The Secretory Pathway of Bovine Platelets
By James G. White

Human platelets contain tortuous channels in their cytoplasm, the surface-connected or open canicular system (OCS), that communicate directly with the surrounding medium through openings on the surface membrane. Some workers have suggested that the OCS serves as the egress route for products secreted during the release reaction. Others have proposed alternate secretory pathways. Since bovine platelets lack the OCS found in human cells, the present study has examined the secretory mechanism of these cells to see whether it can shed light on the mystery of human platelet secretion. Bovine platelet granules, in contrast to human granules, are located more peripherally in resting cells (often in contact with the plasma membrane), most do not move centrally following thrombin stimulation as do human platelet granules, and many fuse directly with the external plasma membrane without any intermediate channel. The lack of peripheral location of human granules, their central rather than peripheral movement during secretion, and the presence of extensive channels are all consistent with the larger importance of the secretory channel to human platelets. Thus, though studies of bovine secretion do show that platelets can secrete their granules by direct fusion of granule and surface membranes, other differences from human platelets emphasize that this pathway, although important to bovine platelet secretion, is less important in human platelets. Studies of bovine platelets also show that the OCS is more dynamic than might have been considered from human studies and can form rapidly in response to stimulation. Such newly formed channels are used as a conduit for secretion of granule contents. The finding emphasizes the importance of channels for granule secretion in platelets generally and puts a new perspective on the ability of these cells to form channels rapidly in response to stimulation.

A recent study of bovine platelets has suggested the existence of a model for examining the two different concepts of platelet secretion.17 Zucker-Franklin et al17 have shown that bovine cells lack the OCS found in human cells. Therefore, cattle platelets must have some mechanism other than channels of the OCS for discharging products from secretory granules to the outside.18 The present study has searched for the secretory pathway in bovine platelets with the aid of freeze-fracture and electron-dense tracers.

MATERIALS AND METHODS

Blood for the present study was obtained from healthy adult cattle housed under direct supervision of the School of Veterinary Sciences at the University of Minnesota. Samples aspirated from the external jugular vein were mixed immediately with citrate-citric acid-dextrose (CCD) (93 mmol/L trisodium citrate, 70 mmol/L citric acid, 140 mmol/L dextrose, pH 6.5) in a ratio of nine parts blood to one part anticoagulant.19 Blood was sedimented at room temperature under a force of 200 g for 20 minutes. Platelet-rich plasma (C-PRP) above theuffy coat was aspirated and transferred to separate tubes. After determining the platelet count, the samples of C-PRP in each tube were combined with equal volumes of the CCD anticoagulant and centrifuged to pellets. The plasma was replaced with Hanks’ balanced salt solution (HBSS) containing 0.1 mmol/L EDTA. After 30 minutes at 37°C, the pellets were resuspended in HBSS with or without EDTA to produce a platelet count of 500,000/μL and returned to the 37°C water bath.20 Samples were fixed for study in the electron microscope at 30 seconds and 1, 3, and 5 minutes after exposure to thrombin at concentrations of 1, 3, and 5 U/mL. Each thrombin concentration and time of exposure was examined in four to seven experiments.

Fixation was accomplished by combining the sample at the appropriate time with an equal volume of 0.1% glutaraldehyde in White’s saline (a 10% solution of a 1:1 mixture of (1) 2.4 mmol/L NaCl, 0.1 mmol/L KCl, 46 mmol/L MgSO4, and 64 mmol/L Ca (NO3)2 · 4H2O and (2) 0.13 mol/L NaHCO3, 8.4 mmol/L NaH2PO4, and 0.1 g/L of phenol red, pH 7.4). After 15 minutes the samples were centrifuged to pellets and the supernatant fixative removed and replaced with 3% glutaraldehyde in the same buffer.19 The samples resuspended in the second aldehyde fixative were maintained at 4°C for 30 minutes and then sedimented to pellets. The supernatant was removed and replaced with either 1% osmic acid, 140 mmol/L dextrose, pH 6.5) in a ratio of nine parts blood to one part anticoagulant.19

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Submitted July 9, 1986; accepted October 14, 1986.

Supported by US Public Health Service Grants HL-11880, CA-21737, and GM-22167 and Grant MO1 1-866 from the March of Dimes.

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0006-4971/87/6903-0029$3.00/0

acid in Zetterquist's buffer or 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for one hour at 4 °C.21

Tannic acid has been used in our previous studies as an electron-dense stain for the OCS of human platelets.12,13 In the present study the method was modified from the procedure reported by Somlyo14 to stain the secretory pathway of bovine platelets. Bovine cells washed and resuspended as described before and exposed to thrombin in the presence or absence of EDTA were fixed through our usual sequence of 0.1% glutaraldehyde in White's saline followed by sedimentation to a pellet, removal of the supernatant, and replacement with 3% glutaraldehyde in the same buffer for two hours at 4 °C. After completion of initial fixation steps, the pellets were washed three times and stored overnight in 0.1 mmol/L cacodylate buffer, pH 7.2. The buffer was decanted the following day and replaced with 4% tannic acid in 0.1 mmol/L cacodylate buffer, pH 7.2, for four hours at room temperature. The supernatant was decanted, the pellets washed in cacodylate buffer, and the samples postfixed in 2% osmic acid alone or combined with 1.5% potassium ferrocyanide for two hours at 4 °C.

All samples were dehydrated in a graded series of alcohols and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate to enhance the contrast. All examinations were made on a Phillips 301 electron microscope.

Some of the aldehyde-fixed control and activated platelet samples from each experiment were combined with glycerol, frozen in liquid nitrogen, and freeze-fractured under high vacuum.25 Carbon platinum replicas prepared from exposed surfaces of the fractured platelet pellets were cleaned in detergent and examined in the transmission electron microscope.

RESULTS

Resting Bovine Platelets

The fine structure of bovine platelets was described previously by Myers et al18 and by Zucker-Franklin and her colleagues.17 Bovine platelets are similar to human cells in many respects. Like platelets of humans, cattle platelets are discoid in form, and their lentiform appearance is supported by granules two to three times larger than those in human platelets.12,13 Many of the large granules are spindle shaped and often appear to contact the inside of the surface membrane (Fig 2). An extensive network of tortuous channels communicating with the cell surface and referred to as the OCS in human platelets12,13 was not found in bovine cells (Figs 3 and 4). This confirmed the work of others showing that cationized ferritin, an electron-dense tracer, easily entered the channel system of human platelets but did not appear in bovine platelet cytoplasm.17 Replicas of freeze-fractured bovine platelets also failed to reveal the typical pits formed by channels of the OCS communicating with the surface of platelets from humans (Fig 3).

Thin sections of resting platelets prepared by our usual fixation procedures in the present study also failed to reveal the well-defined system of channels evident in platelets from humans (Figs 1 and 2). Bovine platelets stained with tannic acid lacked an OCS, and replicas of freeze-fractured cattle platelets contained only rare dimples or pits comparable to the openings of the OCS on the surface membrane of human cells until after stimulation by thrombin (Figs 3 to 6). However, small protrusions about 0.2 μm in diameter and devoid of intramembranous particles were frequently present on the P face of freeze-fractured resting bovine cells (Fig 3). Fractures through the membrane into the cytoplasm often revealed contact between membranes of the large granules and the undersurface of the cell wall at sites resembling the protrusions or indentations seen on the P face (Fig 4).

Elements of a second system of channels, the dense tubular system (DTS), were fully developed in bovine platelets. However, membrane complexes formed by the interaction of canaliculi from the OCS and DTS were absent (Figs 1 and 2).12 The presence of the platelet-specific peroxidase26 localized to the DTS channels of human platelets confirmed the presence of a DTS in bovine platelets. Thus cell cells contain the DTS but are deficient in the specialized membrane complexes found in human cells and in platelets from other species.

Thrombin-Stimulated Bovine Platelets

Aggregates. Thin sections of aggregated bovine platelets bore a superficial resemblance to human platelet thrombi. Shape change was evident, and the groups of cells were closely molded to each other as in hemostatic plugs. Dense spots resembling the contracted masses of actomyosin present in thrombin-stimulated human cells were present in many aggregated bovine platelets. However, the central concentration of organelles and enclosure within constricted rings of microtubules and microfilaments, regularly present in activated human platelets,2 were rarely evident in bovine platelets. Instead, the granules remained dispersed near the cell periphery. Tannic acid staining of the bovine platelet aggregates revealed electron-dense tracer outlining the spaces between aggregated platelets (Figs 7 and 8). In some cells the tannic acid–osmium product appeared in channel-like structures penetrating into the cytoplasm of the clumped platelets close to the dense spots of actomyosin. Some granules were also stained by tannic acid–osmium, which indicated they were connected to the cell wall or channels reaching the platelet surfaces.

Activated platelets. Thin sections of thrombin-stimulated but not aggregated bovine platelets revealed additional differences in their response compared with their human counterpart (Figs 9 to 20). Spikelike pseudopods extending from shape-changed bovine platelets were thinner and straighter than those formed on activated human platelets. Occasional microtubules are evident in human platelet pseudopods, but most surface projections lack the polymer. Almost every pseudopod extending from bovine platelets contained one or several microtubules interspersed with microfilaments. Microtubules were also present in the cytoplasm of activated bovine platelets (Fig 9). However, instead of being organized in constricted rings around centrally concentrated organelles, the tubules were irregularly dispersed in bundles or loose arrangements. The granules in
Fig 1. Resting bovine platelet. A circumferential microtubule (MT) coiled just under the surface membrane supports the lentiform appearance of the unstimulated cell. Granules (G), occasional mitochondria (M) and a few dense bodies (DB) together with glycogen (Gly) particles are randomly dispersed in the cytoplasm. The resting cell does not appear to contain channels of the open canalicular OCS or dense tubular systems (DTS). (Original magnification ×24,000.) Fig 2. Cross section of discoid cattle platelet. Granules are significantly larger than those in human platelets. They often appear to be in close association (1) or contact with the platelet membrane. (Original magnification ×28,000.) Fig 3. Replica of freeze-fractured bovine platelet. The surface membrane of a discoid cell has been split by the procedure, thereby revealing the outside of the inner layer, or “P” face. Indentations representing sites of continuity between channels of the open canalicular and the surface membrane in human cells are rarely seen in replicas of resting bovine platelets. Dimples (1) or protrusions free of the intramembranous particles covering the rest of the exposed surface are evident on most of the replicas. They may represent sites of contact between granules in the cytoplasm and the inner surface of the cell membrane. (Original magnification ×22,000.) Fig 4. Replica of discoid bovine platelet. Granules (G) in the cytoplasm are in close proximity to depressions in the surface membranes (1) at several points. (Original magnification ×28,000.) Fig 5. Replica of washed bovine platelet exposed to thrombin (3 U/mL, five minutes) in the presence of EDTA. The “P” face contains several indentations (1) similar, if not identical, to the openings of the OCS on replicas of resting human platelets. (Original magnification ×28,000.) Fig 6. Thrombin-activated bovine platelet (1 U/mL, three minutes). Shape change is under way in this cell, but the granules remain peripherally oriented. One organelle (G) has caused the surface membrane to protrude. This may precede extrusion of organelle contents. A small channel (1) is also apparent close to the cell membrane in this cell. (Original magnification ×28,000.)

Most platelets were peripherally oriented near the cell membrane rather than concentrated in cell centers.

Although tannic acid failed to stain channels of the OCS or granules in resting bovine platelets, it did so in thrombin-activated cells. Tannic acid appeared to react selectively with some product in platelet granules, which resulted in a heavy deposit of osmium black (Figs 7 to 12). The electron-dense stain appeared to reach granules through connections between the organelles and the cell surface. The mechanism for development of the openings permitting penetration of
Fig 7. Thrombin-aggregated platelets stained during fixation with tannic acid. Osmic acid deposited as an electron-dense stain by tannic acid coats the cell surface, fills channels penetrating into the cells, and has entered some granules (G) similar in size to unstained organelles. Two of them are in direct continuity with the cell surface through stain-filled channels (C). (Original magnification ×17,000.) Fig 8. Thrombin-aggregated bovine platelets. The preparation is the same as that shown in Fig 7. Tannic acid coats the surface of clumped platelets and fills a channel (C) penetrating into one of the cells. One granule (G) is in direct continuity with the channel, and another adjacent to it is unstained. (Original magnification ×28,000.) Fig 9. Thrombin-activated platelet (1 U/mL, three minutes). Little evidence of shape change is apparent in this cell. Microtubules are still evident at the platelet periphery and adjacent pseudopods. One granule (1) stained by tannic acid appears unconnected to the cell surface, another organelle (2) is very close to it, and a third (3) is open to the surrounding medium. Serial sections of this cell (not shown) reveal that organelles 1 and 2 are also directly connected to the platelet surface. (Original magnification ×34,000.) Fig 10. Thrombin-activated platelets (5 U/mL, one minute). The microtubules (MT) in this cell are in three separate groups. One granule (1) is filled with stain and appears unconnected to the cell surface. However, it must be connected above or below the plane of section to the surface membrane in order to take up tannic acid. Another granule (2) is connected to the cell surface, and its contents appear to be in the process of extrusion. (Original magnification ×30,000.) Fig 11. Thrombin-activated platelet (5 U/mL, one minute). Every pseudopod (P) extending from this platelet or adjacent cells contains microtubules. A granule filled with tannic acid-stained material appears to be in the process of discharge from the platelet. (Original magnification ×30,000.) Fig 12. Thrombin-activated platelet. This cell appears to have reacted maximally to thrombin stimulation. All of the granules appear to have fused with the cell surface and discharged their contents to the exterior. Tannic acid–stained material most likely represents granule contents fixed in situ during extrusion. The dense spot remaining in the cell center is a contracted gel (CG) of actomyosin. Microtubules fill adjacent pseudopods (P). (Original magnification ×24,000.)
tannic acid into granules was not clear. However, it must be related to the action of thrombin because connections were not evident in control platelets. The discharge of stained granule contents from thrombin-activated platelets appeared to follow two routes. Most of the granules close to the surface fused with the membrane and secreted directly to the outside medium (Figs 9 to 12). Some examples suggest that granule products are actively extruded (Figs 10 to 12). Internal contraction leading to the formation of dense spots of contractile gel in thrombin-activated bovine platelets may provide the force for expulsion (Fig 12).

In addition to demonstrating the interaction between granules and the surface membrane in thrombin-activated bovine platelets, tannic acid staining also revealed the devel-
opment of a primitive channel system in activated cells (Figs 13 to 20). The suggestion that channels were newly developed came from observations made on replicas of freeze-fractured platelets. Apertures of the OCS onto the surface membrane of resting and activated human platelets were rarely seen on discoid bovine cells (Fig 3). They were regularly present, however, following exposure of bovine platelets to thrombin (Fig 5). In thin sections of tannic acid-stained bovine platelets, electron-dense product penetrated into fragile channels extending for variable distances into the cytoplasm. Communication between stain-filled channels and granules was easily identified. The channels appeared fragile and lacked the tortuosity and extensive distribution of the OCS in human platelets (Fig 20). Channels were also apparent in replicas of thrombin-stimulated bovine platelets (Figs 6 and 21 to 24). Some branching was noted in replicas, but, in general, the channels were very simple structures.

**DISCUSSION**

The present investigation has used ultrastructural techniques to search for the secretory pathway in bovine platelets. Cattle cells resemble human platelets closely. Resting platelets from both species have a characteristic discoid shape supported by a circumferential microtubule. The cytoplasm of human and bovine platelets is filled with secretory organelles including dense bodies and alpha granules. Mitochondria and glycogen particles are common in both cells. There are significant differences, however. Bovine platelets are slightly smaller than human cells and more elliptical in form. Dense bodies are less frequent, and the granules are two to three times larger than comparable organelles in human platelets. Granules in cattle platelets often appear to impinge on the surface membrane, whereas this close relationship does not occur in cells from humans.

The major difference between human and bovine cells lies in the membrane systems. An extensive system of channels communicating with the surface membrane and with each other is a characteristic feature of human platelets. Zucker-Franklin and her colleagues used an electron-dense tracer, cationized ferritin, and freeze-fracture to demonstrate that bovine platelets lack the surface-connected OCS. Examination of tannic acid–stained platelets and replicas of freeze-etched cells in this study confirmed the absence of a definitive OCS in resting cells from cattle.

The findings suggested that the bovine platelet could serve as a useful model to resolve conflicting concepts regarding the mechanism of platelet secretion. A search for the secretory pathway of activated human platelets had shown that granules fuse with channels of the OCS and are extruded from the cell through these conduits during platelet shape change and internal contraction. Other workers reached a different conclusion. Ginsberg et al used immunocytochemistry and electron microscopy to follow secretion in human cells. Centrally concentrated organelles in stimulated platelets fused together to form closed sacs. The sacs of secretory products migrated to the platelet surface, attached to the membrane, and extruded their contents to the outside.

Since bovine platelets lack the OCS, the concept of secretion in which granules fuse with channels and use the canalicular for discharge of products did not seem reasonable for cattle cells. Some variation of the hypothesis proposed by Ginsberg and colleagues would seem more tenable. However, the movement of granules toward platelet centers that precedes their intimate association and possible fusion into closed sacs occurs infrequently in bovine platelets.

In the present study thrombin-induced aggregation and activation of bovine cells caused shape change, pseudopod formation, and the development of stickiness, responses closely resembling those of human cells. The internal reaction of cattle platelets, however, was quite different. Central movement of randomly dispersed organelles and their inclusion within tight-fitting rings of microtubules and microfilaments, a regular feature of stimulated human cells, occurred rarely in bovine platelets. Instead the microtubule coil appeared to remain peripherally located or fractured during shape change, which resulted in random bundles in the cytoplasm and one to several polymers in each spikelike pseudopod. Granules remained dispersed and close to the inside surface of the cell membrane in thrombin-activated and -aggregated bovine platelets.

Examination of freeze-fractured bovine platelets after exposure to thrombin revealed a marked change from resting cells. Dimples indicating sites of communication between channels of the OCS and the surface were virtually absent from discoid platelets. However, pinpoint depressions, slightly smaller than the openings of the OCS on human platelets, were present on nearly every activated bovine platelet. Short invaginations resembling channels communicating with the surface membrane were present in the cytoplasm of freeze-fractured bovine platelets prepared after exposure to thrombin.

Tannic acid staining of thrombin-aggregated and -activated platelets also revealed dramatic changes from the appearance of resting bovine platelets. Electron-dense stain was deposited in many organelles with the size and shape of granules in activated cells. Some of these were in direct continuity with the irregular platelet surface. Other stained organelles were connected to the cell wall and to each other by narrow channels filled with electron-dense material. In many examples the stain appeared to catch granule products fixed during the process of extrusion from thrombin-stimulated bovine platelets.

The close relationship between the large granules and the surface membrane of bovine platelets would seem to obviate the need for OCS channels found in platelets from humans, pigs, rats, whales, horses, rabbits, and other species. It is surprising, therefore, that bovine platelets either have a few elements of OCS invisible in resting cells or develop them during shape change and internal contraction. Electron-dense stain entered channels communicating with the surface in activated platelets. The channels were narrow, compared with elements of the OCS in human cells, and usually short and unbranched. Some, however, extended far into platelet cytoplasm to form a direct connection to stain-filled
Fig 19. Thrombin-activated platelet (5 U/mL, five minutes). A fully transformed platelet is evident in this illustration. Shape change and contraction of actomyosin gel into a dense spot are advanced. At least two channels contain tannic acid-stained residue, which suggests that they are granules or carry granule products to the surface. (Original magnification x25,000.) Fig 20. Thrombin-activated human platelet (3 U/mL, three minutes). This cell has been exposed to the same conditions as bovine acells in the present study. A channel of the OCS in continuity with the surface membrane is dilated by tannic acid-stained material. The tortuous channels are connected directly to at least two granules (G), which are also stained by tannic acid. The constricted microtubule (MT) coils are cut in cross section in this example. (Original magnification x23,000.) Fig 21. Replica of thrombin-activated bovine platelet (3 U/mL, one minute). A fine channel (↑) extends from the surface of the stimulated cell deep into the cytoplasm. (Original magnification x28,000.) Fig 22. Replica of thrombin-activated platelet (3 U/mL, one minute). Two short channels (↑) are evidently extending inward from the surface membrane of the activated cell. (Original magnification x30,000.) Fig 23. Replica of thrombin-activated platelet (3 U/mL, one minute). Two small channels (↑) appear to connect granules to the cell surface on the left. On the right, a channel (C) with two openings on the surface may provide a pathway for secretion of some granule products. (Original magnification x28,000.) Fig 24. Replica of thrombin-activated platelet. One edge of this cell has three openings (↑) into a single channel of the OCS, which extends deeper into the cytoplasm close to a granule (G). (Original magnification x30,000.)

granules. It is possible that the channels developed in activated platelets were the result of infoldings or creases in the membrane or a collapse of granule walls following secretion of their contents. However, except for the extensive distribution of the OCS in human cells and the tortuosity and interconnection of the canaliculi, the appearance of the channels and relationship to granules and the cell membrane in bovine platelets were quite similar.

Thus, the difference in the mechanism of secretion in bovine compared with human cells is not as great as it might at first seem. Bovine platelets do not have a well-defined OCS. As a result, they use the surface membrane as the primary route for discharge of products from secretory granules. Human platelets have an extensive system of internalized surface membrane formed into channels of the OCS. It is used as the primary route of secretion in human cells. Bovine platelets can develop primitive canaliculi following activation, and granule products may leave the cell
through these conduits. Yet it uses the cell surface as a preferential route for exocytosis. The basis for the species variations in platelet structure that result in different preferred routes for secretion of granule products remains unknown.

REFERENCES


ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr Janine Breton-Gorius, CHU Henri Mordor, 94010 Creteil, France, who localized the peroxidase activity in the DTS of bovine platelets.
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