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ALDEHYDE FIXATION CAUSES MEMBRANE VESICULATION DURING PLATELET EXOCYTOSIS: A FREEZE-SUBSTITUTION STUDY

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Abstract

Despite a plethora of reports on the ultrastructure of secretory granule release by exocytosis, the release of coagulant activity from stimulated platelets is still being attributed to membrane vesiculation. Membrane vesiculation and the formation of myelin figures have been shown to be artifacts of glutaraldehyde GA fixation. Cells fixed by direct osmium or rapid freezing are free of such structures. Yet there is still doubt that rapid freezing interferes with vesiculation process. This study has addressed this issue by examining: (1) whether freezing and freeze-substitution affects membrane vesiculation, (2) whether paraformaldehyde-fixation also induces the phenomenon, and (3) whether the aldehyde concentration is of influence. Aldehyde fixation was carried out prior to impact freezing and freeze-substitution. In thrombin-stimulated platelets, membrane vesiculation and myelin figures were found. Glutaraldehyde induced multivesicular structures, paraformaldehyde or low aldehyde concentrations only blebs on the platelet surface. The membrane vesicles were in continuity with the cytoplasmic matrix. Unstimulated platelets did not show vesiculation or myelin figures. Control samples, without aldehyde fixation, showed instead of membrane vesiculation, granule fusion with the plasmalemma, or, instead of myelin figures, compound granules. This confirms that membrane vesiculation and the formation of myelin figures are artifacts induced by the failure of aldehydes to arrest lipid mobility within membranes undergoing rapid changes in structure. Although the presence of membrane vesiculation and myelin figures in platelets indicates that exocytotic processes were occurring at the moment of aldehyde fixation, the finding of membrane vesiculation in aldehyde-fixed platelets does not indicate a separate type of exocytosis.

Key Words: Aldehyde fixation, exocytosis, fixation artifact, membrane vesiculation, platelet, rapid freezing, freeze-substitution.

Introduction

The formation of abundant and polymorphic vesicles was observed in various cell types during cell functions which are accompanied by membrane fusion: fibroblasts (Hasty and Hay, 1978), mast cells (Lagunoff, 1973, Lawson *et al.*, 1977), glandular cells (Tanaka *et al.*, 1980), myoblasts (Kalderon and Gilula, 1979) as well as stimulated platelets (Warren and Vales, 1972 a and b, Behnke and Tranum-Jensen, 1986, Polasek *et al.*, 1987). Moreover, since some coagulant activity of stimulated platelets was described to be associated with released membranous vesicles (Sandberg *et al.*, 1985, Fox *et al.*, 1990), the vesiculation of membranes observed after aldehyde fixation is still discussed as a separate secretory pathway in these cells (e.g. Polasek, 1989). On the other hand, membrane vesiculation was attributed to aldehyde fixation in comparative studies with glutaraldehyde fixation vs rapid-freezing and freeze-substitution in cells during exocytosis using sea urchin eggs (Chandler, 1984) and more recently using platelets (Morgenstern and Edelmann, 1989). This study addresses the question, whether the use of a cryo-technique may prevent the demonstration of membrane vesiculation and myelin figures. The platelets were fixed with two aldehydes at different concentrations prior to rapid-freezing and freeze-substitution.

Materials and Methods

Human platelets in citrated blood plasma were obtained as described previously (Morgenstern and Edelmann, 1989). Exocytosis was triggered by addition of 1 IU/ml of bovine thrombin (Behring/Marburg, FRG) for 30 sec at 37°C. EDTA ($5x10^{-3}$ M) was added prior to stimulation in order to avoid the formation of platelet aggregates. Stimulated and unstimulated platelets were: (1) rapidly frozen without prior fixation, (2) fixed for 30 min with glutaraldehyde (final concentration 0.2%) or paraformaldehyde (final concentration 0.5%). Then the

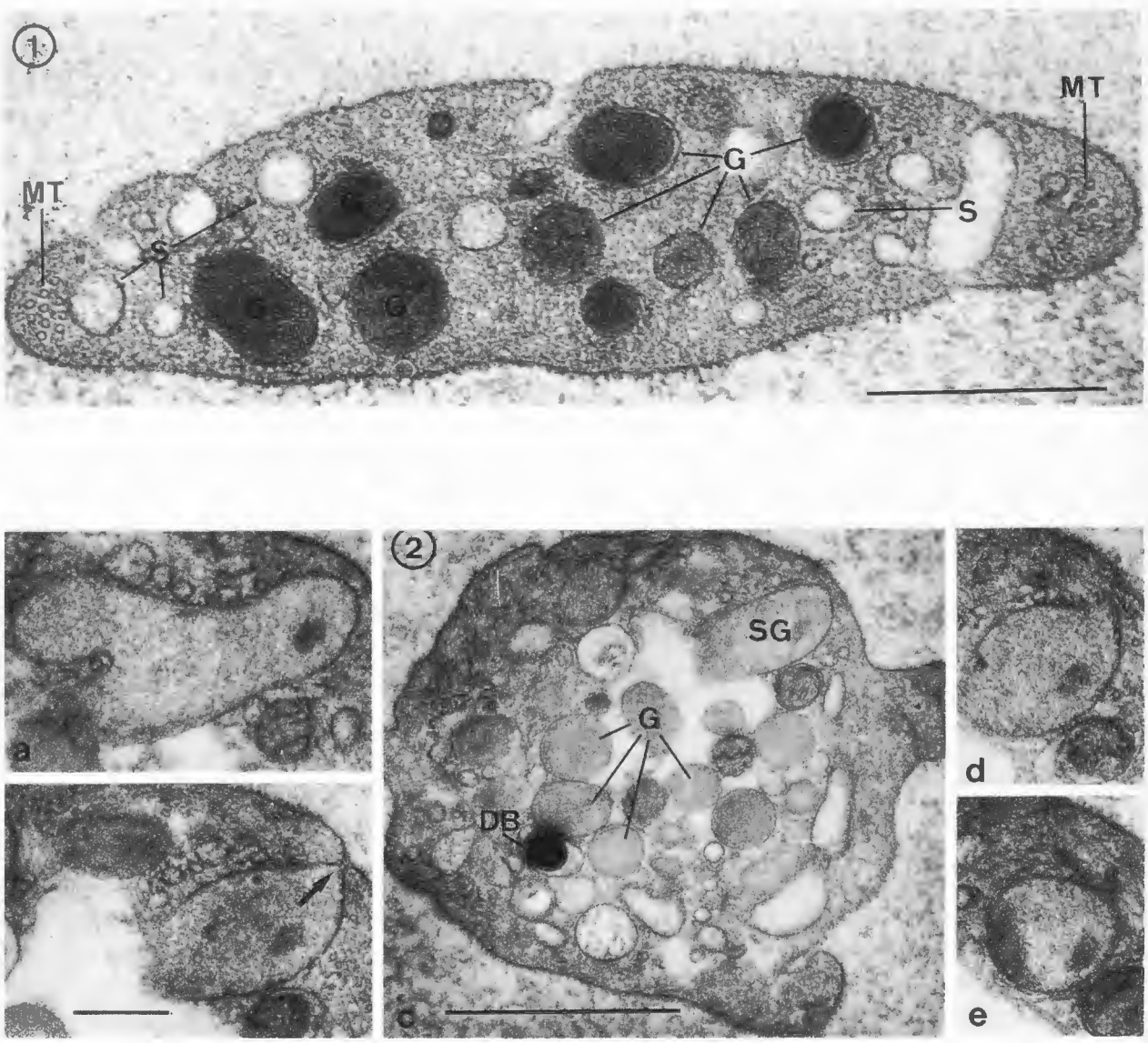

Figure 1. Section of a 0.2% glutaraldehydefixed, rapidly frozen platelet; the section profile, the marginal bundle of microtubules (MT) and the individually situated, unaltered α-granules (G) characterizes the unstimulated platelets. S = surface connected system. Bar = 0.5 μm.

Figure 2. Consecutive serial sections of a stimulated and rapidly frozen platelet. In 2c the section shows the membranes of centralized α-granules (G) and a dense body (DB) in apposition. Bar = 1 μm.

A swollen organelle with a slightly dispersed matrix (SG) is shown in four sections, 2a, b, d, and e. The membrane of this organelle forms a minute fusion pore (arrow) with the plasmalemma only in the section demonstrated in 2b (cf the adjacent sections in 2c, d and e). In a further section the organelle branches out into a compound granule (2a). Bar = 0.25 μm.

suspensions of fixed platelets were rapidly frozen with the metal-mirror attachment MM 80 to the KF 80 cryo-fixation unit (Reichert-Jung/Vienna) as described in Morgenstern and Edelmann (1989). (3) Other samples were centrifuged after (2) and the pellets were fixed for 30 min up to 60 min in 2% buffered paraformaldehyde or in 5% buffered glutaraldehyde at room temperature, washed in buffer (30 min) and frozen. The samples (1), (2) and (3) were then freeze-substituted in 4% osmium tetroxide - acetone medium for 48 h at 193K with the CS auto cryo-substitution unit (ReichertJung/Vienna) and embedded in Araldite after rewarming.

Results

The ultrastructure of prefixed unstimulated platelets (Fig. 1) was found to be quite comparable to that of rapidly frozen and freeze-substituted cells (described in Morgenstern *et al.*, 1987). The secretory organelles (α-granules and dense bodies) of platelets were situated predominantly in the central part of the cell and separately located without membrane contact. Membrane vesiculation or the other phenomena described below were not observed in unstimulated platelets.

Rapidly frozen and freeze-substituted platelets

The ultrastructural alterations of stimulated platelets, using freezing methods, were described in previous studies and compared with the results of conventionally fixed platelets (Morgenstern *et al.*, 1987, Morgenstern and Edelmann, 1989). In brief, in the stimulated platelets, the membrane of secretory organelles was seen to be in close contact (apposition) with the plasmalemma. Furthermore, membranes of the organelles were found to be apposed to each other (Fig. 2d) by the action of a constricting sphere formed by the contractile cytoskeleton. These appositions preceded the formation of fusion pores and the process of compound exocytosis (Fig. 2a-e). Stimulated platelets formed small pores in the apposed plasmalemmal and organelle membranes (Fig. 2d). During this event, fusing organelles were often observed to be situated in bulging parts of platelets as demonstrated in Morgenstern and Edelmann (1989). Sequential fusion of apposed organelles led to the formation of compound granules (Figs. 2a-e). Membrane vesiculation or formation of myelin figures were never seen after freeze-fixation and freeze-substitution.

Fixation with low concentrations of glutaraldehyde (0.2%) or paraformaldehyde (0.5%) prior to rapid freezing

Stimulated platelets showed blebs budding off from their surface (Fig. 3a) and from the pseudopodial membrane (Fig. 3b). The membrane of the blebs was found to be in continuity with the plasmalemma. A membranous demarcation to the cytoplasmic matrix did not exist. These blebs were filled with amorphous material of low electron density but did not contain vesicular substructures (Fig. 3a and b).

Fixation with glutaraldehyde (5% for 30-60 min) or with paraformaldehyde (2% for 30-60 min) prior to rapid freezing

Stimulated platelets showed the formation of blebs and multivesicular structures on their surface (Figs. 4a-d, 5a and b, 6a-d, and 7a and b). The membrane of the blebs was found to be in continuity with the plasmalemma. A membranous demarcation to the cytoplasmic matrix could not be observed in serial sections (Fig. 6a-c). The multivesicular structures revealed an electron lucent content (Fig. 5a and b, 6b-d). Using serial sections the described structures could be traced to the central part of the platelets (Fig. 4a-d), which were found to be in various states of degranulation. Within the degranulating platelets, myelin figures were seen associated with the membranes of vacuoles (Fig. 8a and b). These phenomena were observed both after glutaraldehyde and paraformaldehyde fixation. In contrast to glutaraldehyde, the paraformaldehyde fixed platelets showed no multitude of small vesicles within larger blebs (Figs. 7a and b).

Discussion

Two characteristic effects are induced in the presence of aldehydes during exocytotic processes in platelets: membrane vesiculation and the rise of myelin figures. Both of the alterations are situated at those sites, where membrane fusion takes place in native rapidly frozen, stimulated platelets: on the surface or in compound granules. As shown in the experiments with prior aldehydefixation, rapid-freezing and freeze-substitution, they do not influence the formation of such structures. The occurrence of membrane vesiculation on pseudopodial membranes is explained by the fusion of peripherally located organelles observed after rapid freezing (cf Morgenstern and Edelmann, 1989). The formation of myelin figures in compound granules may be attributed to the high content of phospholipids within the α-granular matrix (Joist *et al.*, 1974, Broekman *et al.*, 1976). These phenomena are not observed in aldehyde-fixed, unstimulated platelets, where the secretory organelles are not in appositional contact. Rapid-freezing prevents membrane vesiculation and myelin figures.

Some pecularities are connected with membrane vesicles, which were suggested to represent an alternative pathway of platelet exocytosis (Polasek, 1989). Firstly, they in fact appear only after platelet stimulation. Secondly, it was shown with glutaraldehyde-fixed platelets that, within the membranes of the blebs, intramembrane particles were absent (Behnke and Tranum-Jensen, 1986). It is known, that the clearing of intramembrane particles in membranes undergoing fusion does not occur, when exocytosis is arrested by rapid-freezing (Chandler and Heuser, 1980, Ornberg and Reese 1981). Thirdly, as in Chandler's comparative study on cortical granule exocytosis of sea-urchin eggs, the glutaraldehyde-induced effects do not appear in platelets after lipid-stabilizing fixation with osmium tetroxide (White and Krivit, 1966). Fourthly, the content of the vesicles is seen to be in continuity with the

Figure 3 (facing page). Two sections of stimulated, 0.2% glutaraldehyde-fixed and rapidly frozen platelets with an irregular profile and pseudopods (P). They are degranulated in 3a or in 3b some cell organelles are seen in the cell center surrounded by electron dense masses of the constricted cytoskeleton (CG). Blebs with electron lucent, amorphous content budding from the surface are indicated by arrows. In 3b the bleb seems to bud from the surface of a pseudopodium. Bar = 1 μm.

Figures 4 - 6 (facing page). Serial sections of stimulated, 5% glutaraldehyde-fixed and rapidly frozen platelets. The bleb in 4a (arrow) can be traced to the platelet interior in the sections shown in 4b-d (asterisks). F = polymerized fibrin. Bar = 0.5 μm.
In 5a and 5b blebs filled with vesicles bud from the platelet surface (arrows) are shown. Bar = 0.5 μm.
In 6a-d the formation of multivesicular structures (asterisks) and myelin figures (filled arrow heads in 6a) are shown in consecutive sections. In 6a-c the continuity between the vesicle content and the ground plasm is indicated by open arrow heads. Bar = 0.5 μm.

ground plasma. Moreover, the microvesicles contain cytoskeletal and contractile proteins (Crawford, 1971, Fox *et al.*, 1990).

The present results with platelets do not differ from those observed on other cells (Chandler, 1984). The observation that this artifact, restricted to membrane areas of transient increase in membrane lability (see Plattner 1989), is attributed to the inability of glutaraldehyde to arrest lipid movement, even though membrane-associated proteins are crosslinked (see Chandler, 1984 for discussion). Paraformaldehyde induces comparable phenomena to those demonstrated with glutaraldehyde. The absence of microvesicles, arising additionally within the larger blebs, may be caused by poor crosslinking of lipid-associated proteins. Paraformaldehyde may stabilize such structures less readily than glutar(di)aldehyde. The effects of low aldehyde concentration may be similarly explained. In this context, procoagulantrich microvesicles were found in supernatants from platelets, when the association between the plasmalemma and the submembranous cytoskeleton was disrupted (Fox *et al.*, 1990). In the latter study using calpain activating agents (ionophore A23187 and dibucain), it was suggested (without morphological examination) that microvesicles shed from the plasmalemma. After induction of platelet exocytosis with A23187, freeze-fixation and freeze-substitution, we did not observe microvesiculation, but formation of fusion pores, as described in this paper for thrombin-stimulated platelets (Patscheke and Morgenstern, unpublished results). The dibucaine induced membrane alterations should therefore also be investigated on rapidly frozen platelets.

This present study on platelets during exocytosis confirms that membrane vesiculation and the formation of myelin figures is an artifact induced by the failure of aldehydes to arrest lipid mobility within membranes that are undergoing rapid changes in structure. On the other hand, the presence of membrane vesiculation and myelin figures in platelets indicates that exocytosis occurs at the moment of aldehyde fixation. Thus, the finding of membrane vesiculation in aldehyde-fixed platelets does not provide proof for the existence of a separate type of exocytosis.

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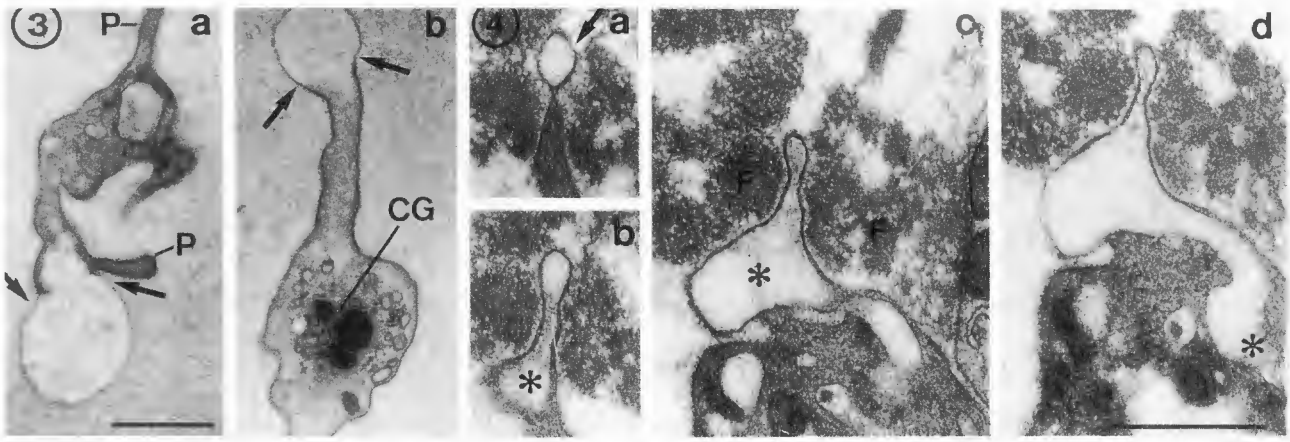
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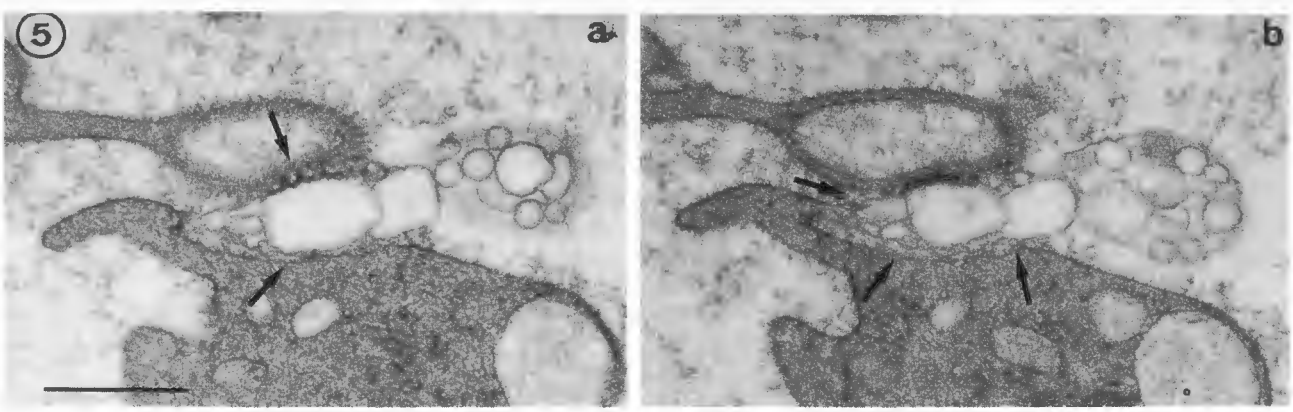
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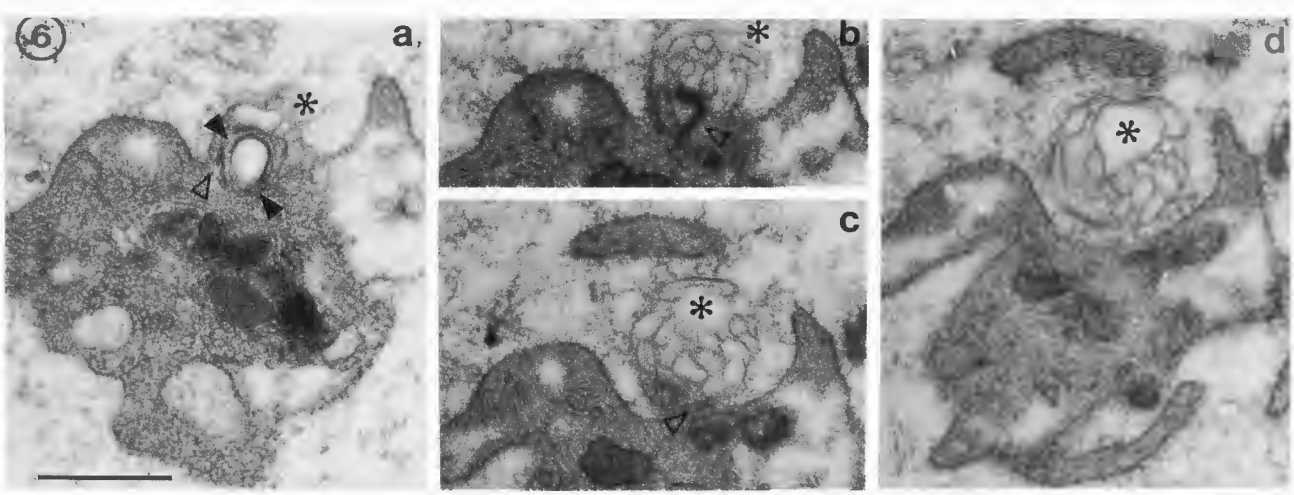
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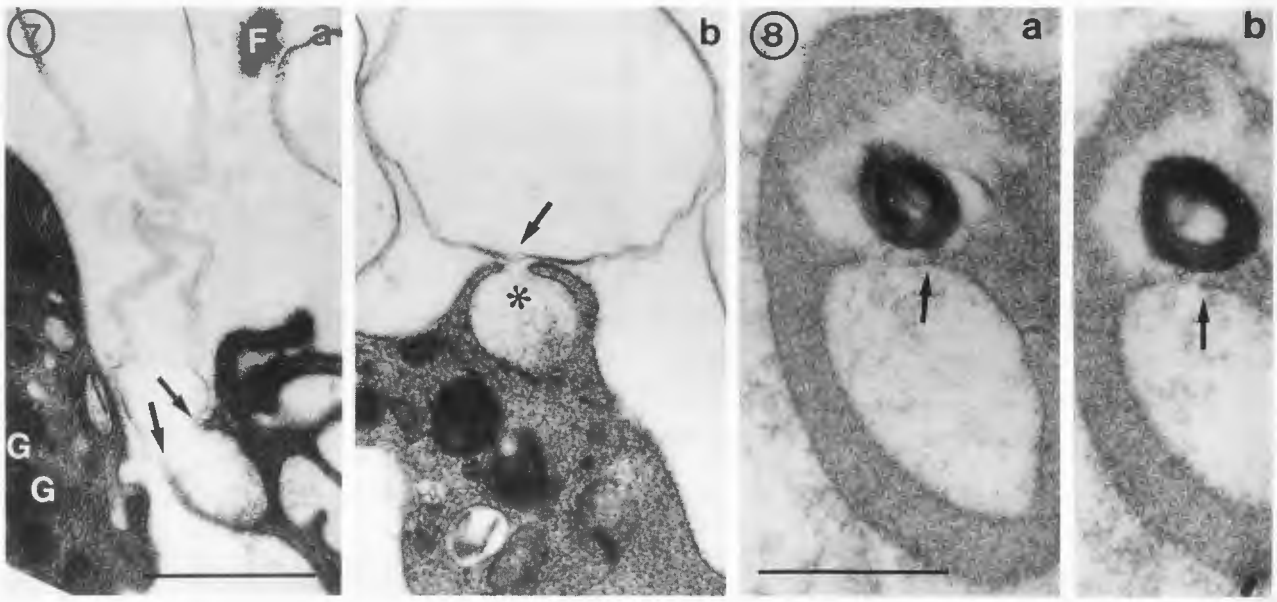

Figures 7 - 8. Serial sections of stimulated, 2% paraformaldehyde-fixed and rapidly frozen platelets.
In 7a the flattened bleb (arrows) is seen budding from the plasmalemma and a cellular cavity. α-Granules (G) are in membrane apposition within a neighbouring platelet The Ω-shaped secretory organelle (asterisk) in 7b is in narrow vicinity with large extracellular blebs (arrow). Bar = 0.5 μm.
In 8a and b myelin figures are indicated with arrows on the site of compound granule formation. Bar = 0.5 μm.

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Discussion with Reviewers

R.A. Steinbrecht: Have you tried to omit osmium tetroxide from the substitution medium? It would be of interest whether acetone alone can stabilize a highly dynamic membrane configuration by its gentle dehydration. Freeze-substitution by acetone may often preserve membranes quite well, but it is likely that exocytosis processes alone need stronger cross-linking of the lipids for stabilization during freeze-substitution.
Author: When stimulated platelets were rapidly frozen and freeze-substituted without osmium tetroxide in acetone (or methanol) - and also after addition of glutaraldehyde to the medium they revealed similar morphological characteristics concerning the exocytosis as shown in Fig. 2 of this study (Morgenstern and Edelmann, unpublished results). Whether the morphological details of membrane structures, which are involved in exocytotic processes, are exactly the same when osmium was omitted cannot been judged: staining of preparations which have been substituted in pure acetone and embedded after rewarming is rather poor.

J. Tranum-Jensen: Are the blebs/vesicles that bulges as a single membrane structure from plain plasmamembrane and the multivesicular structures, often seen enclosed by an outer membrane layer one and the same thing ~ The blebs are clearly in broad continuity with the cytoplasmic matrix and are easily explained by a simple bulging of the plasma membrane, but is less clear how the structure relate to the plasma membrane. Can you exclude that the blebs and the multivesicular structures have different origins, e.g. that multivesicular formations arise at sites of granule exocytosis?
Author: The described artifacts obviously have the same origin: The interior of the blebs and the interior of the multivesicular structures showed continuity with the cytoplasmic matrix. This has been detected by the present investigation of section series. Blebs as well as multivesicular structures arised at sites which are clearly involved in exocytotic processes. The artificial process during aldehyde fixation starts with the formation of an - initially small - bleb at the site of apposition of the secretory organelle and the plasmalemma. This was demonstrated in an earlier study to this subject (cf Morgenstern and Edelmann, 1989, Fig. 1 a and b). The formation of multivesicles or myelin figures obviously depends on the fixation mode and may depend on the availability of further lipids from the organelle contents as discussed in the study.

J. Tranum-Jensen: Was the period of activation before fixation the same in the series fixed at low aldehyde concentrations relative to that fixed at high aldehyde concentrations? If not, would you then consider the possibility that the phenomena of vesiculation developed with the time lapsed before onset of fixation?
Author: The period of platelet stimulation with thrombin was similar in all cases. Experiments with platelets which have been cryofixed after longer periods of incubation have also been carried out (cf Morgenstern *et al.*, 1987) but vesiculation has never been observed. It can be concluded that (1) vesiculation does not occur if aldehyde fixation is avoided (e.g. by osmium fixation, cf White and Krivit, 1966) and (2) that vesiculation which is induced by aldehyde fixation can be preserved by freeze-substitution. Therefore, cryo-techniques which are able to arrest membrane instability and which can preserve existing artifacts are suited to investigate the phenomenon membrane vesiculation, but not the aldehyde-fixation.