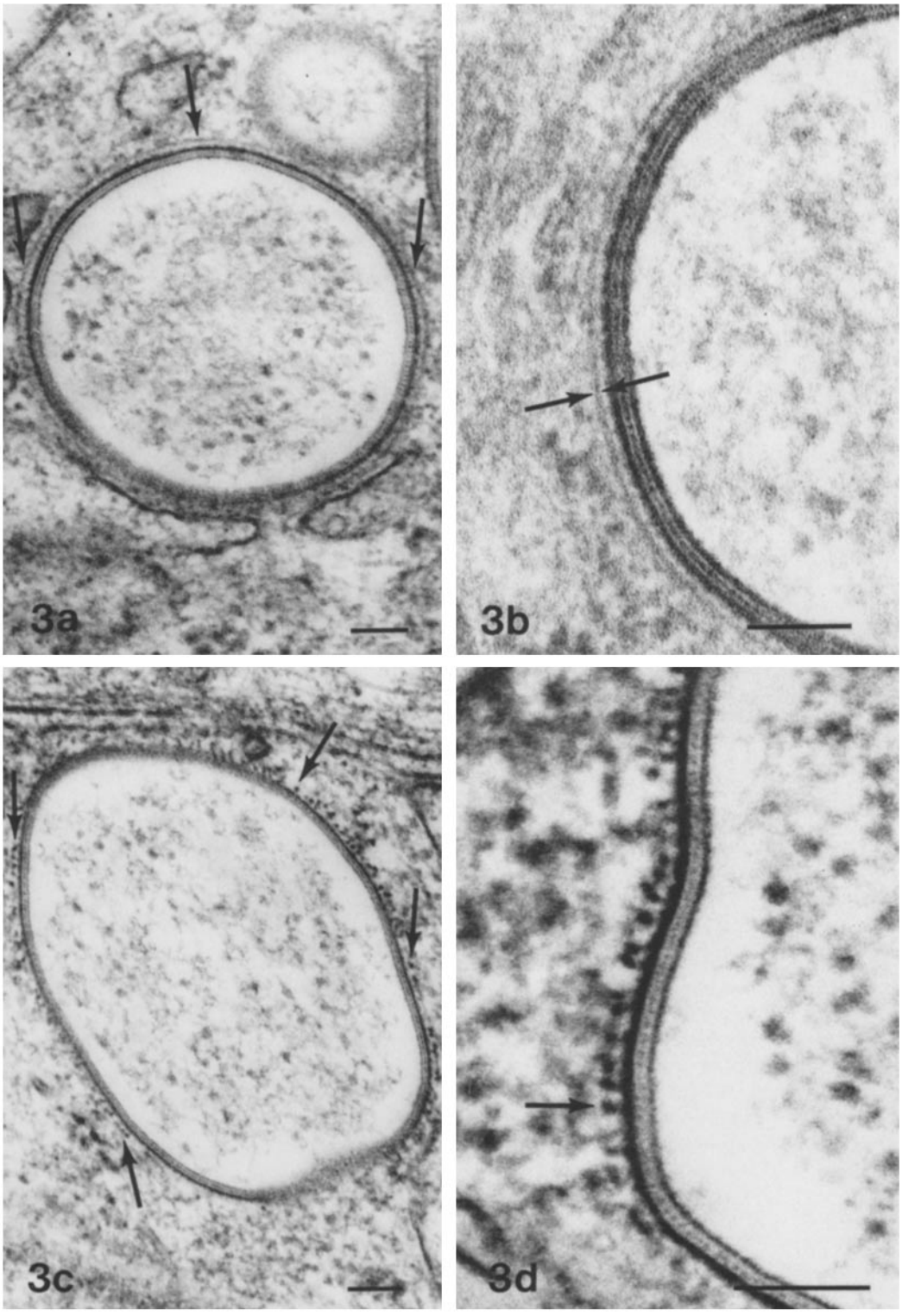


FIGURE 3 Sectioned cytoplasmic gap junction vesicles in rabbit granulosa cells. (*a*) Low-power view reveals an electron-dense halo around most of vesicle profile circumference (arrows). × 87,700. (*b*) Higher magnification micrograph demonstrating 4- to 7-nm electron-dense halo (between arrows) ~10 nm from the junctional membrane profile. × 158,000. (*c*) Low-power micrograph of sectioned gap junction vesicles surrounded by electron-dense dots (arrows). × 82,500. (*d*) High magnification view of gap junction vesicles with associated densities (arrow) observed in the same location as the electron-dense line surrounding the vesicle membrane in *a* and *b*. × 207,200. Bars, 0.1 μm.

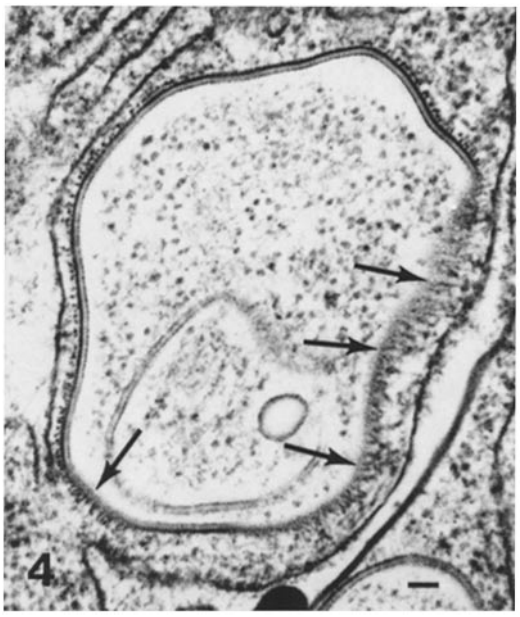


FIGURE 4 Sectioned gap junction vesicle in rabbit granulosa cell. In areas where membrane is sectioned tangentially (arrows), apparent filaments are closely applied to vesicle surface. Bar, 0.1 μm. × 40,500.

S-1 Decoration of Filaments Associated with Cytoplasmic Gap Junctional Vesicles

Arrowhead-decorated filaments were frequently observed in close proximity to sections of cytoplasmic gap junction vesicles after incubation in a salt solution containing S-1 of HMM. Longitudinal sections of these filaments often appeared at the periphery of the annular junctional profile in the same location as the 4- to 7-nm-thick filaments observed in untreated specimens (Fig. 7 *a*). These decorated structures, however, never formed a continuous belt completely circumscribing the annular profile. More frequently, grazing sections of the gap junction vesicle revealed groups of arrowhead-decorated filaments closely applied to the outer surface of the gap junction vesicle in regions where 4- to 7-nm filaments were observed in untreated tissues (Fig. 7 *b–f*). In cases where the arrowheads were clearly demonstrated on filaments emanating from junctional membrane, they usually appeared to be directed away from the membrane (Fig. 7 *d*). In one case, however, arrowheads were directed toward the junctional membrane (Fig. 7 *c*). The likelihood that these arrowhead-decorated filaments are composed of F-actin was supported by the finding that the thin filaments of rabbit cardiac muscle treated with S-1 along with the granulosa cells were also decorated with identical arrowhead structures.

Structures Associated with Gap Junction Membrane at the Cell Surface

Since it seemed possible that the apparent cytoplasmic gap junction vesicles arose through the internalization of cell-cell gap junction membrane, we also examined gap junction membrane at the cell surface for evidence of interactions with cytoskeletal or cytomuscular structures. We discovered that small blebs of surface gap junction membranes 150–300 nm in diameter were frequently coated with short periodic bristles (Fig. 8 *a–d*, *f*, and *g*), whereas straight or slowly curving gap

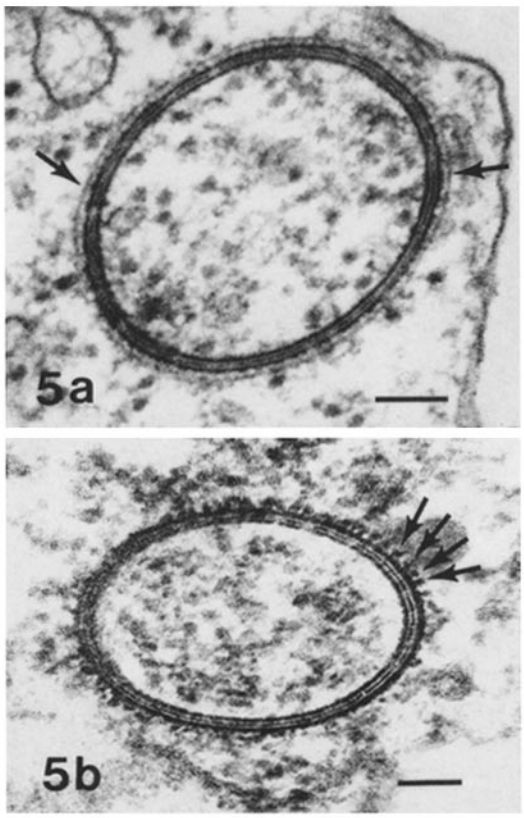


FIGURE 5 Sectioned gap junction vesicle in (*a*) floating SW-13 cell, arrows denote apparent 4- to 7-nm filament at periphery of profile × 90,700; and (*b*) floating B-16 melanoma cell, arrows designate periodic densities similar to those observed surrounding gap junction vesicles in rabbit granulosa cells, × 83,000. Bars, 0.1 μm.

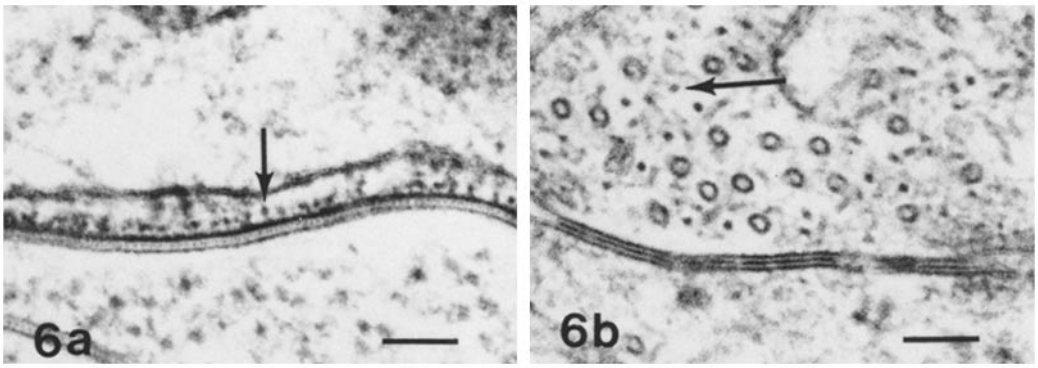


FIGURE 6 Micrographs of two filament types in rabbit granulosa cells at the same magnification. The largest of the smaller filaments indicated by the arrow in *a* is similar in size to the smallest of the larger filaments shown in *b*. The relationship of larger filaments and microtubules with gap junction membrane illustrated in *b* is rare. Bars, 0.1 μm. × 99,500.

junction membranes were devoid of such bristles (Fig. 8 *a–c*, *f*, and *g*). These bristles were ~5–10 nm thick and were spaced ~20 nm apart. They occurred only on the convex side of the gap junctional bleb. Their density, size, and organization were similar to structures surrounding single-membrane vesicles in the same cells (Fig. 8 *d* and *e*). Although some areas of coated gap junction membrane appeared to be continuous with junctional membrane at the cell surface (Fig. 8 *a* and *b*), such connections were not as distinct in other cases (Fig. 8 *c*, *f*, and *g*). *En face* sections of vesicles or blebs in the vicinity of cell-cell gap junctions revealed polygonal substructure (Fig. 9 *a* and *b*), but it was not possible to determine whether these structures were located on junctional or nonjunctional membrane. Cytoplasm in the vicinity of gap junction membrane coated with short bristles often appeared to contain electron-dense particles or short sections of apparent filaments (Fig. 8 *f* and *g*). In some cases these accumulations completely surrounded convoluted profiles of gap junction membrane at the cell surface (Fig. 10).

Occasionally profiles of microtubules and filaments measuring ~10 nm in diameter (Fig. 6 *b*) were visualized in the vicinity of gap junctions still connected to the cell surface.

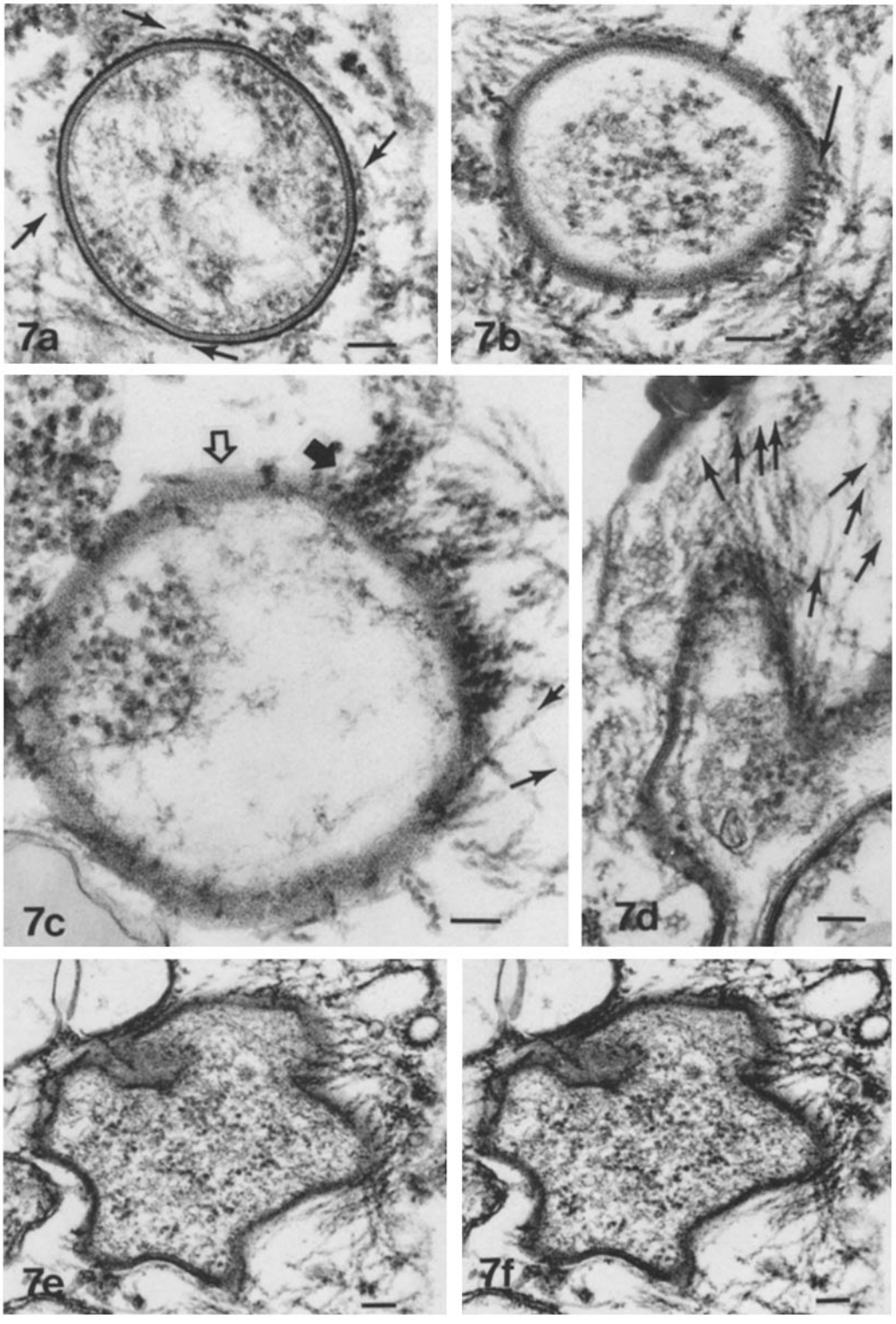
DISCUSSION

In this report, we present evidence of an association between organized microfilaments and cytoplasmic gap junction vesicles, and the occurrence of bristle coats similar to those occurring on single membrane-coated vesicles or pits (19) on gap junction invaginations from the cell surface. Although the role these structures play in the function of gap junction membrane is still unclear, several recent studies support the possibility that their activity may be directly related to the endocytosis of gap junction membrane. This hypothesis is supported by the results of other studies suggesting that a contractile apparatus homologous with that described in skeletal muscle fibers is responsible for the movements of specific cellular membranes including those involved in phagocytotic activity (16, 17, 44).

Actomyosin-Mediated Membrane Movements in Endocytosis

Direct evidence for the functional interaction of an actomyosin contractile apparatus with cellular membranes has been provided by ultrastructural and antibody studies revealing the association of contractile proteins with specific cell membranes (1, 9, 22, 27, 30, 31, 34, 35, 36, 42). The inhibition or stimulation of membrane movements by agents affecting the activity of some of these contractile elements has provided more indirect evidence for this postulated relationship (1, 8, 18, 29, 48).

Evidence for the action of an actomyosin system in the endocytosis of cell membrane has been derived from similar studies (44). Filaments thought to be (on the basis of size) actin-containing microfilaments have been demonstrated in the vicinity of phagocytotic membrane in mononuclear phagocytes (37) and in *Amoeba proteus* (45) and filaments in the peripheral cytoplasm of phagocytosing macrophages have been demonstrated to bind HMM (4).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There is also evidence for the association of another contractile element with endocytotic vesicles. Recently, α-actinin has been extracted from purified preparations of coated vesicles from bovine brain (41). Since α-actinin appears to represent a major component of the Z disk of striated muscle (25), it has been argued that membrane-associated α-actinin may provide attachment sites for actin microfilaments (13, 30). It therefore seems possible that α-actinin on the coated vesicle membrane could anchor microfilaments involved in the endocytotic process.

Less direct support for the action of an actomyosin system in endocytosis has been provided by experiments demonstrating that cytochalasin B, a drug capable of affecting the integrity of microfilaments can inhibit the phagocytotic activity of macrophages (3, 4).

Endocytosis of Gap Junction Membrane

The observation of bimembranous gap junction vesicles within the cytoplasm of cells of many different types has stimulated the speculation that gap junctions at the cell surface may be internalized through an endocytotic mechanism (2, 5, 11, 14, 23, 24, 28). The lack of continuity with the cell surface of some cytoplasmic annular junction profiles is supported by the lanthanum tracer studies of Garant (14) and Merk et al. (28), and by the serial sectioning studies of Merk et al. (28) and Espey and Stutts (11). The presence of numerous annular gap junction profiles within the cytoplasm of single floating SW-13 and B-16 cells as shown in the present study, is indirect evidence that these structures are isolated from the cell surface. Further circumstantial support for the endocytosis of gap junctions is based on evidence suggesting that annular junction profiles may represent a stage in the degradation of gap junction membrane. We have recently demonstrated the presence of acid phosphatase activity within the matrix of these apparent vesicles in rabbit granulosa cells (24) and SW-13 cells (S. A. Murray and W. Larsen, unpublished observations). In addition, Ginzberg and Gilula (15) have demonstrated the presence of annular gap junctional profiles within phagolysosomes of developing chick otocysts.

The most likely mechanism of removal of gap junction membrane from the cell surface, therefore, involves the invagination of one cell into another at the gap junction, followed by constriction and pinching off of the invagination, thus isolating both junctional membranes within the cytoplasm of one cell (23).

Organization of Filaments Surrounding Gap Junction Vesicles

Our study has revealed a basket of organized microfilaments surrounding gap junction vesicles located within the cytoplasm of three cell types. This finding is significant, in part because it is the first direct evidence obtained of an association between an organized aggregation or network of microfilaments and endocytotic membrane. The movement of membrane in several other cellular processes has also been shown to involve organized aggregations of microfilaments rather than single randomly distributed filaments (1, 30, 32, 38, 46, 50).

Although profiles of gap junction vesicles completely encircled by longitudinally sectioned filaments argue for the likelihood that some filamentous baskets are completely closed, evidence of groups of filaments apparently emanating from a small area of the vesicle surface has also been obtained. In addition, clear cross-section profiles of the filaments do not always completely encircle the annular junctional profile. Evidence of completely closed baskets of decorated microfilaments has not yet been obtained in S-1-treated prepara-

FIGURE 7 Sections of S-1-decorated filaments at periphery of cytoplasmic gap junction vesicles in rabbit granulosa cells. (*a*) Apparent longitudinal sections of S-1-decorated filaments at vesicle profile periphery (arrows). × 71,500. (*b*) Tangential section of apparent cytoplasmic vesicle with numerous filament sections at one pole (arrow). × 77,900. (*c*) In this micrograph, the typical gap junctional lattice pattern is more clearly visualized (open arrow) in tangential section of apparent cytoplasmic gap junction vesicle. Numerous decorated filaments are intimately applied to the surface of the vesicle (large closed arrow). The polarity of two of these filaments is indicated by small arrows. × 77,600. (*d*) S-1 arrowheads all point away from points of attachment to this cytoplasmic gap junction vesicle. × 71,700. (*e* and *f*) Stereo pair of gap junction vesicle demonstrating that decorated filaments at right closely follow the contour of the vesicle membrane. × 49,400. Bars, 0.1 μm.

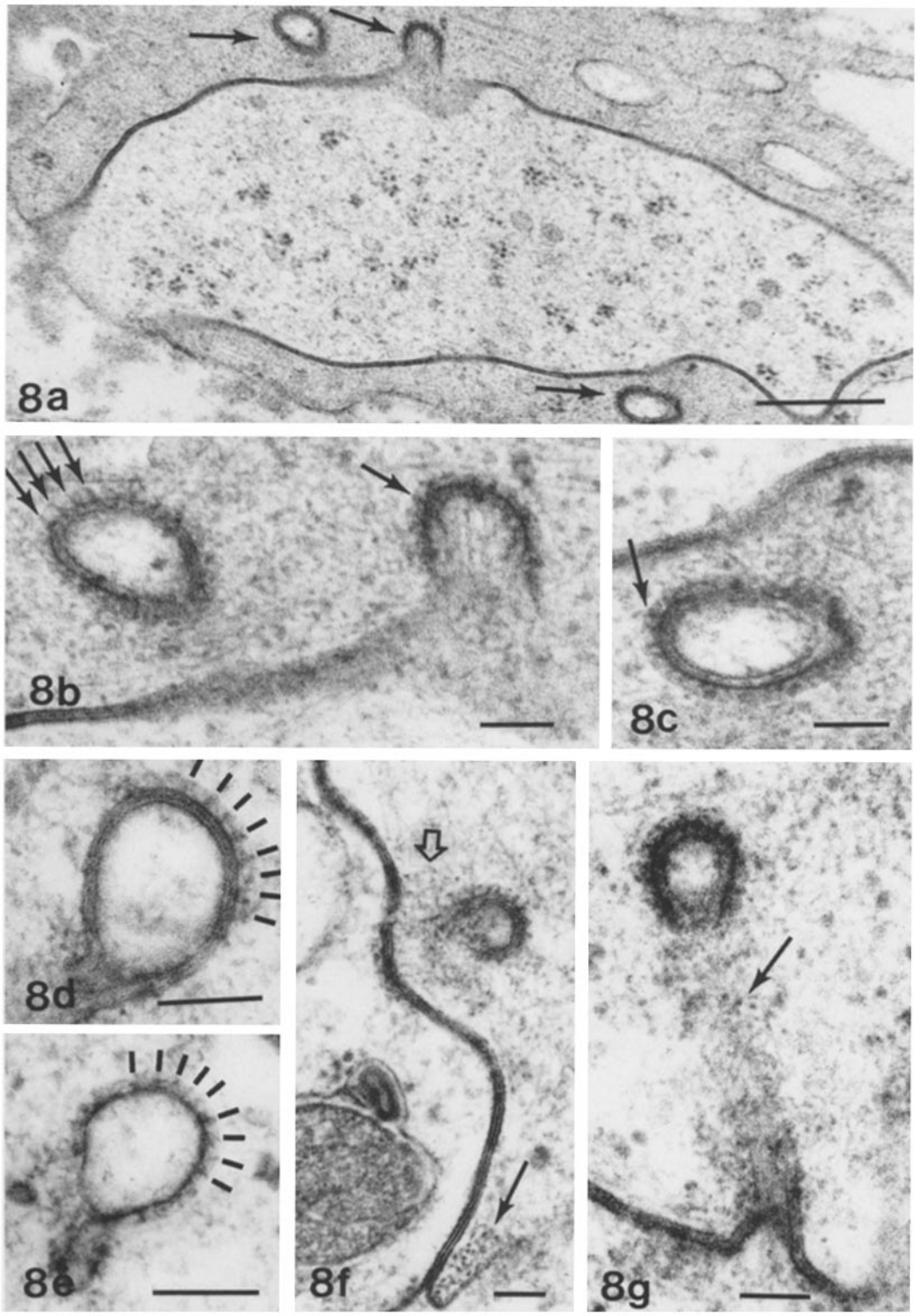

FIGURE 8 (*a*) Large granulosa cell gap junction invagination with small bleb and two small apparent gap junction vesicles in immediate vicinity (arrows). Bar, 0.5 μm. × 38,200. (*b* and *c*) High magnification views of bleb and small vesicles shown in *a* demonstrating 25-nm-thick bristle coats (arrows). Note that rest of gap junction membrane is devoid of bristles. Bars, 0.1 μm. × 110,000. (*d* and *e*) Bristles on gap junction bleb (*d*) have similar periodicity to bristles on single membrane vesicle (*e*) in same cell. Bars, 0.1 μm. × 116,000. (*f*) A small granulosa gap junction bleb with bristles at cytoplasmic pole and discrete cytoplasmic densities at opposite pole (open arrow). Small arrow denotes apparent filament sections at gap junction edge. Bar, 0.1 μm. × 76,500. (*g*) This bristle-coated gap junction vesicle may be connected to surface gap junction membrane by thin neck in region of small arrow. Note also discrete 5-nm-diameter densities in this area. Bar, 0.1 μm. × 107,600.

tions, and we do not know whether or not this is the result of partial disruption of the basket by glycerol or S-1 treatment. We also cannot exclude the possibility that the basket could be totally disrupted and randomly replaced during treatment by bundles of decorated microfilaments from different locations within the cell. None of the organized microfilament configurations just described, however, have ever been observed to enclose other membrane vesicles or organelles in the cells studied, and so we believe that they represent specific associations with gap junction membrane.

Identification and Polarity of Actin Filaments Associated with Gap Junction Vesicles

The identification as microfilaments of the filaments composing the basket is based upon measurements of their diameter and the results of S-1-binding experiments. Tannic acid-glutaraldehyde fixation of granulosa cells treated with S-1 has provided unequivocal evidence for the intimate interaction of arrowhead-decorated filaments and gap junction vesicles.

It has been suggested that the movement of structures generated by attached microfilaments is directed toward the points of arrowhead structures produced by HMM or S-1 decoration (13, 30). Where it can be clearly determined, arrowheads on many of the microfilaments associated with gap junction vesicles point away from their apparent point of attachment with the membrane (refer to Fig. 7*d*). How this configuration may be structurally related to junctional invaginations in the process of endocytosis, however, is not yet clear.

Structures Associated with Gap Junction Membrane at the Cell Surface

Although the short bristles emanating from small invaginations of junctional membrane at the cell surface bear some similarity to microfilaments, comparison of these structures with the covering of coated single-membrane vesicles in the same cell (Fig. 8*d* and *e*) has prompted an alternative explanation of their composition. It seems possible that these structures may represent cross-sectional views of the same clathrin coat surrounding coated vesicles described most extensively in brain (19, 33, 41). Unfortunately, it has not yet been possible to unequivocally identify polygonal substructure in definitive *en face* sections of gap junction membrane invaginations at the cell surface. Suspected microfilaments observed in areas of invaginated junctional membrane (Fig. 10) do not, however, appear to be attached to the membrane or to the small coated gap junctional vesicles located nearby.

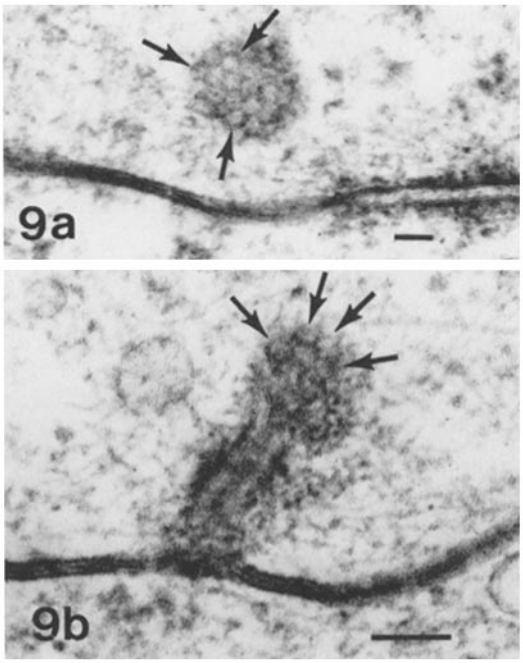

FIGURE 9 (*a*) Apparent *en face* view of coated vesicle in vicinity of gap junction membrane. × 50,000. (*b*) A structure displaying a polygonal pattern similar to that illustrated in *a* appears to be connected to gap junction membrane at the cell surface. × 100,000. Bars, 0.1 μm.

Since the association with gap junction membrane of apparent 10-nm filaments and microtubules is rare, we are uncertain of the possible functional implications of these relationships.

CONCLUSION

This report documents an interaction between an organized basket of microfilaments and gap junctional membrane apparently removed from the cell surface through an endocytotic process. We believe that our evidence favors the hypothesis that actin microfilaments play a central role in the endocytosis of gap junction membrane. We have also documented the presence of a bristle coat on surface invaginations of gap junction membrane structurally identical to that observed on nonjunctional endocytotic membrane, thus providing additional support for the hypothesis that gap junctional regions may be internalized through an

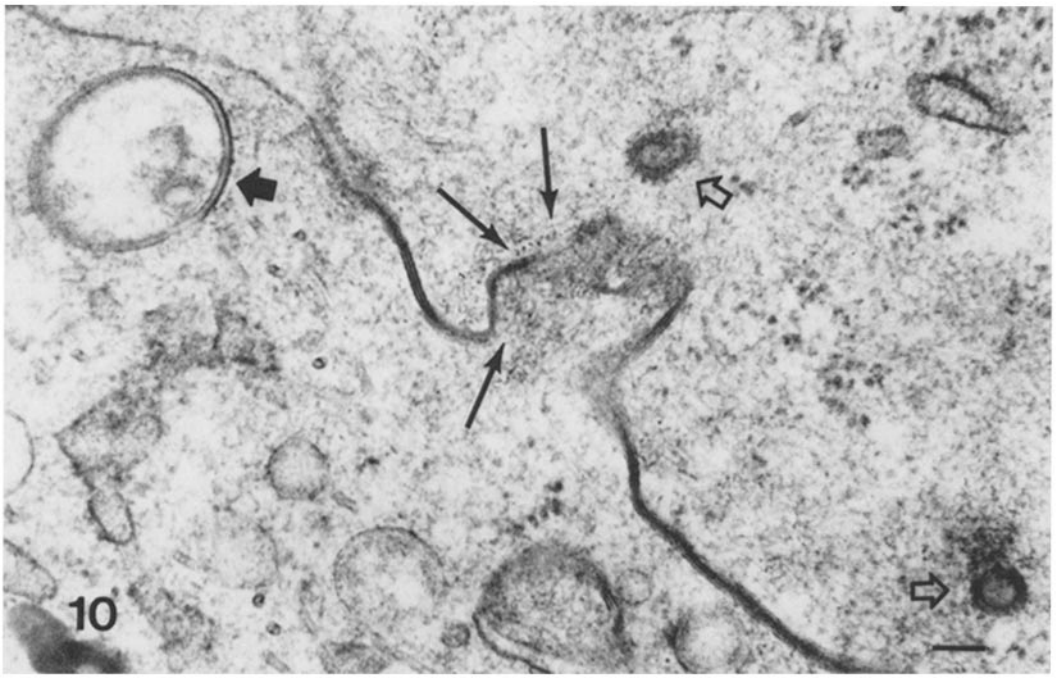

FIGURE 10 Convoluted area of cell-cell gap junction membrane with associated filaments (thin black arrows), bristle-coated gap junctional blebs or vesicles (open arrows), and large, cytoplasmic gap junction vesicle (thick closed arrow). Bar, 0.1 μm. × 61,500.

endocytotic mechanism. How these bristles may be related to the basket of microfilaments surrounding the cytoplasmic junctional vesicles, however, is still unclear.

Ongoing studies are directed to a possible relationship between the microfilamentous basket and bristles associated with gap junction membrane. We will be particularly interested in determining whether or not microfilaments interact in predictable patterns with gap junctional invaginations not yet separated from the cell surface and in attempting to localize other contractile elements, including α-actinin and myosin with these membranes.

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