Endocytosis by Human Platelets: 
Metabolic and Freeze-fracture Studies

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ABSTRACT The mechanism by which platelets endocytose or release particulate or soluble substances is poorly understood. Engulfed materials enter the open canalicular system (OCS) by a process akin to phagocytosis, but fusion of platelet granules with the OCS is rarely observed. Secretion of granule contents, a concomitant of the “release reaction” which occurs during platelet aggregation, does not take place by extrusion at the surface membrane as is true for other secretory cells. Some substances may be secreted without obvious granule loss. To examine whether structural properties of the platelet membrane could account for this unusual behavior, thin section and freeze-fracture analyses were performed on platelets which had undergone endocytosis under a variety of experimental conditions. After freeze-cleavage, most of the intramembranous particles (IMP) remain associated with the outer leaflet of the platelet plasma membrane. The sites where the OCS reaches the surface membrane are marked by pits on the cytoplasmic leaflet (P face) and by complementary protrusions on the outer leaflet (E face) of the membrane. Endocytosis of small particles and solutes takes place via these structures. This process is not energy dependent but arrested at 4°C. Distension of the OCS does not appear to affect the size or number of the pits. On the other hand, large particles are taken up by membrane invagination without redistribution of IMP’s and independent of the pits. This process is sensitive to metabolic inhibition. Thus, the studies have demonstrated the existence of two different pathways for platelet endocytosis which are postulated to be also involved in secretion. The selective release of substances contained in different granules may be related to the “inside-out” structure of the plasma and OCS membranes.

Platelets are able to take up particles as well as solutes against high concentration gradients and are known to retain and transport such substances for prolonged periods of time (5, 7, 18, 24, 33). However, in contrast to cells with amoeboid motion, platelets do not extend pseudopods, nor do they display any obvious chemotaxis towards the object to be engulfed. Instead, random collision followed by specific or nonspecific adsorption to the plasma membrane appears to lead inevitably to internalization. A large number of studies have shown that internalized substances enter into the “open” canalicular system (OCS), a system of channels believed to be formed by invagination of the plasma membrane (2). The uptake of particles is energy dependent and only arrested when both oxidative phosphorylation and glycolysis are inhibited (9, 18, 19). The metabolic requirements for the uptake of solutes, e.g., peroxidase, or very small particles, e.g., ferritin, have been less thoroughly analyzed. It is not at all clear whether these latter substances require adsorption to the membrane as a prerequisite for entry into the OCS or whether they may percolate through the OCS pari passu with the extracellular medium without adhering to the plasma membrane. Moreover, the uptake of soluble substances, such as serotonin, does not result in degranulation, whereas internalization of large particles is usually associated with the release of granule contents into the extracellular medium. This raises the possibility that particulates and solutes are endocytosed by different mechanisms, that they may have different routes of entry, and that they may be taken into different subcellular compartments. These questions are particularly cogent in view of reports showing that incubation of rabbit platelets with serotonin increases the number of dense granules, the organelles believed to store this amine (1, 6, 26). Yet continuity between the granules and the OCS has never been convincingly demonstrated.

These considerations, taken together with numerous reports claiming that the platelet membrane differs biochemically from that of other blood cells, have stimulated us to perform freeze-fracture studies on platelets which had been allowed to endocytose soluble or particulate substances in the absence or...
presence of metabolic inhibitors. It was hoped that these manipulations would reveal alterations in membrane organization unique to platelets. Indeed, we observed that the sites where the channels of the OCS reach the plasma membrane are characterized by pits on the cytoplasmic half and by complementary protrusions on the external side of the membrane. The size and number of these junctions did not change significantly in response to alterations in a variety of experimental conditions, even though marked distention of the cisternae of the OCS was evident under some circumstances. Large particles did not appear to be taken up via the pits, whereas macromolecules continued to enter even during metabolic inhibition. Therefore, it is possible that the junctions of the OCS with the plasma membrane represent specialized structures which may play a role in selective endo- or exocytosis that is distinctive from phagocytosis of large particulates or the massive degranulation associated with irreversible aggregation.

MATERIALS AND METHODS

Blood was obtained from normal subjects and anticoagulated with heparin (5 U/ml) or 3.8% trisodium citrate (0.1 ml/ml). Platelet-rich plasma (PRP) was prepared as previously reported (34). Experiments were conducted with 2-ml aliquots of platelet-rich plasma with platelet counts ranging from 400,000 to 800,000/mm³. Materials for endocytosis consisted of the following: (a) horseradish peroxidase (HRP) (Sigma Chemical Co., St. Louis, Mo.), 5 mg/ml. The interiorized peroxidase was localized with the substrates H₂O₂ and 3,3′diaminobenzidine (14) as described in detail elsewhere (34). (b) Cationized ferritin (Miles Biochemicals, Elkhart, Ind.), 2 mg/ml final concentration. (c) Latex particles 0.312-µm diameter (Dow Chemicals, Midland, Mich.) used in a concentration of 30 µl suspended in 2 ml of autologous plasma.

Conditions of Incubation

Incubation periods ranged from 10 min to 2 h, except for platelets which had been fixed with glutaraldehyde before incubation with tracers. In a few instances, glutaraldehyde-fixed platelets were incubated with ferritin or HRP for 24 h. Experiments were conducted at 4, 20, and 37°C. Metabolic inhibition was brought about by the addition to the platelet suspension of 10⁻³ M NaN₃, 10⁻² M 2-deoxyglucose, and 10⁻¹ M iodoacetic acid. These inhibitors were added to the platelet suspension 30 min before addition of the substances to be engulfed. For experiments conducted at 4°C, the platelet suspension was cooled in ice for 30 min before addition of the test particles.

Freeze-fracture and Electron Microscopy

The incubation periods were terminated by the addition of 3% phosphate-buffered glutaraldehyde. Aliquots were fixed for 2 h or overnight and then

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**Figure 1** Platelet prepared from a specimen of heparinized blood incubated with peroxidase for 30 min at 37°C. The electron opaque reaction product is seen on the plasma membrane (short arrow) and within the canalicular system (long arrow). Note that the cisternae are not particularly distended and that there are few, if any, pinocytotic vesicles. The granules are heterogeneous in size and content. × 22,500.
processed for transmission electron microscopy (TEM). Other aliquots were sedimented, washed in distilled water, and suspended in 25% glycerol for 2 h at room temperature to effect cryoprotection for subsequent freeze-fracture. The glycerinated specimens were sedimented in a Beckman microfuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) for 30 s at 11,000 rpm, after which the specimens were transferred to Balzers specimen holders (Balzers High Vacuum Corp., Santa Ana, Calif.), quick frozen with Freon 22 and further cooled with liquid N₂. Membrane cleavage was executed in a Balzers High Vacuum Freeze Etch unit BAF-300 at a vacuum of 10⁻⁶ and temperature of -100°C. In some experiments, etching was permitted for various time periods ranging from 30 s to 30 min. The cleaved surfaces were shadow-cast with platinum and carbon at angles of 45° and 90° respectively. After thawing, the replicas were cleared of proteinaceous debris with Chlorox overnight and mounted on Parlodion-covered electron microscope grids. The specimens used for TEM were postfixed with osmium tetroxide for 2 h, followed by staining en bloc with 0.5% uranyl acetate in saline for 1 h. Dehydration and embedding with Epon 812 were carried out as described elsewhere (34). An LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) was used to cut thin sections which were contrasted with uranyl acetate and lead citrate. A Siemens Elmiskop I electron microscope was the instrument used for all studies.

RESULTS

As in previous studies (33, 34), platelets which had been incubated with horseradish peroxidase or with cationized ferritin for 30 min at 37°C showed the OCS filled with these substances (Fig. 1). It should be pointed out that both horseradish peroxidase and ferritin were also seen on the plasma membrane of the cells. Therefore, on the basis of these images, it is impossible to decide whether adhering substances are interiorized by membrane flow or whether they would also be taken up with the extracellular medium without attachment to the membrane. As can be seen in Fig. 1, the canaliculi did not appear to be distended when the specimens were anticoagulated with heparin. Freeze-fracture replicas of platelets incubated with ferritin or peroxidase were indistinguishable from those of platelets incubated without addition of these agents.

Although several descriptions of freeze-fractured human...
platelets have appeared in the literature (3, 13, 23, 27, 29), for the purpose of our study it is necessary to reiterate several points. As a general rule, at low magnification the cytoplasmic aspect of the membrane bilayer (P face) appears convex, and the complementary outer leaflet (E face) appears concave. In contrast to the membranes of most other cells analyzed to date, cleavage of the platelet plasma membrane causes a much larger number of intramembranous particles (IMP) to remain associated with the outer leaflet of the bilayer (E face) than with the protoplasmic aspect (P face) (Figs. 2 and 3). Moreover, the IMP's are heterogeneous in size and shape. This heterogeneity is also more noteworthy on the E face, where some of the particles appear almost filamentous or vermiform (inset in Fig. 3), being ~10 nm long and 5 nm in width. The P face of untreated platelets shows a variable number of pits or pores which measure ±25 nm in diameter and are fairly uniform in size when platelets are not activated. The E face shows complementary protrusions (Figs. 2–5). In fortuitous replicas, the pits are seen in continuity with the canalicular system (Fig. 4). However, it is not unusual to find the structures concentrated in one area as though the cell were polarized (Fig. 5a and b; reference 3). Whether the “pit” represents a real opening could not be ascertained by deep etching. In platelets which had fractured through the cytoplasm, the granules, mitochondria, microtubules, and the OCS were easily identified (Figs. 2 and 4). Freeze-fracture replicas of platelets that had been incubated with peroxidase or cationized ferritin did not differ from those of control platelets.

FIGURE 3 Replica of a freeze-fractured platelet illustrates primarily the concave E face of the plasma membrane. The IMP's are more numerous than on the P face (see Fig. 2) and the pits seen on the P face appear here as protrusions (P). Elongate and fused IMP's are often seen on the outer leaflet. This is better appreciated at higher magnification of a detail of an E face within the inset. The cytoplasm shows different cleavage planes of canalicular membranes (C). \( \times 36,000 \). Inset, \( \times 70,000 \).
**Uptake of Particles**

As illustrated in previous studies, large (1 μm) and smaller (0.3 μm) latex particles are readily taken up by the cells (33, 36). In sectioned platelets, the particles appear to invaginate the plasma membrane and come to occupy membrane bound spaces which are believed to form part of the OCS (Fig. 6). On the basis of thin sections, it is not possible to conclude whether the pits, described above, enlarge to accommodate particles the size of latex spheres, or whether the uptake of large particulates occurs without involving these structures. The freeze-fracture analyses may have provided an answer to this question. As illustrated in Fig. 7, the invagination of the plasma membrane by latex particles does not affect the size of the pores of the P face or the protrusions on the E face of the platelet membrane. Moreover, there appears to be no redistribution of IMP's in the region where the plasma membrane bulges inward as has been shown to be the case in other cells during membrane invagination or evagination associated with endo- or exocytosis (4, 15, 20, 22).

**Metabolic Inhibition**

Since previous studies in our laboratory have demonstrated that complete metabolic inhibition, i.e., of glycolysis and oxidative phosphorylation, causes marked distention of the canalicular system (33; Fig. 8) and since others have shown that such treatment prevents the uptake of latex particles (18), we examined what effect such treatment would have on the size and number of the pits seen in the membranes of freeze-cleaved platelets. These experiments yielded two unexpected results. Firstly, although platelets were no longer able to take up latex particles, the interiorization of peroxidase and cationized ferritin was not impaired by metabolic inhibition (Fig. 9). Sec-
FIGURE 6  Detail of platelets from a specimen incubated with latex particles for 10 min. The specimen has been anticoagulated with EDTA to prevent aggregation. Three particles are seen to invaginate the plasma membrane while other particles have already been internalized. Granule (G) discharge into the OCS analogous to phagolysosome formation in leukocytes (31) has not been observed. Note microtubules (R) and submembranous filaments (F). Mitochondrion (M). × 84,000.

...or protrusions on the E face had not perceptibly changed in size or number (Fig. 10). These observations suggest that uptake of solutes or particles smaller than the diameter of the pits is not necessarily energy dependent. They did not explain, however, whether such substances must first react with the plasma membrane to be internalized by membrane flow via the pits, or whether the extracellular medium may percolate passively through the OCS at all times. In an attempt to answer these questions, PRP or whole blood was kept at 4°C for 30 min before addition of peroxidase or ferritin, after which incubation was continued at this temperature for 1 to 24 h with and without agitation of the specimens. Similar experiments were also carried out with glutaraldehyde-fixed platelets. It was postulated that, if the pits represented openings of the OCS to the exterior, incubation of fixed platelets for 36 h with agitation would permit some entry of solutes into the canaliculi by diffusion of the medium. As illustrated in Fig. 11, although the OCS was found markedly distended and the tracer was attached to the plasma membrane, no ferritin had been internalized. A few markedly swollen platelets displayed infoldings of the plasma membrane which resulted in reduplication of the ferritin layer (Fig. 11). Such folds did not extend deeply into the body of the cells and did not appear to coalesce with the OCS. The relationship of such membrane infolding to the pits could not be ascertained. Experiments conducted with peroxidase yielded identical results. Fixation of platelets before addition of the tracers followed by prolonged incubation also failed to reveal any interiorization of the extracellular material. Finally, it is noteworthy that none of the conditions used in these experiments caused a redistribution of the IMP's which has been reported to occur when platelets participate in clot retraction (27).

DISCUSSION

The far-reaching physiologic and pathologic implications of the platelet release reaction as well as platelet endocytosis have stimulated innumerable studies. The majority of these analyses have been concerned with the biochemical aspects of platelet physiology (8, 16), while the morphologic counterparts of the reactions have only been looked at by a relatively small number of investigators (28).
Therefore, it is perhaps not astonishing that our understanding of the mechanisms whereby uptake and discharge of platelet serotonin, adenine nucleotides, and a large variety of other substances takes place, has remained fragmentary. Based on the analogy with other phagocytic or secretory cells, it has generally been assumed that platelet endocytosis involves surface membrane invagination with subsequent fusion, and that degranulation occurs by the same process in reverse. However, the data presented in this communication indicate that this generalization may not be entirely correct. The illustrations presented in this report suggest that more than one mechanism of endocytosis is operative in platelets. Replicas of freeze-cleaved membranes have clearly shown that large particles are taken up by membrane invagination apparently independent of the location of pits believed to represent entrances to the OCS. This process is akin to phagocytosis by leukocytes. It is energy dependent and may take place anywhere along the surface of the cell. Of note, however, is our failure to observe any fusion of platelet granules with the vacuole formed by the invaginated plasma membrane even though we have been able to study this phenomenon extensively in other cells (31, 32). The observation that the freeze-cleaved plasma membrane of the platelet has an “inside-out” configuration may be relevant here. It has been shown that enzymes important in the transmission of biological signals often have an asymmetric distribution within the membrane conferring “sidedness.” In most cell membranes examined, such enzymes are located in the cytoplasmic leaflet. This holds true particularly for enzymes involved in the methylation of phospholipids which play a role in membrane fusion (12). If such enzymes would be associated with the outer leaflet of the platelet membrane rather than with its cytoplasmic aspect as is the case in other cells, the invaginated plasma membrane may not “face” the granule membrane correctly to bring about the “signal” necessary to initiate fusion. Biochemical evidence supporting this view appears to be available (12). The same theory would explain the lack of granule discharge at the surface of the cell and the observation that the IMP’s do not clear the region of membrane invagination. The findings provide sufficient reason to suspect that an alternative pathway for degranulation must exist.

The studies reported here have yielded clear evidence that an alternative mechanism, at least for endocytosis, is available. Solutes and small particles adherent to the platelet membrane enter into the OCS by what appears to be a process of membrane flow. This process is not subject to metabolic inhibition but is arrested at 4°C. Since the pits in the platelet plasma membrane have been shown to be in continuity with the OCS (Fig. 4; references 3 and 27), it is more than likely that this

**FIGURE 7** Replica of freeze-fractured platelets from the same specimen as illustrated in Fig. 6. Note areas where latex spheres have indented surface or canalicular membranes. At L1, a latex particle must have invaginated the plasma membrane comparable to the three contiguous particles depicted in Fig. 6. The P face of the invaginated membrane is seen. L2 also illustrates the inner leaflet (P face) of a cistern which was probably occupied by three particles. On the other hand, the arrow directs attention to the E face of an invaginated membrane area as evidenced by the larger number of IMP’s and the protrusion which has been invaginated as part of the membrane. × 42,000.
variant of endocytosis takes place via these structures.

When platelets were treated with metabolic inhibitors at 37°C, the canalicular cisternae became markedly distended with endocytosed material while the size of the pits did not change significantly (Fig. 10). Distention of the OCS also occurred at 4°C when the cisternae remained "empty" (Fig. 11). We have proposed previously that the distribution of the cytoskeletal elements along the canalicular membranes suggests that the cisternae are contractile (34). This would explain their lack of "tone" under conditions of metabolic inhibition and at low temperatures. The pits, on the other hand, were not affected by these changes. In this context, it should be pointed out that the morphologic and functional similarity which these structures bear to the caveolar openings in the plasmalemma of striated muscle cells is striking (11, 21, 30). On freeze-fracture of muscle fibers, the caveolae which are known to be in continuity with the transverse tubular system have also been reported to be relatively constant in size and number and not subject to metabolic inhibition (10).

While this study has shown beyond reasonable doubt that
the pits are specialized structures which play an absorptive function distinctive from large particle endocytosis, the question remains whether these structures also play a role in secretion. We should like to conjecture that a reversal of membrane flow via the pits also occurs, and that this takes place not only under conditions of normal homeostasis, but that such flow may be accelerated during stimulated release. This would account for the secretion of substances without gross degranulation and provide an explanation for the increase in the number of binding sites for fibrinogen (17) and thrombin (25) when platelets are activated. It will require considerable ingenuity to design experiments that will substantiate this postulate.

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