Rapid Ultrastructural Changes in the Dense Tubular System Following Platelet Activation

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The dense tubular system (DTS) functions to regulate platelet activation by sequestering or releasing calcium, similar to the sarcotubules of skeletal muscle. In resting platelets, the DTS exists as thin elongated membranes. Within 10 seconds of the addition of thrombin, platelets show a major ultrastructural change in their DTS: from the thin elongated form to a rounded vesicular form. These morphologic changes were demonstrated with two different stains and two different fixation methods. Platelets exposed to the calcium ionophore A23187 showed the same ultrastructural changes in the DTS. In contrast, the DTS remains in a thin elongated form when platelets are stimulated by the protein kinase C activators phorbol 12-myristate 13-acetate (PMA) and oleoylacylglycerol (OAG). These morphologic changes may be related to the discharge of calcium from the DTS because this is stimulated by thrombin and A23187, but not by PMA. Preincubation of the platelets with the intracellular calcium chelator 5,5' -dimethyl-bis-[O-aminophenoxy]-ethane-N,N,N',N'-tetra acetic acid (BAPTA) largely prevented both the thrombin-induced rise in intracellular calcium and the changes in DTS morphology, suggesting that the changes in DTS morphology are secondary to the increase in cytosolic calcium. The results provide a morphologic correlate to existing biochemical evidence showing that the DTS is involved early during platelet activation.

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MATERIALS AND METHODS

Collection of blood. Following informed consent, blood was collected by venipuncture from normal donors who had not taken any medication for the previous 10 days. The venous blood was anticoagulated with 1.9 mL acid-citrate-dextrose per 8.1 mL blood.

Preparation of washed platelets. Anticoagulated venous blood was centrifuged at 800g for 5 minutes. The platelet-rich plasma was removed and centrifuged at 800g for 10 minutes. The platelet pellet was gently resuspended in modified Hanks' Balanced Salt Solution (GIBCO, Grand Island, NY) pH 7.4 with 0.1% bovine serum albumin. A platelet count was performed and adjusted to a concentration of 250,000/mL. Ten minutes before activation CaCl₂ was added to 1.0 mL samples to give a final concentration of 1.0 mmol/L.
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Fig 1. Diaminobenzidine staining of the DTS of platelets. (a) The DTS (↑) of the resting platelet is primarily thin and elongated. (b) The DTS (↑) of a platelet activated by thrombin for 10 seconds is rounded and vesicular, as is the DTS in a platelet (c) exposed to thrombin for 120 seconds (↑). Bar = 1 μm.

The washed platelets were stimulated by adding 1 U/mL thrombin (Sigma, St Louis, MO) dissolved in double distilled water, or 40 nmol/L PMA (Sigma), 100 μg/mL OAG (Serday, London, Ontario) or 1.0 μmol/L A23187 (Sigma) each dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was not greater than 0.5%. The reactions were stopped with the appropriate fixative. Where BAPTA was used, it was incubated with the platelets for 15 minutes (as BAPTA-AM) at a concentration of 15 μmol/L. The platelets were then washed and resuspended in Hanks' Balanced Salt Solution as above.

Measurement of intracellular free calcium concentration. Platelet cytosolic free calcium levels were measured using fura-2. Platelet-rich plasma was incubated with 3 μmol/L Fura 2 AM at 37°C for 45 minutes, centrifuged at 800g for 10 minutes, and resuspended in physiologic saline (145 mmol/L NaCl; 5 mmol/L KCl; 1 mmol/L MgCl₂; 10 mmol/L HEPES; 10 mmol/L dextrose, pH 7.4). Aliquots (3 mL; 5 × 10⁷ platelets/mL) were dispensed into quartz cuvettes, 1 mmol/L CaCl₂ added and, while the sample was stirred, the fluorescence was monitored continuously at 339 excitation and 500 nm emission wavelengths in a Shimadzu-5000 spectrofluorometer (Tekscience, Oakville, Ontario, Canada). Cytosolic free calcium levels were quantified by the method of Pollock et al.22

Glucose-6-phosphatase stain. A modified method of Nichols et al18 was used to stain the DTS. Because cold temperature disrupts the microtubular system,23 the platelets were kept at room temperature during the preparation of the washed platelets and fixation. The activated platelets were fixed by adding equal volumes of 1.5% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer (pH 7.4) with 1% sucrose for 10 minutes. The platelets were then washed with 0.2 mol/L cacodylate buffer pH 7.4 and incubated in the glucose-6-phosphatase stain at 30°C for 3 hours. After staining, the platelets were further fixed in 1% osmium tetroxide in 0.2 mol/L sodium cacodylate for one hour at 4°C followed by dehydration and embedding in epon/araldite for electron microscope evaluation (Phillips EM400, Eindhoven, The Netherlands).24

Endogenous peroxidase stain. A modified method of White,1 Graham and Karnovsky,19 and Breton-Gorius and Guichard20 was used to stain for endogenous peroxidase within the DTS. The reported variability to show the endogenous peroxidase of platelets has been attributed to inhibition of the peroxidase by glutaralde-
Therefore, only a short fixation with 0.1% glutaraldehyde was used. The activation of the platelet samples was stopped by adding an equal volume of 0.1% glutaraldehyde, 1% sucrose in 0.1 mol/L cacodylate buffer, pH 7.2, for 15 minutes. The platelet samples were further fixed in 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, pH 7.2, for 30 minutes. The platelets were then incubated in the medium of Graham and Karnovsky,19 25 mg of DAB (diaminobenzadine) in 10 mL of 0.05 mol/L tris-HCl buffer, pH 7.2, to which 0.20 mL of freshly prepared 3% H2O2 and 0.10 mL DMSO had been added, for 3 hours at room temperature. The platelets were further fixed with 8% paraformaldehyde in 0.1 mol/L cacodylate, pH 7.2, for 30 minutes at room temperature, followed by 1% osmium tetroxide for 1 hour at 4°C. The samples were then dehydrated in a series of ethanol concentrations and embedded in spur.

On electron microscopic examination of the cut sections, platelets were randomly selected and photographed at ~5,800 magnification. Using a 1 mm grid, the morphology of the DTS was quantified by counting the number of intersects that overlaid the stained DTS and assigning the structure observed to one of three categories of morphology: narrowly elongated (E), rounded vesicular (R), or indeterminate (I). At least 15 platelets were analyzed per condition with this method.

Statistical analysis was performed using the SAS statistical package general linear models procedure for analysis of variance of samples with repeated measures.

RESULTS

The quiescent platelet is a discoid shaped anucleate cell. The endogenous peroxidase stain showed that the ultrastructural morphology of the DTS was predominately thin and elongated in the resting platelet (Fig 1a). Following addition of thrombin, the platelets extended pseudopods and centralized their granules. Pseudopod extension was evident at 15 seconds and fully developed by 60 seconds. Granule centralization began at 10 seconds and was complete by 30 seconds. As early as 10 seconds after thrombin stimulation, the DTS became a predominantly rounded vesicular structure (Fig 1b and c). Similarly, with the glucose-6-phosphatase stain resting platelets showed a predominantly thin, elongated DTS (Fig 2a), whereas thrombin-activated platelets contained a predominantly rounded DTS (Fig 2b). The size of the vesicles was somewhat smaller with the glucose-6-phosphatase stain than with the peroxidase stain.
Fig 5. DTS in platelets incubated with 100 μg/mL OAG. The glucose-6-phosphatase stain was used. Although the platelets show granule membrane fusion, vesicle formation, and pseudopod extension, the DTS remains thin and elongated. The platelet in this figure shows the elongated DTS (†) interweaving among the granules and vesicles (V). Bar = 1 μm.

Quantitatively, in the unstimulated platelet 68% ± 3% of the DTS is in a thin elongated form (Fig 3). At 10 seconds after thrombin stimulation, this thin elongated form of the DTS decreased to 18% ± 6%, and the rounded vesicular form increased from a relatively small proportion up to 64% ± 7% (P < .0001). At 120 seconds, 87% ± 6% of the DTS within platelets were in the rounded vesicular form.

Stimulation of platelets with 40 nmol/L PMA induced swelling of the platelet granules and membrane fusion, as previously described by Estensen and White. The DTS remained predominantly a compressed thin elongated structure at 1, 3, and 5 minutes after stimulation (Fig 4). In control samples, 0.5% DMSO did not induce swelling, fusion of the granules, or ultrastructural changes in the DTS. The addition of 100 μg/mL OAG to platelets, as noted previously, was associated with changes very similar to those seen with PMA. As with PMA, the DTS remained thin and elongated after OAG (Fig 5).

The calcium ionophore A23187 induced ultrastructural changes identical to those seen with thrombin, including extension of pseudopods and granule centralization. The DTS stained with the glucose-6-phosphatase method, also became rounded and vesicular 2 minutes after stimulation (Fig 6). As with thrombin, the rounded structures were not as large with the glucose-6-phosphatase as with the endogenous peroxidase stain.

Platelets were incubated with BAPTA-AM to further evaluate the relation between changes in intracellular calcium and changes in DTS. BAPTA-AM is taken up by platelets and then hydrolyzed intracellularly to give the active calcium chelator BAPTA. Resting intracellular calcium in the platelets was 105 nmol/L. At 10 seconds after thrombin the intracellular calcium was 666 nmol/L. In samples pretreated with BAPTA before thrombin, the level of intracellular free calcium 10 seconds after thrombin addition was 131 nmol/L. At the same time, platelets incubated with BAPTA and then thrombin showed a predominantly thin and elongated DTS (Fig 7).

DISCUSSION

The platelet DTS is a smooth endoplasmic reticulum that has been shown to be the major site of synthesis of prostaglandin endoperoxides and thromboxane A2, and the critical site of sequestration of Ca2+. Calcium is released from the DTS following stimulation with agonists such as thrombin. The calcium release is mediated at least partly by inositol triphosphate, which serves to stimulate a variety of processes associated with platelet activation. Thus, the DTS plays a crucial role in platelet activation.

Our results suggest that DTS is involved in the early activation of platelets. Major morphologic changes in the DTS occurred within 10 seconds of stimulation with thrombin. The DTS changed from a thin elongated structure to a
The platelets remained discoid in the presence of the Intracellular calcium chelator BAPTA and the DTS remained thin and elongated. Their studies showed three morphologic types of platelets: "reticular" cells with the polygonal dense tubular network; dark metallophilic cells; pale metallophilic cells with microvesicles. On stimulation with thrombin, the number of "reticular" cells decreased and the number of pale metallophilic cells increased. Interestingly, similar changes in the morphology of the smooth endoplasmic reticulum (SER) of axons has been reported. The SER changes from a thin elongated structure to a vesicular one with the formation of synaptic vesicles during fast axonal transport. Therefore, the phenomenon reported in this report may represent a general structural change that occurs following the activation of nonmuscle cells. Whether the morphologic change has functional significance for the DTS or whether it is solely a morphologic marker of DTS or endoplasmic reticulum activation is unknown.

We explored the mechanism of the change in the ultrastructure of the DTS with PMA, OAG, and A23187. Phorbol esters such as PMA stimulate platelets by activating protein kinase C\(^{31}\) but without elevating cytosolic Ca\(^{2+}\),\(^6\) or elevating it to a much lesser extent than thrombin.\(^{16}\) PMA and OAG have been shown at an ultrastructural level to induce swelling of platelet granules and fusion of the granule membranes with membranes of the surface connected canalicular system.\(^{14,24,26,27}\) In our present study, we observed the previously reported effects of PMA and OAG. However, the DTS remained predominantly a compressed elongated structure, even at 5 minutes after PMA stimulation. Clearly the changes in the DTS morphology are not attributable to protein kinase C stimulation.

The ionophore A23187 transports calcium across cellular membranes and releases calcium ions from the DTS, inducing the same ultrastructural changes as those produced by thrombin.\(^{15,32}\) In our study we found that stimulation of platelets with A23187 changes the ultrastructure of the DTS from a thin elongated form to a rounded vesicular form, as was the case with thrombin. We speculate that the change in the DTS from an elongated to a rounded form is caused by the release of Ca\(^{2+}\) from the DTS. Thrombin and A23187 discharge calcium from the DTS, but PMA and OAG do not. Indeed PMA can promote sequestration of calcium in the DTS and prevent release of calcium from the DTS.\(^{17,33}\)

One possibility is that the calcium found within the DTS serves as a cross-bridge to link proteins and phospholipids on opposing bilayer membranes. When the calcium is released, the DTS changes from a compressed elongated structure to a vesicular one. Alternatively, Rittenhouse-Simmons\(^{34}\) has observed phospholipid hydrolysis in A23187 stimulated platelets by activation of phospholipase A\(_2\), an enzyme that Legarde et al\(^{35}\) have localized to the DTS. Thus, the effect that we see may result from the activation of a phospholipase leading to disruption of the phospholipids within the bilayer producing the change in the DTS ultrastructure.

To better understand which mechanism was operative, platelets containing the intracellular chelator BAPTA were studied. BAPTA should not prevent the discharge of calcium from the DTS, but should, through chelation, block the effects of released calcium to promote subsequent processes such as cytoskeletal assembly and phospholipase activation. Inhibition of the rise in intracellular calcium by BAPTA did block the change in DTS morphology. The result suggests that the change in the DTS, from a thin and elongated form to a rounded vesicular form, is secondary to an increase in cytoplasmic calcium, perhaps, as discussed above, because of activation of phospholipases and changes in the phospholipid structure of the DTS.

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