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Phosphoinositide 3-kinase induced activation and cytoskeletal translocation of protein kinase CK2 in protease activated receptor 1-stimulated platelets

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ABSTRACT

CK2 is a highly conserved protein kinase involved in several cellular events. CK2 is expressed in platelets but its role in platelet activation remains poorly understood. In the present study, we tested the hypothesis that CK2 plays a role in platelet activation, particularly in the PAR1-dependent signal transduction pathway. The effect of CK2 and PI 3-kinase inhibitors on aggregation of platelets, activation of GPIIb/IIIa, activation and translocation of CK2 was examined. Platelets were incubated with the cell permeable CK2 inhibitors, DRB, DMAT and TBB and stimulated with the PAR1-AP (SFLLRNP). CK2 inhibitors showed the specific inhibitory pattern of platelet aggregation, characterized by a primary phase of aggregation followed by progressive disaggregation. CK2 inhibitors suppressed the activation of GPIIb/IIIa. PAR1-AP induced two-fold increase in CK2 activity and stimulated the translocation of CK2 from Triton X-100-soluble to -insoluble fraction. Preincubation of platelets with the PI 3-kinase inhibitor, wortmannin or LY294002, impaired PAR1-AP-induced aggregation of platelets. PAR1-AP-induced increase in CK2 activity and translocation of CK2 were inhibited by these treatments. Taken together, the present study demonstrated, for the first time, that PI 3-kinase-CK2 pathway plays an important role in the mechanism of PAR1-dependent platelet aggregation.

Introduction

Protein kinase CK2 is a highly conserved, pleiotropic, protein serine/threonine kinase that can be isolated in an active form from cytoplasm, nuclei and mitochondria [1]. CK2 has been implicated in diverse physiological processes such as gene expression, protein synthesis, cell cycle and proliferation, as well as in various pathological states including carcinogenesis and viral tumorigenesis [2]. CK2 is usually found as a tetramer with an ααββ, αα'ββ or α'α'ββ form. The α and α' subunits possess catalytic activity while the β subunit exerts a regulatory function. Unlike the majority of protein kinases, CK2 utilizes both ATP and GTP as phosphate donors and is inhibited by heparin [1]. In addition, there is increasing evidence that CK2 is involved in the maintenance of cell morphology, cell polarity and in the regulation of actin and tubulin of the cytoskeleton. CK2 is also expressed in platelets [3] but its role in platelet activation is unclear.

Human platelets have two PARs, PAR1 and PAR4, which are members of the G protein-coupled receptor family containing seven transmembrane domains [4]. Thrombin cleaves extracellular regions of PAR1 and PAR4 at the amino-terminal exodomain and unmasks tethered sequences, PAR1-AP and PAR4-AP, respectively, subsequent bindings of these ligand sequences to the receptors are coupled with transmembrane signalings, leading to rapid activation of GPIIb/IIIa that induces aggregation of the platelets. Although PAR1 is activated at the low concentration of thrombin, higher concentration of thrombin is required to activate PAR4. Subsequent signaling is coupled with an activation of heterotrimeric G proteins. Activation of Gαi is coupled with activation of PI 3-kinase [5] and inhibition of adenyl cyclase. PI 3-kinase translocates to the cytoskeleton in

Abbreviations: DMAT, 2-dimethylamino-4,5,6,7-tetrabromo-benzimidazole; DRB, 5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole; GP, glycoprotein; PAR, protease activated receptor; PAR1-AP, PAR1-activation peptide; PI 3-kinase, phosphoinositide 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PRP, platelet-rich plasma; TBB, 4,5,6,7-tetrabromo-2-azabenzimidazole.

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thrombin-activated platelets [6,7]. It has been reported that PI 3-kinase is involved in prolonged GPIIb/IIIa activation and that its inhibition causes disaggregation of platelets [8].

In the present study, we examined whether CK2 is activated by PAR1-AP in platelets. Our results showed that inhibitors of CK2 and PI 3-kinase impaired PAR1-AP-induced aggregation of platelets and that CK2 is active even in resting platelets but increased about two-fold its activity by treating platelets with thrombin or PAR1-AP. Furthermore, we showed that cytoskeletal translocation of CK2 occurs in a PI 3-kinase-dependent manner in PAR1-AP-stimulated platelets. Taken together, these observations suggest that CK2 may play an important role in the prolonged activation of GPIIb/IIIa and sustained aggregation of platelets.

Materials and Methods

Materials

Polyclonal antibodies against CK2α and CK2α' were purchased from Santa Cruz Biotech (Santa Cruz, CA), FITC-labelled monoclonal antibody against activated GPIIb/IIIa (PAC-1) [9] from Becton Dickinson (San Jose, CA) and protein G-Sepharose 4 Fast Flow from Amersham Pharmacia Biotech (Amersham, United Kingdom). PAR1-AP (SFLLRNP) was from SIGMA GENOSYS (Hokkaido, Japan), wortmannin, LY294002 and DRB [10] were from Biomol (Plymouth, PA) and DMAT [11] was purchased from CALBIOCHEM JAPAN(Tokyo, Japan). Casein, TBB [12] and leupeptin were from Sigma (St Louis, MO), phenylmethylsulfonyl fluoride (PMSF), EGTA, Na_3VO_4 and β-glycerophosphate were obtained from Nacalai Chemicals (Kyoto, Japan). [γ-^{32}P]ATP (3,000 Ci/mmol) was from GE Healthcare Bio-Sciences (Piscataway, NJ), [γ-^{32}P]GTP (3,000 Ci/mmol) from PerkinElmer (Yokohama, Japan) and *Complete* tablets from Roche (Mannheim, Germany).

Preparation of platelets

Blood was drawn from drug-free volunteers into plastic syringes containing 0.1 volume of 3.8% trisodium citrate and was centrifuged at 70×*g* for 20 min at room temperature. The supernatant (PRP) was either used for platelet aggregation or for preparation of washed platelets. For preparation of washed platelets, PRP was acidified to pH 6.5 with 1 M citric acid and aliquot of 1 ml of PRP was then centrifuged (5000 rpm, 1 min). After supernatant was removed, platelets were resuspended in 100 μl of Ca^{2+}-free Tyrode buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.4) at a concentration of 2.0×10^9 cells/ml, kept at 37 °C and finally Ca^{2+} was added.

Platelet Aggregation

PRP was preincubated with various concentrations of DRB, TBB, DMAT, wortmannin or LY294002 for 15 min in the dark. Following stimulation with 15 μM PAR1-AP, the change in light transmission was monitored with HEMA TRACER 212 (MC Medical, Tokyo, Japan).

GPIIb/IIIa activation

Human platelet GPIIb/IIIa activation was assessed using fluorescence activated cell sorting (FACS) [13]. The platelets in these samples were pre-treated for 15 minutes with various concentrations of inhibitors. PAC-1 antibody (10 μl each) was added to 5 μl of suspension. Platelets were activated with 15 μM PAR1-AP, incubated for 10 minutes at room temperature without stirring, then fixed for 10 min by addition of 1 ml of 2% formaldehyde. The platelets were analyzed using the single fluorochrome FACS method, and were identified by their side- and forward-light scatter profile. The platelets were gated, and 10,000 cells were analyzed for FITC fluorescence to quantify the amount of platelet-bound PAC-1.

Immunoprecipitation

After 1 min activation with 15 μM PAR1-AP, washed platelets were solubilized with an equal volume of 2× ice-cold lysis buffer (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EGTA, 2 mM PMSF, 2 mM Na_3VO_4, 100 μg/ml leupeptin, 2% Triton X-100, and 1 tablet of *Complete* in 25 ml lysis buffer) and kept on ice for 30 minutes. All subsequent steps were performed at 4 °C. The lysates were centrifuged at 15,000 rpm for 5 minutes and the resulting supernatants were precleared by incubating with protein G-Sepharose for 30 minutes. The supernatants were then incubated with the appropriate antibodies, followed by the addition of protein G-Sepharose. The precipitates obtained after centrifugation were washed 3 times with 1× lysis buffer and processed for renaturable kinase assay.

Subcellular fractionation of platelets

Platelets were stimulated with 15 μM PAR1-AP for 1 min and the reaction was stopped by adding 2 x extraction buffer (1 x buffer; 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 50 mM β-glycerophosphate, 6 mM DTT, 0.1 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF, 0.01% leupeptin and 1% Triton-X 100)(4×10^9 cells/ml of extraction buffer) on ice for 60 min. Cell lysate was centrifuged (8,000×*g*) for 10 min. The supernatant was used as platelet extract for CK2 holoenzyme assay. The pellet was solubilized with 1 x Laemmli buffer and used as cytoskeleton fraction for renaturable protein kinase assay.

CK2 holoenzyme assay

The CK2 assay was based on the transfer of ^{32}P from [γ-^{32}P]GTP to casein. For the CK2 assay [14,15], 10 μl of platelet extract was used. The reaction was initiated by the addition of [γ-^{32}P]GTP to reaction mixture with casein in the amount of 40 μg and incubated at 30 °C for 4 min. The reaction was terminated by adding 200 μl of 15% (w/v) trichloroacetic acid and 50 μl of 0.16% (w/v) bovine serum albumin, precipitated and then washed with 10% (w/v) trichloroacetic acid for 3 times. The radioactivity of protein in the pellet was measured with Cherenkov radiation.

Renaturable protein kinase assay

Renaturable protein kinase assay was performed as described previously [16–18]. Casein (0.5 mg/ml) was added to the polyacrylamide gel solution just prior to polymerization. After SDS-PAGE using casein-containing 10% (w/v) polyacrylamide slab gels (0.1×7×10 cm), SDS was removed from the gel by washing with 20% (v/v) 2-propanol in 50 mM Tris-HCl, pH 8.0 (30 min×2) followed by washing with 50 mM Tris-HCl, pH 8.0, 50 mM 2-mercaptoethanol (30 min ×2) at room temperature. The gel was then treated with 50 mM Tris-HCl, pH 8.0, 50 mM 2-mercaptoethanol and 6 M guanidine HCl (30 min×2) at room temperature and renatured for 16 h with several changes of ice-cold buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM 2-mercaptoethanol and 0.04% (v/v) Tween 40. The gel was then preincubated in reaction buffer (10 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EGTA, 5 mM $MgCl_2$) for 30 min. Protein kinases were detected by incubating each gel at room temperature for 60 min in 10 ml reaction buffer containing 2 μM [γ-^{32}P]ATP (10 μCi/mL). Finally, the gels were washed extensively with 5% (w/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate. The gels were dried and processed for autoradiography.