Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 277-283

EFFECT OF A COLLAGEN-DERIVED OCTAPEPTIDE ON PHOSPHOINOSITIDE TURNOVER AND 43K PROTEIN PHOSPHORYLATION IN COLLAGEN-ACTIVATED PLATELETS

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Received June 1, 1987

SUMMARY: A collagen-derived octapeptide KPGEPGPK which specifically inhibits the activation of platelets by collagen has been tested for its ability to affect the collagen-induced phosphoinositide breakdown and protein phosphorylations. Collagen produced a transient decrease followed by a rapid resynthesis of [32P]-phosphatidyl 4-5 bisphosphate (PIP2) and 4-mono phosphate (PIP). Octapeptide, at a concentration preventing aggregation but allowing shape change, did not impair the phosphoinositide breakdown, whereas the P43 phosphorylation was strongly inhibited. Higher concentrations of peptide which did not permit any shape change were needed to hinder the PIP2 and PIP decrease. Therefore, the octapeptide appears to affect early events of the collagen-induced platelet activation involving the P43 phosphorylation, independently of its effect on the receptor-stimulated phosphoinositide hydrolysis.

The collagen derived octapeptide KPGEPGPK is a specific inhibitor of the collagen induced platelet aggregation and serotonin release (1,2). BEVERS et al (3) showed that it hinders the prothrombin converting activity expressed secondarily to an exposure of negatively charged phosphatidylserine on the outer leaflet of the membrane stimulated by collagen or the combined action of collagen and thrombin. In contrast, it fails to inhibit this procoagulant

Abbrevations: PIP2, phosphatidylinositol 4,5 bis-phosphate; PIP, phosphatidylinositol 4 mono-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; DG, diacylglycerol; IP3, inositol triphosphate; P43 and P20, proteins with 43 and 20 kilodalton molecular weights; PS, phosphatidylserine.

0006-291X/87 $1.50

activity evoked by ionophore or thrombin alone. This peptide also impairs the binding of fibrinogen to platelets activated by collagen but not by ADP (4). Owing to the fact that no inhibition was observed in presence of agonists other than collagen, it appears that the octapeptide does not directly affect the fibrinogen binding to exposed receptors or the prothrombinase activity on a negatively charged surface. It was therefore suggested that it interferes with an activation step which precedes the expression of fibrinogen receptors and the generation of a procoagulant surface. In view of this, we investigated the effect of the octapeptide on early steps of the activation process involving the collagen-induced turnover of the inositol phospholipids. A key event in the initiation of platelet activation is the signal dependent hydrolysis of PIP2 by a specific phospholipase C to produce the second messengers IP3 and DG (for review, see 5, 6). IP3 triggers the mobilization of calcium from the dense tubular system, which leads to the phosphorylation of the myosin light chain (P20). DG initiates the activation of protein kinase C which is responsible for the phosphorylation of the 43K protein (P43). The present study describes the inhibitory effect of the octapeptide on collagen-induced platelet aggregation and P43 phosphorylation, independently of its effect on PIP2 breakdown.

MATERIAL AND METHODS

Platelets in PRP were obtained from ACD-C anticoagulated blood and incubated with [32P]-Pi (370 MBq/ml, Oris, France) for phosholipid metabolism and protein phosphorylations. After 90 min of incubation at 37°C platelets were isolated on metrizamide gradients as previously described (7). The platelets thus obtained were aliquoted. All samples were prewarmed for 2 min with either saline or octapeptide before transfer to an aggregometer cuvette. Platelets were stimulated by addition of 1-3 ug/ml collagen (STAGO). At the indicated times, reactions were terminated by transfer of samples into glass tubes containing 1.5 ml of chloroform/ methanol/12N HCl/0.1M EDTA (20:40:1:2 v/v/v/v/) at 4°C. The mixtures were partitioned into two phases by addition of 1.25 vol each of chloroform and distilled water. Separation of

phases was completed by centrifugation at 200g for 10 min.: the proteins were concentrated at the interphase and the extracted lipids recovered in the lower chloroformic phase. This method allows to analyse lipids and proteins on the same platelet sample.

[32]P-phospholipids were separated by one dimensional thin layer chromatography on silica plates according to Jolles et al (8). [32P] -labeled PIP2, PIP, PI and PA were visualized by autoradiography, recovered by scraping the identified spots, and the radioactivity was counted by liquid scintillation.

[32P]-proteins were solubilized by one hour incubation at 60°C in a buffer containing 2 % SDS (w/v), 20 % glycérol (v/v), 0.01 % bromophenol blue (w/v) and 625 mM Tris-HCl, pH = 10. After reduction with 5 % (v/v) beta-mercapto-ethanol, [32P]-proteins were analyzed by SDS-PAGE using a 13 % (w/v) acrylamide gel. After staining with coomassie blue, gels were dried, and exposed to Kodak XAR. Autoradiograms were scanned with a LKB ultroscan.

RESULTS

Turnover of [32P]-polyphosphoinositides : The variations of [32P] associated with polyphosphoinositides are shown in Figure 1. Activation of platelets by collagen was associated with an hydrolysis of polyphosphoinositides. The breakdown of [32P]-PIP2 occured at 35 seconds, i.e. immediately preceding the beginning of shape change and reached a maximum decline of 22 % of control values. This hydrolysis was followed by a rapid resynthesis up to 24.5 % of control values. A transient decrease in PIP which lagged behind that in PIP2 reached a maximum at 60 sec.. No significant changes in PI during collagen-induced platelet stimulation, could be observed at least in the first two minutes (data not shown). Pretreatment of platelets with octapeptide at low concentrations which did not completely inhibit the aggregation (17 % of intensity), delayed the breakdown of PIP2 by 10 to 20 sec. ; this delay was associated with a prolonged aggregation lag phase. At concentrations of octapeptide which completely prevented aggregation but allowed the shape change, collagen still induced the PPI breakdown ; however, no resynthesis was observed and both PIP2 and PIP remained at these significantly reduced levels. Only higher concentrations of octapeptide which completely inhibited

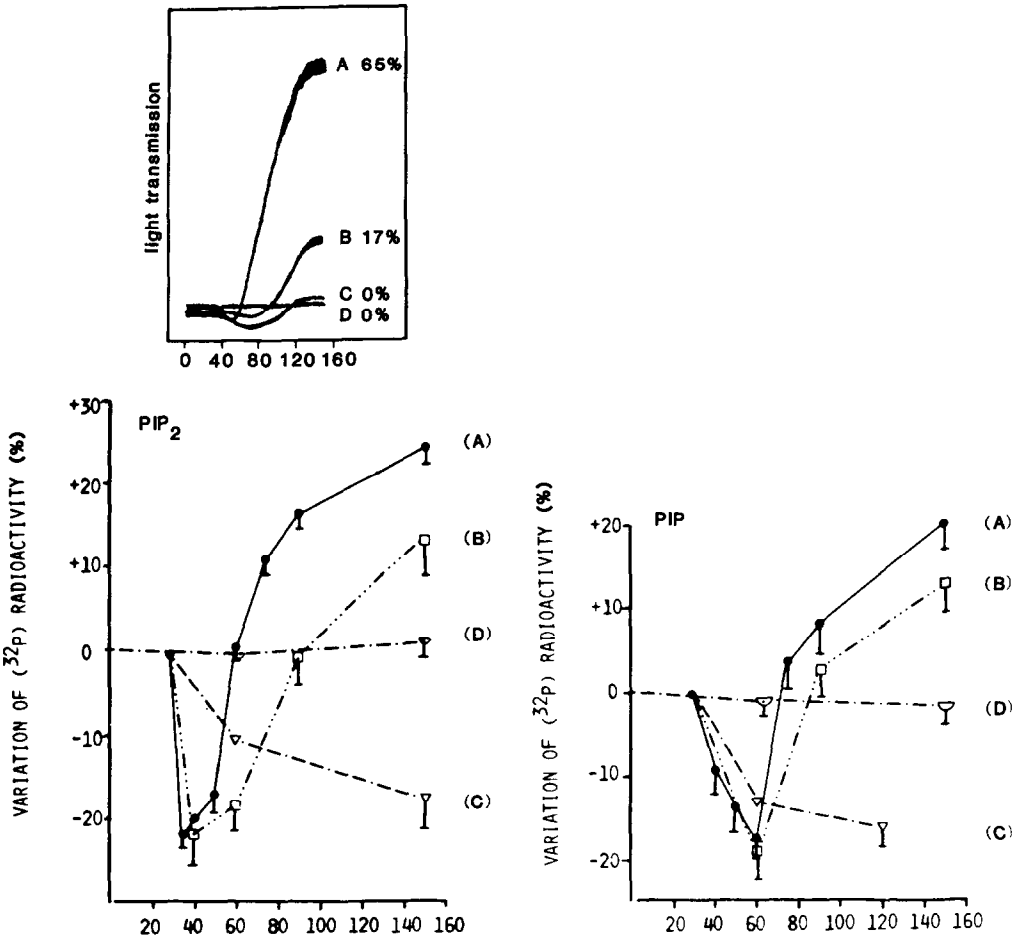
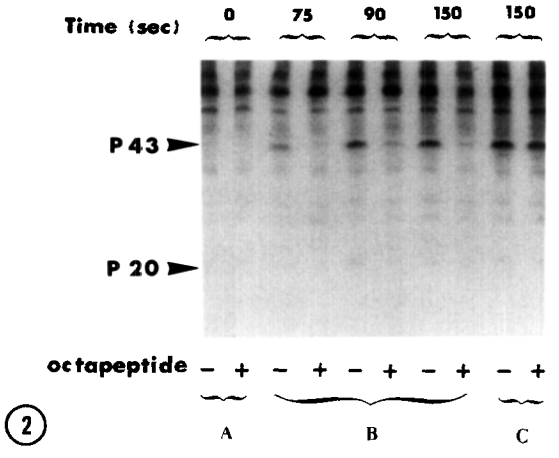


Figure 1. Effect of octapeptide on [32P] - PIP2 and -PIP in platelets exposed to collagen (1-3)ug/ml). Time-course was performed in absence (A) and in presence of 1.5 mM (B), 2.5 mM (C) and 3.5 mM (D) octapeptide. Results are means ± SEM of 5-7 experiments.
Insert : platelet shape change and aggregation tracings in absence (A) or in presence of octapeptide (B, C, D). Percent of intensity are indicated for each concentration.

aggregation as well as shape change totally inhibited any inositol phospholipids metabolism.

Protein phosphorylations : Collagen induced the phosphorylation of P 20 and P 43 (figure 2). The phosphorylation of P 43 was time-dependent. It increased in parallel with the aggregation up to 12.5 % of the total [32]P-radioactivity incorporated into proteins, reaching the same level as observed with thrombin. After octapeptide pre-incubation, collagen-induced phosphorylation of



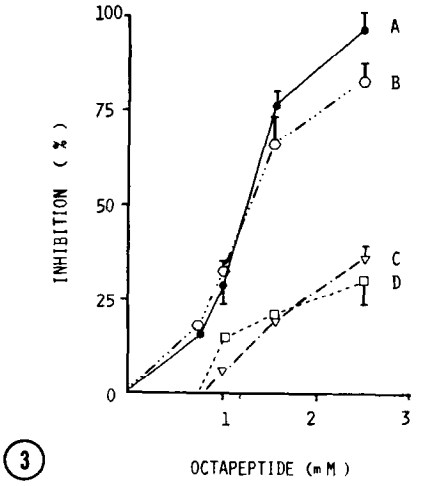


Figure 2. Autoradiogram of P43 and P20 phosphorylations induced either by collagen or thrombin: effect of octapeptide
A) unstimulated platelets, B) platelets stimulated by collagen (1-3 ug/ml) and C) platelets stimulated by thrombin (0.05 u/ml) in absence (-) or in presence (+) of octapeptide (2.5 mM).

Figure 3. Dose-dependent effect of octapeptide on collagen induced platelet aggregation (A), P43 (B) and P20 (C) phosphorylations and phosphatidate formation (D).

P43 was markedly inhibited. In contrast, phosphorylation of P20 was very low and never extended 3 % of the total radioactivity even 150 seconds after collagen addition. Therefore it was difficult to visualize a strong inhibitory effect of octapeptide. As the auto-radiogram clearly showed, the peptide had no effect on thrombin induced phosphorylations.

Differential action of octapeptide : Figure 3 compares the dose-dependent inhibitory effect of octapeptide on different platelet responses. Inhibition of P43 phosphorylation was well correlated with that of aggregration, being inhibited by up to 82 % when aggregation was abolished. In contrast, the collagen-induced PA formation which reached 2-3 times the basal level of the unstimulated platelets (not shown) was only slightly decreased by 30 % $\pm$ 1.5, as was the low level of P20 phosphorylation (35 $\pm$ 6 % of inhibition). Both PA formation and P20 phosphorylation needed high doses of octapeptide to be significantly inhibited.

DISCUSSION

Collagen-induced platelet activation is mediated through the phosphodiesterasic cleavage of pohyphosphoinositides which results in the phosphorylation of P43 and P20. The present study demonstrates that the collagen derived octapeptide strongly reduces this collagen-induced phosphorylation of P43, concomitantly with aggregation whereas shape change and polyphophoinositides breakdown still persisted. These results strongly suggest that octapeptide inhibits the platelet response through either another pathway than polyphosphoinositides turnover or through a mechanism subsequent to the hydrolysis of polyphosphoinositides. In view of this , it is interesting to remember that, whereas the calcium mobilizing IP3 is released to the cytosol, DG operates within the plane of the membrane; the DG-induced activation of protein kinase C depends on its interaction with PS in the membrane phospholipid bilayer (rev.9). Since octapeptide impairs the procoagulant activity at the platelet surface (3) in a process which also involves PS it is not unlikely that it could affect this PS dependent activation of protein kinase C and consequently the P43 phosphorylation.

Collagen provoked a transient breakdown of PIP2 and PIP, while no changes in PI were observed, in agreement with a previous report (10). These data are consistent with the demonstration that PIP2 and PIP but not PI are the substrates for the Ca-independent phospholipase C following receptor activation (11). The octapeptide at concentration which impaired any shape change, inhibited this activation of Ca-independent polyphosphoinositide specific phospholipase C, and hence also the P43 phosphorylation.

In conclusion, independently of its effect on the receptor-stimulated phosphoinositide metabolism, the octapeptide appears to affect P43 phosphorylation which results from a

membrane process . This action on the initial steps of platelet activation would explain its inhibitory effect on the later expression of specific fibrinogen receptors and procoagulant activity on the membrane surface (3,4).

ACKNOWLEDGMENTS

The authors wish to thank Dr. LEFRANCIER and Mr DERRIEN (Institut CHOAY, Montrouge, France) for preparing and donating the octapeptide. This work was supported by Grant n° 82 L 0748 from the Ministère de la Recherche et de l' Industrie.

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