CONCISE REPORT

Absence of a Surface-Connected Canalicular System in Bovine Platelets

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Human platelets possess a surface-connected canalicular system (SCCS) which has been postulated to subserve endocytosis as well as secretion. Platelets of most animal species studied to date, including those of cattle, function similarly. However, ultrastructural analysis of freeze-fractured bovine platelets or thin sectioned bovine platelets treated with electron-dense tracers failed to delineate a SCCS. This observation may throw an entirely new light on the role of this organelle.

SPECULATIONS regarding the function of the surface-connected canalicular system (SCCS) of mammalian blood platelets are numerous, but data supporting these are sparse. Most investigators, including ourselves, have ascribed an endocytic function to this membrane system.[1-3] Others have suggested that the SCCS plays a role in secretion and the platelet release phenomenon.[4] The SCCS has also been looked upon as a storage organelle. The possibility that it may be involved in the transport of Ca^{++} has invited comparison with the sarcoplasmic reticulum of muscle cells.[5] Metabolic and freeze-fracture studies carried out in this laboratory have shown that transport of solutes and small particulates along the membranes of the SCCS does not require energy, but that it is arrested at 4 °C.[3] Such inward membrane flow appears to take place via 25-nm pits, which are not resolved in thin sections, but which, on freeze-fracture, appear to be in continuity with the SCCS. On the other hand, the interiorization of large particles is energy dependent and takes place by invagination of the plasma membrane independent of the location of the pits. The fact that the release phenomenon as well as the interiorization of large particles is energy dependent, whereas internalization into the SCCS is not, puts the role of the SCCS in these platelet activities in question. In any event, the observations raise the possibility that the SCCS may not be very important to normal platelet function. Alternatively, this organelle could be looked upon as a remnant of the demarcation membrane system of megakaryocytes, where its primary role appears to subserve thrombocytopoiesis. This concept has gained support from the inadvertant discovery that bovine platelets possess only a very rudimentary SCCS or none at all. Nevertheless, bovine platelets are secretory and function essentially like those of humans.[6,7] The purpose of this report is to document this observation and to consider its possible implications.

MATERIALS AND METHODS

Human platelets were prepared from heparinized (5 U/mL) or citrated (0.1 mL 3.8% trisodium citrate/mL) peripheral blood obtained from normal subjects as described in detail previously.[8] Blood was centrifuged at 400 g and experiments were conducted with 2-mL aliquots of platelet-rich plasma (PRP), with platelet counts ranging from 400,000 to 800,000 per microliter. Bovine platelets were essentially prepared as reported previously.[6] Two specimens were derived from normal cattle maintained at Washington State University. To ascertain that the observations were not limited to a particular strain of cattle, two specimens were collected from random Holstein dairy cows kept on a New England farm.

Tracer and Freeze-Fracture Studies

Following three washes in citrated Hanks' saline, the platelets were incubated with cationized ferritin (2 mg/mL) (Miles Biochemicals, Elkhart, Ind) for ten minutes. Following this, they were fixed by addition of 3% phosphate-buffered glutaraldehyde and processed for electron microscopy.[8] For freeze-fracture studies, aliquots of the specimens were fixed in glutaraldehyde, washed, and suspended in 25% glycerol for two hours at room temperature to effect cryoprotection. The glycerinated specimens were sedimented in a Beckman microfuge (Beckman Instruments, Spinco Division, Palo Alto, Calif) for 30 seconds at 11,000 rpm, after which the specimens were transferred to Balzers specimen holders, quick-frozen with Freon 22, and further cooled with liquid N_2. Membrane cleavage was executed in a Balzers High Vacuum Freeze Etch unit BFA-300 at a vacuum of 10^{-6} and temperature of −100 °C. The cleaved surfaces were shadow-cast with platinum and carbon at angles of 43° and 90°, respectively. The replicas were cleared of proteinaceous debris with Clorox overnight and mounted on parlodion-covered electron microscope grids. The specimens for thin sections were post-fixed with osmium tetroxide for two hours, stained en bloc with 0.5% uranyl acetate in saline for one hour, dehydrated, and embedded in Poly/Bed 812 as described.[3] Thin sections were contrasted with uranyl acetate and lead citrate. A Siemens Elmiskop I electron microscope was used for all studies.

RESULTS

The ultrastructural appearance of human platelets has been described extensively and need not be reiter-

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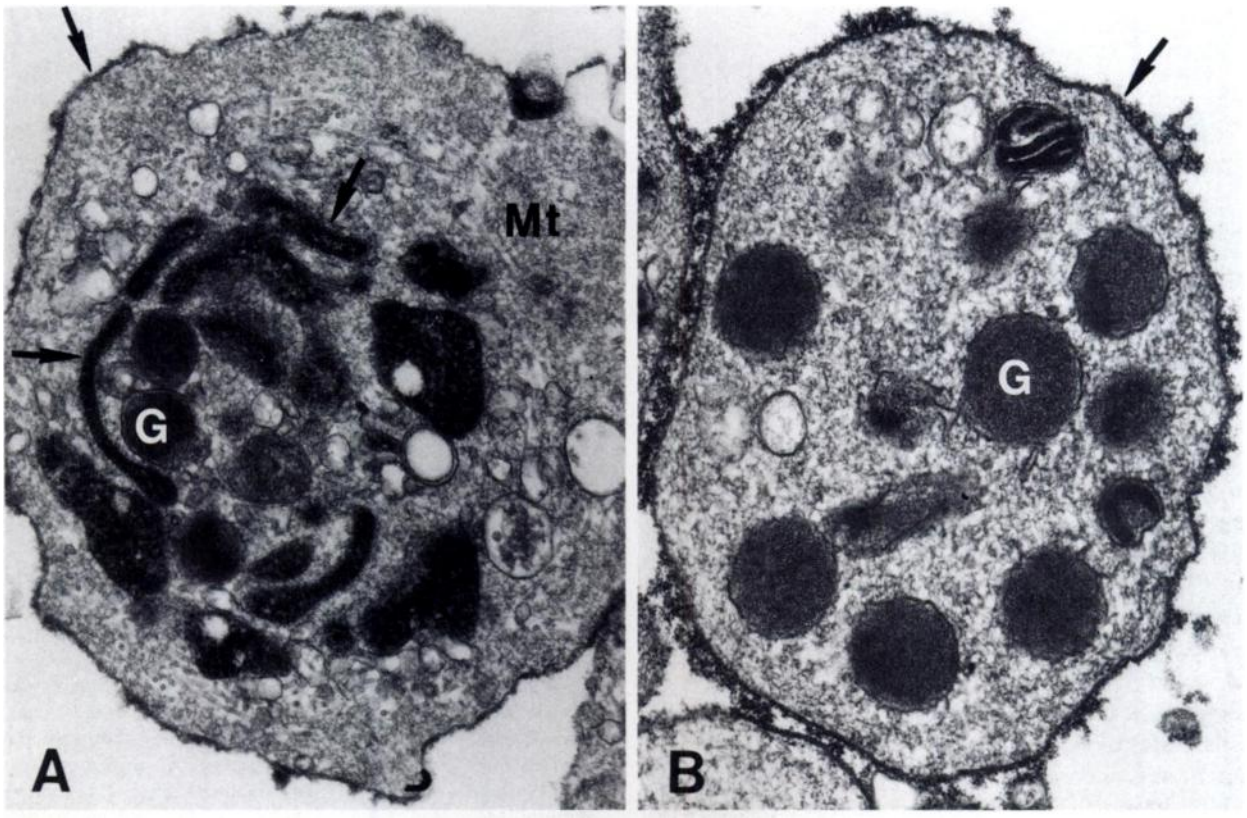

Fig 1. (A) Human platelet incubated with cationized ferritin. The tracer coats the plasma membrane and fills the canalicular system (arrows). G, α-granules; Mt, microtubules. Magnification ×32,000. (B) Bovine platelet treated under identical conditions as human platelet depicted in Fig. 1(A). Cationized ferritin coats the plasma membrane (arrow) but has not been interiorized. Note that the α-granules (G) are much larger. Magnification ×42,000.

ated here. Bovine platelets have fewer α-granules, which are about twice as large as their human counterpart[9] (Fig 1).

As reported previously,[3] when human platelets are incubated with cationized ferritin at 37 °C or room temperature, the tracer adheres to the plasma membrane and enters the canalicular system in less than ten minutes (Fig 1A). Because of its strongly positive charge, prolonged incubation with this tracer causes aggregation,[10] degranulation, and coalescence of various compartments of the SCCS. Treatment of bovine platelets with cationized ferritin under the same conditions also resulted in adherence of the tracer to the plasma membrane, but no interiorization occurred even after two hours of incubation (Fig 1B). The use of ruthenium red or peroxidase as tracers yielded similar results (data not shown). Because of the observation that human platelets internalized solutes and small particulates into the SCCS via pits resolved on replicas of freeze-cleaved membranes (Fig 2A), bovine platelets were freeze-fractured to determine whether they possessed similar structures. The structures are believed to represent openings into the SCCS. Despite considerable searching involving hundreds of replicas, bovine platelet plasma membranes proved to be virtually devoid of such pits (Fig 2B). Complementary protrusions seen on the external leaflet (E face) of human platelet membranes (Fig 2A) were also missing from the E face of bovine platelet plasma membranes (Fig 2B). Moreover, freeze-fracture images of canalicular membranes of human platelets also reveal these structures,[3] whereas they were not seen to be associated with any of the internal membranes of bovine platelets. Thus, occasional vacuoles seen in thin sectioned bovine platelets probably do not represent distended canaliculi, since their freeze-fracture replicas are also devoid of pits or protrusions.

DISCUSSION

The observations described here suggest that bovine platelets do not have a surface-connected canalicular membrane system, which is a prominent morphologic feature of human platelets. This conclusion is based on the finding that tracer substances like cationized ferritin and ruthenium red, which readily adhere to the plasma membrane of bovine platelets, fail to be interiorized. In human platelets, the SCCS became delineated by these agents within minutes of incubation, whereas no tracer was seen in bovine platelets even

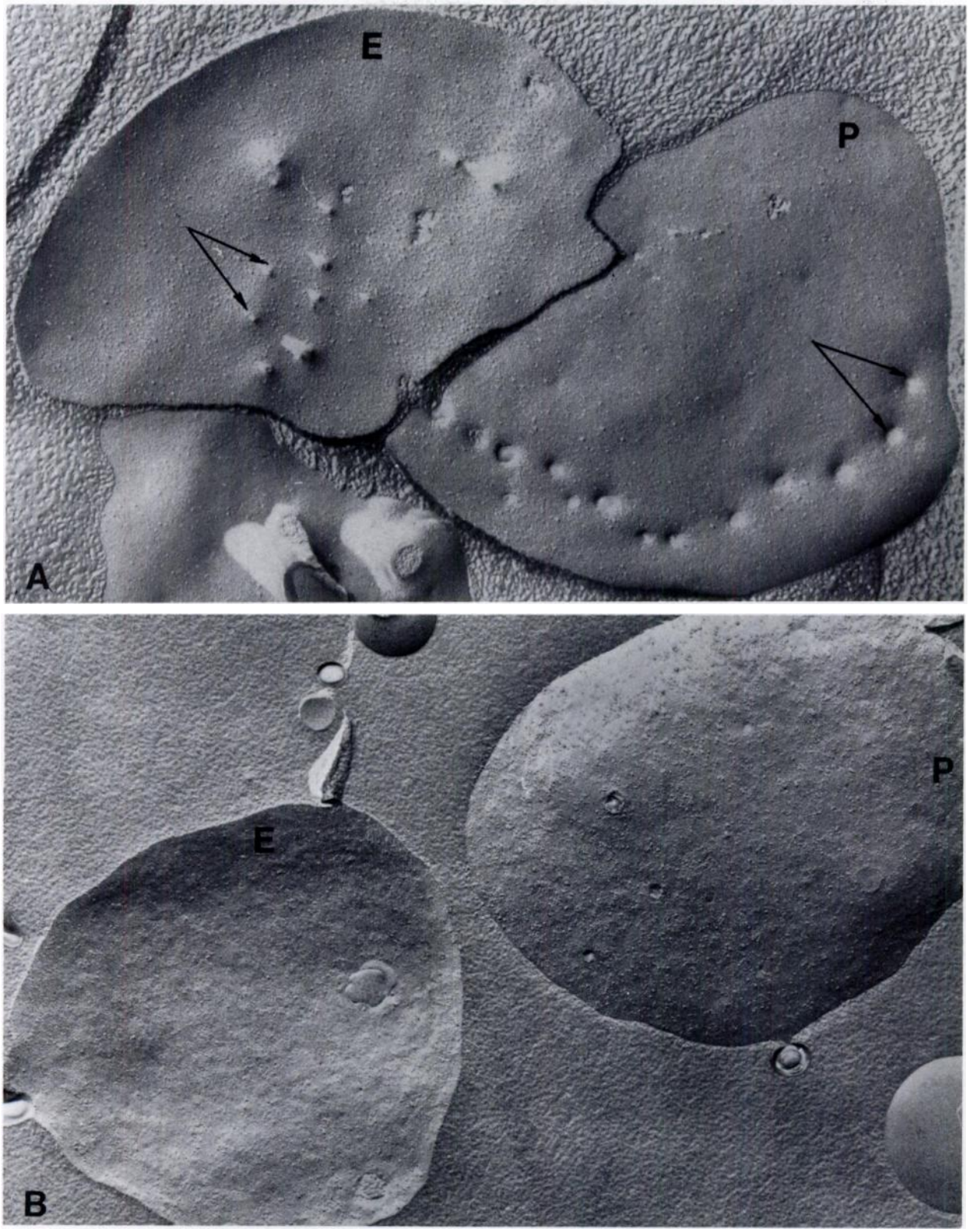

Fig 2. (A) Freeze-fracture replicas of human platelets. The external leaflet (E face) on the left shows protrusions (arrows), while the protoplasmic leaflet (P face) shows pits (arrows). These structures are known to be in continuity with the canalicular system. The intramembranous particles have been described in detail elsewhere.[3] Magnification ×36,000. (B) Freeze-fracture replicas of bovine platelets. The external leaflet (E face) on the left lacks protrusions and the protoplasmic leaflet (P face) on the right is devoid of pits. The depressions seen on the P face, which are probably fractured processes, should not be confused with characteristic pits. Magnification ×36,000.

after two hours. Furthermore, replicas of freeze-cleaved plasma membranes of bovine platelets did not exhibit pits or complementary protrusions, structures which in human platelets are believed to represent surface openings of the canalicular system.[3] However, apart from minor species variations in the total content of granule constituents,[6] the platelets of cattle and humans function similarly in hemostasis. Bovine platelets, like their human counterpart, release adenosine triphosphate, adenosine diphosphate, serotonin, and divalent cations in response to treatment with aggregating agents.[6] Does this imply that the SCCS is not significantly involved in secretory activity or does the release mechanism in human platelets differ from that manifested by the bovine cells? The release phenomenon is accompanied by a centralization of granules in human platelets.[8] This is not the case in bovine platelets, in which granules remain distributed peripherally, even when the cells are treated with thrombin (unpublished observation made independently by K.M.M. and D.Z.-F.). Thus, the theory that the granules discharge their content into the SCCS during the release reaction (a theory not fully proven for human platelets) does not appear to be applicable to bovine cells. The SCCS of human platelets is often considered analogous to the demarcation membrane system (DMS) of megakaryocytes. Both membrane systems are in continuity with the extracellular space and both are involved in endocytosis of solutes and small particulates. However, whether this aspect of platelet physiology is relevant to its role in hemostasis could be questioned. In the cytoplasm of megakaryocytes, the DMS delineates "platelet fields" and is thus believed to play an important role in thrombocytopoiesis. The so-called giant platelets, which are seen in the peripheral circulation under a variety of conditions, also consist of several "platelet fields" delineated by similar membranes (see Fig 11, p 569[11]). Fragmentation of such giant platelets into smaller subunits is likely to take place along this membrane system. The absence of a SCCS in bovine platelets not only suggests that thrombocytopoiesis may be different in cattle than in humans, but it also implies that the canaliculi may not play a crucial role in platelet function. Ultrastructural analysis of bovine megakaryocytes and platelets during accelerated thrombocytopoiesis and after-treatment with various aggregating agents will be necessary to answer these questions.

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