Anchorage of Secretion-competent Dense Granules on the Plasma Membrane of Bovine Platelets in the Absence of Secretory Stimulation

Takako Morimoto, Satoshi Ogihara,* and Haruhiko Takisawa

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka, 560, Japan; and *Department of Biology, College of General Education, Osaka University, Toyonaka, Osaka, 560, Japan

Abstract. The ultrastructural changes in electropermeabilized bovine platelets that accompany the Ca^{2+}-induced secretion of serotonin were investigated in ultra-thin sections of chemically fixed cells. Such preparations permitted us to study both the localization of and the structures associated with serotonin-containing dense granules. Localization of dense granules within cells was examined by measuring the shortest distances between the granular membranes and the plasma membrane. About 40% of total granules were located close to the plasma membrane at an average distance of 10.8 ± 1.6 nm. 71% of the total number of granules were localized at a similar average distance of 12.5 ± 2.7 nm in intact platelets. The percentage of granules apposed to the plasma membrane corresponded closely to the percentage of total serotonin that was maximally secreted after stimulation of the permeabilized (38 ± 4.9%) and the intact platelets (72 ± 3.6%). Furthermore, the percentage of granules anchored to the membrane, but not of those in other regions of permeabilized cells, decreased markedly when cells were stimulated for 30 s by extracellularly added Ca^{2+}. The decrease in the numbers of granules in the vicinity of the plasma membrane corresponded to ~22% of the total numbers of dense granules that were used for measurements of the distances between the two membranes and corresponded roughly to the overall decrease (15%) in the average number of the granules per cell. Most dense granules were found to be associated with meshwork structures of microfilaments. Upon secretory stimulation, nonfilamentous, amorphous structures found between the plasma membrane and the apposed granules formed a bridge-like structure that connected both membranes without any obvious accompanying changes in the microfilament structures. These results suggest that the dense granules that are susceptible to secretory stimulation are anchored to the plasma membrane before stimulation, and that the formation of the bridge-like structure may participate in the Ca^{2+}-regulated exocytosis.

In response to various extracellular stimuli, components of intracellular granules are released into the extracellular milieu through exocytotic processes, which include the fusion of the granular membrane and the plasma membrane. Regulated exocytosis, such as that involved in the release of neurotransmitters and hormones, is generally triggered by a rise in the cytosolic concentration of calcium (1, 4, 20). In previous studies, two models have been proposed for the mechanism of calcium-regulated exocytosis. In one model, secretory granules bound to the end of an actin filament can move to the plasma membrane as a result of the contraction of actomyosin that is activated by calcium ions (7). In the other model, the granules bound to the network of actin filaments underneath the plasma membrane are able to fuse with the plasma membrane as a result of disassembly of the network by calcium ions (4, 15). At present, few experimental results have been presented in support of either model.

The early stages of Ca^{2+}-regulated exocytosis, in particular the membrane fusion processes, were revealed by Chandler and Heuser in ultrastructural studies using a quick-freezing technique (6). They observed the initial fusion event: formation of a pore, 10–50 nm in diameter, that connects the interior of the granule with the extracellular space. In addition to the formation of the pore, they occasionally observed that small fibrils, which connect the plasma membrane with an unfused secretory granule, lie just beneath the plasma membrane. Chandler proposed that the membrane fusion is preceded by the reorganization of such a small fibrillar cytoskeleton (5). However, neither the identity of the cytoskeleton nor the movement of secretory granules distal to the plasma membrane has been clarified.

In this study, we examined the localization of and the structures associated with serotonin-containing secretory granules (dense granules) in electropermeabilized and intact platelets. In permeabilized platelets, the secretion of serotonin can be initiated by extracellularly added Ca^{2+} (13, 20), which permits us to investigate various stages of calcium-regulated exocytosis with special attention to ultrastructural changes. Our results indicate that secretion-competent gran-

ules are located in the close proximity to the plasma membrane in the absence of any stimulation and that nonfilamentous structures between the granular membrane and the plasma membrane may participate in the exocytotic process.

Materials and Methods

Preparation of Electropermeabilized Platelets

Platelets were prepared from bovine blood as described previously (18). Isolated platelets were suspended in a medium that contained 95 mM NaCl, 12.6 mM KCl, 22.6 mM glucose, 10.8 mM citric acid, and 14.1 mM trisodium citrate at pH 7.2, the pH being adjusted with Tris (Tris-ACD). The platelets were electropermeabilized by the following procedure. Platelets suspended in Tris-ACD were centrifuged at 500 *g* for 2 min. The pellet was resuspended in a medium that consisted of 280 mM glycine, 10 mM glutamate, 1 mM EGTA, and 20 mM Pipes at pH 7.0 (glycine buffer). The suspension of platelets was placed in a chamber in which plane platinum electrodes were located 1 mm apart, and the chamber was subjected to seven electric discharges from a 4-μF capacitor charged at 1.2 kV (13).

Preparation of Samples for EM

The procedure used for the processing of platelets for EM was a modification of that used by Bennett and Condeelis for examination of the cell cortices of *Dictiostelium discoidium* amebae (3). Intact platelets in Tris-ACD, or electropermeabilized platelets in suspension in the glycine buffer supplemented with 1 mM ATP or 1 mM Mg-ATP and 1 mM $CaCl_2$ (free concentration of Ca^{2+} of ~4 μM) at 35°C were fixed by mixing with 10 vol of a medium that consisted of 2% glutaraldehyde, 0.2% tannic acid, and 60 mM phosphate buffer at pH 7.0. After 30 min at room temperature, platelets were collected by centrifugation at 3,000 *g* for 5 min, washed three times with 40 mM phosphate buffer at pH 7.0, and resuspended in the same buffer. The suspension was treated with 0.5% osmium tetroxide at 0°C for 5 min. Fixed platelets were then collected by centrifugation at 3,500 *g* for 3 min, washed twice with ice-cold water, and stained with 2% uranyl acetate in water for 15 min at room temperature. The stained platelets were overlaid on 0.5% lukewarm, liquid agarose, sedimented onto the bottom of the agarose layer by centrifugation at 10,000 *g* for a few seconds, and then the mixture was solidified on ice. The solidified samples were cut into pieces and dehydrated in a graded ethanol series to 100% ethanol. After replacement of ethanol by propyrenoxide, the samples were embedded in a 50:50 mixture of EPON and Araldite. Ultra-thin, silver-gray sections were cut and picked up on 300-mesh grids. Sections on grids were stained with uranyl acetate and Reynold's lead citrate, and photographed at 80 kV on a JEM-100S (JEOL, Ltd., Tokyo, Japan). Techniques for measuring micrographs are described in Results and the test for significance or differences between two values was performed using a x^2-distribution.

Measurements of Secretion of Serotonin from Platelets

Intact platelets in Tris-ACD were stimulated by addition of 5 U/ml thrombin at 35°C, while electropermeabilized platelets in the glycine buffer were stimulated in the presence of 1 mM Mg-ATP by extracellular addition of 4 μM free calcium ions at 35°C. After stimulation for 3 min, the platelets were removed by centrifugation at 10,000 *g* for 3 min. The supernatant was mixed with HCl to give a final concentration of 3.6 M, and the amount of serotonin in the supernatant was determined with a spectrofluorometer (MPF-4, Hitachi Ltd., Tokyo, Japan) by measuring the fluorescence intensity of excitation and emission wavelengths at 295 and 550 nm, respectively (22).

Chemicals

Bovine blood was obtained at a slaughterhouse. Tannic acid was obtained from Mallinckrodt (Paris, KY). Uranyl acetate was obtained from E. Merck Japan, Ltd. (Tokyo, Japan). Bovine thrombin was obtained from Mochida Pharmaceutical, Co., Ltd. (Tokyo, Japan). All the other chemicals were of reagent grade.

Results

Intact and electropermeabilized bovine platelets were chemically fixed with 2% glutaraldehyde in the presence of 0.2% tannic acid, which has been reported to improve the visibility of actin (2, 16). In addition, the fixed cells were exposed to osmium tetroxide for a short time to preserve the intracellular structures (3). In Fig. 1, the ultrastructure of intact platelets (*a*) is compared with that of electropermeabilized platelets (*b*). In most sections, intact platelets were oval in shape, and bundles of microtubules (23 nm in diameter) were seen on both longitudinal edges of the cells. Such bundles of microtubules, running along the longitudinal edges of cells serve to maintain the characteristic discoid shape of the intact platelets (10). In preparations of electropermeabilized platelets, most cells were round in shape (~3.0 μm in maximum diameter). The bundles of microtubules which were observed in intact platelets disappeared, and fragmented microtubules were seen to be dispersed in the permeabilized cells. The ultrastructural differences between intact and permeabilized platelets suggest that the electropermeabilization procedure changed the shape of the cell from discoid to spherical.

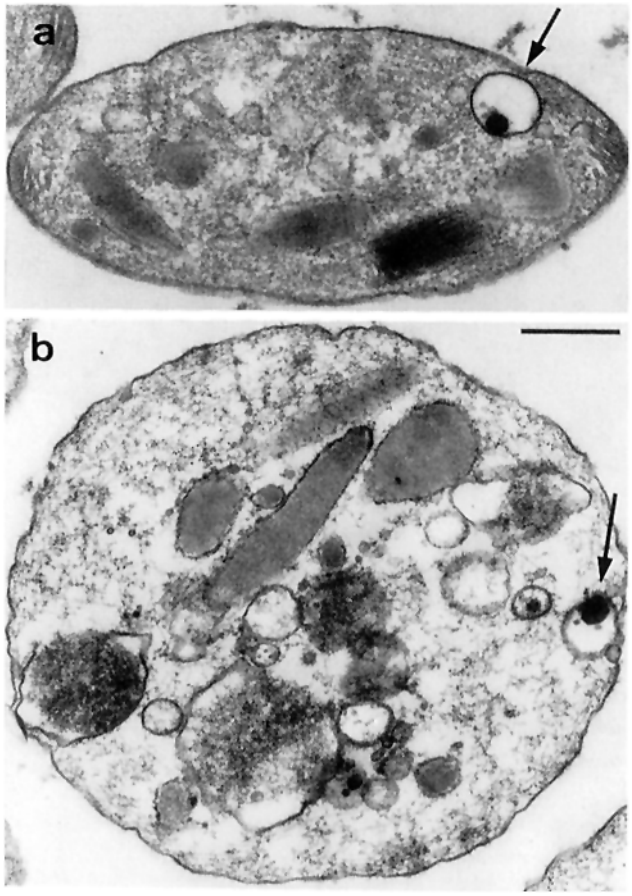

Figure 1. Electron micrographs of bovine platelets. Intact (*a*) and electropermeabilized (*b*) platelets were chemically fixed, sectioned, and observed as described in Materials and Methods. The granular organelles having "bull's eyes" (*arrow*) are dense granules that contain serotonin. Bar, 0.5 μm.

Various types of granular organelle were identified from micrographs similar to that in Fig. 1. Dense granules that contained serotonin were easily identified by the presence of an electron-dense "bull's eye" (10, 21). In Fig. 1, *a* and *b*, the

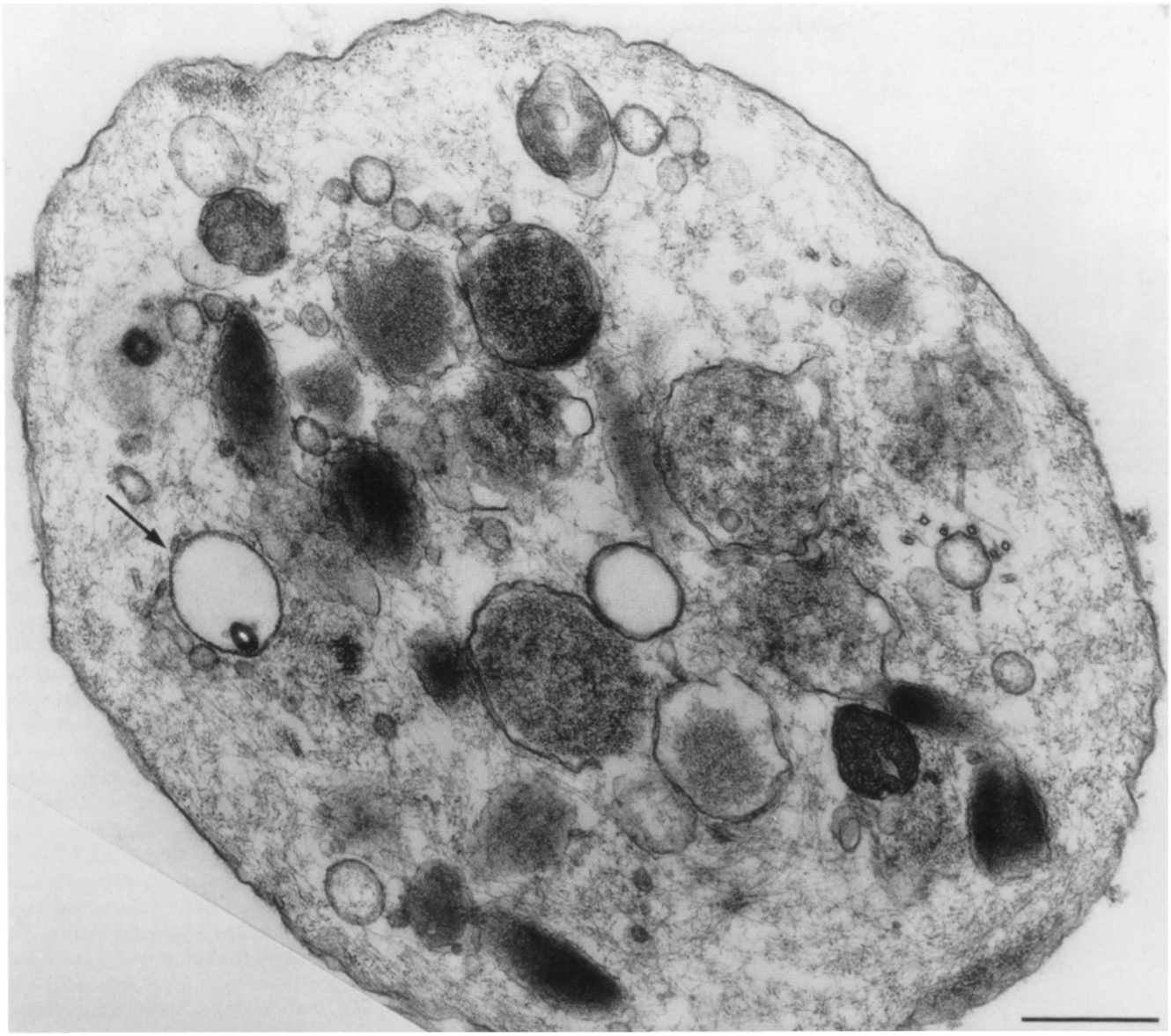

Figure 2. A high-magnification view of an electropermeabilized platelet. Microfilaments spread throughout the cell, including the regions around the granular organelles, such as the dense granules (*arrow*) and the alpha granules. Longitudinal and cross sections of microfilaments are seen near the plasma membrane, indicative of the presence of a highly ordered mesh of microfilaments. Bar, 0.5 μm.

dense granules are located close to the plasma membrane. In intact platelets, dense granules were surrounded by dense fibrous materials, without any visibly distinct structures. The electropermeabilization of the platelets resulted in an increase in the visibility of the intracellular structures, especially the organization of microfilaments, probably as a result of the higher permeability to the fixatives and the loss of some soluble cytoplasm. No changes could be detected in the shape of granular organelles.

Images at high resolution (Fig. 2) revealed that microfilaments were spread throughout the cell. Cross sections of microfilaments near the plasma membrane indicated the presence of a highly ordered mesh of microfilaments underneath the membrane. Granular organelles, including dense granules, were surrounded by microfilaments.

The permeabilized platelets still retained the ability to secrete serotonin, although they did not respond to thrombin, a potent agonist of the secretion of serotonin in intact cells (13, 20). The secretion of serotonin from the permeabilized cells was evoked by extracellularly added Ca^{2+}, which penetrated the membrane easily. In the presence of 1 mM Mg-ATP, increases in the extracellular concentration of free Ca^{2+} up to 4 μM resulted in the maximal release of serotonin (38 $\pm$ 4.9% of the total serotonin content of the cells). By contrast, stimulation by thrombin of the intact platelets resulted in release of 72 $\pm$ 3.6% of the total serotonin. These results indicate that the ultrastructure as well as the functions required for the exocytosis of the dense granules are preserved after electropermeabilization of the cell membrane.

We next examined the intracellular localization of the dense granules in intact and electropermeabilized platelets. Dense granules can be easily distinguished from the other

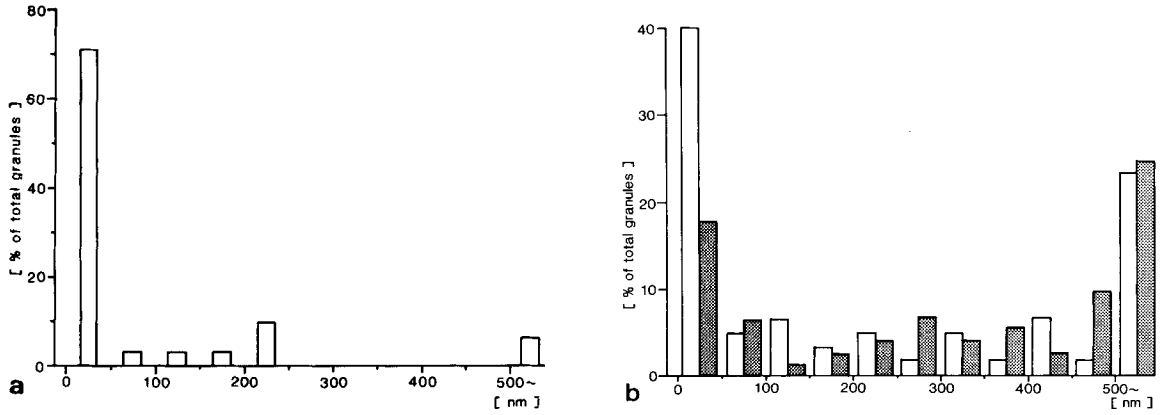

Figure 3. Distribution of the distances between dense granules and the plasma membrane in intact (*a*) and electropermeabilized (*b*) platelets. The localization of dense granules is expressed in terms of the shortest distance from the plasma membrane. Numbers of granules were counted and the percentages of the total granules are represented in each column at 50-nm intervals. In *a*, 31 granules were analyzed for measurements of the distance. In *b*, the open and the meshed columns represent the percentages of the granules before and 30 s after stimulation with Ca^{2+}, respectively. 60 and 63 granules were analyzed, respectively.

types of granules because both the bull's eye and the membrane of these granules appear particularly electron dense (see Fig. 1). The average number of dense granules calculated in this way using ultra-thin sections (two to four granules per cell) closely corresponded to that observed directly with fluorescent microscopy (two to five granules per cell, data not shown); dense granules fluoresce due to the fluorescence of acriflavine, incorporated into dense granules as a serotonin analogue. The location of the granules was quantified by measurement of the shortest distance between the membranes of the dense granules and the plasma membrane. For measurements of the distance, we chose the dense granules and plasma membrane in exact profile, i.e., as a unit membrane. Fig. 3 shows the distribution of distances between dense granules and the plasma membrane in intact (*a*) and electropermeabilized platelets (*b*). The distances are illustrated schematically at 50-nm intervals. To describe our results more precisely, there was a sharp peak of intermembrane distances at ~10 nm, and the fraction of granules associated with this peak could easily be distinguished from the rest (>50 nm). The dense granules located close to the plasma membrane represented 71 and 40% of the total number of granules at an average distance of 12 ± 2.7 nm and 11 ± 1.6 nm, in intact and electropermeabilized platelets (n = 22, 24), respectively. Dense granules found in the other regions of the cell (>50 nm) appeared to be randomly distributed, as shown in Fig. 3.

The distribution by distance of dense granules in electropermeabilized platelets did not change significantly after secretion of serotonin except for the percentage of granules closest to the membrane (Fig. 3 *b*), at 0–50 nm ($P < 0.02$). Permeabilized platelets were stimulated in the presence of 1 mM Mg-ATP by extracellularly added Ca^{2+} (4 μM), and they were fixed for EM after 30 s. The number and the localization of the granules were examined. The average number of the dense granules per cell was estimated by dividing the total number of granules by the total number of cells observed in the sections. The average number of granules per cell decreased from 0.34 (471/1379) to 0.29 (393/1338) after stimulation. These two values are significantly different ($P < 0.01$). In other words, the number of dense granules after stimulation decreased to 85% of that before the stimulation. The distribution in terms of distance from the plasma membrane of the granules before and after stimulation is compared in Fig. 3 *b*; the total number of granules counted at 30 s was divided by 0.85 so that the number of the granules that were lost by exocytosis would be taken into account. The percentage of the total number of granules located in the 0–50-nm range decreased from 40 to 18% after stimulation. The average distance for granules in the 0–50-nm range was not significantly changed upon stimulation (11 ± 3.1 nm). The distribution of dense granules in the other regions did not appear to be altered in a meaningful way.

The structures associated with dense granules were examined in electropermeabilized platelets. Fig. 4 shows the close apposition of the dense granules to the plasma membrane in ultra-thin sections when cells were cut at various angles. The granules were associated with a meshwork of microfilaments but they were not entirely covered with the filaments. Fig. 5 shows sections of the granular and the plasma membrane in exact profile. Both the granular and the plasma membrane showed a bilayer structure. In every section, an electron-dense amorphous structure could be observed between the two membranes. No filamentous structure was seen in this narrow gap. The other regions of the surface of dense granules were not naked but they were in part associated with microfilaments and in part coated with fluffy material. Note that the outer surface of the plasma membrane, to which dense granules were attached from inside the cell, had some protrusions (*arrowheads*), typical of sugar chains of glycoproteins. These protrusions seemed more concentrated in this region than in other regions of the cell surface.

Upon exocytotic stimulation, the amorphous structures observed between dense granules and the plasma membrane formed bridge-like structures that connected the plasma membrane and the granule. Fig. 6, *a* and *b* show that such bridge-like structures were formed as early as 5 s after the addition of Ca^{2+}. The inset in Fig. 6 shows that electron-dense, dot-like structures of ~32 nm in diameter occurred at intervals of ~48 nm in a somewhat circular arrangement. Those structures appeared to be located on the surface of a dense granule, as suggested by ultra-thin sections cut at an

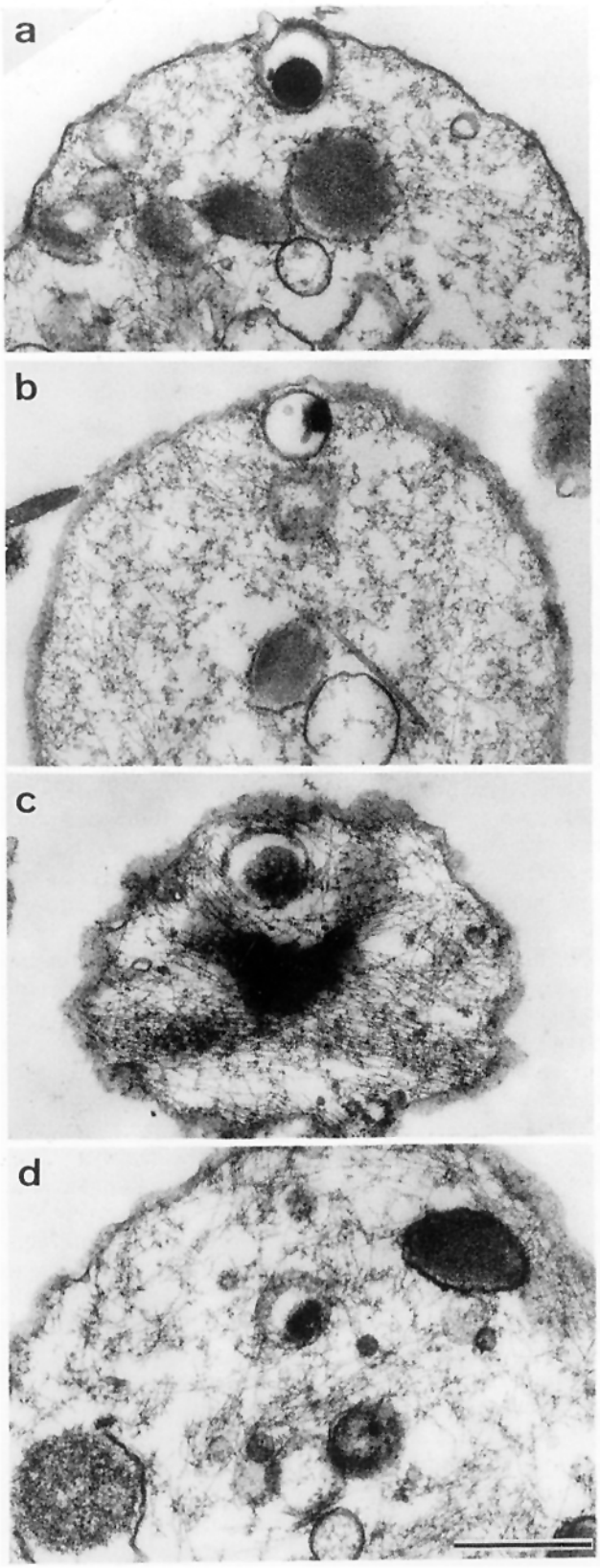

Figure 4. Oblique sections of dense granules in electropermeabilized platelets before stimulation with Ca^{2+}. Dense granules beneath the plasma membrane (*a–c*) and inside the cell (*d*) are associated with networks of microfilament. Bar, 0.5 μm.

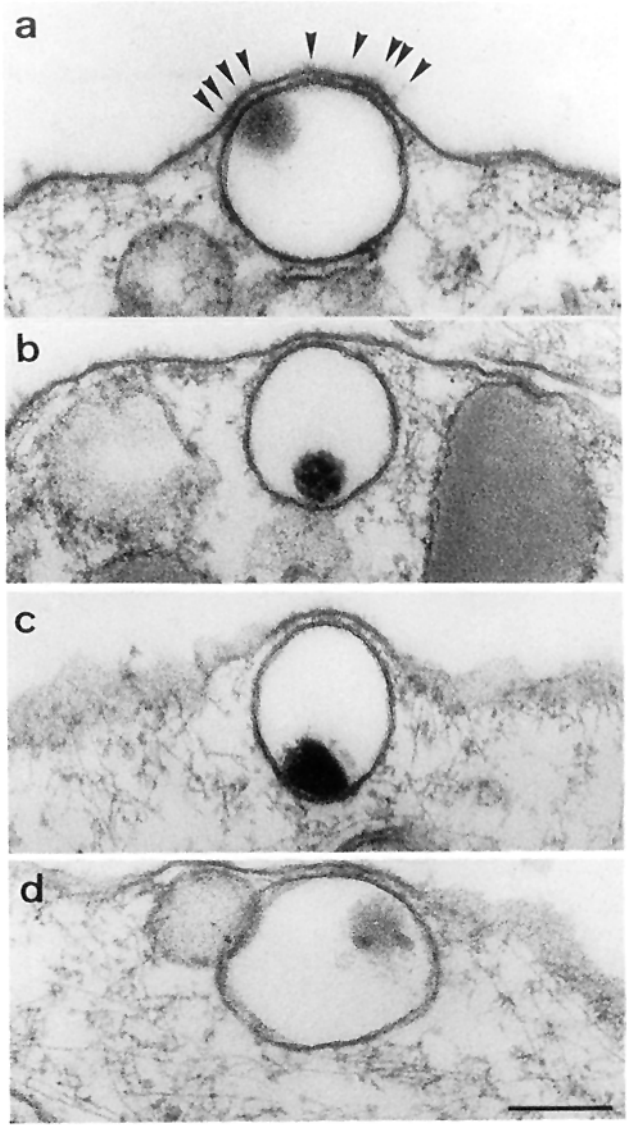

Figure 5. Typical views of dense granules anchored to the plasma membrane in the absence of stimulation. In these sections, both the granular and the plasma membranes are in exact profile. Nonfilamentous, amorphous structures are seen between the two membranes. Arrowheads indicate protrusions on the outer surface in regions of the plasma membrane where amorphous structures are seen inside the cell. Bar, 0.2 μm.

angle. They appear to be identical to the bridge-like structures seen in sections in exact profile, for example, in Fig. 6, because of the similarities in the size of the dot-like structures to that of the bridge and in the 48-nm periodicity of the structures on the membrane of the dense granules. A clear and regular array of bridge-like structures (11.6-nm interval and 2-nm width) was observed in a specimen fixed 30 s after stimulation with Ca^{2+} (Fig. 6 *c*); >80% of the dense granules that were docking to the plasma membrane were found to have the bridge-like structures by 30 s after the stimulation. As shown in Fig. 3, the distance between the dense granules and the plasma membrane did not change, even when the bridge-like structures were formed (11 nm).

No changes could be observed in the organization of the

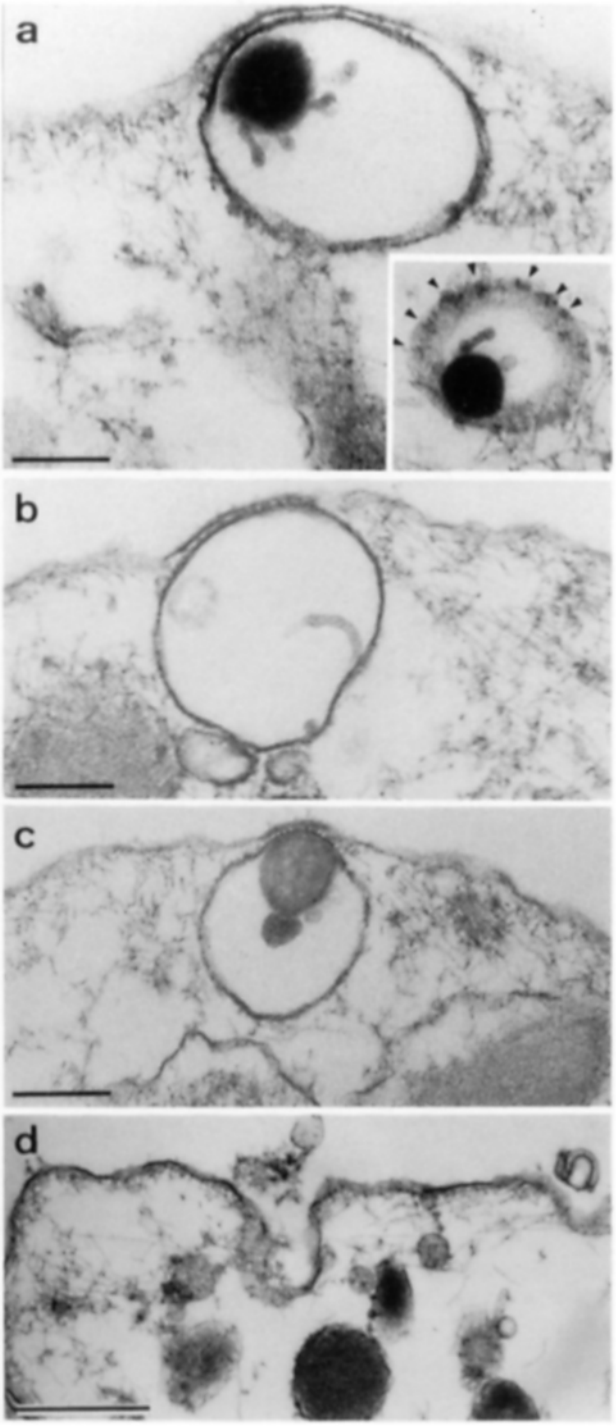

Figure 6. Electropermeabilized platelets were fixed 5 s (*a* and *b*) and 30 s (*c* and *d*) after stimulation with Ca^{2+}. Bridge-like structures between the plasma membrane and the anchored granules are observed in *a–c*. Arrowheads in the inset show the dot-like structures that are thought to be identical to the bridge-like structures. In *d*, an Ω shape formed after the exocytosis of a dense granule is shown. Bars: (*a–c*) 0.2 μm; (*d*) 0.5 μm.

microfilaments associated with dense granules after the stimulation. Fig. 6 shows that microfilamentous structures were associated with the dense granules and the microfilamentous mesh underneath the plasma membrane was well-preserved 5 or 30 s after exocytotic stimulation. The microfilamentous structure was also preserved in the vicinity of the Ω shape, which has been suggested to indicate the site at which the exocytotic release of dense granules occurred (Fig. 6 *d*).

The morphology of the plasma membrane appeared to be greatly altered during the exocytotic process. In unstimulated platelets, the area of the plasma membrane to which dense granules were attached and the neighboring areas appeared clearly as a bilayer structure in most sections. However, while the part of the plasma membrane that was above the bridge-like structure retained the bilayer structure after stimulation, the other part of the plasma membrane was often found to have been obliquely sectioned. This result indicates that most areas of the plasma membrane become more fluid upon stimulation than the plasma membrane of unstimulated platelets, and it suggests that stimulation by Ca^{2+} may affect the membrane cytoskeleton to increase the fluidity of the plasma membrane.

Discussion

An increase in the concentration of intracellular Ca^{2+} is known to trigger most of regulated exocytosis (1, 4, 20). Structural aspects of the exocytotic processes, in particular the process of membrane fusion, have been clarified by EM of quick-frozen preparations of mast cells (5, 6). In the present study, we examined the ultrastructural features of Ca^{2+}-regulated release of serotonin from bovine platelets. Platelets as an experimental system possess the following advantages over other endocrine cells (*a*) easy identification of secretory granules on ultra-thin sections of the specimens; (*b*) high efficiency of release of serotonin in response to stimulation; and (*c*) strict dependence of the release of serotonin on Ca^{2+}.

We first investigated the intracellular localization of dense granules. Fig. 3 shows that 71 and 40% of dense granules in intact and electropermeabilized platelets, respectively, are already located fairly close to the plasma membrane even before stimulation. The average distance between the plasma membrane and the anchored dense granules was ~11 nm, which corresponds to roughly twice the thickness of the lipid bilayer. In response to stimulation, a maximum of 72 and 38% of the serotonin in the cell was released from the intact and the electropermeabilized platelets, respectively. The percentage of dense granules anchored to the plasma membrane was virtually equal to the percentage of the total amount of serotonin in the cell that was released maximally from either preparations of cells. These results, therefore, readily suggest the possibility that those granules apposed to the plasma membrane are preferentially released upon stimulation.

This possibility was further supported by the intermediate distribution of dense granules in the electropermeabilized platelets fixed 30 s after stimulation with Ca^{2+} (Fig. 2 *b*). The fraction of dense granules attaching to the plasma membrane decreased from 40 to 18% within these 30 s. This decrease (22%) can be explained by the decrease in the average number of dense granules per cell (15%). In addition, very few dense granules attaching to the plasma membrane could be found in the platelets fixed 3 min after stimulation with Ca^{2+}, when the release of serotonin reached a maximum. Anchorage of secretory granules to the plasma membrane has been reported in bovine platelets (23) and other types of

cell, for example, in chromaffin cells and cultured PC12 cells (19), and in the presynaptic region of nerve cells (12). Cultured PC12 cells have most of their secretory granules aligned just underneath the plasma membrane. However, it has not yet been determined whether the granules are attached to the plasma membrane or whether they are at distance from the plasma membrane at the electron microscopic level of resolution. In addition, nerve terminals and chromaffin cells have secretory granules not only in close proximity to the plasma membrane, but also deep in the cytoplasm. Thus, it is difficult to determine the precise location of the granules subjected to exocytosis. Our results from secretion-competent, electropermeabilized bovine platelets strongly suggest, however, that secretion-competent dense granules are already attached to the inner surface of the plasma membrane before stimulation.

What kind of intracellular structure is involved in anchoring the release-competent dense granules to the plasma membrane? Possible candidates are the microfilaments. It is well known that secretory granules are generally associated with microfilaments both in vivo (14, 17) and in vitro (8, 9). As shown in Figs. 4 and 5, microfilaments seem to be associated with the dense granules; microfilaments appear to attach to the surface of the granules sideways. The length and orientation of the microfilaments around the granules, however, do not seem to be uniform. Moreover, those microfilaments lack straightness in many cases. Therefore, it is not certain if the microfilaments are linking the dense granules to the plasma membrane. Another candidate is the amorphous structure found between the plasma membrane and the anchored dense granule. These structures are of similar width, and they extend over relatively large areas on the surface of granules in many instances, thereby, forming gaps of relatively constant width. It is of particular interest to know whether these amorphous structures occur only in restricted regions or they are distributed evenly on the entire inner surface of the plasma membrane; we do not know if such fluffy materials coating regions of the plasma membrane other than the gaps as observed in Figs. 2, 4, and 5 are identical to the amorphous structures. At present, the biochemical nature of such amorphous structures is not known.

Maintenance of the dense granules just beneath the plasma membrane may require ATP. It is well-known that the Ca^{2+}-dependent release of serotonin requires millimolar concentrations of Mg-ATP in the permeabilized platelets (13, 20). The presence of millimolar levels of ATP also prevents the spontaneous inactivation of the Ca^{2+}-dependent release of serotonin in electropermeabilized platelets (our unpublished observations). In addition, we observed that the percentage of the total number of dense granules anchored to the plasma membrane decreased to 26% in specimens fixed in the absence of exogenously added ATP (data not shown). At present, it has not been determined whether or not the attachment of dense granules to the plasma membrane is strictly dependent on the presence of ATP, but results of a recent study with permeabilized chromaffin cells also suggest that Mg-ATP is required for maintaining the primed state necessary for exocytosis (11). These results together suggest that ATP may play some role in maintaining the secretion-competent granules in regions close to the plasma membrane.

As a model for the regulation of secretion by Ca^{2+}, it has recently been proposed that the network of microfilaments blocks the movement of secretory granules, thereby, preventing granules from fusing with the plasma membrane (4, 15). Disassembly of the microfilamentous structures by Ca^{2+} facilitates the fusion of membranes. In the present study, no filamentous structures, but rather amorphous structures, were found between the plasma membrane and the anchored dense granules. In addition, stimulation by Ca^{2+} did not significantly alter the organization of microfilaments within the cells, but bridge-like structures connecting granules and the plasma membrane were newly formed after stimulation with Ca^{2+}. Considering the above results, we propose a new model for Ca^{2+}-regulated exocytosis. In this model, secretion-competent granules are anchored to the plasma membrane independently of subsequent stimulation, and the Ca^{2+} signal reorganizes the nonfilamentous cytoskeletal structures between the plasma membrane and the granular membranes to permit the fusion of the two membranes. Such a model seems to provide an explanation for the rapid release of neurotransmitter upon electric stimulation. At the same time, this model accommodates the observations of the exocytotic process obtained by a quick-freezing technique, namely, that there are unfused secretory granules located just beneath the plasma membrane (5, 6). Further biochemical studies on these junctional structures will enable us to clarify the validity of the present model and the exact mechanism of Ca^{2+}-regulated exocytosis.

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