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CALYCULIN A INHIBITS THE EXPOSURE OF FIBRINOGEN RECEPTOR IN THROMBIN-STIMULATED PLATELETS

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Calyculin A (CLA) and okadaic acid (OA), specific and potent inhibitors of protein phosphatase 1/2A,inhibit platelet aggregation. However, their inhibitory mechanisms remain unknown. We investigated the effects of CLA on the exposure of fibrinogen receptor in thrombin-stimulated platelets, using flow cytometry with a monoclonal antibody against the fibrinogen receptor of activated glycoprotein(Gp)IIb/IIIa complex (PAC-1). CLA inhibited the exposure of fibrinogen receptor in a dose related manner when added either before or 3 min after thrombin stimulation. In contrast, CLA had no significant effect when the expression of GpIIb/IIIa complex was examined in resting platelets, using a monoclonal antibody recognizing non-activated GpIIb/IIIa complex (NNKY1-32). These results suggest that protein phosphatase 1/2A may be directly involved in the exposure of platelet fibrinogen receptor.

The binding of fibrinogen to activated glycoprotein (Gp) IIb/IIIa complex (fibrinogen receptor) plays a key role in platelet aggregation (1,2). Although Ca++ is essential for the formation of GpIIb/IIIa complex(3), the regulatory mechanism(s) in the exposure of fibrinogen receptor has not been well elucidated yet. We recently reported that okadaic acid (OA) or calyculin A (CLA), potent inhibitors of type 1 and 2A protein phosphatases, suppressed thrombin induced platelet reaction such as aggregation, ATP secretion and Ca++ influx (4,5). However, the involvement of these protein phosphatases in platelet reaction remains unknown. As GpIIb/IIIa complex is also known to be involved in Ca++ influx in platelets(6), we investigated the effects of CLA on the exposure of fibrinogen receptor (activated GpIIb/IIIa complex) in thrombin stimulated platelets, using flow cytometry with monoclonal antibodies against different epitopes of GpIIb/IIIa complex.

MATERIALS AND METHODS

Materials:
Calyculin A was purchased from Wako Pure Chemicals Co (Osaka, Japan). Thrombin (bovine) was donated by Mochida Pharmaceutical Co. (Osaka, Japan). PAC-1, a murine monoclonal antibody against the activated form of GpIIb/IIIa complex (fibrinogen receptor) was generously supplied by Dr. Shattil, University of Pennsylvania (Philadelphia,PA)(7). NNKY1-32, a murine monoclonal antibody which recognizes an

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epitope other than the fibrinogen receptor of GpIIb/IIIa complex was kindly gifted from Dr. Shousaku Nomura, Kansai Medical College (Osaka, Japan)(8). PD-10 column was obtained from Pharmacia.

Thrombin-induced platelet activation and treatment with CLA:
Washed platelets were prepared from venous blood drawn from healthy adult donors. Briefly, platelet rich plasma was prepared from citrated blood (40 ml) by centrifugation at 170xg for 15 min and platelets were washed twice with HEPES glucose buffer (10 mM HEPES, pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 5 mM glucose). After 5 min incubation with CLA or vehicle (ethanol, final concentration: 0.5%), platelets ($5x10^8$ /ml) were stimulated by thrombin (0.1 U/ml) for 5 min at 37°C. In some experiments, CLA or vehicle was added 3 min after thrombin stimulation. Platelets were then fixed with 1% paraformaldehyde and subjected to flow cytometric analysis.

FITC labeling of PAC-1:
One ml of PAC-1 solution (1 mg/ml) was dialyzed overnight against 4 liter of carbonate buffer (0.16M Na_2CO_3, 0.33 M $NaHCO_3$, pH 9.5) and mixed with 200 μg of FITC, dissolved in dimethyl sulfoxide (10 mg/ml). The mixture was kept in the dark at room temperature for 4 hours. It was applied on a PD-10 column previously equilibrated with phosphate buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4), and was eluted with the same buffer. Fluorescein isothiocyanate (FITC)/protein molar ratio of each fraction was determined by measuring optical density at 280 nm and 495 nm. FITC/IgM ratio of PAC-1 was 2.3. FITC-conjugated PAC-1 antibody was stored until use at 4 °C in the presence of 0.1% sodium azide.

Flow cytometric analysis:
Platelets treated with 1% paraformaldehyde were incubated with FITC-conjugated PAC-1 (final concentration; 30 μg/ml) at room temperature for 30 min. The samples (100 μl) were then diluted with 1 ml of phosphate buffered saline (pH 7.5) and analyzed using a Becton Dickinson FACScan flow cytometer (San Jose, CA). The FACScan equipped with a 5 W argon laser was operated at 550 mW at a wavelength of 448 nm. Data were analyzed by FACScan Research software. To exclude the background scatter of electronic noise, the platelet gate was set at the left hand border of unstimulated platelets. Ten thousand FITC positive platelets were analyzed in each sample.
In experiments with NNKY1-32, fixed platelets (100 μl) were incubated with NNKY1-32 (10 μg/ml) for 30 min and then with FITC-labeled goat anti-mouse IgG (20 μg/ml) for another 30 min. The samples (112.5 μl) were then diluted with 1 ml of phosphate buffered saline (pH 7.5) and analyzed by a FACScan flow cytometer as described above.

RESULTS

When the exposure of fibrinogen receptor (activated GpIIb/IIIa complex) was examined using flow cytometry with PAC-1, a single peak of intensive fluorescence was observed in thrombin (0.1 U/ml) stimulated platelets (Fig 1, upper panels). Preincubation with 20 nM CLA significantly suppressed this peak of fluorescence (Fig 1, lower panels). The fibrinogen receptor was fully exposed at 30 sec after thrombin (0.1 U/ml) stimulation and its exposure was not altered for at least 5 min thereafter. CLA inhibited thrombin induced exposure of fibrinogen receptor in a dose related manner (Fig 2). In contrast, when the exposure of non-activated GpIIb/IIIa complex was examined in resting platelets using NNKY1-32, CLA did not show any inhibitory effects (Fig 3).

As CLA inhibits thrombin induced platelet activation, the inhibitory effect of CLA might result from the inhibition of activation process leading to the exposure of fibrinogen receptor. To investigate this possibility, CLA was added 3 min after thrombin stimulation, at which the fibrinogen receptor is fully exposed. The addition of CLA after

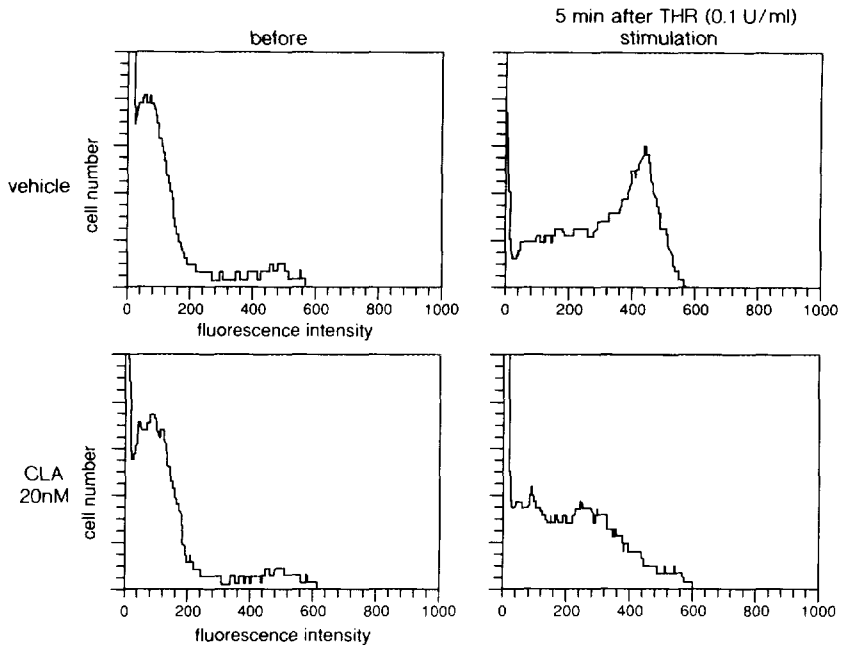

Figure 1. The effect of CLA on the exposure of fibrinogen receptor in thrombin stimulated platelets.
Washed platelets were incubated with 20 nM CLA or vehicle (ethanol, final concentration, 0.5% ethanol) for 5 min and stimulated by thrombin (0.1U/ml). The exposure of fibrinogen receptor was determined by flow cytometric analysis with PAC-1 as described in the text.

thrombin stimulation also suppressed the exposure of fibrinogen receptor in a dose related manner (Fig 4).

DISCUSSION

We demonstrated that CLA, a potent and specific inhibitor of protein phosphatase 1/2A, inhibited the exposure of fibrinogen receptor in thrombin stimulated platelets. This

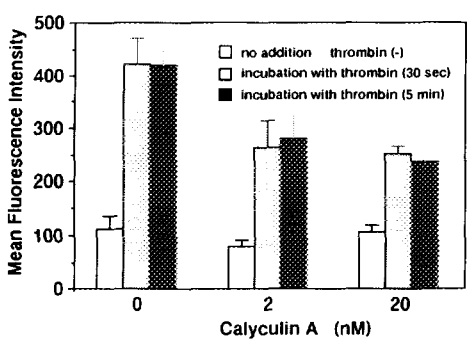

Figure 2. The dose dependent effect of CLA on the exposure of fibrinogen receptor in thrombin stimulated platelets.
Washed platelets were incubated with CLA for 5 min and thrombin induced exposure of fibrinogen receptor was determined by flow cytometric analysis with PAC-1 as described in the text. Data represent means ± SD of three different experiments.

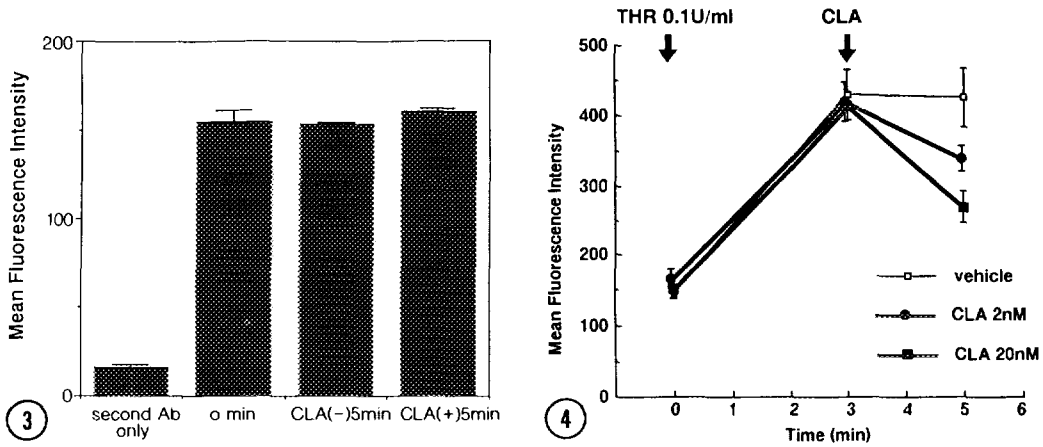

Figure 3. The effect of CLA on the exposure of GpIIb/IIIa in resting platelets.
Washed platelets were incubated for 5 min with or without 20 nM CLA and the exposure of GpIIb/IIIa was determined by flow cytometric analysis with NNKY1-32 as described in the text. Data represent mean ± SD of three different experiments.

Figure 4. The effect of CLA on fibrinogen receptor exposed 3 min after thrombin stimulation.
Washed platelets were stimulated by thrombin (0.1 U/ml) for 3 min and then CLA or vehicle (ethanol, final concentration, 0.5%) was added. The exposure of fibrinogen receptor was determined by flow cytometric analysis with PAC-1 as described in the text. Data represent means ± SD of three different experiments.

inhibitory effect does not seem to result from the nonspecific effect on platelet membrane because CLA had no significant effects on the expression of GpIIb/IIIa complex when examined using NNKY1-32, a monoclonal antibody recognizing an epitope different from the fibrinogen receptor of GpIIb/IIIa complex. Although CLA suppressed thrombin induced platelet reaction (3,4,9), its inhibitory effect on the exposure of fibrinogen receptor may not result simply from the inhibition of platelet activation because CLA exerted its effect even after thrombin stimulation (Fig 4). Similar inhibitory effects were also observed with OA, another potent inhibitor of protein phosphatase 1/2A (data not shown). Furthermore, CLA (20 nM) had no significant effect on the membrane fluidity of platelets when examined using 1-anilino-8-naphthalene-sulfonate (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH)(unpublished data). These results suggest that in addition to Ca++ dependent mechanisms (2,3), protein phosphatase 1/2A may be directly involved in the exposure of fibrinogen receptor in activated platelets.

The cytoplasmic domain of GpIIIa is phosphorylated on serine and threonine residues by protein kinase C or Ca++ / calmodulin-dependent kinase II (10,11). Therefore, the phosphorylation and dephosphorylation of these residues may regulate the exposure of fibrinogen receptor by modulating the extracellular structure of GpIIb/IIIa complex. However, this possibility is unlikely, considering the fact that only a few percent of GpIIb/IIIa complex was phosphorylated following platelet stimulation (11). On the other hand, GpIIb/IIIa complex is associated with actin filament and some actin binding proteins are well-known substrates of kinases and phosphatases. For example,

talin, an actin binding protein involved in the linkage between GpIIb/IIIa complex and actin filament, is markedly phosphorylated when non-stimulated platelets are treated with CLA (5). Filamin is also phosphorylated by cyclic AMP dependent kinase and this blocks cytoskeletal reorganization by inhibiting calpain mediated proteolysis (12). Therefore, the phosphorylation of these cytoskeletal proteins may regulate the extracellular structure of GpIIb/IIIa complex.

In conclusion, the present study suggest that protein phosphatase 1/2A may be directly involved in the exposure of fibrinogen receptor in agonist stimulated platelets.

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