Synergistic Functions of Protein Phosphorylation and Calcium Mobilization in Platelet Activation*

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When human platelets were stimulated by synthetic diacylglycerol, such as 1-oleoyl-2-acetyl-glycerol, which was a potent activator in vitro of Ca\(^{2+}\)-activated, phospholipid-dependent protein kinase (protein kinase C) (Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y., and Fujikura, T. (1982) J. Biochem. (Tokyo) 91, 427–431), a protein having \(M\_v \sim 40,000\) (40-kilodalton protein) was rapidly phosphorylated, just as it was by natural extracellular messengers such as thrombin. Fingerprint analysis appeared to indicate that protein kinase C was indeed responsible for this 40-kilodalton protein phosphorylation in intact platelets. Under these conditions, neither inositol phospholipid breakdown nor endogenous diacylglycerol formation was observed, indicating that the synthetic diacylglycerol intercalated into the membrane and directly activated protein kinase C without interaction with cell surface receptors. During this process, the diacylglycerol was converted in situ to the corresponding phosphatidate, 1-oleoyl-2-acetyl-glycerol-3-phosphoric acid. Experiments with the synthetic diacylglycerol and Ca\(^{2+}\) ionophore A23187 suggested that the protein phosphorylation catalyzed by protein kinase C was a prerequisite requirement for the release of serotonin, and that the receptor-linked protein phosphorylation and Ca\(^{2+}\) mobilization acted synergistically to elicit the full physiological cellular response.

A wide variety of neurotransmitters, peptide hormones, secretagogues, and many other biologically active substances have been repeatedly shown to provoke inositol phospholipid turnover in their target tissues (see for reviews, Refs. 1–3). In general, the stimulation of most of these receptors immediately mobilizes Ca\(^{2+}\), and this divalent cation appears to play crucial roles in the cellular response to these extracellular messengers. A series of recent studies in our laboratories has suggested that diacylglycerol derived from this inositol phospholipid breakdown is directly involved in the transmembrane control of protein phosphorylation through activation of Ca\(^{2+}\)-activated, phospholipid-dependent protein kinase (protein kinase C) (4–9). Nevertheless, neither the relationship between the phospholipid degradation and Ca\(^{2+}\) mobilization nor the biological role of this receptor-linked protein phosphorylation has yet been definitely established. This communication will describe that under appropriate conditions a synthetic diacylglycerol exogenously added to intact platelets may intercalate into membranes and directly activates protein kinase C without inducing the phospholipid degradation and Ca\(^{2+}\) mobilization. Thus, using diacylglycerol and Ca\(^{2+}\) ionophore, it is possible to show that either the protein phosphorylation or Ca\(^{2+}\) mobilization alone is a prerequisite but not a complete requirement, and both are synergistically effective for causing the full physiological cellular response such as release of serotonin.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Human platelet-rich plasma and washed platelets were prepared by the method of Baenziager and Majerus (10). OAG 1 and AOG were synthesized as described previously (11). 1-Oleoyl-2-acetyl-sn-glycerol-3-phosphoric acid was synthesized from 1-oleoyl-2-acetyl-sn-glycerol by the method of Lammers and Van Boom (12). These synthetic products were chromatographically pure. 

Bovine thrombin was obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). A23187 was a product of Calbiochem. [\(^{3}H\)]Arachidonic acid (78.1 Ci/mmol), carrier-free H\(^{32}\)PO\(_4\), and [\(^{14}C\)]Serotonin (58 mCi/mmol) were obtained from New England Nuclear, Japan Radiation Association, and Amersham, respectively. \([\gamma\_\!-^{32}P]ATP\) and calf thymus H1 histone were prepared as described earlier (13). Other materials and chemicals were obtained from commercial sources.

Assay for Lipid Metabolism—The platelet-rich plasma (36 ml) was labeled with 25 \(\mu\)Ci of \([\gamma\_\!-^{32}P]ATP\) under the conditions described by Rittenhouse-Simmons (14), and platelets were isolated and washed as described (7–9). The radioactive platelets were stimulated by either thrombin, synthetic diacylglycerol, or A23187 as indicated in each experiment. The incubation was terminated by the addition of chloroform/methanol (1:2), and the radioactive lipids were directly extracted by the method of Bligh and Dyer (15). Phospholipids, diacylglycerol, and arachidonic acid metabolites were separated by Silica Gel G plate thin layer chromatography. The solvent systems employed were methyl acetate, n-propanol, chloroform, methanol, water (95:2:10) for HETE and HHT. The area corresponding to each lipid was scraped into a vial, and the radioactivity was determined.

In another set of experiments, the washed platelets (4 \(\times\) 10\(^{10}\) cells) were labeled with 1 mCi of carrier-free \[^{32}P\] under the conditions described by Lyova et al. (16). The radioactive platelets were then stimulated by thrombin or synthetic diacylglycerol and the reaction was terminated by the addition of chloroform/methanol (1:2). The radioactive phospholipids were directly extracted, and separated by two-dimensional Silica Gel G plate thin layer chromatography using

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§ Other materials and chemicals were obtained from commercial sources.

\(^{1}\) The abbreviations used are: OAG, 1-oleoyl-2-acetyl-glycerol; AOG, 1-acyetyl-2-oleoyl-glycerol; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatetraenoic acid; SDS, sodium dodecyl sulfate.

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chboroform, methanol, 7 N ammonia water (12:7:1) as a 1st dimension solvent system, and chboroform/methanol/acetic acid/water (25:15:4:2) as a 2nd dimension solvent system. Under these conditions, 1-oleoyl-2-acetyl-glyceryl-3-phosphoric acid was well separated from other phospholipids. The plate was then exposed to a Kodak Royal X-Omat film to prepare an autoradiograph.

**Assay for Protein Phosphorylation**—The washed platelets were labeled with [32P] as described above, and stimulated by either thrombin, synthetic diacylglycerol, or A23187 under the conditions specified in each experiment. The radioactive platelets were then directly subjected to SDS-polyacrylamide gel electrophoresis, stained, dried, on a Whatman No. 1 filter paper, and exposed to a Kodak Royal X-Omat film to prepare an autoradiograph. The relative intensity of each band was quantitated by densitometric tracing at 430 nm using a Shimadzu dual wavelength chromatogram scanner. Model CS-910.

**Assay for Serotonin Release**—The platelet-rich plasma (20 ml) was incubated with 1 μCi of [3H]serotonin as described by Haslam and Lynham (17), and platelets were isolated and washed as described earlier (7-9). The radioactive platelets were stimulated by either thrombin, synthetic diacylglycerol, or A23187. The incubation was terminated by formaldehyde followed by centrifugation at 10,000 × g for 40 s, and the radioactive serotonin released was determined as described by Costa and Murphy (18).

**Other Procedures**—Fingerprint analysis of the [32P]-labeled platelet proteins was carried out under the conditions described previously (8,9). Two-dimensional mapping of the tryptic phosphopeptides was made by the method of Beemon and Hunter (19). Protein kinase C was routinely assayed with calf thymus H1 histone as a phosphate acceptor (20). Lactate dehydrogenase was assayed by the method of Kornberg (21). The radioactivity of 3H-, 32P-, and 14C-labeled samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320.

**RESULTS AND DISCUSSION**

Protein kinase C absolutely requires Ca2+ and phospholipid for enzymatic activity, and it was shown that unsaturated diacylglycerol increased its affinity for Ca2+ dramatically to the 10-7 M range, and thereby rendered this enzyme fully active at physiologically lower concentrations of Ca2+ (5, 6, 22). It was also described that various synthetic diacylglycerols such as OAG, AOG, and diolein were equally active in this capacity when tested in *in vitro* enzymatic reactions (11). In the experiment shown in Fig. 1A, OAG was suspended in a small amount of 1% dimethylsulfoxide solution, sonicated to prepare micelles, and then directly added to intact platelets. It was found that an endogenous protein having an approx-
Dalton (20-kilodalton protein) was also phosphorylated (7-9, an additional protein having a molecular mass of 20,000).

A specific calmodulin-dependent protein kinase was proposed shown that, when platelets were activated by natural messengers

Port enzymatic reaction 16,17). This protein was identified as myosin light chain, and both preparations were subjected to fingerprint analysis. The mapping patterns of the tryptic phosphopeptides thus obtained were identical with the pattern obtained from the 40-kilodalton protein preparation which was phosphorylated in vitro by a homogeneous preparation of protein kinase C in the presence of Ca++, phospholipid, and diacylglycerol.

The results presented above seem to indicate that OAG may intercalate into the phospholipid bilayer and directly activates protein kinase C without interaction with any of cell surface receptors. The observed effect of OAG did not appear to be simply due to the damage of platelet membranes, since cytoplasmic enzymes such as lactic dehydrogenase did not leak into the medium. It was noted that the exogenously added diacylglycerol was rapidly converted in situ to the corresponding phosphatidate, that is 1-oleoyl-2-acetyl-glycerol-3-phosphoric acid, presumably by the action of diacylglycerol kinase. The large spot shown by an arrow in Fig. 2A was identified as such by comparison with an authentic sample of the synthetic product. This unique compound was not found in the resting platelets nor in the platelets stimulated by thrombin (Fig. 2, B and C). The rapid phosphorylation of the 40-kilodalton protein in platelets was also observed with AOG instead of OAG. Diacylglycerols possessing two long fatty acyl moieties such as diolein were practically ineffective to induce 40-kilodalton protein phosphorylation in vivo, presumably due to their inability to intercalate into the membrane, although this diacylglycerol was highly active to support enzymatic reaction in vitro as described earlier (5, 6, 11).

The next series of experiments was designed to examine if the protein phosphorylation catalyzed by protein kinase C or Ca++ mobilization or both were essential for eliciting physiological response such as release of serotonin. It was repeatedly shown that, when platelets were activated by natural messengers such as thrombin, collagen, and platelet-activating factor, an additional protein having a molecular mass of 20,000 dalton (20-kilodalton protein) was also phosphorylated (7-9, 16, 17). This protein was identified as myosin light chain, and a specific calmodulin-dependent protein kinase was proposed to be responsible for its phosphorylation (23). In fact, as shown in Fig. 3A, thrombin induced the phosphorylation of both 40-kilodalton and 20-kilodalton proteins in a parallel manner, indicating that the natural messenger provokedinositol phospholipid breakdown as well as Ca++ mobilization. If, however, platelets were stimulated by low concentrations of A23187, only 20-kilodalton protein was significantly phosphorylated as shown in Fig. 3B. This ionophore at lower concentrations did not induce 40-kilodalton protein phosphorylation nor produced endogenous diacylglycerol. On the other hand, OAG induced the phosphorylation of 40-kilodalton protein to the extent that was induced by thrombin, whereas 20-kilodalton protein was not phosphorylated under the conditions given in Fig. 3C. These results seem to provide the rationale that, under the given conditions, the activation of protein kinase C and mobilization of Ca++ may be independently induced by the exogenous addition of the synthetic diacylglycerol and Ca++ ionophore, respectively. In the experiments shown in Fig. 4, human platelets were stimulated by OAG in the presence or absence of a low concentration of A23187. It was found that the 40-kilodalton protein phosphorylation reaction proceeded by the addition of OAG irrespective of the presence and absence of the Ca++ ionophore (Fig. 4A). In a marked contrast to this protein phosphorylation, serotonin was not sufficiently released by the addition of OAG alone, and the full physiological response was observed when both OAG and A23187 were added (Fig. 4B). This ionophore per se at the concentration employed (0.4 μM) did not induce endogenous diacylglycerol formation and 40-kilodalton protein phosphorylation nor caused serotonin release. At higher concentrations (more than 0.5 μM), A23187 alone caused the phosphorylation of 40-kilodalton protein in addition to 20-kilodalton protein, and released serotonin concomitantly. This is presumably due to nonspecific degradation of phospholipids and/or to activation of protein kinase C by a large increase in the Ca++ concentration (5, 6, 22). Likewise, OAG alone at higher concentrations (more than 50 μg/ml) caused the release of a significant quantity of serotonin. The exact reason for this enhancement is not known, but it is possible

![Fig. 3. Effects of thrombin, A23187, and OAG concentrations on phosphorylation of 40-kilodalton and 20-kilodalton proteins. The platelets prelabeled with 32P, were stimulated at 37 °C for 1 min by various amounts of either thrombin, A23187, or OAG. The final concentration of dimethylsulfoxide was fixed at 0.1%. The phosphorylation of 40-kilodalton and 20-kilodalton proteins was assayed as described under "Experimental Procedures." A, with thrombin; B, with A23187; C, OAG. ](http://www.jbc.org/lookup/rights/6703/00219524/H03020012X/fig03.jpg)

![Fig. 4. Synergistic effects of synthetic diacylglycerol and Ca++ ionophore on platelet activation. The platelets, which were labeled with 32P, or [14C]serotonin, were preincubated with various concentrations of OAG as indicated for 1 min at 37 °C, and then stimulated by 2 mM Ca++ plus 0.4 μM A23187 at 37 °C for 30 s. The final concentration of dimethylsulfoxide was 0.1%. The phosphorylation of 40-kilodalton and 20-kilodalton proteins and serotonin release were determined as described under "Experimental Procedures." A, 40-kilodalton protein phosphorylation; B, serotonin release; C, 20-kilodalton protein phosphorylation. ](http://www.jbc.org/lookup/rights/6703/00219524/H03020012X/fig04.jpg)
that the diacylglycerol may act as a membrane fusigen in this
exocytotic process (24). Under the given conditions, the phos-
phorylation of 20-kilodalton protein induced by Ca++ iono-
phore was not significantly affected by the addition of
OAG (Fig. 4C).

The present studies seem to provide additional evidence
that the receptor-mediated breakdown of inositol phospho-
lipid is directly coupled to the activation of protein kinase C,
which is responsible for the phosphorylation of 40-kilodalton
protein. It is evident that the phosphorylation of this protein,
if not a sole target of protein kinase C, is a prerequisite for
the full physiological response that is release of serotonin,
although the precise role of 40-kilodalton protein in platelet
activation remains unknown. It is also conceivable that
thrombin, like various other extracellular messengers which
provoke inositol phospholipid turnover, immediately mobi-
lizes Ca++ as judged by the phosphorylation of 20-kilodalton
protein. Obviously, Ca++ may play diverse roles in the acti-
vation of cellular functions, but the present experiments with
synthetic diacylglycerol and Ca++ ionophore strongly suggest
that the receptor-linked protein phosphorylation and Ca++
mobilization act synergistically to elicit the full physiological
response. Nevertheless, the evidence described above is insuf-
ficient to discuss the relationship between the phos-
pholipid degradation and Ca++ mobilization. The event occur-
ing immediately after stimulation of the receptors also re-
mains unknown.

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