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Interactions between the Activation of Phospholipase A_2(PLA$_2$) and C(PLC) Induced by Thrombin in Human Platelets: Evidence for the Separate Activation of PLA$_2$ and PLC

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Summary. **We investigated the relationship between the activation of phospholipase A_2 (PLA$_2$) and C (PLC) induced by thrombin in human platelets. Lysophosphatidylinositol (LPI) formation, an indicator of PLA$_2$ activation, was completely inhibited by pretreatment with pertussis toxin, but inositol-1, 4, 5-triphosphate (IP$_3$) formation, an indicator of PLC activation, was reduced by about 50%. Prostaglandin E_1 inhibited IP$_3$ formation completely, but only reduced LPI formation by about 50%. However, cholera toxin did not affect either formation. *Conclusion*: 1) The differential sensitivity of PLA$_2$ and PLC to pertussis toxin and PGE$_1$ strongly suggests that the activity of both enzymes is modulated by distinct GTP- binding proteins; 2) pertussis toxin sensitive G-protein is involved in the thrombin-induced platelet activation process, but cholera toxin is not.**

Key words—phospholipase A_2, phospholipase C, G-proteins, human platelets.

INTRODUCTION

Thrombin is one of the most powerful platelet aggregating agents. The human thrombin receptor has been thought to be formed by a single polypeptide chain with seven transmembrane domains and an extracellular N terminus, suggesting that it belongs to the superfamily of G protein-coupled receptors.[1,2] When thrombin binds its receptor via the extracellular N terminus, it cleaves the receptor at residues between Arg^{41} and Ser^{42}, and the newly exposed amino terminus then functions as a tethered peptide ligand, resulting in receptor activation. Furthermore, a 14-amino acid polypeptide corresponding to residues Ser^{42} through Phe^{55} (SFLLRNPNDKYEPF, TRP42/55) can evoke cellular responses, including the activation of phospholipase C (PLC), inhibition of adenylate cyclase, increase in the cytosolic free Ca^{2+} concentration, and increase in tyrosine kinase and phosphatidylinositol-3-kinase activity.[1-9] This suggests that the newly exposed N terminus of the receptor may itself be the true "ligand" for the receptor.

However, the signal transduction distal to the receptor activation and cross talk of the platelet activation pathway remains unclear. In intracellular thrombin-induced platelet activation process, PLC activation has been believed to play a central role in regulating platelet function, since it induces the breakdown of phosphatidylinositol-4, 5-bisphosphate (PIP$_2$) to produce diacylglycerol (DG) and inositol-1, 4, 5-triphosphate (IP$_3$). DG stimulates protein kinase C to phosphorylate, a 47 KD a protein known as pleckstrin,[10] and the liberated IP$_3$ induces Ca^{2+} mobilization from intracellular stores, which then regulates Ca^{2+}-related functions.

In addition to these events, thrombin induces arachidonic acid (AA) release from the platelet membrane. As for the pathway of the AA release, at least three pathways have been believed to exist in human platelets: PLA$_2$ phospholipase D (PLD), and DG/monoglyceride (MG) lipases. There are two types of PLA$_2$ in human platelets, type II PLA$_2$ (sPLA$_2$) and type IV PLA$_2$ (cPLA$_2$). The former enzyme is released from stimulated platelets,[11,12] but whether it contributes to the AA release is still a matter of debate. On the other hand, the involvement of cPLA$_2$

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on the receptor-coupled release of AA has been well documented in platelets.[13,14] PLD also has been reported to be activated by several agonists, including thrombin[15,16] via G-protein (G_E).[17] However, in intact human platelets, PLD activity accounts for only 10-20% of the total phosphatidic acid that accumulates after stimulation by thrombin.[18] Furthermore, the DG/MG lipase pathway has been reported to be unessential for thrombin-induced AA release.[19] These reports suggest that $cPLA_2$ activation is the main pathway which contributes to AA release in thrombin-induced platelets.

However, the relationship between the activation of PLA_2 and PLC induced by thrombin has not been fully elucidated. To clarify this point, we investigated the effect of prostaglandin E_1 (PGE_1), pertussis toxin, and cholera toxin on thrombin- induced inositol-1, 4, 5-triphosphate (IP_3) and lysophosphatidylinositol (LPI) formation as an indicator of PLC and PLA_2 activation, respectively.

MATERIALS AND METHODS

Preparation of washed platelet suspension

Venous blood anticoagulated with one tenth volume of 3.8% sodium citrate was centrifuged at 150 g for 10 min at room temperature to obtain PRP. PGE_1 (Sigma) was added to PRP at a concentration of 0.5 μg/ml to prevent platelet aggregation during the subsequent manipulation. Platelets were then pelleted from PRP by centrifugation at 850 g for 15 min. The platelet pellet was washed with 10 mM Tris/1 mM EDTA/saline buffer (TES buffer), pH 7.5 twice, and was finally resuspended in Ca^{2+}, Mg^{2+} free Tyrode buffer, pH 7.5.

Measurement of $[^3H]$-IP_3 and $[^3H]$-LPI formation induced by thrombin

The washed platelet suspensions (2 to 2.5×10^9/ml) were incubated with $[2\text{-}^3H(N)]$ inositol (20 Ci/mmol, purchased from NEN) at 37℃ for 3 h. The platelet suspension was centrifuged at 850 g for 10 min and the pellet was washed twice with TES buffer. The labeled platelet pellet was resuspended in Ca^{2+}, Mg^{2+}-free Tyrode buffer at a concentration of 1×10^9 platelets/ml. After the $[^3H]$-inositol labeled platelets were preincubated with different concentrations of PGE_1 for three minutes, one milliliter of inositol labeled platelet suspension (1×10^9 platelets containing 5 to 7.2×10^4 disintegrations per minute) was stimulated with thrombin (0.5 IU/ml) at 37℃ for varying times. The reaction was terminated with 3.75 ml of $CHCl_3/CH_3OH/HCl$ (50 : 100 : 1) followed by 1.25 ml of $CHCl_3$ and 1.25 ml of water. The tubes were vortexed and centrifuged at 800 g for 10 min. The aqueous phase was neutralized with 1 M Tris-base and then applied to a Dowex-1-X8 (Sigma, Cl-form) column (0.7×2 cm). The column was eluted stepwise with increasing concentrations of HCl as described by Griffin and Hawthorne.[20] Briefly, $[2\text{-}^3H]$-inositol-1-phosphate (IP_1), $[2\text{-}^3H]$-inositol-1, 4-bisphosphate (IP_2), and $[2\text{-}^3H]$-inositol-1, 4, 5-triphosphate (IP_3) were collected in 2 ml of 30 mM HCl, 90 mM HCl, and 500 mM HCl, respectively, and the radioactivity was determined with a liquid scintillation counter. Triplicate experiments were performed. The results are expressed as percent of controls, because the radioactivity incorporated into inositol phospholipids varied from experiment to experiment. The lower phase (chloroform phase) was removed with a Pasteur pipette and dried under a stream of nitrogen at 25℃; the extracted lipids were separated on silica gel 60 plates with $CHCl_3/CH_3OH/4\ N\ NH_4OH$ (45/35/10 vol/vol) as the solvent system. Spots were identified by comigration with authentic standards. The areas corresponding to phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), phosphatidyilinositol-4, 5-bisphosphate (PIP_2), and lysophosphatidylinositol (LPI) were scraped into vials and the radioactivity was determined in a liquid scintillation counter.

Measurement of $[^3H]$-IP_3 and $[^3H]$-LPI formation induced by thrombin in saponin permeabilized platelets

The $[^3H]$ inositol-labeled platelets were finally suspended in a reaction buffer (140 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.2 mM NAD, 1.5 mM ATP, 10 mM Hepes, pH 7.4). Pertussis toxin and cholera toxin (List Biological Lab. Inc.) were preactivated by incubation for 60 min at 25℃ with dithiothreitol.[21] The platelets were permeabilized with 11 μg/ml of saponin (Sigma) for 5 min in the presence of preactivated pertussis toxin or cholera toxin, and after the addition of thrombin (0.5 IU/ml), incubated for the indicated times. The $[^3H]$-IP_3 and the $[^3H]$ LPI formation were determined by the same method.

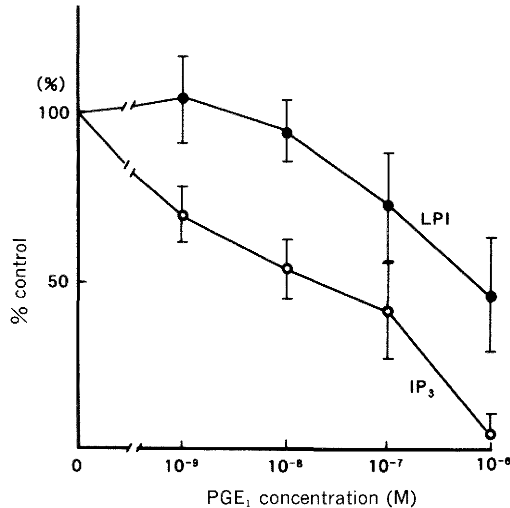

Fig. 1. Effect of PGE_1 on thrombin-induced LPI and IP_3 formation. [^{3}H]-inositol labeled platelets (1×10^9/ml) were pretreated for three minutes with the indicated concentrations of PGE_1 before activation with thrombin (0.5 IU/ml). The reaction was terminated after 15 sec and 2 min of activation of thrombin for IP_3 and LPI determination, respectively. Each was assayed as described in the Materials and Methods and is expressed as percentage inhibition of the control values in the absence of PGE_1. Mean values and standard deviations are shown in the Figure (n=4). (●—●); LPI, (○—○); IP_3.

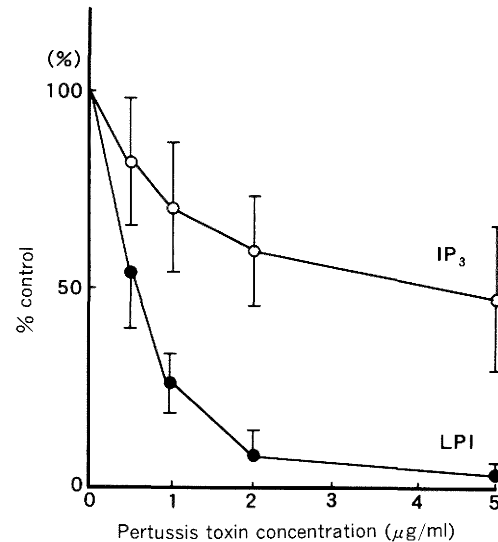

Fig. 2. Effect of pertussis toxin on thrombin-induced LPI and IP_3 formation. [^{3}H]-inositol labeled platelets (1×10^9/ml) were permeabilized with saponin (11 μg/ml) in the presence of various concentrations of preactivated pertussis toxin. After 5 min of incubation, platelets were stimulated with thrombin (0.5 IU/ml). The reaction was terminated at 15 sec and 2 min for IP_3 and LPI determination, respectively. The following details are the same as described in Fig. 1. (●—●); LPI, (○—○); IP_3.

RESULTS

1) Time course of thrombin-induced IP_3 formation and LPI formation

IP_3 formation rapidly increased to reach a plateau at 15 sec after the addition of thrombin, and then rapidly decreased. IP_2 formation reached a plateau at about 1 min, and IP_1 formation gradually increased.

The breakdown of PIP_2, which reflects the formation of IP_3, was rapid, followed by the breakdown of PIP and PI, which reflects IP_2 and IP_1 formation respectively. LPI formation, which is a direct indicator of PLA_2 reached a plateau at 2 min after addition of thrombin. Therefore, we stopped the reaction at 15 sec and 2 min to determine the level of IP_3 and LPI formation, respectively, in the following experiments (data not shown).

2) Effect of PGE_1 on thrombin-induced IP_3 and LPI formation

PGE_1 inhibited thrombin-induced IP_3 formation in a dose-dependent manner. The IP_3 formation was

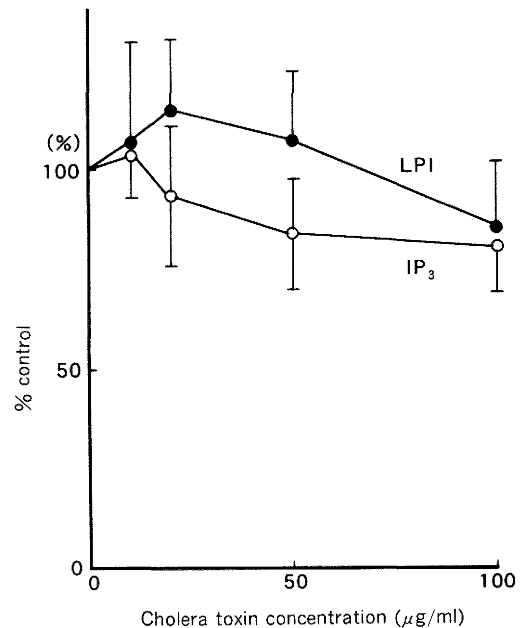

Fig. 3. Effect of cholera toxin on thrombin-induced LPI and IP_3 formation. Details are the same as described for Fig. 3. (●—●); LPI, (○—○); IP_3.

almost completely inhibited at 10^{-6} M PGE_1. However, thrombin- induced LPI formation was much less susceptible to PGE_1, and only decreased by about 50% at 10^{-6} M PGE_1 (Fig. 1).

3) Effect of pertussis toxin and cholera toxin on thrombin-induced IP_3 and LPI formation in permeabilized platelets

Pertussis toxin completely inhibited thrombin-induced LPI formation at more than 2 μg/ml, but only reduced IP_3 formation by about 50%, even at 5 μg/ml (Fig. 2). Cholera toxin did not affect either formation (Fig. 3).

DISCUSSION

We investigated the effect of PGE_1, pertussis toxin, and cholera toxin on thrombin- induced IP_3 and LPI formation.

PGE_1, at 10^{-8} M, inhibited thrombin-induced IP_3 formation by about 50%, but did not affect LPI formation. Even at 10^{-6} M, LPI formation was still found (about 50%) in spite of the complete inhibition of IP_3 formation. Crouch et al.[22] reported that PGI_2 (100 ng/ml), an A-kinase activator as is PGE_1, completely inhibited α-thrombin-induced AA release, but only reduced IP_3 and PA formation by about 50%. The difference from our findings may come from different kinds and concentrations of thrombin or the assay system for the evaluation of PLA_2. The 50% reduction of LPI formation with pretreatment of 10^{-6} M PGE_1 can be explained by the total inhibition of PLC activity. It causes defective IP_3 formation and decreased intracelular Ca^{2+} mobilization which results in reduced $cPLA_2$ activity, since it requires submicromolar concentrations of Ca^{2+}[14]. Furthermore, $cPLA_2$ must be phosphorylated to exhibit full activation.[14] Since the involvement of protein kinase C in the regulation of $cPLA_2$ has also been suggested for many other cells,[23-26] the decreased protein kinase C activation may also account for the decreased PLA_2 activity. However, the most striking finding is that about 50% of LPI formation was still found even in the absence of IP_3 formation. This strongly suggests that PLA_2 can be activated even in the absence of PLC activation.

Pertussis toxin almost completely inhibited thrombin-induced LPI formation at 2 μg/ml, but only reduced IP_3 formation by about 50%. There have been reports that thrombin- induced AA release is abolished by pertussis toxin treatment in human[27] and rabbit platelets.[28] On the other hand, Brass et al.[29,30] reported that about 70% of thrombin-induced phosphatidic acid formation was inhibited by the treatment of pertussis toxin, suggesting that pertussis toxin sensitive G-protein is involved in thrombin-induced phosphoinositide hydrolysis. Although the results are controversial, our findings suggest that thrombin-induced activation of PLA_2 is mediated by the pertussis toxin sensitive G- protein. Furthermore, it strongly suggests that the activity of PLA_2 and PLC is modulated by distinct G-proteins, since pertussis toxin affected differently the LPI and IP_3 formation.

However, cholera toxin did not affect thrombin-induced LPI and IP_3 formation. This suggests that cholera toxin sensitive G-protein is not involved in the thrombin-induced platelet activation pathway.

We measured LPI formation instead of lysophosphatidylcholine (LPC) or lysophosphatidylethanolamine (LPE) formation as an indicator of PLA_2. As for the substrate of PLA_2, phosphatidylcholine (PC) has been believed to be the prominent source of released AA.[31,32] However, both PC and PI have been reported to be hydrolyzed when presented to the partially purified $cPLA_2$ enzyme in Triton X-100 mixed micelles.[33] Based on this fact, and since this assay system has the advantage of simultaneous evaluation of PLA_2 and PLC activation, we chose LPI formation as an indicator of PLA_2 activation.

Taken together, our findings strongly suggest that PLA_2 activation can occur separately from PLC activation. PLA_2 activation is also mediated by a distinct G-protein in human platelets, as reported by us and others.[22,27,34-36] The G-protein involved in PLA_2 activation should be further investigated.

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