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PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE-C OF PLATELETS: ASSOCIATION WITH 1,2-DIACYLGLYCEROL-KINASE AND INHIBITION BY CYCLIC-AMP

M. Motasim Billah, Eduardo G. Lapetina and Pedro Cuatrecasas

Department of Molecular Biology, The Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709, U.S.A.

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Summary. Horse platelets prelabeled with [^{14}C]arachidonate (AA) rapidly degrade [^{14}C]phosphatidylinositol (PI) to [^{14}C]1,2-diacylglycerol (DG) upon treatment with deoxycholate (DOC). This phospholipase-C (PLC) activity is specific for PI since other phospholipids or neutral lipids are not affected. Although exogenous Ca^{2+} is not required for activity, EGTA or EDTA abolishes PI degradation. Addition of Mg^{2+} (1 mM) and ATP (1 mM) results in phosphorylation of the DG and production of phosphatidic acid (PA). Higher concentrations of DOC inhibit DG-kinase. These observations, together with the fact that different platelet agonists induce a rapid degradation of PI and production of PA, indicate that PLC and DG-kinase activities are intimately linked. Incubation of platelets with dibutyryl cyclic-AMP, cyclic AMP-phosphodiesterase inhibitors and pyridoxal-5'-phosphate, which prevent platelet aggregation, inhibits the DOC-dependent conversion of PI to DG. The activity of PLC may play a central role in mediating platelet function and aggregation.

Introduction. An important feature of phosphatidylinositol (PI) metabolism in a wide variety of tissues is the rapid turnover of its phosphorylinositol moiety in response to stimulation of a class of receptors which are strongly implicated in the mobilization of calcium ions within cells (1-4). In several tissues (5-9) various ligand-receptor interactions cause a specific breakdown of PI. This breakdown is presumably effected by activation of a C-type phospholipase (PLC) that is specific for PI. Such an activity has been demonstrated in several tissues (10-14).

An increased turnover of PI is also observed in platelets upon activation by thrombin, collagen or ADP (15,16). Platelet activation also results in the release of arachidonic acid (AA) and its cyclooxygenase and lipoxygenase products (9,17-21). AA release is preceded by a rapid increase in the labeling of phosphatidic acid (PA) in platelets prelabeled with [^{14}C]AA, and it has been suggested that these effects result from an early, primary stimulation of a PLC-type activity (9).

Abbreviations: Phosphatidylinositol, PI; 1,2-diacylglycerol, DG; phosphatidic acid, PA; deoxycholate, DOC; phospholipase-C, PLC; arachidonic acid, AA; platelet-rich plasma, PRP.
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The present experiments were aimed at investigating the mechanism of PA formation in platelets. The results demonstrate the existence of a PI-specific PLC which is inhibited by cyclic-AMP and which is very closely linked to a DG-kinase activity.

Materials. Most materials were obtained as described previously (9,18). [1-^{14}C]Arachidonic acid (specific activity 55.2 mCi/mol) was from Amersham, Arlington Heights, Illinois. Pyridoxal-5'-phosphate and C. welchii PLC were from Sigma.

Methods. Preparation of labeled platelets. Washed horse platelets were obtained from 500 ml of acid citrate-dextrose blood. The blood was centrifuged at 300xg at room temperature. The supernatant, platelet-rich plasma (PRP), was removed and centrifuged twice more to remove red cells. Samples of 50 ml of PRP were then incubated at 37°C for 2 h with 2 μCi of [^{14}C]AA. At the end of the incubation period, EDTA was added to a final concentration of 2 mM and platelets were pelleted by centrifugation at 3000xg for 20 min at 0-4°C. The pellet was resuspended gently in 50 ml of NaCl/Tris-HCl/glucose (134 mM/15 mM/ 5 mM) buffer, pH 7.4 (Tris-Saline buffer). The platelets from 500 ml of blood were finally suspended in 20-25 ml buffer and were used within one hour.

Incubation procedures and lipid extraction. Incubations were performed in duplicate or triplicate in a shaking water bath at 37°C for 30 min. The reaction volume was usually 1 ml (occasionally 0.5 ml) and contained platelets equivalent to 3-4 mg protein (22). The basic incubation mixture contained Tris-Saline buffer (pH 7.4) plus appropriate additions as described in the legends to the figures and tables. All experiments presented are one of at least three giving very similar results.

Incubations were stopped by adding 3.75 ml of a chloroform:methanol (1:2, v/v) mixture (23). The phases were split by adding 1.25 ml of chloroform and 1.25 ml of 2M KCl solution. The lower chloroform phase was removed carefully using a pasteur pipet and dried under a flow of nitrogen at 25°C.

Chromatographic Separation of Lipids. The dried lipids were redissolved in chloroform and applied onto silica gel G (for prostaglandins and neutral lipids), activated silica gel H (for phospholipids) or formaldehyde-impregnated paper (for PI separation).

The solvent used for prostaglandins was the upper phase from a mixture of ethylacetate:2,2,4-trimethylpentane:acetic acid:water (90:50:20:100, v/v) which gives a good separation of cyclooxygenase and lipooxygenase products and of PA (9). In this system, DG, obtained from platelet phospholipids by C. welchii PLC migrates between AA and the solvent front. The R_f values for AA and DG were 0.87 and 0.91, respectively. Triglycerides move with the solvent front. Neutral lipids were analyzed (24) using petroleum ether:diethylether: acetic acid (90:10:1, v/v). The R_f values for DG, AA and triacylglycerol were 0.07, 0.25 and 0.44, respectively.

Phospholipids from platelet extracts were analyzed on activated silica gel H plates in a solvent system of chloroform:methanol:acetic acid:water (75:45:12:3, v/v) (25). In this sytem, phosphatidylserine and PI migrate together. PI was separated on formaldehyde-impregnated papers (26). Lipids were localized by radioautography or I_2 vapours and counted in a liquid scintillation counter with Bray's solution.

Results. Horse platelets contain a PLC activity which specifically degrades PI. The present studies use the production of DG from endogenous phospholipids of platelets prelabeled with [^{14}C]AA to detect and measure PLC activity. No PLC activity can be detected when sonicated horse platelets are incubated for 60 min at 37°C. However, substantial amounts of DG are generated

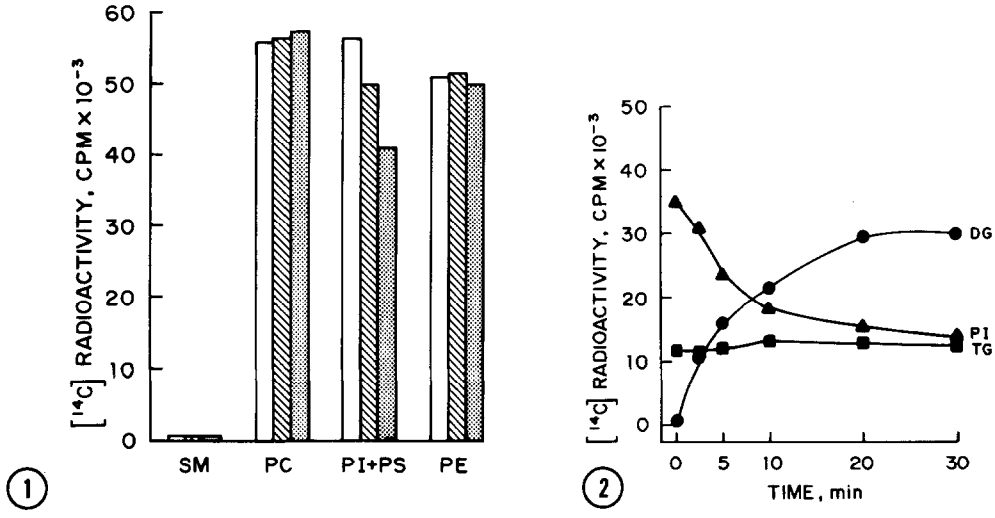

Fig. 1. Effect of DOC on the degradation of various platelet phospholipids. Platelets prelabeled with [^{14}C]AA were treated without DOC (open bars) and with DOC (5 mM) for 5 min (stripped bars) or for 30 min (dotted bars). Platelet suspension contained 3.9 mg protein/ml.

Fig. 2. Time-course of DOC activation of PI-specific PLC activity. Platelets prelabeled with [^{14}C]AA (3.5 mg protein/ml) were incubated with 5 mM DOC for different periods of time. Formaldehyde-impregnated papers (see ref. 26) were used to separate PI from other phospholipids. ▲, PI; ●, DG; ■, triglycerides.

when the incubation is performed in the presence of DOC. DG production is dependent on the DOC concentration, 5 mM being optimal for PLC stimulation. Higher concentrations of DOC are less effective. This endogenous activity is abolished by incubating platelets at 100°C for 2 min.

The phospholipids which are labeled with [^{14}C]AA after incubating PRP with this radioactive fatty acid are phosphatidylcholine, phosphatidylethanolamine and PI (18, Fig. 1). Sphingomyelin and phosphatidylserine are virtually not labeled (18). PI represents only 3% of the total phospholipid in horse platelets (9). DOC does not effect the breakdown of phosphatidylcholine, phosphatidylethanolamine or sphingomyelin (Fig. 1), or of triglyceride (Fig. 2), and degrades only PI (Figs. 1,2). The production of DG parallels the breakdown of PI (individually separated on formaldehyde-impregnated papers). The activity of PLC on endogenous PI is linear for at least 5 min (Fig. 2), and it is dependent on the Ca^{2+} available from endogenous sources after solubilization with DOC since EGTA and EDTA almost completely abolish the activation (Fig. 3).

Association of PLC and DG-kinase activities. In the presence of Mg^{2+} (1 mM) and ATP (1 mM), the quantity of DG produced is reduced and significant amounts of labeled PA accumulate (Fig. 4). These changes presumably reflect the activity of DG-kinase, which catalyzes the phosphorylation of DG to PA. This kinase activity is reduced by higher concentrations of DOC (Fig. 4).

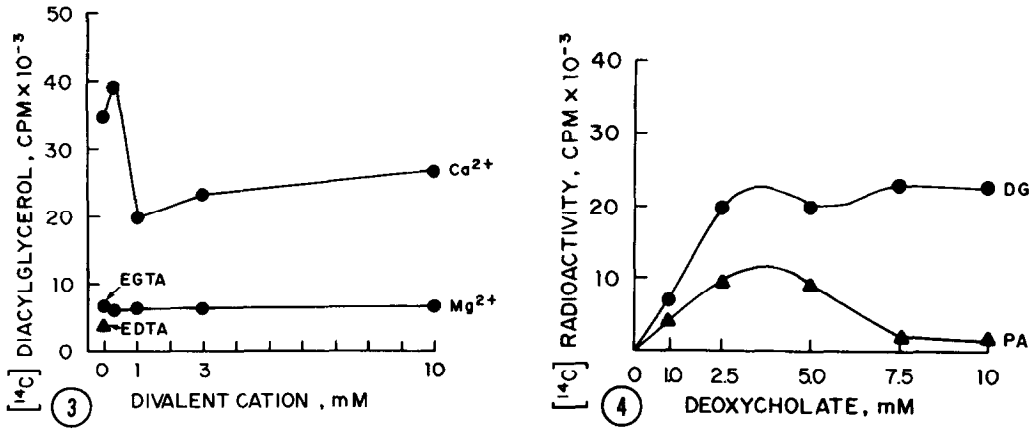

Fig. 3. Effect of Ca^{2+}, Mg^{2+}, EDTA and EGTA on DOC-induced production of DG. Assays contained 3.8 mg of protein/ml and 5 mM DOC. Experiments with Mg^{2+} were performed in the presence of 1 mM EGTA.

Fig. 4. DG produced by treatment is phosphorylated to PA in the presence of $MgATP^{2-}$. Assays contained 3.9 mg protein/ml, 1 mM ATP, 1 mM Mg^{2+}, 0.2 mM Ca^{2+} and various concentrations of DOC. •, DG; ▲, PA.

Brain DG-kinase has also been reported to be profoundly activated by DOC and this activation is also decreased by raising the DOC concentration (27). Parachloromercuribenzoate (0.1 mM) inhibits the phosphorylation of DG while not affecting the activation of PLC by DOC in horse platelets (data not shown).

Inhibition of PLC activity by cyclic-AMP. The DOC-induced activation of PLC is reduced drastically when platelets are preincubated with dibutyryl cyclic-AMP (1 mM) and methylisobutylxanthine (0.22 mM) for 15 min (Fig. 5). Preincubation with cyclic AMP-phosphodiesterase inhibitors (aminophylline plus methylisobutylxanthine) alone is also effective, but to a lesser extent (Fig. 5). Intact platelets are needed to demonstrate these effects of cyclic-AMP. The PLC activity remains inactivated after subsequent dissolution of the platelets with DOC.

Pyridoxal-5'-phosphate inhibits PLC activity. It has been reported recently that pyridoxal-5'-phosphate inhibits platelet aggregation induced by various aggregating agents such as thrombin, ADP and collagen (28). This effect was presumed to be related to an activation of platelet adenylate cyclase (28). The effect of this cofactor on the activation by thrombin and DOC was studied. Preincubation of platelets prelabeled with [^{14}C]AA with pyridoxal-5'-phosphate (3-10 mM) inhibits the thrombin-induced production of PA (70-100%) as well as the release of AA, as shown by a dramatic reduction of the lipoxygenase (65-90%) and cyclooxygenase (35-90%) products. Such treatment

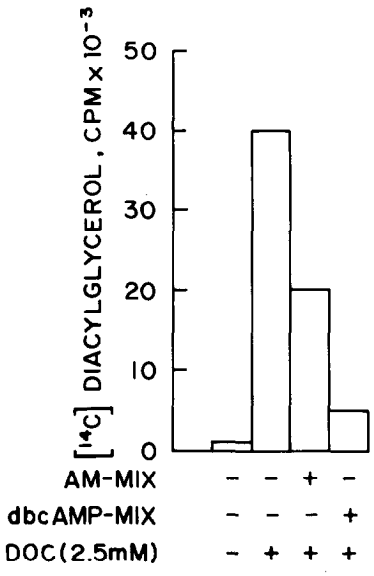

Fig. 5. Effect of dibutyryl cyclic-AMP, aminophylline and methylisobutylxanthine on DOC-induced production of DG. Intact platelets prelabeled with [^{14}C]AA (3.2 mg protein/ml) were preincubated for 20 min with 2.5 mM aminophylline (AM) plus 0.22 mM methylisobutylxanthine (MIX), or with 1 mM dibutyryl cyclic AMP (dbcAMP) plus 0.22 mM MIX, and then with 2.5 mM DOC for 5 min.

with pyridoxal-5'-phosphate also inhibits the DOC-induced expression of PLC activity and thus the production of DG (30-60%). These results are consistent with an action of pyridoxal-5'-phosphate through cyclic-AMP.

Discussion. The breakdown of PI by PLC produces DG, D-myoinositol 1:2-cyclic phosphate and D-myoinositol 1-phosphate (10). PLC specifically cleaves endogenously available PI from amongst a large excess of other phospholipids (Figs. 1,2). In broken cell preparations the activity is detectable only in the presence of DOC, an anionic detergent which has been shown to induce similar activities in several other tissues (10-14). The enzyme shows a specific requirement for calcium ion, the endogenous concentration of which is sufficiently high to elicit maximal activity (Fig. 3). A similar activity observed in the cytosolic fraction of human platelets, which was assayed with exogenous PI as substrate, requires much higher concentrations of Ca^{2+} (i.e., 5 mM) for maximal activation (29,30).

Intact platelets contain only trace amounts of DG, and under our experimental conditions activation with effective concentrations of thrombin, trypsin or ionophore A23187 does not induce detectable increases in the DG content of horse platelets prelabeled with [^{14}C]AA. However, under the same conditions of activation there are marked increases in labeled PA (9). These findings

suggest that stimuli activate an endogenous PLC, and that in vivo the generated DG is immediately and efficiently phosphorylated to PA by a DG-kinase (9,31). Another reflection of the stimulation of PLC is the increased production of [^{32}P]D-myoinositol 1,2-cyclic phosphate in platelets prelabeled with [^{32}-P]ortho-phosphate and exposed to thrombin (Lapetina, unpublished). The present studies demonstrate that DG is produced from PI in the presence of DOC, and conversion to PA occurs when $MgATP^{2-}$ is included in the incubation medium (Fig. 4). These findings suggest a close association between PLC and DG-kinase activities. Such a link appears to be even more effective in intact cells, probably explaining the rapid formation of PA in the absence DG accumulation after platelet stimulation PA formation has previously been considered as reflecting stimulated PI-breakdown (see 9 for references) and thus may provide a simple parameter by which to assess such reactions in intact tissues.

Although calcium appears to be essential for the expression of PLC activity, the involvement of additional factors must also be considered in attempting to explain the mechanism by which this enzyme is activated and regulated by stimuli. In intact platelets, ionophore A23187 is less effective than thrombin in inducing PA production while both agents are equipotent in releasing AA and its metabolites (20). Cell-free preparations exhibit little or no PLC activity even in the presence of a large excess of Ca^{2+}; expression of activity requires DOC. However, a cytosolic PLC from human platelets (30), or from homogenates of horse platelets (unpublished observations), attack exogenously supplied PI in the absence of DOC.

In activated platelets, increased labeling of PA precedes the release of AA (9). Both of these effects require Ca^{2+} (29,30,32,33 and present studies, Fig. 4) and are blocked by cyclic-AMP (9). Cyclic-AMP does not inhibit the release of AA by phospholipase A_2 activity induced by calcium in broken cell preparations (33). However, the effects of cyclic-AMP on PLC persist even after solubilization of the platelets by DOC (Fig. 5). Cyclic-AMP may act primarily or exclusively on PLC, the inhibition of AA release then being secondary to this blockade. The present evidence strongly suggests that the activation of PLC is the first event (9,34) in a sequence of reactions leading to AA release.

Cyclic-AMP (18,19) and pyridoxal-5'-phosphate (28) inhibit platelet aggregation and PLC activity. The kinetics of stimulation, as judged by the increase in labeling of PA (9), closely parallel those of the aggregation and release reactions and suggest an important role for the combined activities of PLC and DG-kinase (and their products) during platelet activation.

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