IN SITU OBSERVATION OF DENSE-BODY RELEASE FROM HYDRATED HUMAN PLATELETS

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ABSTRACT Human-platelet dense bodies (secretory granules) have been visualized by electron microscopy in cells maintained in a hydrated state, and their sequential release after stimulation by thrombin has been observed *in situ.* The pattern of dense body release from individual platelets suggests that a portion of the dense body complement of a single cell can be extruded without appreciable change in the position of the remaining dense bodies.

INTRODUCTION

One vital component of the hemostatic process is believed to be the release reaction of platelets. During this process, platelets release to the extracellular medium the contents of dense bodies—electron-dense granules which serve as storage sites for serotonin, pyrophosphate, adenine nucleotides, and calcium (1–3). Our understanding of the process in normal platelets, although greatly expanded in recent years by a variety of elegant morphological and biochemical studies, has been handicapped by our inability to follow at an ultrastructural level the secretory response of an individual cell (4–9). Utilizing the differentially pumped specimen stage developed previously (10, 11), we have made serial studies of human platelets as they release their dense bodies in response to thrombin stimulation.

METHODS

Human platelets were collected in citrate/EDTA and prepared as platelet-rich plasma, or resuspended in the sodium chloride-Tris-citrate buffer utilized previously (8). In some preparations, 0.35% bovine serum albumin (8) was included in the buffer solution. Aliquots of platelet-rich plasma or resuspended platelets were allowed to settle on carbon-coated grids and blotted in a water-saturated atmosphere, leaving platelets (presumably attached to the carbon substrate) covered with a thin film of liquid. Specimens were viewed and photographed at 100 kV in a Siemens 1A electron microscope equipped with an environmental chamber (10) designed to keep the specimen fully hydrated over a temperature range of −10°C to +60°C (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) The electron-beam current density was reduced with a 10-μm condenser aperture, and the beam dose per micrograph was 5×10^{-5} Cs/cm^2 (measured by screen-current collection calibrated with a Faraday cage). Although no details were visible on the microscope screen at this low current density, images could be recorded on an ultrasensitive film (Kodak No-Screen X-ray film, Eastman Kodak Co., Rochester, N. Y.).

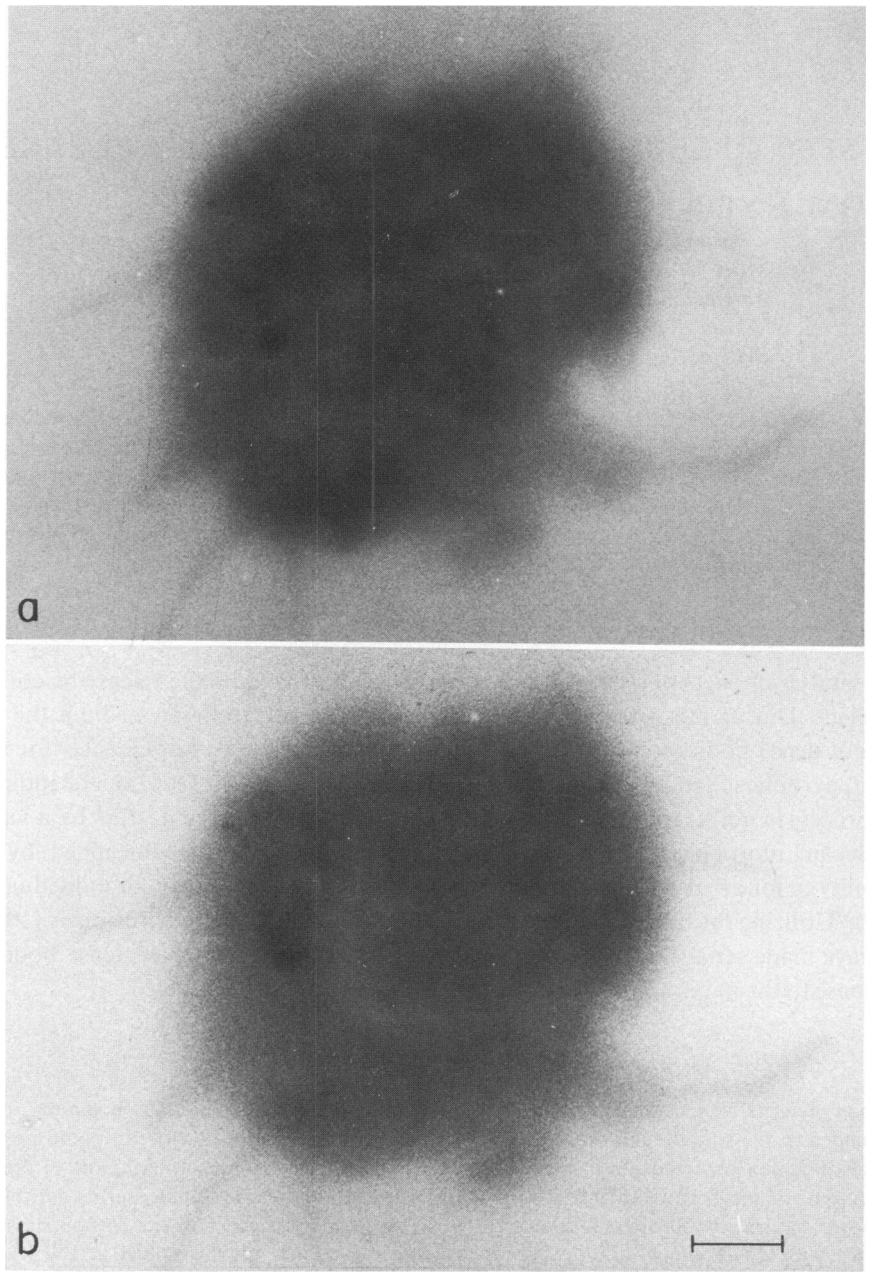

FIGURE 1 Consecutive photographs of a human platelet, taken 50 s apart, showing no detectable movement of the dense bodies (dark circular structures) within the platelet. The platelet is at 25°C and surrounded by an isotonic buffer containing 116 mM NaCl, 4 mM KCl, 1.8 mM KH_2PO_4, 1.1 mM $MgSO_4$, 25 mM Tris, 10 mM sodium citrate, and 5.9 mM dextrose. (bar = 1 μm)

RESULTS

Details of some platelets were obscured by a thick film of water, but dense bodies similar in morphology to those seen in air-dried preparations were clearly visible in a large proportion of the platelets photographed (Fig. 1). Although air-dried platelets can contain more than 20 dense bodies (9), no photographs of hydrated platelets revealed more than 8 distinct dense bodies per platelet. This result may be due to our inability to identify the smallest groups of dense bodies, or possibly because a thick layer of water over certain portions of the hydrated cells obscured a number of dense bodies. Despite this apparent discrepancy, the distribution of unobscured platelets by dense-body content was similar to that noted in air-dried platelets unstimulated by thrombin (Fig. 2*a*, hatched bars). (Platelets obscured by a thick film of water are noted as "not countable" in the figure.) Furthermore, many resuspended platelets examined after treatment for 2 min at 25°C with a saturating concentration of human thrombin (4 U/ml) contained no dense bodies (Fig. 2*a*, lined bars). Other platelets

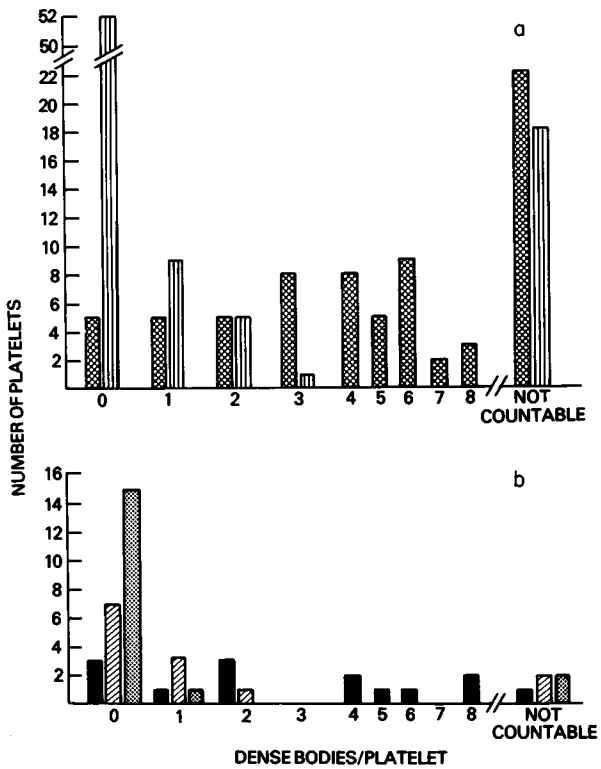

FIGURE 2 (*a*) The distribution of platelets by dense-body content in control (unstimulated) platelet populations (hatched bars), and after treatment at 25°C with 4 U/ml of human thrombin (lined bars). Conditions are as described for Fig. 1. Platelets listed as "not countable" were obscured by a thick film of water. (*b*) Distribution of platelets by dense-body content on a grid treated with thrombin (4 U/ml) at 4°C and photographed at 5°C (solid bars), 15–17° (hatched bars), or 25–27°C (dotted bars).

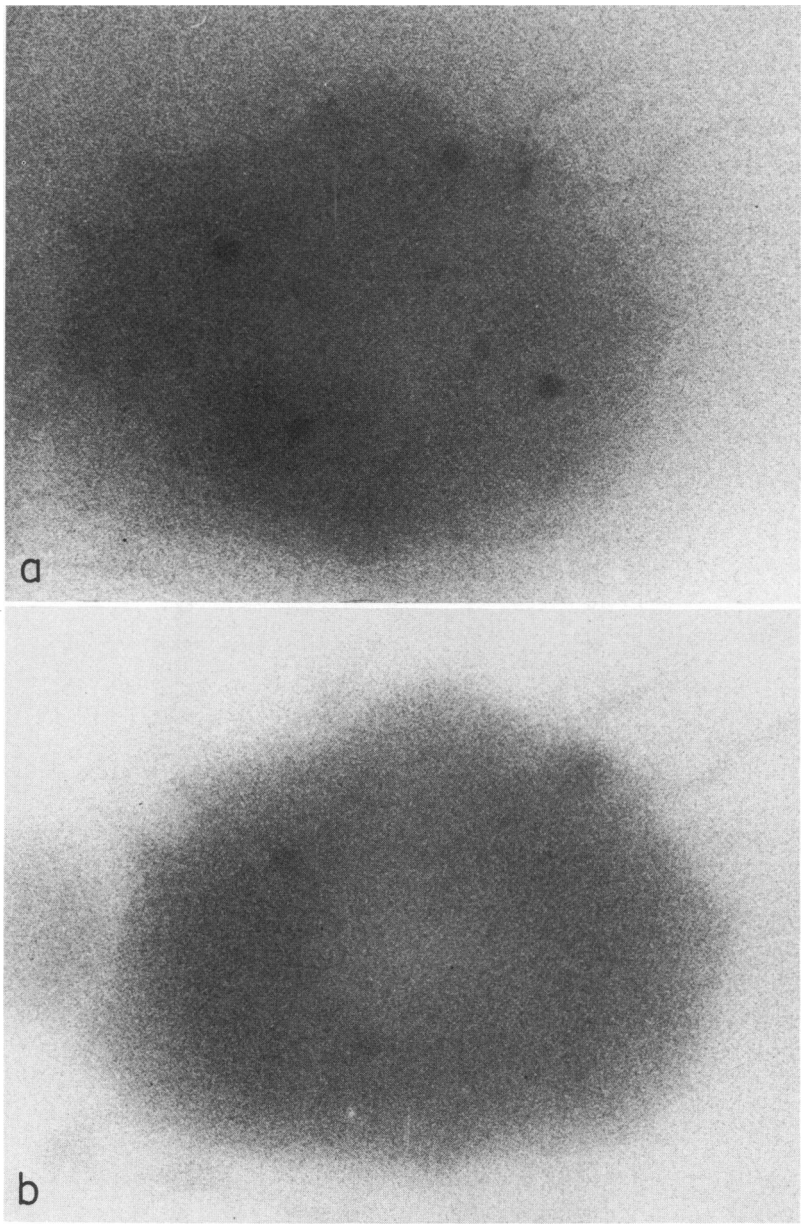

FIGURE 3 Consecutive photographs of a thrombin-treated human platelet taken at 5°C (*a*) and 16°C (*b*). Some of the dense bodies are lost during the warming process; those remaining behind appear not to have changed their location inside the cell. (same magnification as in Fig. 1)

so treated contained a few dense bodies apparently refractory to thrombin, a series of results similar to those noted after thrombin treatment and preparation of air-dried specimens (9). In all specimens examined, no definite motion of the dense bodies was detected in consecutive photographs taken from 5 s to 5 min apart (Fig. 1), although Brownian movement of smaller particles in lipid bilayers has been observed under similar microscope operating conditions (12).

To examine the *in situ* response of the cells to thrombin, we took advantage of the fact that platelets incubated with saturating concentrations of thrombin do not release dense-body constituents at 4°C (5, 13, 14), and show a steady increase in the amount of dense-body constituents released as the temperature is raised from 4°C to 37°C (15). Human thrombin (4 U/ml) was added to resuspended platelets held at 4°C. The thrombin-treated specimen was placed on an ice-cold grid, and excess fluid was removed by blotting under a vapor pressure corresponding to the dew point at 4°C. The specimen was then transferred to the environmental chamber (cooled to 5°C) and photographed at this temperature. After 5 min at 5°C, the specimen was warmed (1°C/min) to 15–17°C, photographed during a 5-min period in this temperature range, then warmed (1°C/min) to 25–27°C, and again photographed over a 5-min period. The electron beam was deflected away from the specimen between micrographs to minimize radiation damage. Unobscured platelets remained unobscured throughout the experiment.

As indicated in Figs. 2 *b* and 3, the thrombin-treated platelets lost progressively more of their dense bodies as the temperature was raised to 27°C. In contrast, unstimulated platelets and platelets incubated with antimycin A (4.1 μg/ml) and 2-deoxyglucose (32.2 mM) for 60 min at 37°C before cooling and thrombin treatment showed no loss of dense bodies during the warming process.

DISCUSSION

Our observation *in situ* of the thrombin-induced loss of platelet dense bodies suggests that, as measured by their capacity to secrete, platelets can survive inside the environmental stage in the microscope column. The thrombin-mediated loss (secretion) of dense bodies, which as studied in air-dried platelets occurs in a dose- and time-dependent fashion (9), appears to be a response particular to living platelets. The release process is arrested by both the air-drying process and by fixation of platelets with glutaraldehyde or formaldehyde (5, 9). In addition, thrombin-induced dense body release as monitored in air-dried cells is negligible from platelets incubated for 60 min at 37°C with the metabolic poisons antimycin A (4.1 μg/ml) and deoxyglucose (32.2 mM) (unpublished studies).

Thus, our data prove that dense bodies similar to those seen in air-dried whole mounts exist in living platelets. In addition, our findings indicate that dense bodies in living platelets attached to a carbon substrate exhibit little horizontal displacement over a 5-min period at 25°C. Futhermore, the release process in a single platelet can proceed in steps as the temperature is raised, verifying our previous speculation that

release from a given platelet might not be all-or-none (9). During this stepwise release, dense bodies not released at a given temperature have not changed position appreciably after release of the remainder of the dense-body complement. This observation suggests that if a platelet "contractile wave" (16) precedes and/or initiates secretion, it is not noticeable at this level before the actual loss of dense bodies from the cell. It also suggests that platelet dense bodies need not all migrate to a specific release site on the platelet membrane to be discharged from the cell.

Although distributions of platelets by dense-body content before and after stimulation have been examined, it has not been possible previously to follow the secretory response of an individual platelet. This technical limitation has greatly complicated the interpretation of the platelet secretory process (9). Utilization of the hydrated specimen stage, which has already permitted the visualization of many interesting biological structures beyond the resolution limit of the light microscope (12, 17), should now permit a more critical examination of the platelet secretory process, and possibly similar release processes in other secretory cells.

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