血小板膜蛋白 GPⅣ（CD36）の機能と発現調節に関する研究

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研究発表

（３）　学会誌等

１．幸村　近、林　由紀子、池田　久實．フローサイトメトリーを用いた抗血小板の検出法．臨床病理　４７巻（１９９９）４４７－４５２

２．幸村　近、池田　久實．フローサイトメトリーによる血小板活性化の評価．Cytometry Research、９巻（１９９９）６７－７２

３．Yukiko Hayashi, Susumu Takenaka, Chikashi Kohmura and Hisami Ikeda
Preparation of discoid washed platelets by differential centrifugation. Clinica Chimica Acta. 275(1998) 99-105

（４）　口頭発表

１．林　由紀子、池田　久實。アラキドン酸による血小板凝集阻害反応．第２３回日本血栓止血学会学術集会．（１９９９）

２．幸村　近、池田　久實．フローサイトメトリーを用いた抗血小板の効果判定．第２３回日本血栓止血学会学術集会．（１９９９）

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４．林　由紀子、幸村　近、池田　久實．CD36 欠損血小板のアラキドン酸取り込みとアラキドン酸による阻害反応．第２２回日本血栓止血学会学術集会．（１９９８）

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文部科学技術省科学研究費基盤研究（C）(2)、０９６７２３４６による平成９年度から平成１２年度までの研究「血小板膜蛋白 CD36(GPIV)の機能と発現調節に関する研究」の成果を関連する雑誌掲載論文別刷とともにまとめて報告する。

CD36 は血小板、単球、マクロファージ、脂肪細胞などに発現されている。血小板では膜蛋白中４番目に多い蛋白であるが CD36 欠損者の止血機能に生理的条件下では正常者との差は見られず、その機能は不明な部分が多い。近年、マクロファージの scavenger receptor として酸化 LDL 取り込みや脂肪細胞での不飽和脂肪酸取り込み機能が報告され心血管障害、炎症、アポトーシスなどとの関連性も報告されている。我々は日本人に比較的多い（３―５％）CD36 欠損者の血小板を用いて血小板情報伝達における CD36 機能の解明を目指した。血小板機能に主要な役割を担っている不飽和脂肪酸であるアラキドン酸との反応の検討から以下の結果を得た。

1）CD36 欠損血小板は細胞内貯蔵カルシウムが減少する条件でのみ種々のアゴニストに対する応答が減少する。特に放出されるアラキドン酸（AA）代謝物である TxA2 により促進される反応が低下する。CD36 欠損血小板の TxA2 への応答は正常なので放出される AA がかなり低濃度の条件下（低カルシウムによる低 PLA2 活性）でのみ取り込まれる AA に依存した正常血小板との差が出現すると考えられた。
2）AA は大部分 CD36 を経由して取り込まれるが、TxA2 などに代謝されない場合（低酸素状態など）それ自身 TxA2 による血小板凝集を抑制することを証明した。すなわち何らかの理由で活性化された一部の血小板から放出された AA が他の一部の細胞では代謝産生される TxA2 による不可逆凝集へといたる過程を制御している。
3）AA による血小板凝集抑制メカニズムは、細胞質内で直接カルシウム非依存性 PKC を活性化して PLCβをリン酸化することで TxA2・receptor―Gqα―PLCβの相互作用を阻害する。従って、血小板凝集に主要な役割を担うカルシウム依存性 PKC が PLC により活性化されず、不可逆凝集にはいたらない。このことはまた、PKC の血小板機能における相反する作用、すなわち不可逆凝集促進、カルシウム動員、放出の抑制などが存在の場を異にするアイソザイムによって分担されていることを意味する。

Arachidonic acid inhibits human platelet aggregation induced by thromboxane A_2 mimetic U46619 through calcium independent PKC

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Summary

In the presence of cyclooxygenase and lypoxygenase inhibitors, arachidonic acid (AA) blocked platelet secretion and aggregation induced by U46619 keeping shape change and calcium mobilization. The IC_{50} value of AA for inhibition of normal platelets was 26.5 ± 12.2 μM and that for CD36 defective platelets was 102 ± 44 μM, respectively. Similar inhibition was brought about by Gö 6976, a selective inhibitor for calcium dependent protein kinase C (PKC). When platelets were treated with AA or a PKC activator, 12-O-tetradecanoilphorbol 13-acetate (TPA), phospholipase Cβ3 (PLCβ3) was serinephosphorylated, which was demonstrated by immunoprecipitation followed by western blotting. All these results suggest that AA incorporated into platelets through CD36 directly activates cytosolic calcium independent PKC leading to serinephosphorylation of PLCβ3. Consequently, one of the two thromboxaneA_2 (TxA_2) receptor mediated signals linked to PLC stimulation was blocked to inhibit platelets aggregation.

Key words: CD36 defective platelets; arachidonic acid; calcium independent PKC

1. Introduction

Platelet membrane glycoprotein CD36 has been suggested to be involved in uptake of long chain fatty acid [1,2]. Both activating and inhibitory effects of cis-unsaturated fatty acids including AA on platelets have been reported, in which PKC has been implicated [3-5]. PKC itself has complex functions in platelet signal transduction: it induces release reaction and aggregation, while it inhibits calcium mobilization and aggregation stimulated by other agonists [6-8]. AA as cis-unsaturated fatty acids directly induces PKC activation in a physiologic environment [9,10]. However, the precise role of PKC activation by the acids in platelet responses is obscure. In order to elucidate explicit roles of AA on platelet responses, we investigated the effects of AA in CD36 defective platelets comparative to normal ones. The results suggested that AA released from activated platelets, if not metabolized, blocked TxA_2 mediated signal transduction through calcium independent PKC in cytosol before calcium dependent PKC activation takes place in membrane.

2. Materials and methods

2.1 Materials

Arachidonic acid, TPA, 5,8,11,14-eicosatetraynoic acid (ETYA), BSA , cis 11,14-eicosadienoic acid, Fura 2-AM, U46619, leupeptin, and PMSF were purchased from Sigma Ardorich (Tokyo,Japan). Indomethacin, Gö6976 , Protein G Plus-Agarose were obtained from Calbiochem-Novabiochem Co (La Jolla, USA). Anti-PLCβ3 antibody was from Santa-Cruze Biotechnology (San Francisco, USA) and anti-phosphoserine antibody was from Zymed Laboratories Inc (San Francisco, USA). The rest of reagents were from Wako-Pure Chemical Industries, Ltd (Osaka, Japan).

2.2 Preparation and measurement of platelets

Platelets were separated from blood of healthy volunteers and then washed by 250 g centrifugation in the 20g/L concentration of BSA according to the method previously reported [11]. Buffy coat were kindly provided from Asahikawa Red Cross Blood Center. For immunoprecipitation and western blotting, platelets from buffy coat were used. They were diluted one and half times with the wash buffer and treated in similar manner as the platelet rich plasma (PRP) from freshly drawn blood. Within 30 hours after blood collections, about 70% of platelets prepared from buffy coat retained the same reactivity to U46619 as those from freshly drawn blood. After incubation with AA or TPA, 200 μl of 7~10 x 10^9/ml platelets were frozen at -80°C for immunoblotting analysis.

CD36 antigen of all the donated blood were routinely examined by flow cytometry COULTER Corporation, (Miami, USA) using FITC- labeled mouse monoclonal antibody to CD36, FITC- OKM5, Ortho Diagnostic Systems, Inc (Tokyo, Japan). About 5% of all samples examined were CD36 deficient.

For intracellular calcium analysis, PRP or diluted buffy coat were preincubated with 5μM Fura-2 for 30min at room temperature and then washed in a similar manner as shown above.

Platelets counting and mean platelet volume (MPV) were measured by Technicon H-1 (Tarry town, USA).

2.3 Immunoprecipitation and western blotting analysis

Immunoprecipitation of PLCβ3 was performed using rabbit polyclonal antibodies to PLCβ3. Briefly, frozen platelets were lysed with 200μl of Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 20 mM NaF, 150 mM NaCl, 2 mM PMSF, 2% Triton-X100, 20 μg/ml aprotinin and leupeptin, followed by addition of 20μl Protein G Plus-Agarose, and placed on ice for 2 hours. Lysate was centrifuged at 5000g for 3 min. 4μl polyclonal antibody to PLCβ3 was added to the supernate. After incubation for 1 hour at 4 °C, 20μl Protein G Plus-Agarose was added and incubated at 4 °C overnight. The immune complexes formed were isolated by centrifugation at 5000g for 3 min followed by 4 times of washing with lysis buffer by centrifuing at 5000g for 3 min. The final pellet was resuspended in 200μl SDS-PAGE electrophoresis buffer (TEFCO, Tokyo Japan) and boiled for 3 min. For western blotting analysis, lysate adjusted to $1.5x10^8$ platelets were run on SDS-PAGE with 3-8% gradient gel, using Xcell II Mini-Cell apparatus (NOVEX electrophoresis, San Diego, USA) at 150 volt constant for 1 hour. Proteins were transferred to nitrocellulose membrane (TEFCO, Kashiwabara, Japan) at 180 mA for 1 hour using a semidry blotting system (FUNAKOSHI, Tokyo, Japan) according to manufacturer's instructions. Blocking was done with 5% BSA in PBS for 2 hours at room temperature. For assay of serine phosphorylation, the transferred membrane was reacted with rabbit polyclonal anti phosphoserine antibody using ABC-POD (R) kit (Wako-Pure Chemical Industries Ltd, Osaka,

Japan) according to manufacturer's instructions.

2.4 Measurement of calcium mobilization and aggregation

Platelet aggregation was studied in modified HEPES-Tyrode buffer containing 3.5g/L (53 μM) BSA, 25μM indomethacin and ETYA, 1mM $CaCl_2$, 2.5-3.0 x 10^8/ml platelets (pH 7.3) using Aagretec TE-500 (Erma Optical Works Ltd, Japan) at 37°C.

Fluorescence measurements of Fura 2-AM loaded platelets for simultaneous calcium mobilization and aggregation studies were performed using Hitachi F-4500 fluorescence spectrophotometer by a F4500 IC program. For measurements of cytosolic Ca^{2+} concentration, excitation was performed at 340 and 380 nm, and emission was at 510 nm. For investigating aggregation, decreases in light scattering of 3.0 x10^8/ml platelets with shape change and aggregation were monitored at excitation 380 nm and emission 400 nm. Early small decrease was corresponded to the small decrease in light transmission which was thought to be produced by the shape change (12, 13), while large decrease caused by irreversible aggregation corresponded to the large increase in the transmission.

All the data show in figures are representative of 4-5 experiments.

3. Results

3.1. Effects of AA on U46619 induced responses in normal and CD36 deficient platelets

In the presence of indomethacin, a cyclooxygenase inhibitor and ETYA, a lypoxygenase inhibitor, both normal and CD36 deficient platelets were equally aggregated by 1μM of TxA_2 mimetic U46619. Unlike preceding reports, AA by itself did not induce any platelet responses in the level to 350 μM. Pretreatment of platelets with AA for 1~5 min at 37 ℃ reduced the aggregation by U46619 in a concentration dependent manner without affect on shape change monitored by light transmission (Fig 1) and light scattering (Fig 2 b). There was difference in the effective concentration of AA between CD36 deficient and normal platelets (Fig 3). U46619 mediates platelet activation through two receptor-effector systems; one linked to PLC activation, resulting aggregation and secretion, the other mediating shape change and an increase in cytosolic calcium [14]. Effects of AA on calcium mobilization were thus studied. When U46619 was added, calcium mobilization in normal and CD36 deficient platelets reached the maximum level within 10 sec, keeping its level until AA concentration exceeded 100μM (Fig 2 a, b). Beyond this level, dose dependent decrease was observed (Fig 4). The difference in effective AA concentration between normal and CD36 deficient platelets was present, however, little as compared with that found in aggregation. In CD36 deficient platelets, discrepancy between decrease of aggregation and calcium mobilization were not so clear because of right hand shift of the IC_{50} of AA (Fig 3 and 4). The IC_{50} of AA for normal platelets was 26.5 ± 12.2 μM (n = 4) and that for CD36 deficient platelets was 102.5 ± 44 μM (n = 5), respectively.

3.2. Effects of AA on thrombin induced responses

Both in normal and CD36 deficient platelets, aggregation and calcium mobilization

induced by 0.05 U/ml thrombin were not inhibited by AA at the concentration below 250μM. Inhibition of aggregation accompanied with calcium mobilization was observed at 380μM AA (Fig 5). At AA of 500 μM, lactate dehydrogenase was found in the supernatant fluid. We thought that decrease in aggregation together with calcium mobilization was not the reaction linked to signal transduction. Rather it may be physico-chemical non-specific modification common to different receptor-effector systems such as membrane perturbation [3]. Hence studies were focused on the initial effects of AA on TxA_2 receptor-effector signals leading to aggregation.

3.3. Calcium independent PKC in responses to U46619

We found that Gö 6976, the selective inhibitor of calcium dependent PKC [15], also prevented platelet aggregation without disturbance of calcium mobilization and shape change (Fig 2 c). In pretreatment of platelets with PKC activator TPA 3 nM, the concentration no response to U46619 (Fig 6 a), AA could not exert any influences on aggregation by U46619 (Fig 6 b). In sharp contrast, Gö 6976 was unaffected by the interference of 3nM TPA (Fig 6 c)

3.4. AA induced phosphorylation of PLCβ3

Fatty acids are unable to activate membrane bound PKC but preferentially activate cytosolic PKC. Therefore, fatty acid induced activation of PKC might occur in the cytosol, where they can phosphorylate a subset of PKC substrates [9,16]. Feedback regulation of PLCβ by PKC through serine phosphorylation was proposed by Sung ho Ryu et al [17]. We thus studied PLCβ phosphorylation by AA through PKC activation. In resting states, there are two PLCβ isoforms in cytosol, PLCβ2 and PLCβ3 [18,19]. The latter is thought to be stimulated by receptor activated Gαq. Hence, immunoprecipitation by anti PLCβ3 antibody

and western blotting with anti phosphoserine antibody of the precipitated protein were undertaken to detect PLCβ3 phosphorylation by AA. Serine phosphorylation of PLCβ3 by AA was demonstrated as well as by TPA (Fig 7).

4. Discussion

Pivotal role of AA in platelet function depends on its metabolite TxA_2. However, many reports have suggested that AA itself as cis unsaturated fatty acids directly regulate signal transduction through PKC activation. Unlike classical model of PKC activation by DAG in conjunction with calcium and phosphatidyl serine, fatty acids preferentially activate PKC independently of Ca^{2+} in the cytosol [9,10,16].

The responses inhibited by AA demonstrated here were equivalent to those mediated by an irreversible binding site of GR32191 [14]. We have confirmed the same inhibition of the responses to U46619 by cis 11,14-eicosadienoic acid without ETYA and indomethacin (data not shown). When the reaction mixture contained 0.25mg/ml fibrinogen, inhibition of the aggregation by AA disappeared. This means the inhibition was caused by lack of α-granule secretion. In contrast, activation of $\alpha_{IIb}\beta_3$ was not altered as long as calcium mobilization was retained. The activation of $\alpha_{IIb}\beta_3$ appeared to be reduced in parallel with calcium mobilization.

The responses to U46619 in CD36 deficient platelets were same to those of normal platelets. The IC_{50} of AA inhibition for the CD36 deficient platelets was, however, 4 times higher than those of normal ones. Accordingly, the inhibition at least seems to be directly brought about by AA transferred through CD36. Fibrinogen also prevented decrease of aggregation by AA at 200 μM but not at 300 μM where the calcium mobilization was lowered to 40 % of control solution (Fig 4). The preventive effects of fibrinogen decreased with decreasing calcium mobilization. Therefore, AA induced inhibition of aggregation and calcium mobilization seemed to be unconnected events in CD36 defective platelets as in normal ones.

Gö6976 also induced the same inhibition (Fig 2 c). When 0.25mg/ml of fibrinogen was added to the reaction mixture, Gö6976 was ineffective as well as AA. Differences in

inhibitory effects between them were revealed in TPA treated platelets (Fig 6). Since TPA effectively stimulates Ca^{2+} dependent PKC than Ca^{2+} independent isotypes [20,21], AA could not inhibit Ca^{2+} dependent PKC activated by 3nM TPA. It seemed that calcium independent PKC was activated by AA. Consequently, one of the TxA_2 receptor mediated signals leading to secretion and aggregation through PLC activation were prevented separately from another signal leading to shape change and calcium mobilization [14].

Among the PKC substrates related to receptor mediated signal transduction to PLC activation (receptor, G-proteinα and PLCβ) only some isotypes of PLCβ are present in the cytosol in resting platelets [18,19]. Several reports on PLC regulation by PKC suggested that G-protein PLC coupling was interrupted through phosphorylation of PLCβ by PKC [7,22]. In response to activation of G-protein coupled receptors, PLCβ3 in cytosol translocates to the cytoskeleton where it interacts with Gαq (19). Although we cannot exclude effects of phosphorylation of other proteins by PKC, phosphorylation of PLCβ3 take place prior to translocation, exerting immediate effects to terminate the essential signal transduction to aggregation.

Recent studies have revealed that Gαq in platelets is essential in the transduction of physiological signals and cannot be replaced by other heterotrimeric G proteins [23,24]. In addition to association with Gαq - PLCβ3, TxA_2 receptors were found to couple $G\alpha_{13}$, which is thought to be linked to extracellular Ca^{2+} influx and Na^+/H^+ exchanger [25]. Pharmacologically discriminated functions of TxA_2 receptor pathways may be attributed to the differences in bound G-proteins. The physiological significance of Ca^{2+} independent PKC probably exists in early interruption of platelet function. Interestingly, AA if it is not metabolized as observed in hypoxia [26, 27], triggers the counteraction of its posterior's role.

5. Acknowledgements

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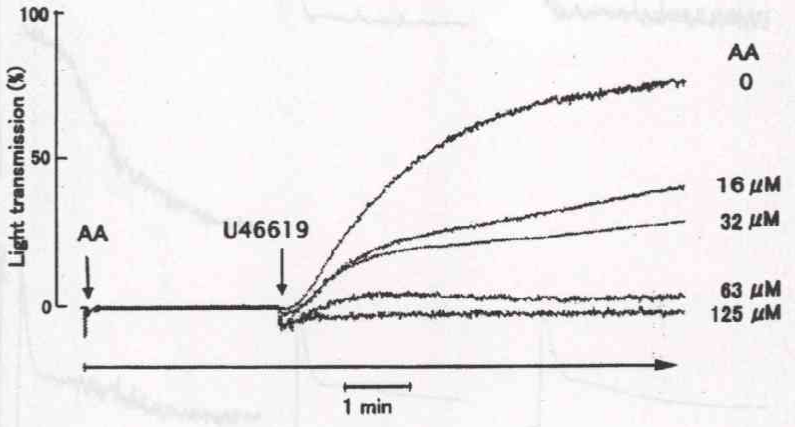

Figure 1. Effect of AA on platelet aggregation observed with aggregometer. $2.5x10^8$/ml platelets separated from blood of healthy volunteers as described under the Methods, were stimulated by 1µM U46619. Reaction buffer containing various concentrations of AA, was incubated for 3 min, at 37°C. Tracings were superimposed on the same time scale.

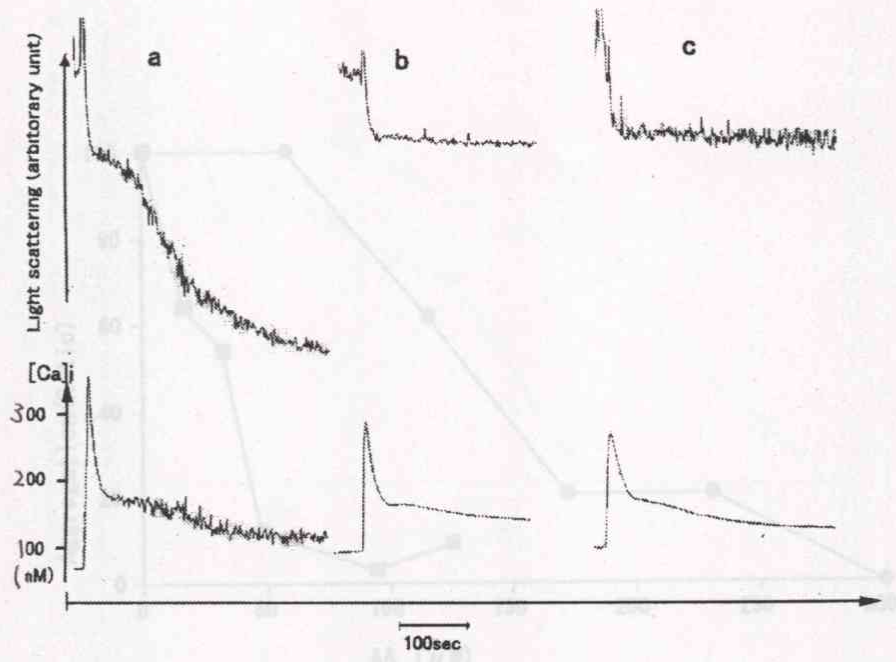

Figure 2. Inhibition of U46619 induced aggregation and calcium mobilization by AA and Gö6976. Fura-2 loaded platelets prepared from buffy coat as described under the Methods were stimulated by addition of 1μM U46619. $3.0x10^8$/ml platelets in reaction buffer were pre-incubated with 25 μM indomethacin and ETYA , 1mM $CaCl_2$ and 95μM AA or 1.1 μM Gö6976 for 5 min, at 37°C. Simultaneous measurements of calcium mobilization and aggregation were recorded as described under the Methods. a: vehicle, b: preincubated with AA, c: preincubated with Gö6976

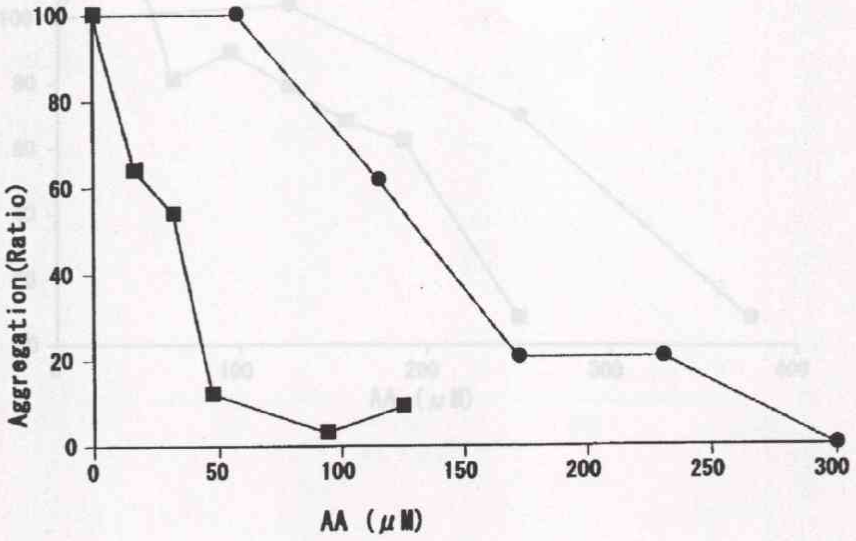

Figure 3. Dose-dependent inhibition of AA on U46619 induced aggregation in normal and CD36 deficient platelets. Platelets from healthy volunteers were aggregated as described under the legend for Figure 1. For the sake of comparison, aggregation was expressed by percentage to those at AA = 0 (100%). ●—●: CD36 deficient, ■—■ : control.

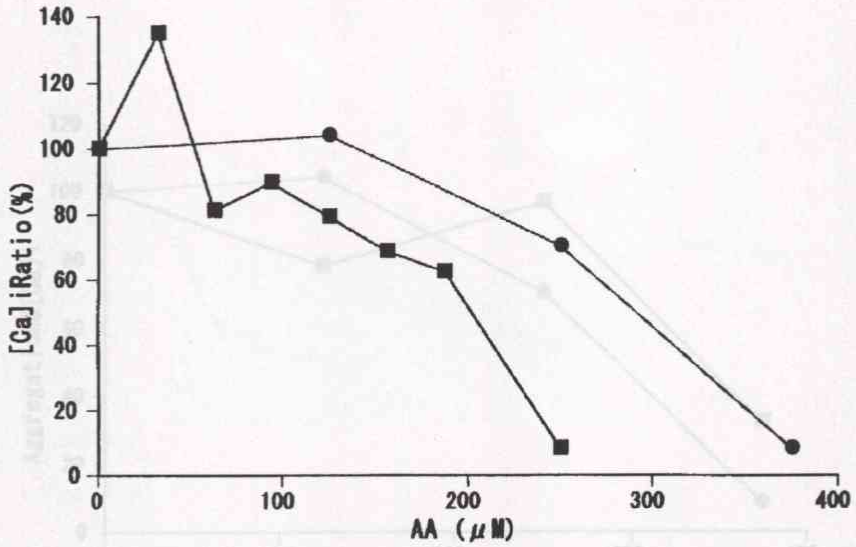

Figure 4. Inhibition of U46619 induced calcium mobilization by AA in normal and CD36 deficient platelets. Fura-2 loaded platelets prepared from healthy volunteers as described under the Methods were incubated with various concentrations of AA and activated by 1μM U46619. For the sake of comparison, calcium mobilization were expressed by percentage to those at AA = 0 (100 %). ●—●: CD36 deficient, ■—■ : control

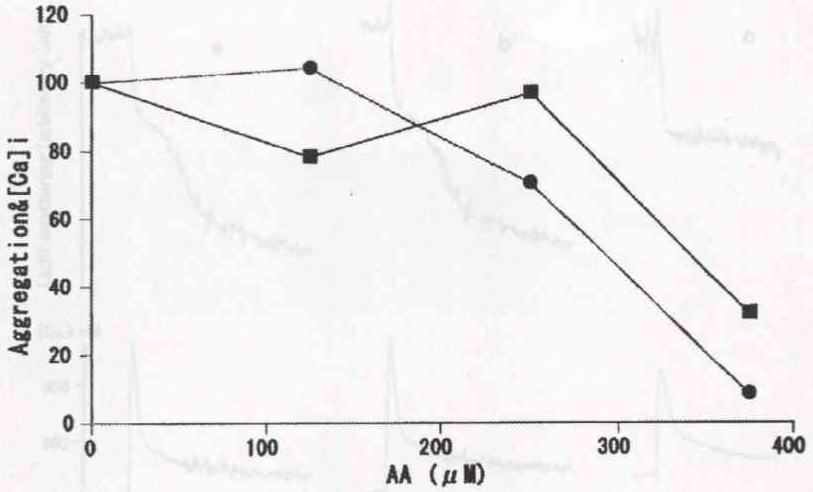

Figure 5. Effects of AA on aggregation and calcium mobilization induced by 0.05U/ml thrombin. CD36 deficient platelets from healthy volunteers were incubated as described under the legend of Fig 4 and stimulated by 0.05U/ml thrombin. Percent aggregation at 5 min after the reagent addition and the maximum calcium concentration were plotted against AA concentration. Values were relative to those at AA =0 (100 %).

●—●: aggregation, ■—■ : maximum calcium concentration. Similar results also observed in normal platelets.

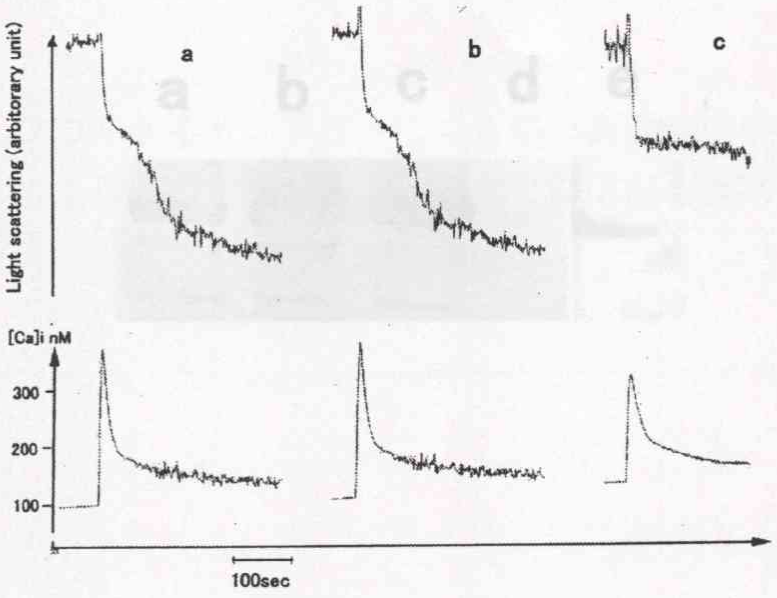

Figure 6. Effects of AA and Gö6976 on platelets treated with TPA. Reactions started by addition of 1μM U46619 to 3 nM TPA treated platelets under the same conditions of Figure 2. a: vehicle, b: AA 95μM, c: Gö6976 1.1 μM

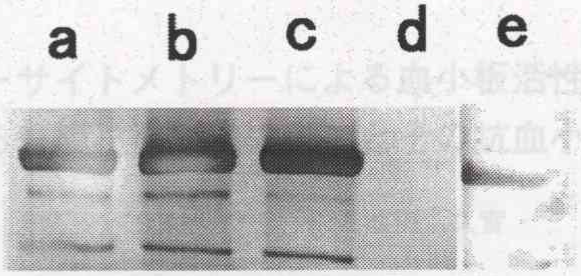

Figure 7. Serine phosphorylation of PLCβ3 by AA and TPA as analyzed on Western blotting. 200 μl of 7~ 9x 10^8/ml platelets from buffy coat in the reaction buffer containing 25 μM indomethacin and ETYA, were incubated with 10nM TPA(a), 164μM AA(b), 123μM AA(c) and vehicle (d) for 5min at 37 ˚C. After immunoprecipitation and SDS PAGE as described under the Methods, proteins were probed by rabbit polyclonal antibody to anti phosphoserine (a~d) and anti PLCβ3 (e). lane a: TPA 10 nM, lane b: AA164 μM, lane c: AA 123μM, lane d: control(platelets in reaction buffer), lane e: control probed with anti PLCβ3.