Ultrastructural Changes in Human Platelets During Arachidonic Acid-induced Aggregation

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This study was undertaken to visualize the sequence of ultrastructural changes occurring in human platelets during aggregation induced by arachidonic acid. Aggregometer recordings were obtained up to the time of adding glutaraldehyde to begin fixation of platelets for electron microscopy. A correlation was shown between the degree of aggregation and changes in the fine structure of human platelets which had been allowed to aggregate for 15, 30, 90, or 180 sec. The pattern of ultrastructural changes found in the platelets aggregating in response to arachidonic acid was similar to that reported previously for other aggregating agents such as collagen or ADP. Amorphous material was observed within the dilated canalicular system of platelets, supporting the thesis that its channels serve as a pathway for the products released during aggregation. The dilatation of the canalicular system of platelets induced by arachidonic acid could be mediated by an intermediate in prostaglandin synthesis.

HUMAN BLOOD PLATELETS synthesize prostaglandins E_2 and $F_{2\alpha}$ during blood clotting[1] and during platelet aggregation in response to aggregating agents such as collagen and epinephrine.[2] Arachidonic acid, an essential fatty acid and the ubiquitous precursor of these prostaglandins, induces platelet aggregation and prostaglandin formation in vitro[3,4] and causes sudden death with platelet microthrombi in the lungs of rabbits after an injection into the ear vein.[5,6] Furthermore, it has been shown that 30 sec after the addition of sodium arachidonate to platelet-rich plasma an intermediate in prostaglandin synthesis is present.[7] The intermediate was also detected at the onset of platelet aggregation induced by collagen. This intermediate is probably one of the two recently isolated from seminal vesicles.[8] It was observed that these compounds could induce platelet aggregation.

The formation of the intermediate, postaglandin synthesis, platelet aggregation, and death of arachidonic acid-treated rabbits may all be inhibited or abolished by aspirin. Aspirin is also known to prolong the bleeding time of humans.[9] These findings suggest that arachidonic acid plays a significant role in hemostasis and thrombosis. Therefore, it was considered important to examine the ultrastructural changes in human platelets undergoing aggregation in response to arachidonic acid and to compare such data with those already published for other aggregating agents.

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MATERIALS AND METHODS

Platelet-rich Plasma (PRP) and Platelet-poor Plasma (PPP)

Blood was withdrawn from the antecubital veins of healthy, fasting donors. Nine volumes were mixed with one volume of 3.8% trisodium citrate, centrifuged at 250 *g* for 15 min at room temperature (20-22°C), and the supernatant PRP was removed and held at room temperature until used. Blood and PRP contacted only siliconized surfaces. PPP was prepared by sedimenting the cells in PRP by centrifugation (900 *g*, 25 min) and removing the clear supernatant fluid.

Experimental Procedure

Arachidonic acid (> 99% pure, Hormel Institute, Austin, Minn. or Nuchek, Elysian, Minn.) was deposited on the bottom of a small test tube and dispersed under nitrogen in PPP to give a final concentration of 50 m*M*. This was freshly prepared just before each experiment.

Experiments were performed in siliconized aggregometer tubes. PRP was stirred at 37°C using an aggregometer (Chronolog Corp., Broomall, Pa.) so that continuous recordings of changes in light transmittance could be observed for 180 sec. Thirty microliters of 50 m*M* archidonic acid in PPP were injected into 1.5 ml of stirred PRP. Fifteen, thirty, ninety, or one hundred eighty seconds later, stirring was stopped, and 1.5 ml of 0.1% glutaraldehyde solution buffered at pH 7.4 with 0.15 *M* $Na_2HPO_4 \cdot NaH_2PO_4 \cdot H_2O$ was added to begin fixation for electron microscopy. Recordings of platelet aggregation for three of these time periods (30, 90, 180 sec) are presented alongside the corresponding electron micrographs in the results. Controls included 1.5 ml PRP to which had been added 30 μl PPP with no stirring and 1.5 ml PRP plus 30 μl PPP with stirring for 180 sec. All experiments were run in duplicate.

Preparation for Electron Microscopy

After stirring was stopped and fixation begun by adding the 0.1% buffered glutaraldehyde solution, the contents of the aggregometer tubes were poured into larger tubes and centrifuged at room temperature for 5 min at 900 *g*. The supernatant solution was discarded, and the sediment was overlayed with 3% glutaraldehyde solution (pH 7.4, buffered as above). After 15 min the platelets were carefully transferred to 1 = ml amounts of the buffered 3% glutaraldehyde solution in the cups of plastic trays (Polysciences, Inc., Warrington, Pa.), and they remained there for an additional 15 min. The pellets of glutaraldehyde-fixed platelets were than sliced into small pieces and postfixed for 90 min at 4°C in 1% osmium tetroxide buffered at pH 7.3 with the 0.15 *M* phosphate buffer. The blocks were washed for 30 min at 4°C in 0.9% saline and stained for 1 hr in 0.5% magnesium uranyl acetate in 0.9% saline at 4°C with shaking. The specimens were dehydrated in ethanol and embedded according to Spurr[10] in a low-viscosity epoxy resin (standard medium) using a cure period of 13 hr at 70°C. The LKB Ultrotome III was used to obtain sections of the plastic-embedded platelets. The thin sections on naked copper grids (300 mesh) were stained with lead citrate for 6 min.[11] The sections were examined with a Siemens Elmiskop I electron microscope at 80 kV with a pointed filament, double condenser containing a 200-μ aperture and a 30-μ silver objective aperture at magnifications of 3000-25,000.

RESULTS

Controls

The controls for the ultrastructural studies were samples from 1.5-ml amounts of PRP to which microliter amounts of PPP had been added and then fixed immediately (Fig. 1) or fixed after stirring in an aggregometer tube for 180 sec. The flattened discoid form of unaltered or circulating platelets, as previously described,[9] is apparent. They show few pseudopods. Perhaps those that had been stirred showed a few more in some micrographs, but in all other respects they resembled the unstirred controls.

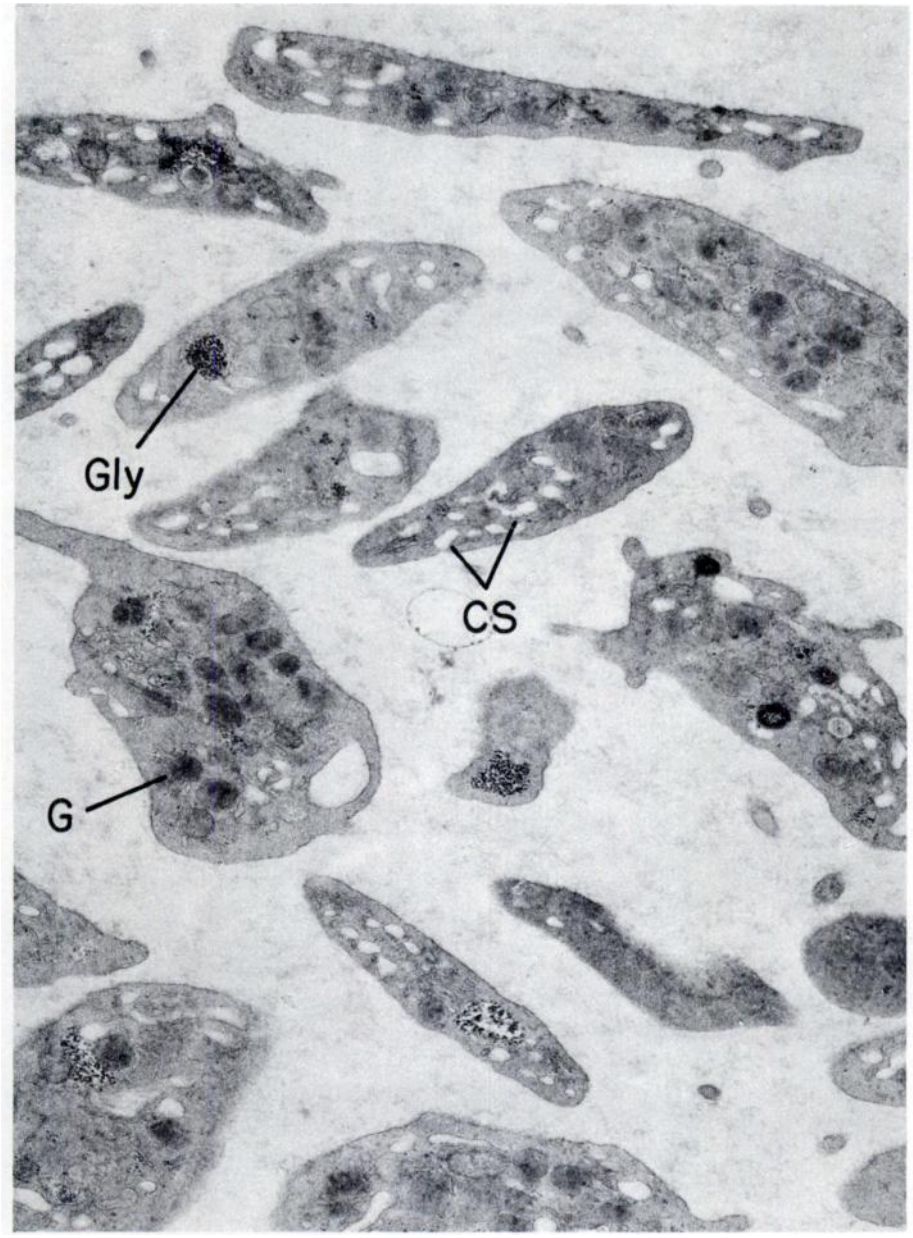

Fig. 1. Platelets from a sample of PRP to which microliter amounts of PPP have been added and fixed immediately. The cells exhibit few pseudopods. Large granules (G), elements of the canalicular system (CS), and presumptive glycogen granules (Gly) are dispersed randomly in the dense cell matrix (× 18,000).

Ultrastructural Changes During the Course of Arachidonic Acid-Induced Platelet Aggregation

Fifteen seconds after addition of arachidonic acid. At this time the platelets showed more pseudopods and fewer disc-shaped forms (Fig. 2). Platelet organelles were randomly distributed. The open-channel system appeared somewhat dilated in most cells.

Thirty seconds. It became evident that platelets were beginning to approach one another, and occasional aggregates consisting of two or more platelets were seen (Fig. 3A). Organelles in some platelets appeared to have centralized with concomitant dilatation of the canalicular system. At this time recordings of light transmittance indicated that platelet aggregation was beginning (Fig. 3B). It is to be recalled that significant amounts of an intermediate in prostaglandin synthesis have been detected at 30 sec under similar conditions.[7]

Ninety seconds. The recording at 90 sec (Fig. 4B) indicated that many platelets had aggregated. This correlated with electron micrographs which showed considerable platelet aggregation (Fig. 4A), although some platelets were not aggregated. The ultrastructural features seen in the aggregated platelets at this time included further centralization of organelles and some breakdown of granules and swelling of pseudopods.

One hundred eighty seconds. Finally, at 180 sec, when the light transmittance recording indicated that aggregation was maximal (Fig. 5B), aggregation of platelets was seen in the electron micrographs (Fig. 5A). Amorphous material, which was not present at earlier times, was now seen within portions of the open canalicular system (Fig. 6).

DISCUSSION

The sequential alterations in cell fine structure accompanying human platelet aggregation induced in vitro by arachidonic acid include the following: (1) a conversion from a discoid shape with a smooth contour to one that is irregularly spherical, (2) the development of many pseudopods, (3) adhesion of some platelets to each other and movement of organelles centrally (the degree of internal reorganization and shape changes varies among the cells in any one sample), (4) fusion of granules and dilation of the canalicular system, and (5) interdigitation of swollen platelet pseudopods within the mass of the aggregate. This pattern of ultrastructural changes is similar to those induced by ADP, collagen, epinephrine, or thrombin (reviewed in reference 12) and, like prostaglandin synthesis, appears to be a characteristic reaction of platelets to aggregating agents.

The present study also shows the expected correlation between increases in light transmittance and the number of platelets seen adhering to each other in electron micrographs.

During the process of aggregation, blood platelets release a number of intragranular substances, such as ADP, serotonin, and hydrolytic enzymes, into the plasma. The morphologic expression of the complete sequence of events accompanying secretion of such products has been elusive. Early in the process of aggregation the randomly dispersed granules become concentrated centrally and fuse, an event easily visualized in electron micrographs. However, the

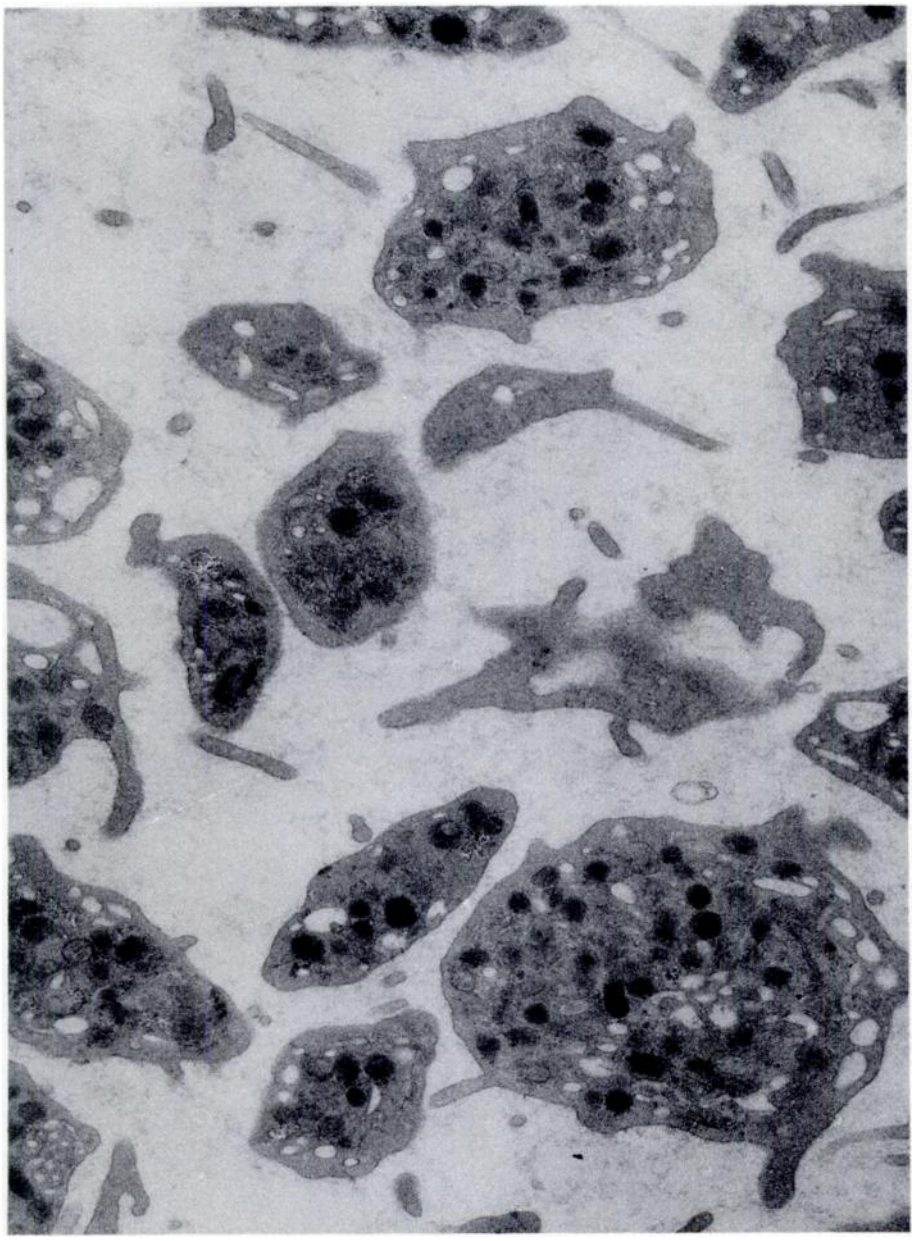

Fig. 2. Platelets fixed 15 sec after the addition of arachidonic acid. Organelles appear randomly distributed for the most part. Platelets exhibit a greater number of pseudopods compared with control preparations without the arachidonic acid. An occasional discoid form is seen (× 18,000).

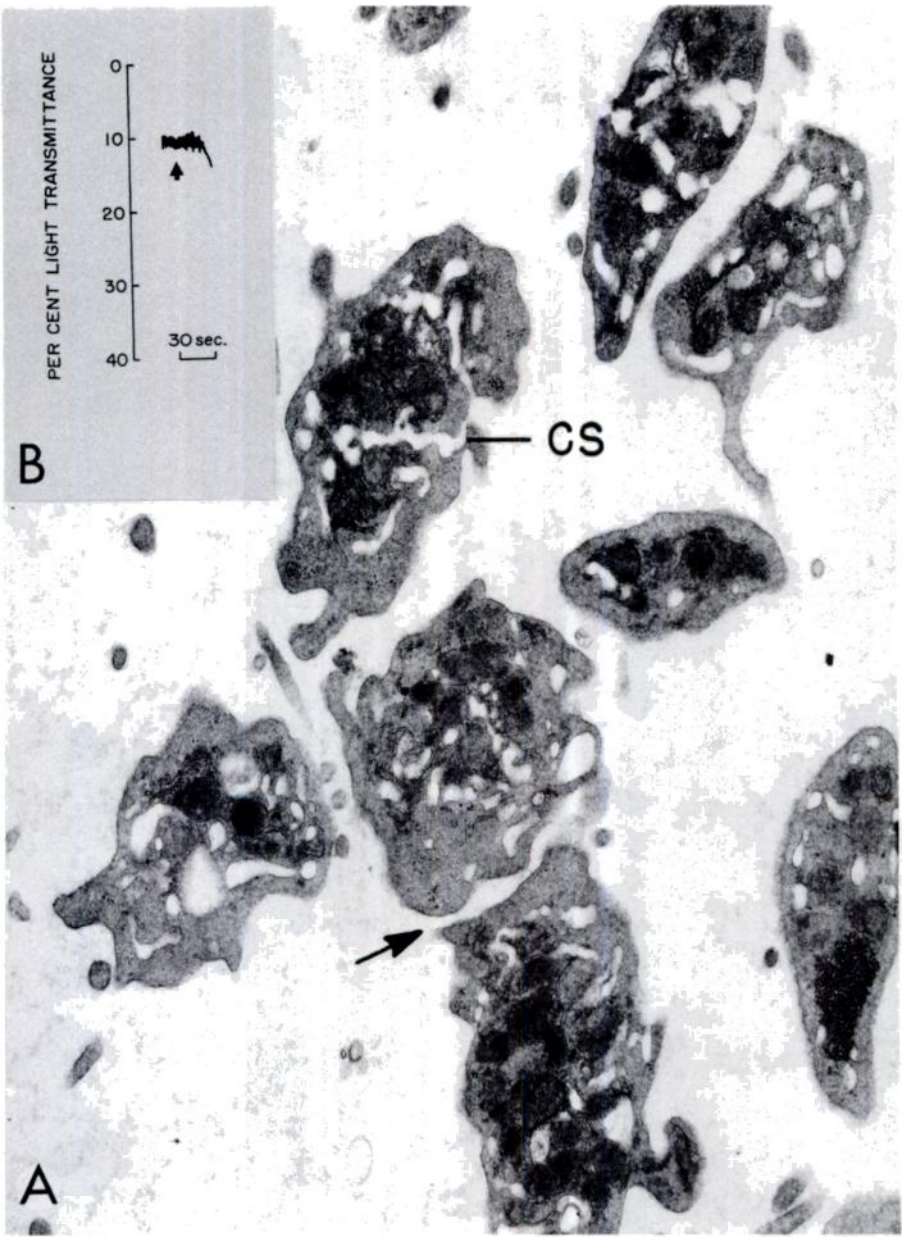

Fig. 3. (A) Platelets fixed 30 sec after exposure to arachidonic acid showing aggregation of a pair of platelets (arrow) (× 18,000). The canalicular system (CS) appears dilated, and organelles have moved toward the center of the cells. (B) Aggregation recording made during this experiment. Note aggregation beginning. Arachidonic acid dispersed in PPP was added (arrow) to PRP to give a final concentration of 1 mM. After stirring for 30 sec glutaraldehyde was added, and platelets were prepared for electron microscopy.

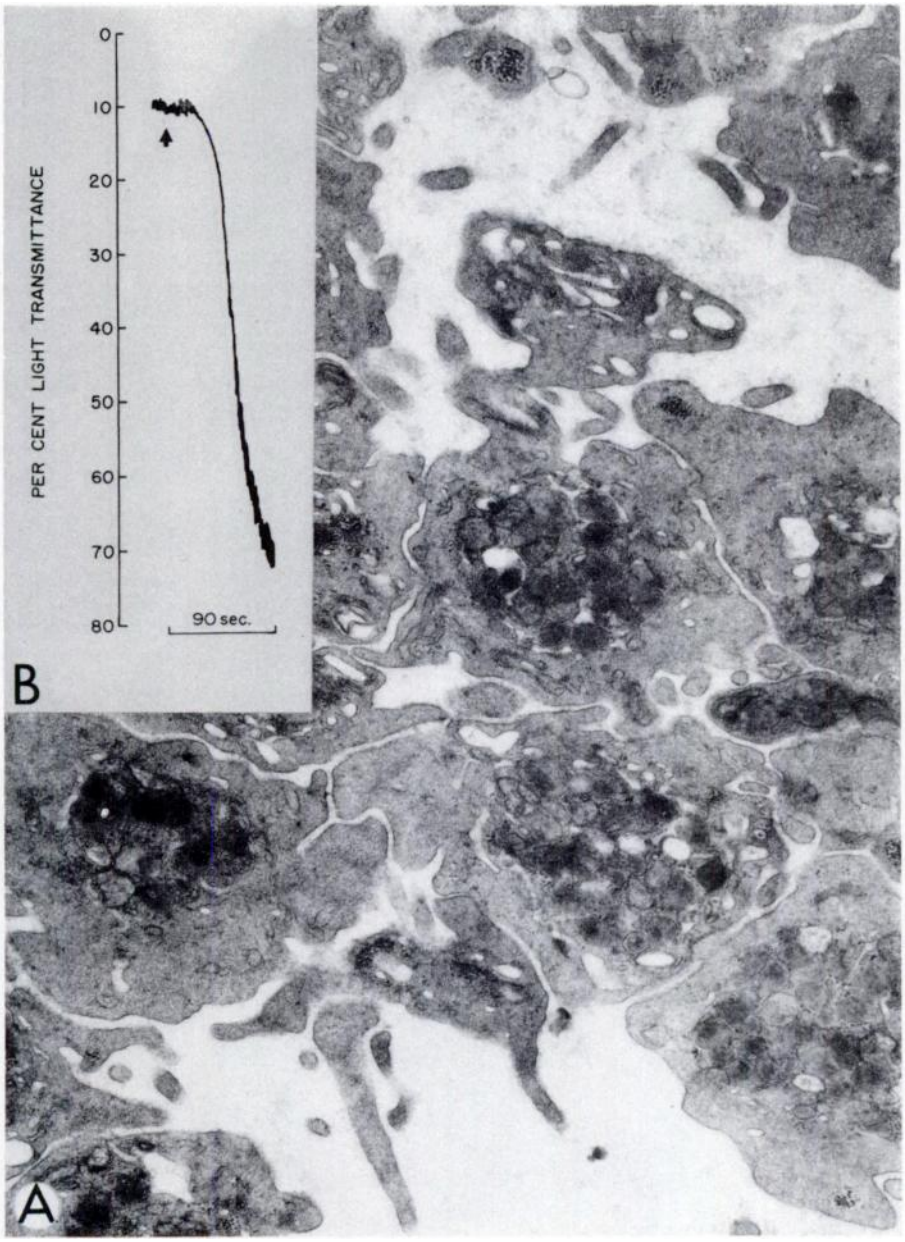

Fig. 4. (A) Platelets from PRP fixed after 90-sec treatment with 1 mM arachidonic acid in PPP stirred at 37°C. An aggregate of a number of cells is seen in the micrograph. The granules in many cells appear concentrated centrally. A loss of contour or a shape change is obvious in the platelets composing the aggregate, including sections through swollen pseudopods (× 18,000). (B) Aggregometer recording made during this experiment. Note that considerable aggregation has taken place. Experimental details similar to those of Fig. 3B except glutaraldehyde added at 90 sec.

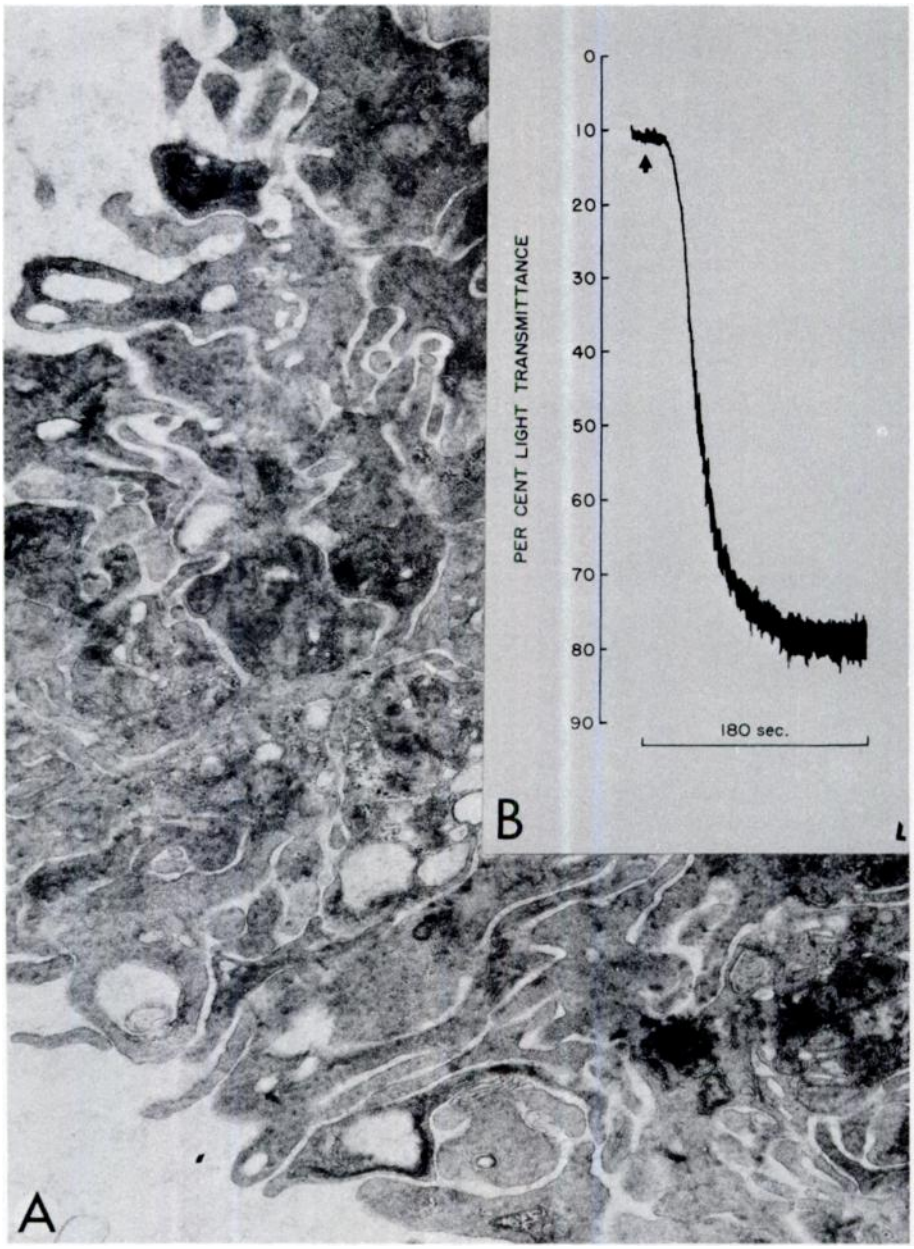

Fig. 5. (A) Platelets from PRP fixed after 180-sec exposure to 1 mM arachidonic acid in PPP stirred at 37°C. Swollen pseudopods are observed at the margins of the aggregate, while those pseudopods within the cell mass are interdigitated and closely approximated to one another. In a number of cells the granules appear to have fused together (× 18,000). (B) Aggregometer recording made during this experiment. Details similar to those of Fig. 3B except glutaraldehyde added at 180 sec. Note maximal aggregation at 180 sec.

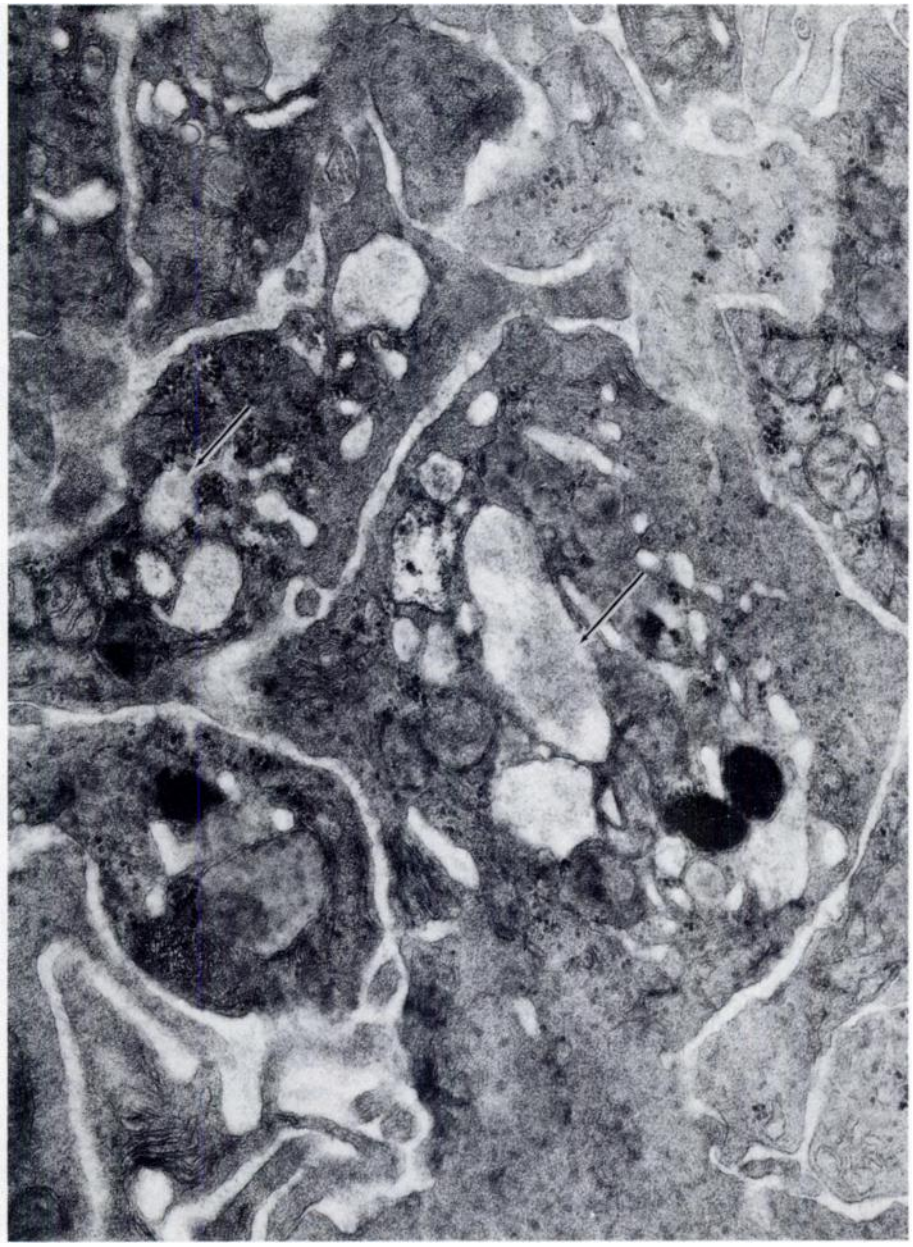

Fig. 6. Platelets from a sample of PRP fixed after a 180-sec treatment with 1 mM arachidonic acid in PPP stirred at 37°C. See Fig. 5B for aggregometer data showing maximal aggregation immediately before fixation of sample. Amorphous material is seen within the dilated canalicular system (↑). This material is not found in control preparations incubated for 180 sec without the addition of arachidonic acid (× 25,000).

actual release of substances into the open canalicular channels has not been observed consistently. Perhaps this is because secreted substances dissolve in the plasma within channels[13] and may be washed away during the preparation procedures. Cationic polypeptides have been used to follow the secretion process by platelets during aggregation, providing evidence that granules are released into the open canalicular system.[13] Under such circumstances the positively charged polypeptides apparently stabilized the granules so that they were extruded intact without dissolution in the plasma. The electron micrographs in the present investigation show amorphous material, presumably released from granules, within the open canalicular system. This supports the thesis that this system acts as a pathway for secretory products released by platelets.

It is noteworthy that dilatation of the open canalicular system begins about 30 sec after the addition of arachidonic acid to stirred platelet-rich plasma. Under similar conditions significant amounts of an intermediate in prostaglandin symthesis were found at this time.[7] The end products of prostaglandin synthesis from arachidonic acid, prostaglandins E_2 and $F_{2\alpha}$, do not induce platelet aggregation.[14] These findings suggest that this intermediate could cause dilatation of the open canalicular system and thus promote the release reaction in platelets. Since the intermediate is also detected during collagen- and epinephrine-induced aggregation,[7] it is possible that the function of the intermediate is dilatation of the canalicular system, regardless of the aggregating agent.

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