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Membrane-integrated Proteins at Preformed Exocytosis Sites[1]

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Paramecium tetraurelia **cells were used for analyzing the crucial but also controversial question regarding the organization of those sites of the cell membrane that undergo fusion during exocytosis. Paramecia are unique in that they display a "rosette" of from eight to ten MIPs (Membrane-intercalated particles, as seen on freeze-fracture replicas) at each of their numerous preformed exocytosis sites. We analyzed these structures by exposing slightly fixed cells to various enzymes and by subsequent freeze-fracturing. We show that "rosette" MIPs are selectively sensitive to proteolytic enzymes. Since "rosettes" are known to be necessary for membrane fusion, this cytochemical result would be in line with the assumption that membrane-integrated proteins may play an active role in regulating exocytotic membrane fusion.**

KEY WORDS: Exocytosis; Freeze-fracture; Intramembranous particles; Membranes; Membrane proteins; *Paramecium*.

Introduction

Although the general sequence of events leading to the fusion of a secretory vesicle with the cell membrane during exocytosis is currently well established (15), there is considerable uncertainty regarding the molecular events that occur during the fusion process itself. A crucial but also very controversial point (see, e.g., refs. 17 and 21) is the fate of membrane-intercalated particles (MIPs), as seen on freeze-fracture replicas, during membrane fusion. There is clearly a need for more information on the chemical composition of molecular constituents occurring at these fusion sites. The occurrence of regularly arranged ultrastructural elements, including "rosettes" of eight to ten MIPs, at exocytosis sites in *Paramecium* cells (18) should greatly facilitate this task. Therefore, we applied cytochemical methods on the ultrastructural level, including exposure to enzymes and subsequent freeze-fracturing, in order to elucidate what takes place in our particular system.

Materials and Methods

Paramecium tetraurelia cells, strain K401, grown monoxenically in a decoction of dry lettuce in the presence of *Enterobacter agglomerans*, were harvested at early stationary phase. Culture conditions were as specified previously (20).

Cells were fixed with glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.0) for only 1 min, followed by 1:10 dilution with buffer for 10 min. This prefixation was required primarily to render the cells just resistant enough for the enzymatic degradation experiments at various pH values (see also Results). After several washes, cells were exposed to various enzymes, as specified in Table 1. We applied pronase, trypsin, chymotrypsin, and esterase (all of highest purity from Merck, Darmstadt, FRG) in free form and occasionally after conjugation to cyanobromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), according to Axén et al. (1). All enzymes were used under their respective optimal conditions. Controls included exposure to the identical conditions (buffer, ionic strength, pH, etc.) but without the particular enzyme added.

The enzymatic activity of proteolytic enzymes was tested by casein splitting tests (4). While the cells were incubated, the enzymatic activities indicated in Table 1 were permanently controlled by parallel spectrophotometric tests, according to ref. (4). Enzyme solutions were renewed when the activity had dropped by more than 25%. This allows the direct comparison of all results presented in Table 1 and Figures 2–4.

Since it was necessary to prefix cells (for the reasons mentioned above and in the Results section), we also performed the common antifreeze impregnation (10, 20, 30% glycerol, 10 min each), before pellets were frozen (by dipping vigorously into liquid Freon 22) and freeze-fractured in a Balzers BAF 300 unit (turbomolecular pump, 45° Pt-C replication). Aliquots for ultrathin sectioning were prepared after the same kind of prefixation and enzyme treatment, but then stained with tannic acid, as reported previously (31).

Results

The system used, *Paramecium tetraurelia* cells, possesses exceptionally well-defined fusion zones (18) (Figures 1–4). These are regularly arranged over most of the cell surface (Figure 1); each fusion zone is encircled by a 300-nm wide—mostly

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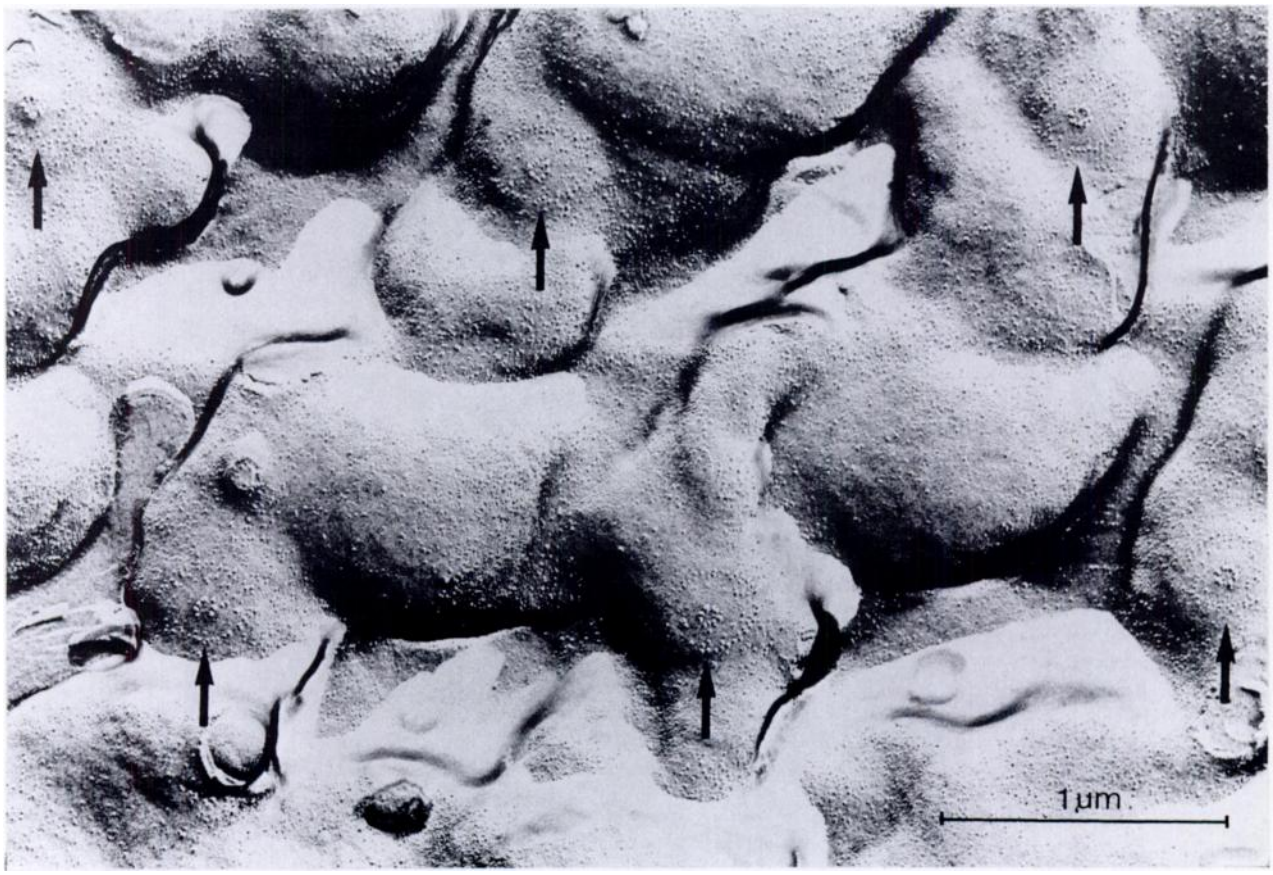
Table 1. *Enzymatic degradation of "rosette" MIPs*

Type of enzyme	Activity (A)[b] × time (t)[c] product	Enzyme activity (A)[b]	Incubation time (t) (N × hours)[c]	Degree of digestion of "rings" and "rosettes"[d]	Figure reference
Controls[a]	0	0	4–48 hr	0	1a
Free pronase	2.5	0.14	18 hr	+ +	1b
	10.8	1.35	2 × 4 hr	+ +	
	21.6	1.35	4 × 4 hr	+ + to + + +	1c
	216.0	13.5	4 × 4 hr	+ + +	
Free trypsin	7.2	0.4	18 hr	0	
	67.2	4.2	4 × 4 hr	+	
	675.2	42.2	4 × 4 hr	+ + to + + +	1d
Free chymotrypsin	0.9	0.05	18 hr	0	
	8.0	0.5	4 × 4 hr	0	
	72.0	4.5	4 × 4 hr	+	
Pronase conjugate	10.6	0.22	48 hr	+ to + +	1e
Free esterase	540.0	30	18 hr	0	1f

[a]Controls: buffers alone or inactivated enzymes.
[b]A = enzyme activity (pronase, trypsin, chymotrypsin, in transmission units (TU), for casein splitting activity, ΔE/mg enzyme/min; esterase activity is indicated as enzyme units).
[c]N = number of changes of enzyme solutions, t = hours of incubation time.
[d]Scale for digestion: 0 = none, + = weak, + + = medium, + + + = strong (almost total) digestion, according to freeze-fracture experiments. Since "rings" and "rosettes" react about in the same way (although "rings" react more slowly), we did not differentiate between these two structures. Conjugates affect "rosette" MIPs selectively (see Results).

Figure 1. Survey of a freeze-fractured control (fixed but undigested); P-face. Arrows point to numerous preformed exocytosis sites, which are arranged at regular intervals on top of elevations along the cell body. These sites are characterized by the presence of about 300 nm wide MIP "rings" with "rosette" MIPs in their center. For a detail see Figure 4a. Shadow casting from bottom to top. Original magnification × 38,000.



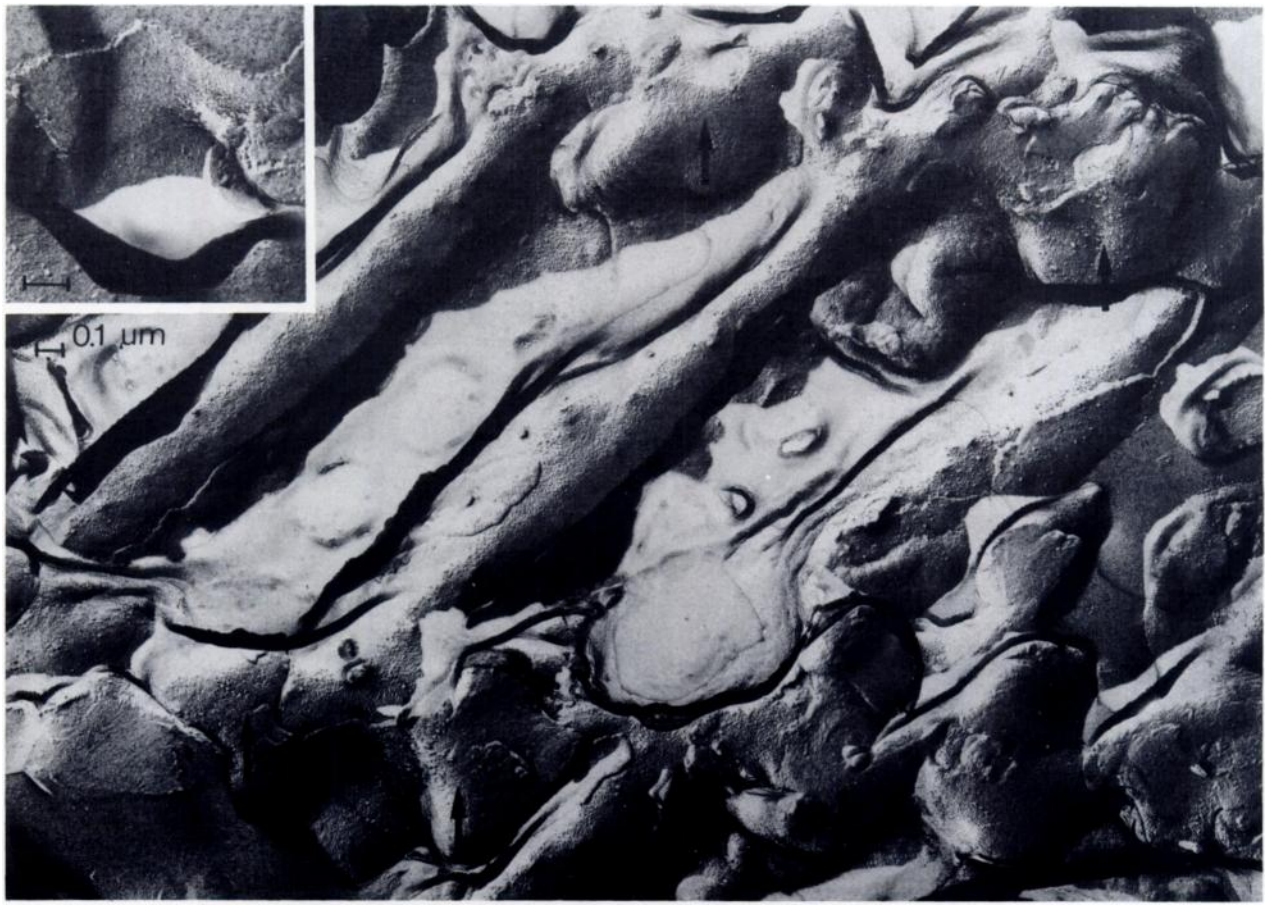


Figure 2. Survey of a freeze-fractured cell after 16 hr pronase treatment; P-face. "Rings" and "rosettes" would be expected at sites labeled by arrows, but appear considerably degraded, as are "free" MIPs outside fusogenic zones. The inset shows that, after protease treatment, all types of MIPs are very scarce on an E-face of a trichocyst attachment site as well. Shadow casting from bottom right to top left. Original magnification ×38,000. Inset. Original magnification ×60,000.

double—"ring" of MIPs and contains an 80-nm large "rosette" of eight to ten MIPs in the center (Figure 4a). Because of its regularity and redundancy this system proved very suitable for analyzing the chemical nature of MIPs in these fusogenic membrane zones.

For the following reasons we applied "standard methods," including chemical prefixation (but for a very brief time) and antifreeze impregnation: a) Cells become sufficiently resistant so that they do not disintegrate during the subsequent enzymatic digestion. b) Cilia become so stiff that they are sheared off by mechanical forces when cells are shaken in the presence of Sepharose–enzyme conjugate beads; only then can conjugated enzymes get into contact with the exocytotic regions of these cells. c) This type of prefixation changes the partition coefficient (as defined in ref. 22) in a way such that all "rosette" MIPs become "fixed"onto the P-face (10) and thus can be most reproducibly analyzed by freeze-fracturing (Figures 1, 2, 4). (The required pretreatments did not justify the use of quick freezing techniques in this case.) We ascertained that the ultrastructural organization of all control samples (see Figures 1 and 4a) was not essentially different from previous results obtained with quick frozen, native cells (18), with the exception of the above-mentioned simultaneous occurrence of both "rosettes" and "rings" on the same fracture face (P-face). This is clearly an advantage for the present analysis, as it allowed us to monitor all experimental changes on one fracture face (Figures 2, 4b–f).

Table 1 and Figures 2 and 4b–e show that the MIPs composing "rings" and "rosettes" are sensitive to proteolytic enzymes. The sensitivity seems to be higher for pronase than for trypsin or chymotrypsin. The final stages of digestion would no longer allow the recognition of any MIPs and are, therefore, not shown. Before total digestion occurs, one can recognize that the number of MIPs decreases and that some MIPs appear swollen, distorted, or rather diffuse, respectively, when cells were exposed to proteolytic enzymes (Figure 4b–e). We also analyzed E-fracture faces (Figure 2, inset); as in controls (see above and ref. 10), we observed no substantial number of "ring" or "rosette" MIPs on E-faces, regardless of the conditions used. Therefore, all micrographs presented in Figure 4 are strictly comparable with regard to the degradation effect

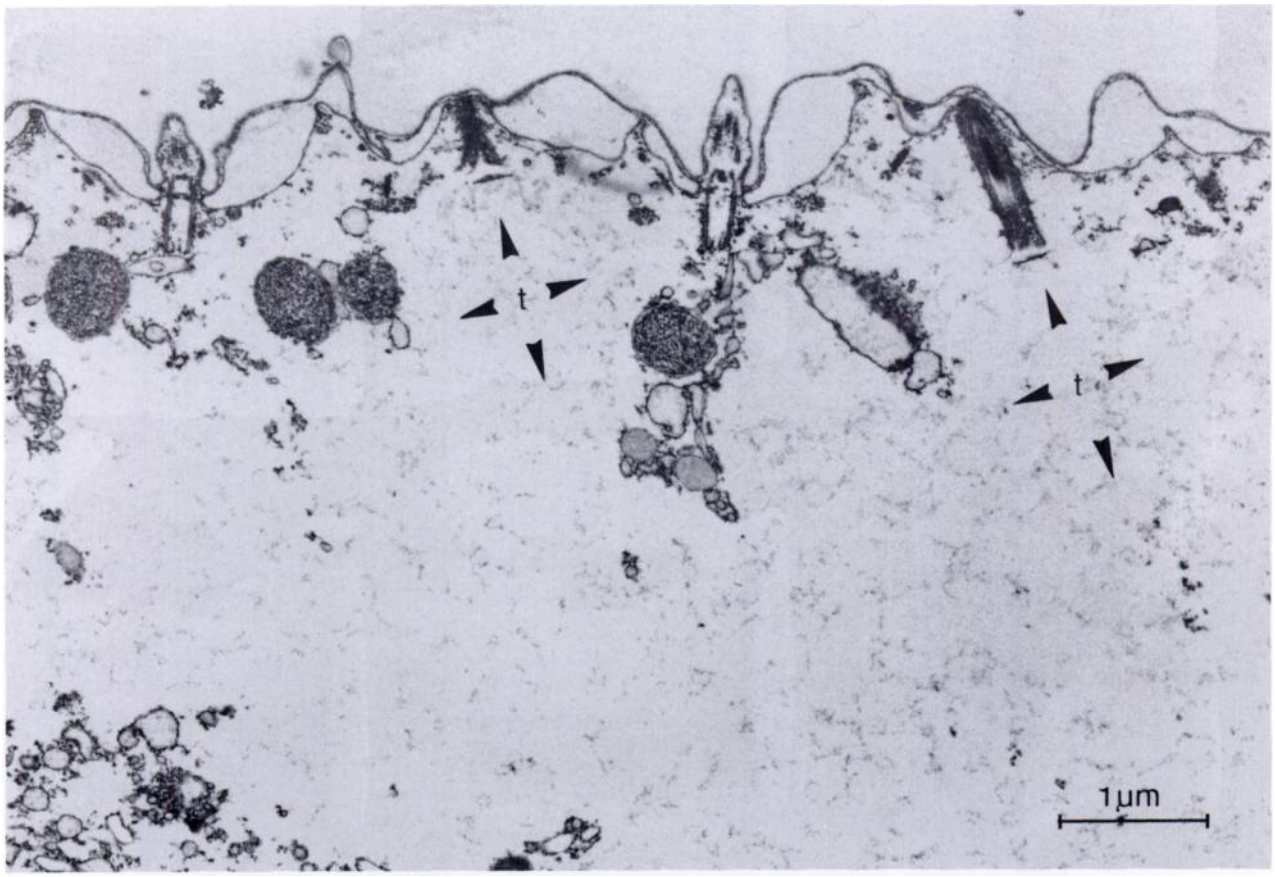


Figure 3. Ultrathin section of a trypsin-digested cell. Note that the secretory proteins contained in "trichocyst" (t) organelles largely disappear; tannic acid staining (31). Original magnification ×20,000.

achieved (for conjugated enzymes see below). Proteolytic enzymes also reduce the number of free MIPs (outside fusogenic zones) on both fracture halves (Figure 2) and they degrade the secretory contents of trichocysts (Figure 3). We also took into account the possibility that the proteolytic enzyme preparations we used could have some esterase side effect (27). However, esterase treatment, even with high activity and over long time periods, did not change the appearance of the "rings" or "rosettes" (Figure 4f). This also holds true for some of the more centrally located "rosette" MIPs (which were alluded to in ref. 16 as possibly being structures analogous to alleged lipid micelles in fusing membranes). These too were sensitive only to proteolytic enzymes.

Proteolytic enzymes, after coupling to Sepharose beads, could apparently reach only those MIPs that are assembled in the "rosettes" (and that are located on top of ridge-like elevations of the cell body surface; cf. ref. 18), but not those of the surrounding "rings" (Figure 4e); these appear less accessible, probably simply for geometric reasons, to the large conjugate beads.

Discussion

In the current literature (as summarized in ref. 17) some authors claim that fusion of membranes during exocytosis would be preceded by a large-scale MIP removal from the membrane regions involved in the fusion process (13). In contrast, other authors, using more advanced freezing techniques (7,8,14) or simply avoiding chemical treatments before freezing (26), find no evidence for such large-scale MIP rearrangements. Another group (16), working with fungal spores, claims that some MIPs would be formed when both membranes come in contact during exocytosis, as a sort of "ignition site" in the form of micellar lipid particles (see also below). It is also noteworthy that work with the ciliated protozoa *Tetrahymena* (23,24) and *Paramecium* (18) has already shown the occurrence of MIP aggregates ("fusion rosettes") specifically in those regions of the cell membrane that are predetermined for exocytosis.

In most of these discussions (except for ref. 16), the MIPs seen after freeze-fracturing were interpreted to be membrane-integrated proteins (possibly with a narrow lipid annulus) within a split lipid bilayer (for review see ref. 32). Most of the above-mentioned freeze-fracture data would then imply that membrane-integrated proteins would have to be removed from relatively large areas before membrane fusion could occur. In contrast, paramecia quite definitely contain "rosette" MIPs at

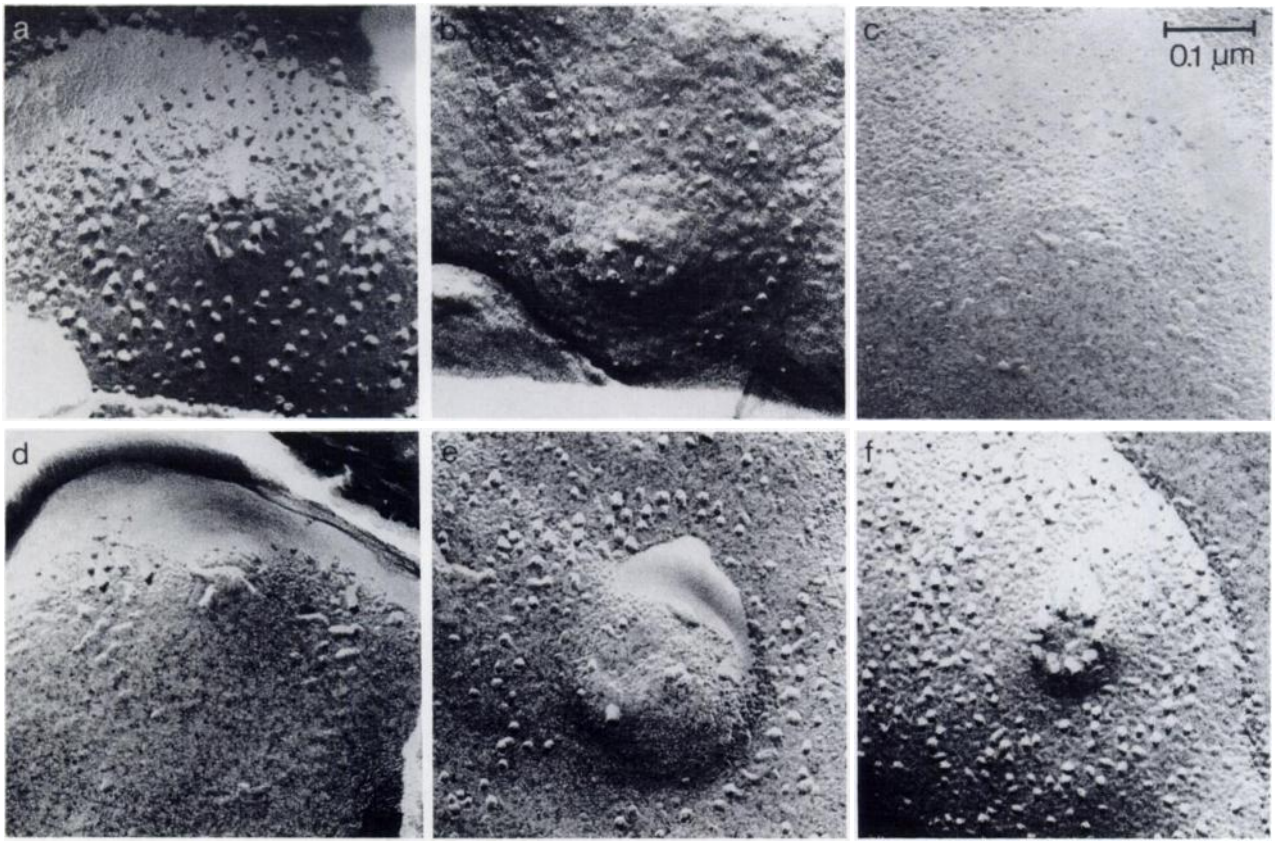


Figure 4. (a) Control: exposed only to buffer (18 hr 0.1 M cacodylate, pH 7.0). P-face of cell membrane over a potential exocytosis site. An ~300-nm double "ring" of ~10 nm membrane-intercalated particles (MIP) surrounds the potential zone of exocytotic membrane fusion. This zone contains a number of scattered 10-nm particles and, in its center, a MIP aggregate called the "rosette." The central region of the "rosette" may be devoid of any MIPs or be occupied by one or two MIPs (in some cases represented by a pit after the MIP was removed during fracturing. (b) Moderate digestion by free pronase (18 hr, 0.14 TU). The number of "rings," "rosettes," and other MIPs is reduced. (c) More extensive digestion by free pronase (16 hr, 13.5 TU) removes most of the MIPs, whereas the remaining MIPs appear swollen and distorted. (d) Extensive treatment with free trypsin (four times for 4 hr, 42.2 TU) leads to the same result as with pronase (see c). (e) Immobilized pronase (48 hr, 022 TU) slowly attacks "ring" and particularly "rosette" MIPs. This selectivity might be due to the better accessibility of "rosettes" (located at the top of protrusions of the cell body surface) to the large Sepharose beads to which pronase was coupled. (f) Free esterase (18 hr, 30 units) exerts no effect on "rings," "rosettes," or other MIPs between them. Within the "rosette" a more centrally located MIP (or a depression thereof) also remains un-affected. Shadow casting from bottom to top. Original magnification ×120,000.

their potential fusion sites in order to accommodate normal exocytosis, since mutant strains without these structural elements are incapable of exocytosis (2,3,9,11,20). Most recently, an alternative model for membrane fusion was proposed, when it was shown that MIPs might also represent inverted lipid micelles of the hexagonal phase H-II (28,29), as inferred from parallel nuclear magnetic resonance and freeze-fracture analysis of fusing pure lipid vesicles (28,29). The above-mentioned globular particles observed at exocytosis sites in fungal spores (16) were also interpreted to be inverted lipid micelles. Ultimately, this led to a zipper model for membrane fusion (16); in the same work, a similar interpretation was also given for some elements of "fusion rosettes" of paramecia.

All these discrepancies prompted us to perform in situ analyses of those distinct MIP aggregates that occur specifically at the well-organized fusogenic zones of paramecia. Our data (Figure 4 and Table 1) show a time- and concentration-dependent sensitivity of "rosette" MIPs to proteolytic enzymes; MIPs composing the "rings" are also (but less) sensitive. Digestion of "rosette" MIPs does not require enzyme penetration into the cells, as shown with conjugates. Therefore, we can also largely exclude any redistribution of MIPs via degradation of intracellular membrane-associated structures. (Indeed, peripheral proteins were also shown to occur on the preformed exocytosis sites (31), where they may be involved in the assembly of membrane-integrated protein "rosettes.") On the other hand, we ascertained that specific protein structures, such as trichocyst contents (25), are also attacked by free proteases (Figure 3).

Although it would appear feasible to examine the effect of extraction or enzymatic breakdown of lipids, attempts to do

so are notoriously hampered by the insensitivity of membrane lipids to such treatments or by structural rearrangement of membrane components (30), as well as, ultimately, by the total abolition of membrane splitting (6,12). Thus, we restricted control experiments to esterase treatment; this was mainly done in order to exclude any side effect of esterase as a possible contaminant (compare ref. 27 with ref. 5). The absence of any such effect also supports our conclusion that "rosette" (and probably also "ring") MIPs are likely to be composed of proteins. Further support comes from the changed partitioning of "rosette" MIPs during freeze-fracturing in response to the protein cross-linking (cf. ref. 32) glutaraldehyde fixative.

Our results do not preclude the possibility of a micellar lipid arrangement during the actual fusion event (see ref. 17), but this would have to be a rather short-lived intermediate stage (different from "rosette" components seen so far), which up to this time has yet to be demonstrated by the freezing techniques applied. The important new aspect of the present analysis is the occurrence of membrane-integrated proteins at the preformed exocytosis sites within the cell membrane, i.e., long before fusion takes place.

Since "rosettes" were found to be a genetically controlled determinant for the fusion capacity in paramecia (2,3,9,11,20), one must now take into consideration a positive modulatory effect of membrane proteins for membrane fusion. Further support along these lines comes from cytochemical work with *Paramecium* cells: It was shown that the preformed exocytosis sites of various strains display a Ca^{2+}-stimulated adenosine triphosphatase activity (19), but only with those mutations that simultaneously contain "rosettes" and are capable of exocytosis (20). Further studies have to clarify the precise chemical identity of the "rosette" MIPs, which at this time we assume to correspond to membrane-integrated proteins.

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