Vol. 197, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1993 Pages 62-67

PROTEIN-TYROSINE KINASE p72^{syk} IS ACTIVATED BY THROMBOXANE A$_2$ MIMETIC U44069 IN PLATELETS

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Received September 11, 1993

We show that p72^{syk} is rapidly activated following the stimulation of thromboxane A2 mimetics, U44069 and STA2 in porcine platelets. The activity of p72^{syk} reached a maximum at 10 s and decreased to a basal level within 60 s after 1 μM U44069 stimulation. This activation was enhanced in a dose-dependent manner and completely canceled by the pretreatment of platelet suspension with ONO3708, a specific antagonist of thromboxane A2. Pretreatment of platelets with aspirin as well as apyrase did not affect the activation of p72^{syk}. When both extra- and intra-cellular Ca^{2+} were depleted, the activation of p72^{syk} was still persistent; in contrast, the deactivation process was completely abrogated even at 120 s after U44069 stimulation. These results suggest that p72^{syk} is a responsible enzyme to the protein-tyrosine phosphorylation events, and that p72^{syk} functions mainly before Ca^{2+} recruitment in thromboxane A2-stimulated platelets.

Thromboxane A2 (TXA2) is one of the potent stimulators of platelets and a constrictor of vascular and respiratory smooth muscles. Because of such potent biological activities and its formation in response to various stimuli, it has been implicated as a mediator in many pathophysiological conditions such as stroke, myocardial infarction, tumor metastasis, pulmonary hypertension in sepsis, and anaphylaxis (1).

TXA2 also induces platelet aggregation and secretion (2). It is generated by the TXA2 synthase catalysis from unstable prostaglandin endoperoxides that are products of cyclooxygenase from arachidonic acid that is released by phospholipases upon platelets activation (3, 4). TXA2 has a central role not only in mediating platelet activation but also in amplifying it, especially in the case of weak agonists, by being secondarily liberated into the extracellular space to stimulate inositol phospholipid hydrolysis, Ca^{2+} mobilization, and fibrinogen receptor exposure that can facilitate full activation of platelets (4).

Protein-tyrosine phosphorylation is suggested to be involved in the molecular mechanism in platelet activation, because it was shown that protein-tyrosine phosphorylation of multiple cellular

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Abbreviations: TXA2, thromboxane A2; PTK, protein-tyrosine kinase; SH2 and SH3, *src* homology region 2 and 3, respectively.

0006-291X/93 $4.00

proteins increased in the platelets activated by physiological agonists including thrombin, TXA2, and so forth (5-8). Since the receptors of those agonists do not possess the intrinsic protein-tyrosine kinase (PTK) activity (9, 10), non-receptor PTK is supposed to participate in the intracellular signal-transduction pathway after those receptors. At least, seven non-receptor and one cytosolic protein-tyrosine kinases have been identified in platelets: protein products of *src*, *fyn*, *yes*, *hck*, *lyn*, FAK, and *syk* (11-13), and CPTK71 (14), in which only $p72^{syk}$, $p125^{FAK}$, and $p60^{src}$ have been reported to be activated upon platelet stimulation (12, 13, 15).

$p72^{syk}$ is a member of non-receptor PTK and has a unique structural character, possessing the second *src* homology region 2 (SH2) instead of SH3 in its amino-acid sequence (16). It is expressed mainly in hematopoietic cells; splenocytes (17), platelets (18), tonsilocytes (19), polymorphonuclear leukocytes (20), and peripheral blood lymphocytes (21). In those cells, rapid activation of $p72^{syk}$ is observed upon the stimulation by wheat germ agglutinin (17, 18), thrombin (13), anti-IgM antibodies (19), and concanavalin A (20, 21), respectively. In this report, we show that $p72^{syk}$ is activated primarily by TXA2 mimetics, U44069 (22, 23) and STA2 (24), and is negatively regulated through Ca^{2+}.

EXPERIMENTAL PROCEDURES

Materials and Methods --- The sources of materials used in this work were as follows: 9, 11-dideoxy-9α, 11α-epoxymethanoprostaglandin F2α (U44069), apyrase, and EGTA from Sigma, acetylsalicylic acid from Wako Pure Chemicals, and acetoxymethyl ester of 5, 5'-dimethyl-bis-(o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid (BAPTA-AM) from Dojin Chemicals. STA2 and ONO3708 were provided by Ono Pharmaceutical Co., Ltd. Anti-CPTK40 antibodies, raised against the synthetic partial polypeptide of $p72^{syk}$ in rabbits, were prepared as described (16). Porcine blood was obtained at a local slaughterhouse.

Isolation and Stimulation of Platelets --- Porcine platelets were isolated by differential centrifugation as described (13) and were finally suspended at the concentration of 10^9 cells/ml in modified Tyrode-Hepes buffer (135 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 20 mM Hepes/NaOH, pH 7.4) containing 1 mM Ca^{2+}. The supernatant prepared from blood by centrifugation at 1,500 x g for 15 min was used as platelet poor plasma. In the case of Ca^{2+} depletion, platelet suspension was incubated with 15 μM BAPTA-AM for 30 min at 37 °C, then sedimented, and finally suspended in modified Tyrode-Hepes buffer containing 2 mM EGTA. Platelet poor plasma (2 μl/ml) was added in platelet suspension just before stimulation. Platelets were stimulated by the addition of U44069 dissolved in ethanol (final ethanol concentration is below 1 %) for indicated time.

Immunoprecipitation Kinase Assay --- After platelets were stimulated, cells were sedimented by a quick centrifugation and the resulting pellets were lysed with lysis buffer (2 % Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 100 μM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) as described (18). The lysates were immunoprecipitated with anti-CPTK40 antibodies and were phosphorylated as described (18). After separation of samples on an SDS polyacrylamide gel, the activity of $p72^{syk}$ was estimated by autoradiography.

RESULTS

Activation of $p72^{syk}$ by U44069 stimulation in platelets --- U44069 induced a rapid increase in the radioactivity corresponding to a 72-kDa band which reached a maximum at 10 s and then decreased to a basal level within 60 s (Fig. 1). Phosphoamino acid analysis and KOH treatment revealed that the phosphorylated amino acid of the band was exclusively tyrosine in all lanes (Ref. 18 and data not shown). The amount of precipitated $p72^{syk}$ did not change throughout the time

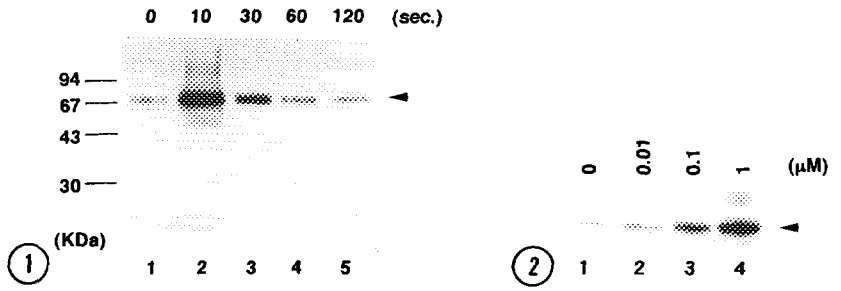

Fig. 1. Time course of p72*syk* activation induced by U44069 stimulation. Platelets were stimulated with 1 μM U44069 for 0 (lane 1), 10 (lane 2), 30 (lane 3), 60 (lane 4), or 120 s (lane 5) and were subjected to immunoprecipitation kinase assay as described under "EXPERIMENTAL PROCEDURES". Positions of molecular mass markers are shown to the left in kilodaltons. An arrow head indicates the position of p72*syk*.

Fig. 2. Dose dependency of p72*syk* activation induced by U44069 stimulation. Platelets were stimulated for 10 s with 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3), or 1 μM (lane 4) of U44069. Then, immunoprecipitation kinase assay was performed as described under "EXPERIMENTAL PROCEDURES". An arrow head indicates the position of p72*syk*.

course judging from the immunoblot analysis (data not shown). As shown in Fig. 2, this activation was enhanced in a dose-dependent manner.

Effect of ONO3708 and aspirin as well as apyrase on the activation of p72syk induced by U44069 or STA2--- To confirm p72*syk* activation induced by other TXA2 agonist, we used STA2, which also induced rapid activation of p72*syk* in the same pattern as U44069 did (Fig. 3a and data not shown). In order to test the specificity of the activation for TXA2, we employed ONO3708, a TXA2 specific antagonist (25). As shown in Fig. 3a, the activation induced by STA2 was abrogated in a dose-dependent manner when ONO3708 was added in the platelet suspension. Next, since platelets are activated not only primarily by an added agonist itself but also by substances secondarily secreted from platelets themselves following platelet activation (4),

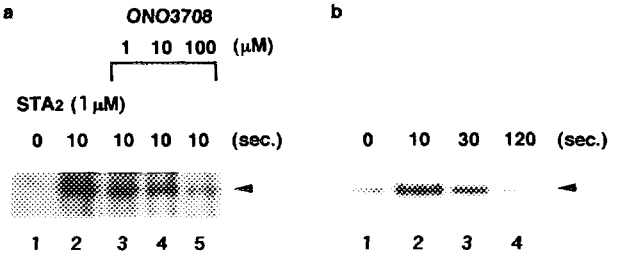

Fig. 3. Effect of ONO3708 and aspirin as well as apyrase on p72*syk* activation induced by U44069 or STA2 stimulation. (a) Platelets were preincubated without (lanes 1 and 2) or with 1 (lane 3), 10 (lane 4), or 100 μM (lane 5) of ONO3708 for 1 min, and stimulated with 1 μM STA2 for indicated time, followed by immunoprecipitation kinase assay. (b) Platelets were preincubated with 1 mM aspirin for 30 min at 37 °C and in addition, 0.5 unit/ml apyrase was added 1 min before stimulation. They were stimulated with 1 μM U44069 for the indicated time and were processed for immunoprecipitation kinase assay. An arrow head indicates the position of p72*syk*.

we employed aspirin and apyrase in order to know whether this activation was primary or not. As shown in Fig. 3b, the activation pattern of $p72^{syk}$ induced by U44069 was affected neither by aspirin pretreatment nor by inclusion of apyrase in platelet suspension. These results suggest that this activation of $p72^{syk}$ is the primary reaction triggered specifically by TXA2 agonists.

Effect of the depletion and replenishment of Ca^{2+} on the activation of $p72^{syk}$ induced by U44069 --- Since the activation of $p72^{syk}$ is independent of Ca^{2+} and is negatively regulated through Ca^{2+} in thrombin-stimulated platelets, we have investigated the relationship between the activation of $p72^{syk}$ and Ca^{2+} in U44069-stimulated platelets. In the Ca^{2+}-depleted condition, the activation of $p72^{syk}$ was still persistent; in contrast, the deactivation was completely abolished even at 120 s (Fig. 4, lanes 1 to 4). However, the replenishment of Ca^{2+} resulted in the deactivation of $p72^{syk}$ (Fig. 4, lanes 5 to 7) in a manner similar to that seen in the Ca^{2+}-rich condition (Fig. 1). These results suggest that the activation process of $p72^{syk}$ is independent of Ca^{2+} but deactivation process requires Ca^{2+} mobilization in U44069-stimulated platelets.

DISCUSSION

TXA2 induces platelet shape change and irreversible aggregation in human platelets that are mediated by a TXA2/prostaglandin endoperoxide receptor (4). The receptor is linked through a pertussis toxin insensitive G-protein to a phospholipase C which hydrolyzes membrane phosphoinositides, resulting in the release of two intracellular messengers; inositol-1,4,5-triphosphate and diacylglycerol (26). The former releases calcium from the intracellular storage and the latter stimulates protein kinase C, cooperatively inducing full response of platelet. However, the inhibitor of protein tyrosine kinase was found to inhibit TXA2-induced platelet activation including aggregation, secretion, and phospholipid turnover (8). In addition, three non-receptor PTKs, $p72^{syk}$, $p125^{FAK}$, and $p60^{src}$ have been recently shown to be activated in thrombin-stimulated platelets (12, 13, 15), suggesting the involvement of PTK in the signal transduction upon the platelet activation.

In this study, we employed U44069 and STA2 as stable TXA2 mimetics (22-24), to demonstrate that $p72^{syk}$ was rapidly activated after U44069 stimulation and the activation was the

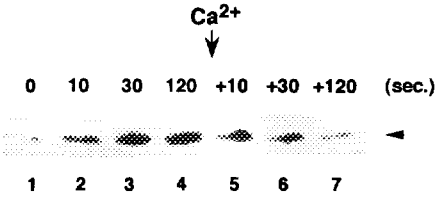

Fig. 4. Effect of depletion and replenishment of Ca^{2+} on $p72^{syk}$ activation induced by U44069 stimulation. Ca^{2+}-depleted platelets were stimulated by 1 μM U44069 for 0 (lane 1), 10 (lane 2), 30 (lane 3), or 120 s (lane 4). In lanes 5-7, 5 mM Ca^{2+} was added after a 120-s stimulation and the incubation was continued for additional 10 (lane 5), 30 (lane 6), or 120 s (lane 7). Immunoprecipitation kinase assay was performed as described under "EXPERIMENTAL PROCEDURES". An arrow indicates the addition of Ca^{2+} and an arrow head does the position of $p72^{syk}$.

primary reaction triggered specifically by TXA2 agonists in porcine platelets. Furthermore, the activation process of $p72^{syk}$ was independent of Ca^{2+} but the deactivation process required Ca^{2+} mobilization in U44069-stimulated platelets. These results suggest that $p72^{syk}$ is participating in the U44069-induced increase of intracellular Ca^{2+} and that $p72^{syk}$ is negatively regulated through Ca^{2+} by feed back mechanism.

The $p125^{FAK}$ has also been reported to be activated upon platelet stimulation (12). However, the activation of $p125^{FAK}$ is evoked rather slowly than that of $p72^{syk}$ and is dependent on the platelet aggregation and the events induced by fibrinogen binding to GP IIb-IIIa, that require Ca^{2+} recruitment in platelets (4). Thus, the activation of $p125^{FAK}$ is likely to occur downstream of the platelet aggregation mediated by GP IIb-IIIa (12). In the case of $p72^{syk}$, the activation occurs rapidly following stimulation with the peak at 10 s (Fig. 1 and Ref. 13) and is observed even in the Ca^{2+}-depleted condition (Fig. 4), in which the aggregation is absent. These lines of evidence suggest that $p72^{syk}$ and $p125^{FAK}$ share distinct roles; the former functions in an earlier phase of platelets activation, upstream of Ca^{2+} mobilization, and the latter does downstream of integrin-dependent events that accompany platelet aggregation.

Acknowledgments: We are grateful to Dr. Yutaka Fujii for advice on U44069, STA2, and ONO3708. This study was supported by Grant-in-Aids for General Scientific Research and for Co-operative Research from the Ministry of Education, Science and Culture, Japan, and Yamanouchi Foundation for Research on Metabolic Disorders.

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