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A POSSIBLE ROLE OF PROTEIN KINASE C IN SIGNAL-INDUCED LYSOSOMAL ENZYME RELEASE*

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SUMMARY: In platelets, activation of protein kinase C and mobilization of Ca^{2+} were selectively induced by the addition of 1-oleoyl-2-acetyl-glycerol and a low concentration of A23187, respectively (Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701-6704). Using this procedure evidence was obtained suggesting that the protein phosphorylation and Ca^{2+} mobilization were both essential and synergistically effective to cause release of lysosomal acid hydrolases such as N-acetylglucosaminidase. A similar observation was made for the lysosomal enzyme release from rat neutrophils.

Upon stimulation of receptors platelets normally release many constituents of various granules including lysosomes, α-granules and dense bodies into the medium (for reviews, see Ref. 1,2). Although the release of lysosomal enzymes such as NAGase[1/] may be observed at a slightly higher concentration of thrombin or collagen than that needed for serotonin release from dense bodies (3), it has been suggested that the increase in cytosolic Ca^{2+} is essential

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1/ Abbreviations used are: NAGase, N-acetylglucosaminidase; protein kinase C, Ca^{2+}-activated, phospholipid-dependent protein kinase; OAG, 1-oleoyl-2-acetyl-glycerol; and SDS, sodium dodecyl sulfate.

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for the release reactions from various platelet granules (2). Preceding reports from our laboratory (4,5) have shown that signal-dependent mobilization of Ca^{2+} and protein phosphorylation catalyzed by protein kinase C are synergistically effective for the release of serotonin. Extending these observations, the present communication will describe that a similar conclusion may be made for the lysosomal enzyme release from rabbit platelets and perhaps for the enzyme release from rat neutrophils as well. It is suggestive that the receptor-linked activation of protein kinase C is a prerequisite requirement and may lie on a common pathway for the release reactions of various constituents of different subcellular granules.

EXPERIMENTAL PROCEDURES

Washed rabbit platelets and rat neutrophils were prepared by the method of Baenziger and Majerus (6) and Johnson and Varani (7), respectively. OAG was synthesized as described previously (8). Bovine thrombin and p-nitrophenyl-N-acetyl-β-glucosaminide were obtained from Mochida Pharmaceutical Co. and Sigma, respectively. Ionophore A23187 was a product of Calbiochem. [^{3}H]Arachidonic acid (78.2 Ci/mmol), carrier-free $H_3{}^{32}PO_4$ and [2-^{14}C]serotonin (58 mCi/mmol) were purchased from New England Nuclear, Japan Radioisotope Association and Amersham, respectively. Other chemicals and materials were obtained from commercial sources. The platelets were prelabeled with either [^{3}H]arachidonic acid, $H_3{}^{32}PO_4$ or [2-^{14}C]serotonin under the conditions described by Rittenhouse-Simmons (9), Haslam et al. (10) and Haslam and Lynham (11), respectively. The radioactive platelets (6 x 10^8 cells/ml) were stimulated as indicated in each experiment. The radioactive lipids were directly extracted from the platelets by the method of Bligh and Dyer (12), subjected to silica gel G plate thin layer chromatography, and quantitated as described by Rittenhouse-Simmons (9). The radioactive platelet proteins were applied to SDS-polyacrylamide slab gel electrophoresis. The gel was stained, dried on a filter paper, and then exposed to an X-ray film to prepare an autoradiograph. The electrophoresis was carried out under the conditions specified by Laemmli (13). The relative intensity of each band was quantitated by densitometric tracing at 430 nm with a Shimadzu chromatogram scanner, Model CS-910. The release of radioactive serotonin from the platelets was determined by the method of Costa and Murphy (14). NAGase was assayed colorimetrically with p-nitrophenyl-N-acetyl-β-glucosaminide as a substrate by the method of Dangelmaier and Holmsen (15). Other detailed procedures were specified earlier (5).

RESULTS AND DISCUSSION

When rabbit platelets were stimulated by thrombin, diacylglycerol was rapidly and transiently produced, and protein kinase C was concomitantly activated as judged by the phosphorylation of its

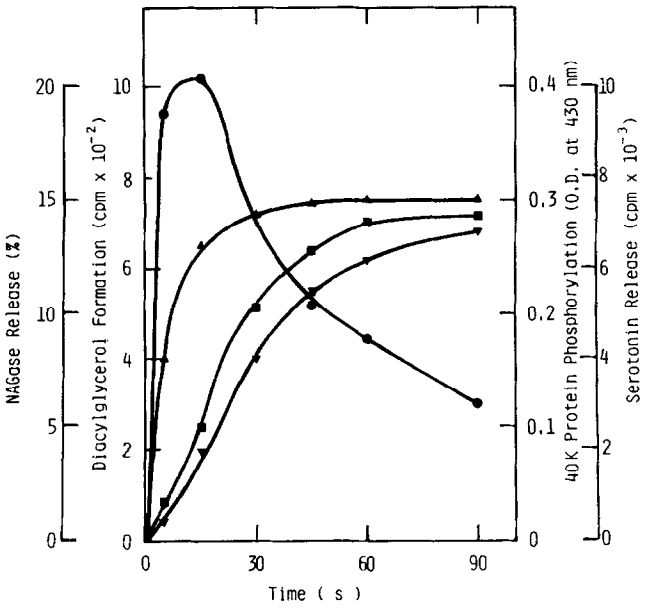

Fig. 1 Time courses of formation of diacylglycerol, phosphorylation of 40K protein and release reactions during platelet activation. The platelets, which were prelabeled with either [^{3}H]arachidonic acid, ^{32}Pi or [^{14}C]serotonin, were stimulated at 37°C by thrombin (0.2 unit/ml) for various periods of time as indicated. Diacylglycerol, radioactive 40K protein, serotonin and NAGase were determined as described under "Experimental Procedures". (●——●), formation of diacylglycerol; (▲——▲), phosphorylation of 40K protein; (■——■), release of serotonin; (▼——▼), release of NAGase.

specific endogenous substrate protein that has a molecular weight of about 40,000[2/] (40K protein) as described for human platelets (17,18). This reaction was immediately followed by the release of serotonin and lysosomal enzymes such as NAGase as shown in Fig. 1. Stimulation of the receptor concurrently caused a rapid increase in the Ca^{2+} concentration, as judged by the phosphorylation of a distinct protein having a molecular weight of 20,000 (20K protein). This protein has been identified as myosin light chain, and its phosphorylation is absolutely dependent on Ca^{2+} and calmodulin (19). It was shown earlier (4,5) that synthetic diacylglycerol such as OAG is intercalated into the membrane and directly activates protein kinase C in intact platelets without interaction with cell

2/ Imaoka et al. (16) have recently reported that this 40K protein shows the molecular weight of 47,000, and comprises 7-9 phosphorylated forms with different pI values but same mobility on SDS-polyacrylamide gel electrophoresis.

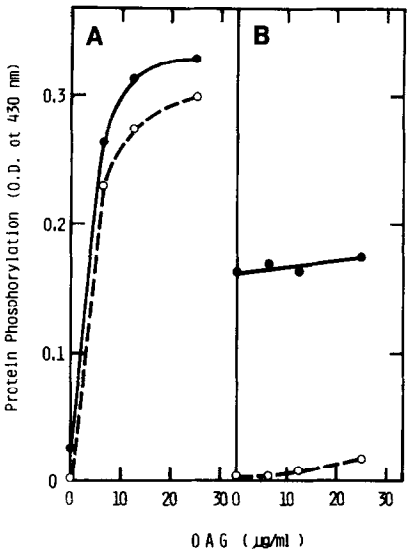

Fig.2 Effect of OAG and A23187 on phosphorylation of 40K and 20K proteins. The platelets labeled with ^{32}Pi were incubated for 2 min at 37°C with various concentrations of OAG, and then stimulated for 1 min by A23187 (0.6 μM) in the presence of $CaCl_2$ (1 mM). Protein phosphorylation was determined as described under "Experimental Procedures". A, phosphorylation of 40K protein; B, phosphorylation of 20K protein. (●——●), stimulated by OAG and A23187; (○- - -○), stimulated by OAG alone.

surface receptors. An analysis with various concentrations of OAG revealed that the phosphorylation of 40K protein was induced maximally by this synthetic diacylglycerol alone, and that the reaction rate was not affected greatly by the addition of Ca^{2+} ionophore at lower concentrations as shown in Fig. 2A. Although protein kinase C absolutely required Ca^{2+} for enzymatic activity, diacylglycerol such as OAG dramatically increased the affinity of this enzyme for Ca^{2+}, and thereby rendered the enzyme fully active without a net increase in Ca^{2+} concentration (20-22). Inversely, the phosphorylation of 20K protein was induced by a low concentration (0.6 μM) of A23187 alone, and was not affected to measurable extents by the addition of OAG (Fig. 2B). Thus, under appropriate conditions 40K and 20K proteins were phosphorylated selectively and independently by OAG and A23187, respectively. On the other hand, these proteins were phosphorylated simultaneously when platelets were stimulated by natural

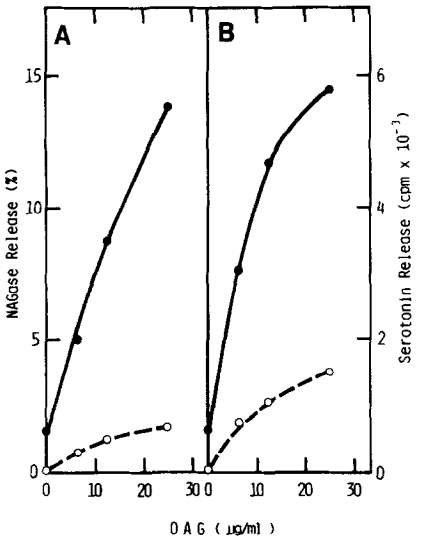

Fig. 3 Synergistic effects of OAG and A23187 on platelet release reactions. The platelets labeled with [^{14}C]serotonin were incubated for 2 min at 37°C with various concentrations of OAG, and then stimulated for 1 min by A23187 (0.6 μM) in the presence of $CaCl_2$ (1 mM). NAGase and serotonin were determined as described under "Experimental Procedures". A, release of NAGase; B, release of serotonin. (●——●), stimulated by OAG and A23187; (○- - -○), stimulated by OAG alone.

extracellular messengers such as thrombin as repeatedly described (10,11,17,18).

In the next experiments shown in Fig. 3, it was found that, like serotonin release from dense bodies (4,5), the lysosomal enzyme was not released sufficiently by the addition of OAG alone, although the 40K protein phosphorylation reaction proceeded rapidly under the same conditions. However, when both OAG and A23187 were added together, NAGase was released as it was by thrombin. This ionophore alone at the concentration used (0.6 μM) induced the release of NAGase and serotonin only slightly. At higher concentrations (more than 1.0 μM), the Ca^{2+} ionophore *per se* caused rapid phosphorylation of 40K protein as well as 20K protein, and released NAGase concomitantly. This is presumably due to the activation of protein kinase C by a large increase in Ca^{2+} concentration and also due to non-specific degradation of phospholipids to produce endogenous diacylglycerol. Likewise, OAG alone at higher concentrations (more than

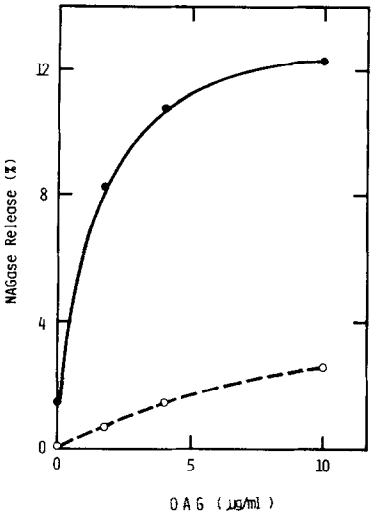

Fig. 4 Synergistic effect of OAG and A23187 on release of NAGase from rat neutrophils. The neutrophils were incubated for 2 min at 37°C with various concentrations of OAG in the presence of cytochalasin B (5 μg/ml), and then stimulated for 10 min by A23187 (0.6 μM) in the presence of $CaCl_2$ (1 mM). NAGase was assayed as described under "Experimental Procedures". (●——●), stimulated by OAG and A23187; (○- - -○), stimulated by OAG alone.

50 μg/ml) caused a significant release of NAGase without measurable phosphorylation of 20K protein. The exact reason for this release is unknown, but it is possible that at higher concentrations diacylglycerol may act as a membrane fusigen in exocytotic processes (23). In another set of experiments using rat neutrophils, an essentially similar pattern of NAGase release was obtained, and the full physiological response was observed when OAG and A23187 were simultaneously added as shown in Fig. 4.

The results described above suggest that Ca^{2+} mobilization and protein phosphorylation catalyzed by protein kinase C are synergistically effective for causing release reactions of various constituents of different granules. However, no unequivocal evidence is available indicating that 40K protein is an essential ingredient of the machinery for the release of NAGase as well as serotonin. It is also uncertain whether 40K protein occurs in neutrophils. Nevertheless, it is attractive to suggest that activation of protein kinase C which is directly linked to signal-induced inositol phos-

pholipid breakdown is a prerequisite requirement for the cellular response, since the release reactions are always accompanied with the activation of this enzyme as monitered by the phosphorylation of its specific endogenous substrate that is 40K protein.

It has been shown that slightly higher concentrations of natural signals such as thrombin and collagen are needed for NAGase release from lysosomes than those needed for serotonin release from dense bodies (3). In an elegant experiment with platelets which are rendered permeable to Ca^{2+} by exposure to a high voltage electric field, Knight et al. (24) have suggested that some factor or factors other than Ca^{2+} may also be involved in the stimulus-response coupling, which may explain the signal selectivity observed for release reactions of different platelet granules mentioned above. Although each of Ca^{2+} and protein kinase C may play diverse roles during the exocytotic processes, it is possible that protein kinase C is an additional factor that is proposed by Knight et al. (24). Further studies are needed on endogenous substrate proteins of protein kinase C for understanding more precise mechanism of various release reactions.

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