The Effects of 1-Oleoyl-2-acetylglycerol on Platelet Protein Phosphorylation and Platelet Ultrastructure

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1-oleoyl-2-acetylglycerol (OAG), an activator of protein kinase C and a synthetic diglyceride, was used in an investigation of the role of diglycerides in platelet stimulus-activation coupling. OAG (20–100 μg/ml) added to platelets resulted in rapid phosphorylation of the 47,000-dalton protein as well as a gradual dose dependent disappearance of alpha granules and dense bodies and the appearance of vacuolar structures containing remnants of granule matrix material. These morphologic changes occurred more slowly than the phosphorylation of 47K, which suggests that if these are related the phosphorylated 47K serves to activate some other mechanism, which is ultimately responsible for the changes observed. These results are most consistent with the role for the phosphorylation of 47K to promote granule labilization. Myosin light chain (MLC) phosphorylation also occurred. An absence of granule centralization suggests that MLC phosphorylation by protein kinase C may not trigger effective actin–myosin contraction. (Am J Pathol 1985, 121:79–87)

STUDIES of the formation of diglycerides as possible intracellular messengers in platelet stimulus-activation coupling are part of a growing interest in the role played by membrane phospholipid turnover in receptor-linked activation of platelets. Thrombin-stimulated diglyceride production is currently believed to result largely from the breakdown of the polyphosphoinositides phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate by phospholipase C.[1,2] Investigation of the functions of diglycerides in intact platelets has been difficult because most diglycerides do not easily penetrate the cell membrane. However, in cell-free assay systems, unsaturated diglycerides have been shown to activate an intracellular protein kinase C to phosphorylate a 47,000-dalton protein (47K).[3,5] Furthermore, a synthetic diglyceride which can penetrate the platelet membrane, 1-oleoyl-2-acetylglycerol (OAG), has been shown to cause aggregation of platelets and secretion of their granule contents in association with such phosphorylation.[6,7] The mechanism by which phosphorylation of 47K might influence granule secretion and aggregation is unknown. In this study we have investigated the role of diglycerides in platelets by evaluating the morphologic effect of OAG on platelets and have correlated the ultrastructural changes with changes in protein phosphorylation.

Materials and Methods

Preparation and Labeling of Platelets

Whole blood was collected, after informed consent was obtained, into citrate anticoagulant (final concentration: 0.7 mM citric acid, 9.3 mM sodium citrate, 13.6 mM dextrose; pH 6.5) and centrifuged at 200*g* for 20 minutes at room temperature. Platelet-rich plasma was collected, and an equal volume of citrate anticoagulant was added. The suspension was held on ice for 5 minutes, then centrifuged at 1000*g* for 10 minutes at 4 C. The platelet pellet was resuspended at 4×10^8 platelets/ml in Tangen HEPES buffer (147 mM NaCl, 5 mM KCl, 0.05 mM $CaCl_2$, 0.1 mM $MgCl_2$, 515 mM dextrose, 5 mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid], 1 mg/ml bovine serum albumin (BSA); pH 7.4). For protein phosphorylation studies, 1.0 mCi/ml phosphorus-32 (as orthophosphoric

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acid in HCl-free aqueous solution, carrier-free; New England Nuclear) was included in the final resuspension volume. Labeled platelets were incubated at 37 C for 1 hour, unlabeled platelets for 10 minutes, then centrifuged at 1000*g* at 4 C for 10 minutes. The supernatants were discarded and the pellets resuspended in the same final volume of modified Hanks' balanced salt solution (HBSS; GIBCO; final concentrations: 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM dextrose, 0.5 mM $MgCl_3$, 3.4 mM $NaHCO_2$, 1 mg/ml BSA; pH 7.4).

Protein Phosphorylation Studies

Samples of labeled platelets were held in siliconized glass aggregometer cuvettes containing a siliconized metal stir bar, in a water bath at 37 C. $CaCl_2$ at a final concentration of 2.5 mM was added to each sample and incubated a minimum of 10 minutes. Cuvettes were placed in an aggregometer at 37 C, stirring at 800 rpm. OAG (1-oleoyl-2-acetylglycerol), a gift from Dr. K. Kaibuchi, Kobe University School of Medicine, Kobe, Japan, was dissolved by sonication in 20 mM Tris HCl, pH 7.4, and added at final concentrations of 20 μg/ml, 50 μg/ml, and 100 μg/ml. In addition, phosphorylation was evaluated on a sample incubated with 1 IU/ml bovine thrombin (Sigma). An aliquot was removed from each cuvette before the addition of OAG (or 20 mM Tris HCl for control) and at 30 seconds, 2 minutes, and 5 minutes after the addition of OAG, and immediately added to 0.5 volume of 3× concentrated denaturing solution (6% sodium dodecyl sulfate (SDS), 6% 2-mercaptoethanol, 30% glycerol, 3 mM ethylenediaminetetraacetic acid (EDTA), 12 mM ethylenebis(oxyethylene-nitrile) tetraacetic acid (EGTA), 0.03% bromophenol blue, 450 mM Tris, pH 6.8) and held at 100 C for 3 minutes. Samples were then frozen at −70 C until electrophoresis.

Proteins in the samples were separated on a 5–18% gradient polyacrylamide–SDS slab gel by a modification of the method of Laemmli.[8] The gel was fixed and stained at 37 C in 25% 2-propanol, 10% acetic acid, 0.05% Coomassie brillant blue R, and destained at 37 C in 10% methanol, 10% acetic acid. The destained gel was transferred to 50% methanol, 5% acetic acid, 1% glycerol at 37 C for a minimum of 14 hours, then dried under vacuum. The dried gel was exposed to Kodak BB-1 X-ray film; phosphorylated protein bands, as identified from the autoradiogram, were scanned for optical density, and peak areas determined, with the use of a Beckman DU-8 spectrophotometer (Gelscan module).

Aggregation and Ultrastructural Studies

Samples of unlabeled platelets were placed in siliconized aggregometer cuvettes containing siliconized metal stir bars and incubated with 2.5 mM $CaCl_2$ for a minimum of 10 minutes at 37 C, then placed in the aggregometer and stimulated with bovine thrombin (1 IU/ml), with OAG at 20, 50, and 100 μg/ml or with 100 μg/ml OAG plus 20 mM EDTA. Samples were fixed in suspension for electron-microscopic study after 30 seconds, 2 minutes, and 5 minutes by addition of an equal volume of 0.1% glutaraldehyde in White's Saline. After 10 minutes at room temperature the suspensions were centrifuged at 1000*g*, the supernatants were removed, and fresh 3% glutaraldehyde in White's Saline was gently layered on top of the pellets. After a minimum of 18 hours the glutaraldehyde was removed, and the pellets were refixed and stained for 1½ hours at 4 C in 1% osmium tetroxide solution (1% OsO_4, 5 mM $CaCl_2$, 1.5% potassium ferrocyanide). Pellets were broken up and washed in water before *en bloc* staining with 3% uranyl acetate at 4 C for a minimum of 18 hours. All samples were then washed in water, dehydrated in a graded series of ethanols, and embedded in Spurr's resin. Thin sections were stained with lead citrate and examined with a Philips EM 400 electron microscope. Electron-microscopic supplies were obtained from J.B. EM Services.

Results

Protein Phosphorylation

Platelets responded to OAG with rapid phosphorylation of the 47K protein. Phosphorylation in platelets stimulated with 20 μg/ml OAG reached a maximum at 2 minutes, followed by a decrease in phosphorylation within 5 minutes. As the concentration of OAG was increased, the drop in phosphorylation decreased, because of both a decline in the maximum level of phosphorylation and an increase in the final level of phosphorylation. At 100 μg/ml OAG, phosphorylation rose to a maximum at 30 seconds, after which phosphorylation neither increased nor decreased notably. Phosphorylation of myosin light chain (MLC) reached a plateau at 50 μg/ml OAG. The initial rate of MLC phosphorylation increased with higher doses of OAG, but overall the maximum phosphorylation and initial rate of phosphorylation of MLC was less than that of the 47K protein (Figure 1).

The phosphorylation of actin-binding protein (ABP) differed, increasing steadily to a maximum after 5 minutes (Figure 2).

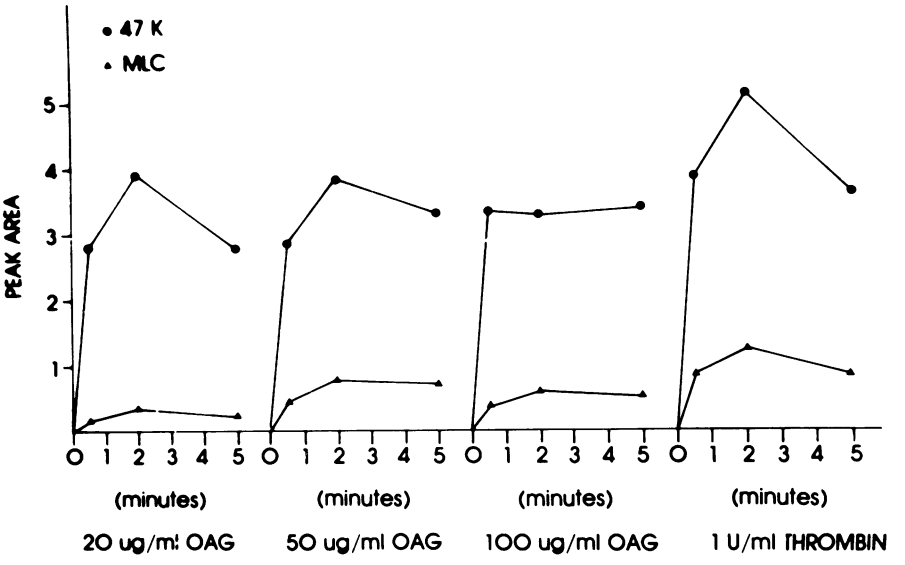


Figure 1—Platelets were stimulated with OAG or thrombin under the conditions described in Materials and Methods. In response to 20–100 μg/ml OAG, rapid phosphorylation of the 47K protein was followed by a partial dephosphorylation which leveled off as OAG concentration increased. MLC was phosphorylated at a slower rate and to a lesser extent. Phosphorylation of both proteins in response to 1 IU/ml thrombin was greater than that due to OAG. While the rate of MLC phosphorylation due to OAG was much less than that due to thrombin, the initial rate of phosphorylation of the 47K protein in response to OAG and to thrombin was virtually identical. ●, 47K protein; ▲, MLC.

In response to 1 IU/ml thrombin, phosphorylation of all three proteins (ABP, 47K, MLC) increased to a maximum at 2 minutes followed by dephosphorylation within 5 minutes. Both the initial rate and total phosphorylation of MLC due to thrombin was much greater than that due to OAG. Thrombin produced only a slight increase in total 47K phosphorylation, compared with OAG, and the initial rates of phosphorylation for 47K in response to OAG and thrombin were virtually identical.

Ultrastructural Studies

The platelet ultrastructural response to OAG was proportional to dosage and to duration of exposure. Thirty seconds after stimulation with 20 μg/ml OAG, most platelets retained their normal discoid shape, circumferential microtubule bands, and random distribution of organelles. Very few pseudopods were observed. The most noticeable response was swelling of the open canalicular system (OCS) and the appearance of a few vacuoles containing an amorphous matrix material (Figure 3a). A few very small aggregates were present. After 2 minutes, many large vacuoles had appeared, the OCS was less prominent, and there were fewer alpha granules evident (Figure 3b and c). Some shape change had occurred, and more small aggregates were observed, but many platelets remained single. Pseudopods were present on some platelets. After 5 minutes, larger aggregates had formed; in most cells the majority of the platelet interior was taken up by distended vacuoles. Intact alpha-granules had virtually disappeared (Figure 3d–f). Dense bodies also appeared to be converted into vacuoles but at a slower rate than alpha granules, possibly because of denser matrix material. Even after aggregation, intact dense bodies were sometimes observed, as well as dense bodies in which a central dense core was surrounded by diluted matrix material (Fig-

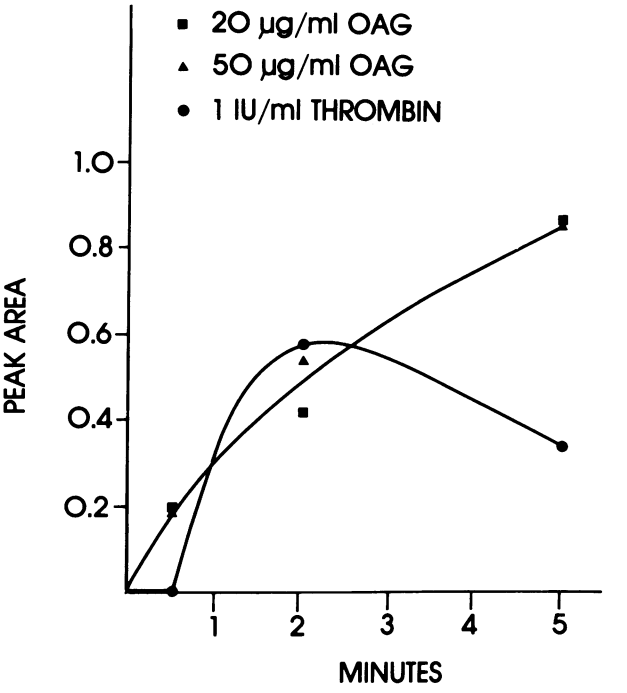
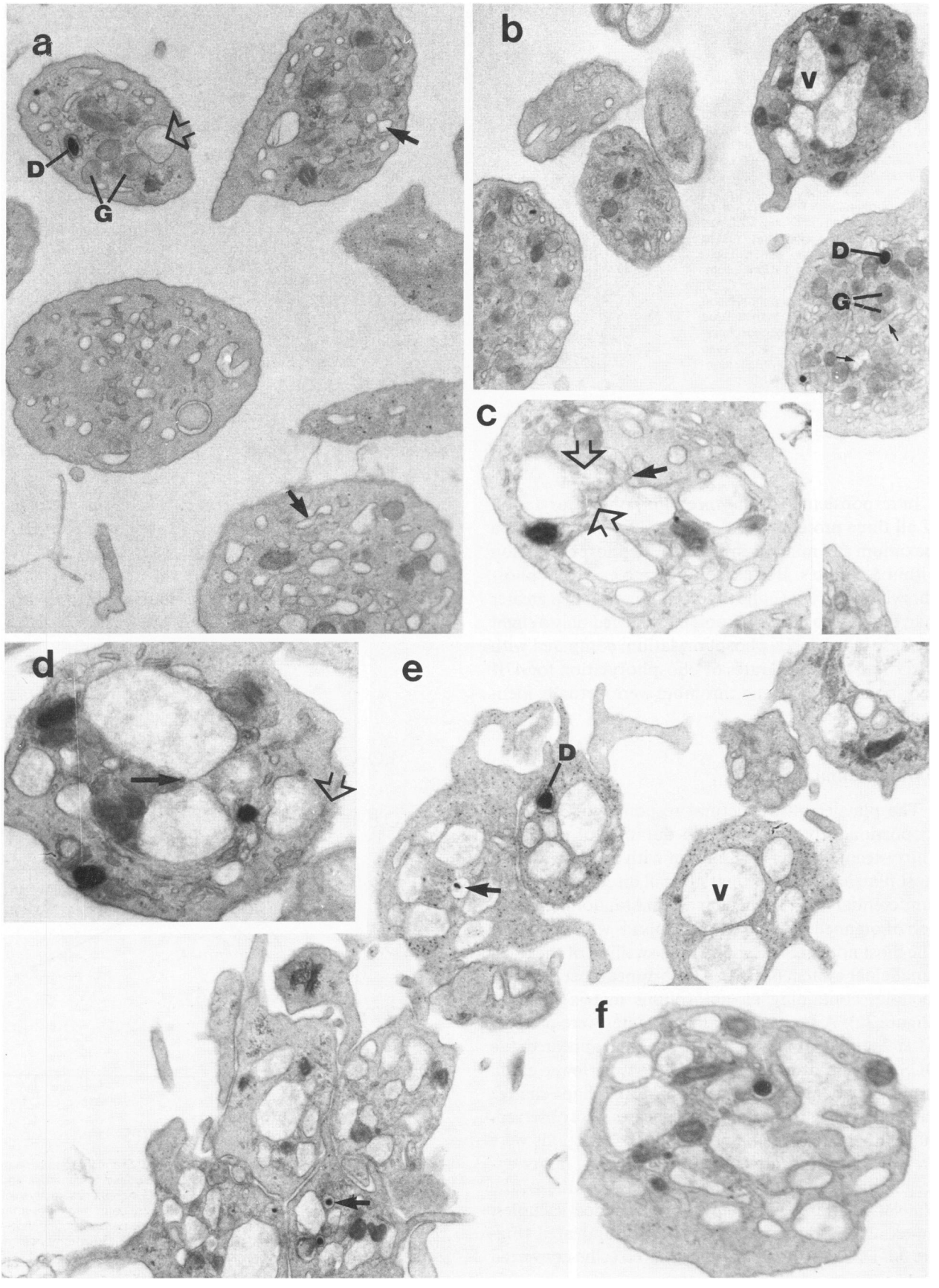


Figure 2—Phosphorylation of ABP in response to OAG and thrombin. Platelets were stimulated with OAG or thrombin as described in Materials and Methods. ABP phosphorylation in response to OAG increased steadily over the time course, whereas phosphorylation in response to thrombin, after an initial lag, rose quickly to a maximum, followed by partial dephosphorylation in a manner similar to the response of 47K and MLC to thrombin stimulation.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ure 3e). As the dose of OAG was increased, these ultrastructural changes developed faster—the number and size of vacuoles increased, and aggregation occurred sooner (Figure 4). In contrast, thrombin-activated platelets responded as described in the literature.[9,10] Platelets lost their discoid shape and extended pseudopods, centralizing granules, and secreting granule contents, quickly forming large aggregates. Vacuolar structures, similar to those produced by OAG, were observed in some thrombin-aggregated platelets. Most thrombin-aggregated cells contained the central mass of contractile gel indicative of internal contraction but no dense bodies, alpha-granules, or vacuolar structures (Figure 5a). Incubation of platelets with EDTA prior to stimulation with OAG prevented aggregation and pseudopod formation but did not alter the ultrastructural effects described above (Figure 5b). Control platelets, 5 minutes after addition of 20 mM Tris, remained in the resting state, unactivated (Figures 5c and d).

Discussion

Activation of protein kinase C and phosphorylation of a 47,000-dalton intracellular protein have been suggested as acting cooperatively with a rise in cytoplasmic calcium in the stimulation of platelet granule secretion.[6,7,11] The rise in cytoplasmic calcium is known to activate (via calmodulin) MLC kinase.[12,13] Phosphorylation of MLC is associated with initiation of actin-myosin contraction and the morphologic changes of granule centralization or internal contraction[14,15] and of shape change.[16] Morphologic changes stimulated by activation of protein kinase C are less well understood, although phorbol myristate acetate (PMA), an activator of protein kinase C in platelets, is known to labilize platelet granules.[17,18]

The physiologic activator of protein kinase C in platelets is presently believed to be diglyceride.[19] Previous experiments suggested that diglycerides also labilize platelet granules; however, large concentrations were needed for any significant effects.[20] It was therefore of considerable interest to study the effect of the synthetic diglyceride, OAG, on platelets. This diglyceride is known to activate protein kinase C; and because of the acetyl group on carbon 2, it can more easily penetrate the platelet membrane, which allows the use of reasonable concentrations.[7]

Platelets initially responded to OAG with swelling of the OCS accompanied by a rapid increase in the phosphorylation of the 47K protein and a less noticeable increase in MLC phosphorylation. As platelet alpha granules and dense bodies were converted into swollen vacuoles, phosphorylation of both 47K and MLC peaked, and the swollen channels of the OCS became less prominent. These alterations occurred without noticeable change in the random organization of platelet organelles, and there was little or no contraction of the circumferential microtubule band. Platelet aggregate size and number were both concentration- and time-dependent. Platelet aggregates often contained dense bodies as well as the large distended vacuoles typical of both aggregated and unaggregated platelets. Most of the aggregation and platelet granule conversion occurred after phosphorylation of 47K and MLC had peaked, although pseudopod formation and phosphorylation of ABP, known to be associated with pseudopod formation,[14] increased steadily over the time course.

The effects of OAG on platelet ultrastructure resembled those described for PMA, a phorbol ester and tumor promoter known to produce irreversible platelet aggregation.[17,18,21] When added to platelets in minute quantities, PMA provokes a similar swelling of the OCS and labilization of granules leading to the formation of distended vacuoles, in the absence of internal contraction.[17] Platelets stimulated by PMA secrete significant amounts of serotonin and adenine nucleotides before aggregation and show little or no shape change until after aggregation.[18] PMA and OAG share a pathway of action through the activation of protein kinase C, which has been shown to be a receptor for PMA.[22] Castagna et al[23] have shown that PMA directly activates protein kinase C, substituting for the diacylglycerol, a product of receptor-mediated phosphati-

Figure 3—Platelets stimulated with 20 μg/ml OAG respond with enlargement of the OCS, followed by vacuole formation. Organelles remain randomly dispersed in platelets which largely retain their discoid shape. (Uranyl acetate and lead citrate) **a**—Platelets first respond to OAG with swelling of the OCS (*small arrows*). Some storage granules have been converted to vacuolar structures containing diluted matrix material (*open arrow*). *D*, dense body; *G*; alpha-granule. (×18,100) **b**—After 2 minutes, large, distended vacuoles (*V*) containing diluted granular matrix have formed in some platelets. Many platelets have not yet formed vacuoles and still contain swollen OCS tubules (*arrows*) and many granules. *D*, dense body; *G*, alpha-granule. (×13,700) **c**—Swollen granules may fuse with each other (*open arrows*) or with elements of the OCS (*small arrow*). (×24,000) **d**—Extremely large vacuoles may form through fusion of smaller vacuoles (*small arrow*). In addition, vacuoles may fuse with the plasma membrane to release their contents (*open arrow*). (×24,200) **e**—Five minutes after OAG stimulation, some small aggregates have formed. Some dense bodies are present (*D*), as well as dense bodies in the process of conversion to vacuoles (*arrows*). Alpha-granules are virtually absent. Swollen canaliculi of the OCS have disappeared, and many large, swollen vacuoles (*V*) are present. Aggregated cells lack the central mass of contractile gel formed in response to thrombin (see Figure 5a), indicating that internal contraction has not occurred. (×14,600) **f**—An interconnected network of swollen canaliculi and vacuoles may be formed. (×25,100)

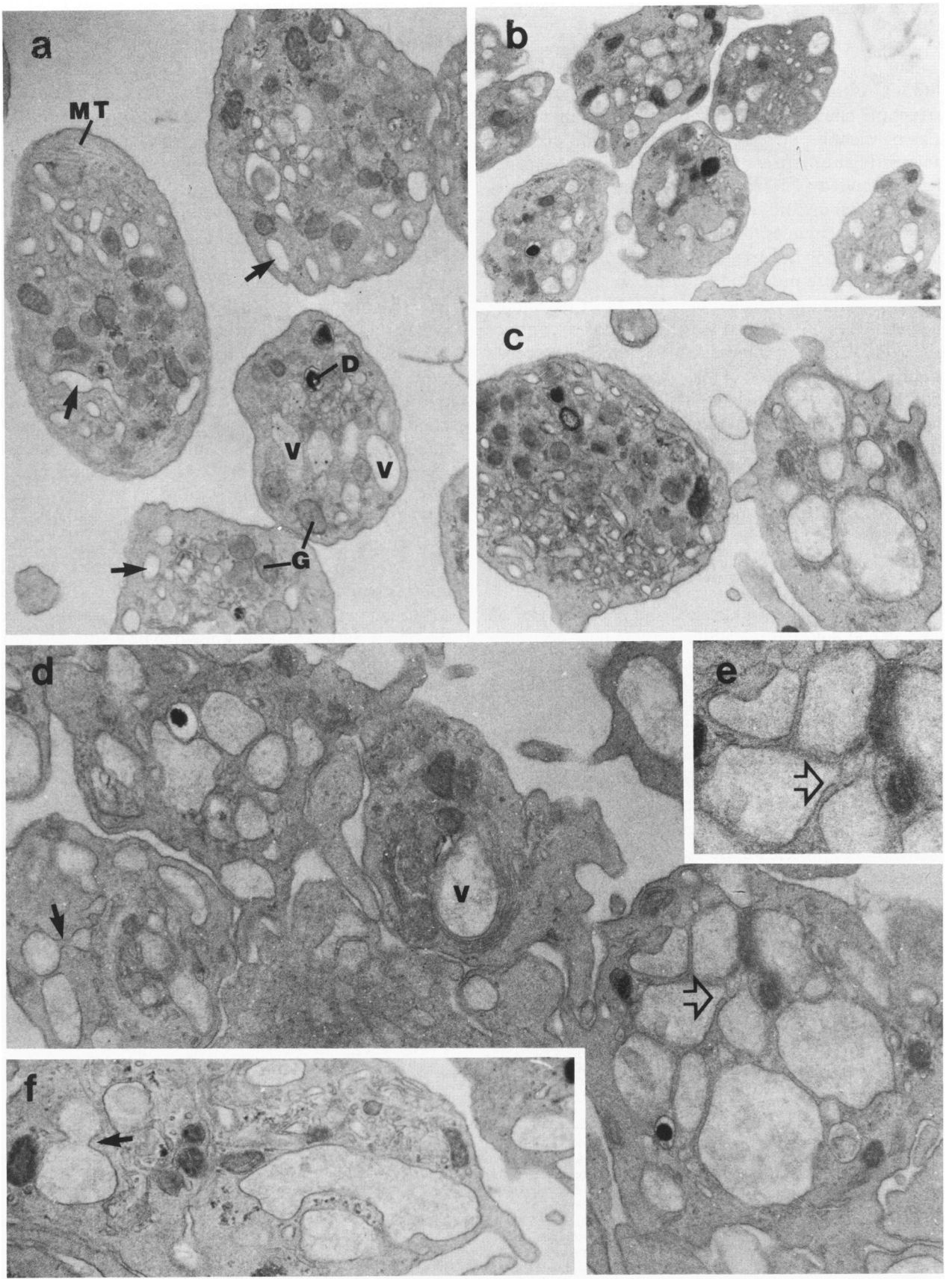

Figure 4—As OAG concentration is increased, internal changes occur more rapidly and to a greater extent. (Uranyl acetate and lead citrate) **a**—Thirty seconds after stimulation with 50 μg/ml OAG. The OCS (*arrows*) is swollen, and many vacuoles (*V*) have formed. *D*, dense body; *G*, alpha-granules; *MT*, microtubules. (×19,000) **b**—Two minutes after stimulation with 100 μg/ml OAG. In most cells the swollen OCS has been replaced by intermediate to large-sized vacuoles. (×11,600) **c**—The formation of large vacuoles usually results in the loss of granules and of the swollen OCS. The platelet on the right has formed large vacuoles while still retaining its discoid shape. (×19,600) **d**—After 5 minutes, platelets exposed to 100 μg/ml OAG have formed large aggregates. Dense bodies may be present, but alpha-granules have virtually disappeared. Many pseudopods have been extended; internal contraction seldom occurred. Vacuoles (*V*) are usually very large and are connected to remnants of the OCS (*small arrow*) and to each other (*open arrow*). (×23,700) The area indicated in **d** with an *open arrow* is shown enlarged in **e**. (×35,100) **f**—Five minutes after stimulation with 50 μg/ml OAG, aggregates are smaller but still contain distended vacuoles. Small vacuoles are fusing in the process of forming larger vacuoles (*arrow*). (×25,100)

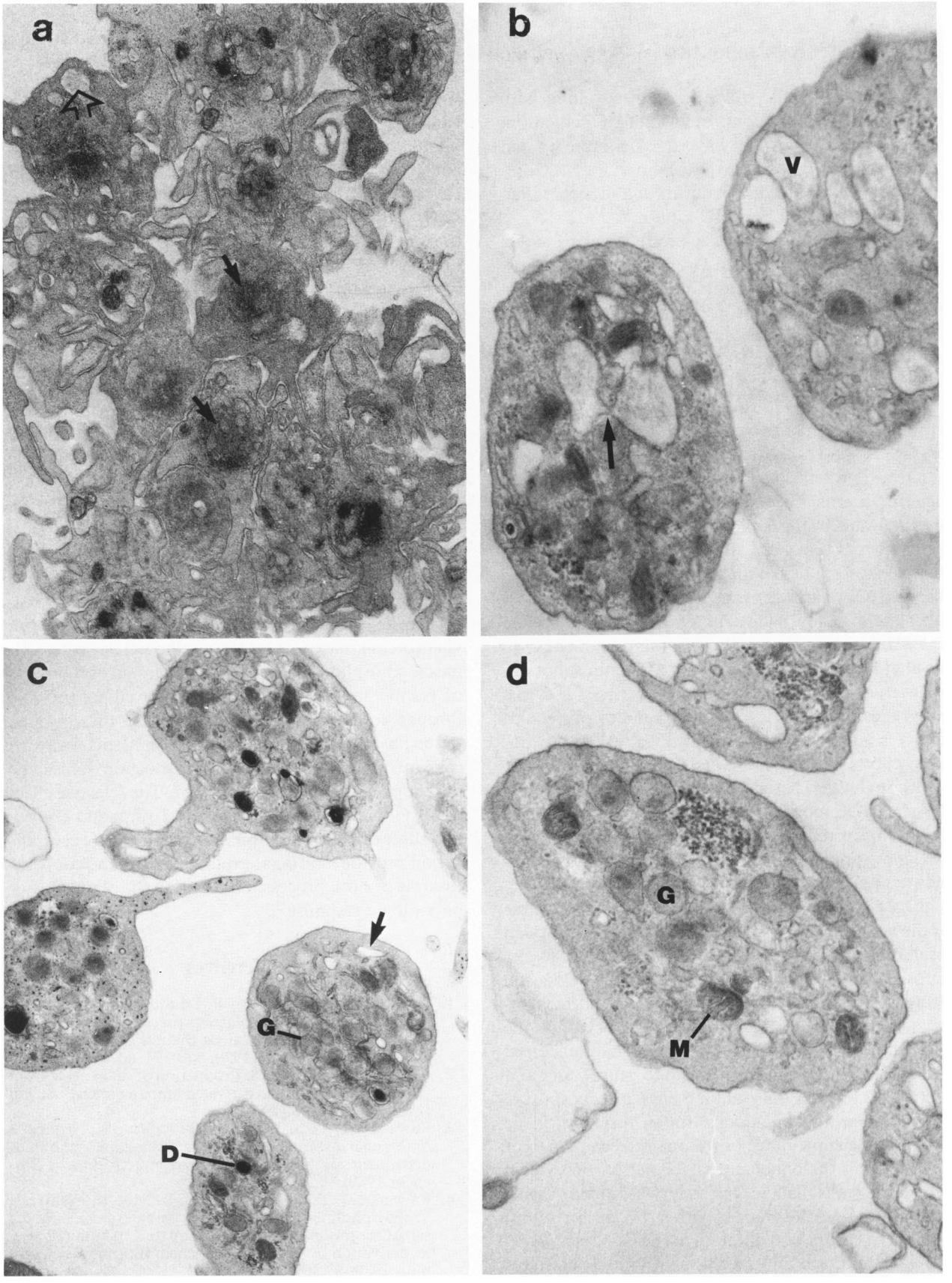

Figure 5a—Within 2 minutes, platelets stimulated with 1 IU/ml thrombin quickly form large aggregates. Internal contraction has occurred, and centralized masses of contractile gel have formed (*small arrows*). Many pseudopods have been extended, and cells do not retain their discoid shape. Dense bodies are absent from the aggregated cells. Some platelets on the periphery of the aggregate contain vacuolar structures similar to those formed after OAG stimulation (*open arrow*). (Uranyl acetate and lead citrate, ×10,900) **b**—Cells treated with 20 mM EDTA prior to stimulation with 100 μg/ml OAG do not aggregate or form pseudopods but still form the large vacuoles typical of OAG-stimulated platelets. Most storage granules have been converted to vacuoles (*V*) filled with diluted matrix material. Vacuoles are connected to each other (*arrow*). (Uranyl acetate and lead citrate, ×28,100) **c**—Control platelets stirred on the aggregometer for 5 minutes. No aggregation has occurred. In some platelets the OCS is slightly enlarged (*small arrow*), but most cells remain in the resting state. Some cells have extended a few pseudopods. *D*, dense body; *G*, alpha-granule. (×17,100) **d**—Most control platelets retained their discoid shape and random distribution of organelles. *G*, alpha-granules; *M*, mitochondrion. (×32,700)

dylinositol hydrolysis, which is required along with phospholipid and Ca^{2+} for protein kinase C activation. Protein kinase C phosphorylates the 47K protein[24] and has recently been shown to phosphorylate MLC, although this phosphorylation occurs at a site other than that phosphorylated by MLC kinase.[25]

How protein kinase C acts to produce the distended vacuoles and other effects of OAG is unknown. Phosphorylation of the 47K protein has previously been linked to platelet secretion.[6,7] Phosphorylation of MLC via MLC kinase is associated with internal contraction and centralization of organelles, but in OAG-stimulated platelets the phosphorylation of this protein is not associated with either of the above events. Diglycerides act to greatly increase the affinity of protein kinase C for Ca^{2+},[26,27] and both OAG and PMA have been shown to activate protein kinase C in the absence of an increase in internal Ca^{2+} levels.[23,28] However, MLC kinase is activated by the Ca^{2+} mobilization associated with agents such as thrombin. This suggests that MLC kinase was not responsible for the MLC phosphorylation observed. OAG may activate protein kinase C to phosphorylate MLC on an alternate site, as demonstrated for PMA by Naka et al.[25] Phosphorylation of MLC on this alternate site may explain why MLC phosphorylation was not accompanied by internal contractile events and suggests that MLC phosphorylated by protein kinase C is inactive and does not contribute to the dramatic effects of OAG on platelet ultrastructure.

Although OAG activates protein kinase C to phosphorylate 47K, most of the major ultrastructural effects of OAG occurred after phosphorylation had peaked. OAG may stimulate other, separate processes as yet unknown; or alternately, phosphorylation of 47K may act to initiate some other processes which are responsible for the labilization of granules to produce large, swollen vacuoles. Fusion of granules to each other and to elements of the OCS may be promoted by destabilization of the lipid membranes or by removal of proteins immediately surrounding the granules on the cytoplasmic side. Dilution of granule contents may have occurred through activation of an ion pump to cause water and electrolytes to enter the granule[30] or by direct exposure to the extracellular medium through fusion with the OCS. The disappearance of the enlarged canaliculi of the OCS and the concomitant formation of swollen vacuoles suggests that the OCS may be recruited in some way for vacuole formation, either by fusion of individual or compound granules to the OCS as a mechanism for dilution and secretion of granule contents or by recruitment of the OCS membrane for the formation of the distended vacuoles.

There are currently two pathways proposed for granule secretion in platelets. In one model, granules fuse with the OCS, releasing their contents through the OCS canaliculi to the platelet exterior.[30] In the second model, granule–granule fusion results in compound granules in which the contents are diluted by an influx of water and electrolytes. These fused granules, with increased flexibility due to dilution of the granule matrix, can slip through the contracting actomyosin to fuse with the outer plasma membrane and release their contents.[9] Our results suggest that both mechanisms may occur. Although granule–granule fusion did occur and the contents of the fused granules were diluted, even aggregated platelets contained large vacuoles which had not fused with the plasma membrane to release their contents. Our results suggest that granule–granule fusion, granule dilution, and granule fusion with the OCS may all be involved in platelet granule secretion.

The dramatic effects of the synthetic diglyceride OAG on platelet ultrastructure and protein phosphorylation implicate diglycerides as important intracellular messengers in the receptor-mediated activation of platelets. The rapid, transient production of diglycerides, known to occur after stimulation by agents such as thrombin, is followed by a rapid increase in 47K phosphorylation via protein kinase C. Our results show that protein kinase C phosphorylation of 47K is followed by a process of granule labilization, fusion, and swelling, perhaps through contact with the OCS, and that these events, in the absence of internal contraction associated with calcium mobilization, lead to the production of the large distended vacuoles described above. The selective effect of OAG on membrane–membrane fusion and a selective effect of calcium mobilization on granule centralization provide a rational explanation for the cooperative role of both processes in eliciting the platelet's full physiologic response.[7,31]

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