

A Defect in Collagen Receptor-Ca^{2+} Signaling System in Platelets from Cattle with Chediak-Higashi Syndrome

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Keywords

Chediak-Higashi syndrome, bovine platelet, Ca^{2+} mobilization, collagen receptor, rhodocytin

Summary

Decreased platelet aggregation to collagen is a cause for bleeding diathesis of Chediak-Higashi syndrome (CHS). We investigated whether the collagen receptor-Ca^{2+} signaling system was impaired in platelets from cattle affected with CHS. A collagen-induced increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was depressed in CHS platelets, which was accompanied by a decrease in the production of inositol 1,4,5-trisphosphate. When the influences of endogenous arachidonic acid metabolites and ADP were excluded, convulxin or collagen-related peptide, which are specific agonists for the collagen receptor GPVI, increased $[Ca^{2+}]_i$ in both normal and CHS platelets. In contrast, rhodocytin, which was thought to activate another collagen receptor GPIa/IIa, increased $[Ca^{2+}]_i$ in CHS platelets to a lesser extent than in normal ones. Cytochalasin D, an actin polymerization inhibitor, depressed the response to collagen or rhodocytin but not the response to convulxin. Adhesion of CHS platelets to acid soluble type I collagen, which was mediated by GPIa/IIa, was similar to that of normal platelets. These results suggest that a defect in the rhodocytin-sensitive pathway is responsible for decreasing the response to collagen in CHS platelets. It remains to be determined which receptor is associated with the mechanism.

Introduction

Chediak-Higashi syndrome (CHS) is an autosomal recessive disease characterized by giant cytoplasmic granules in circulating granulocytes, partial oculocutaneous albinism, increased susceptibility to pyogenic infections, and repeated hemorrhagic episodes (1). CHS in humans, mice, and cattle is ascribed to mutations at the same gene, which encodes a novel 430-kDa protein, LYST (2-4). However, the function of this huge protein for platelet activation has not been elucidated.

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The hemorrhagic tendency in CHS is due to insufficient platelet aggregation (5, 6). Platelets from CHS patients exhibit δ-storage pool deficiency (δ-SPD), which is characterized by reduced and irregular dense bodies, which contain ADP, ATP, serotonin and Ca^{2+} (6-8). Since ADP is secreted from dense bodies by a primary agonist such as collagen, and ADP in turn acts as a secondary activator, δ-SPD has been proposed to be a cause for the impaired aggregation of CHS platelets (7-9). Some studies have revealed additional abnormalities in platelets from patients with CHS, such as increased sensitivity to prostacyclin or a decrease in arachidonic acid metabolism (9, 10).

We previously reported that an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) induced by collagen was depressed in platelets from CHS-affected cattle, whereas the response to ADP was almost normal (11). In contrast to human platelets, the collagen-induced increase in $[Ca^{2+}]_i$ in bovine platelets was only partially inhibited by treatment with aspirin and ADP receptor antagonists (12), suggesting that arachidonic acid metabolites and ADP play only a minor role in the collagen-induced response of bovine platelets. Hence, we think that a main cause for the defective activation by collagen of CHS platelets is an impairment of a collagen receptor-signal transduction system that underlies Ca^{2+} signaling rather than a decreased release of ADP or abnormality in arachidonic acid cascade.

Glycoprotein (GP) Ia/IIa (also termed integrin α2β1) and GPVI are presumably predominant receptors for collagen in platelets (13, 14). Although GPIa/IIa is suggested to be responsible for the adhesion to collagen, and GPVI for platelet activation, the precise role of each receptor and how the two receptor systems interact have not been fully understood. Recently, several proteins, which preferentially act on GPIa/IIa or GPVI, have been reported. Convulxin, isolated from the snake *Crotalus durissus terrificus* venom (15), and collagen-related peptide (CRP, 16), which consists of a glycine-proline-hydroxyproline repeat and is cross-linked via cysteine residues at its C- and N-terminals, have been reported to activate platelets by interacting with GPVI. Additionally, rhodocytin, isolated from the snake *Calloselasma rhodostoma* venom, was suggested to interact with GPIa/IIa (17-19), although this hypothesis has been challenged (20, 21). These substances are useful to address which collagen receptor was impaired in CHS platelets. For this purpose, we investigated the Ca^{2+} signaling produced by collagen, convulxin, CRP or rhodocytin in platelets from normal or CHS-affected cattle. We particularly focused our attention on the Ca^{2+} signaling due to the direct action of collagen under conditions which excluded the influences of endogenous agonists.

Materials and Methods

Materials

Convulxin and rhodocytin were purified from the venom of *Crotalus durissus terrificus* (15) and *Calloselasma rhodostoma* (17), respectively. CRP was synthesized by the method of Morton et al. (16). Adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS), mouse IgG1 κ (MOPC-21), Arg-Gly-Asp-Ser (RGDS), acid soluble type I collagen from calf skin, and cytochalasin D were from Sigma (St. Louis, MO, USA). Native collagen fibrils from equine tendons were purchased from Nycomed (Munich, Germany), monoclonal antibody CD49b (Gi9) from Cosmo Bio (Tokyo, Japan), Ins(1,4,5) P_3 [^{3}H] Radioreceptor Assay Kit from NEN (Boston, MA, USA), and ^{51}Cr as sodium chromate from Daiichi Pure Chemicals (Tokyo, Japan). AR-C66096 was from Astra-Zeneca R&D Charnwood (Loughborough, UK). All other agents were from previously reported sources (12).

Measurement of $[Ca^{2+}]_i$ in Platelets

Healthy Japanese Black cattle have been maintained at Sumiyoshi Ranch, Miyazaki University. CHS-affected Japanese Black cattle were brought from farms in our area to the Veterinary Hospital of Miyazaki University. Washed platelets were prepared as described (12) at a concentration of 3×10^8 platelets/ml in Hepes buffer [136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM Hepes, 5.5 mM glucose and 0.35% (w/v) bovine serum albumin (BSA, fraction V), pH 7.4]. To measure $[Ca^{2+}]_i$, fura-PE3/AM was loaded to washed platelets by incubating them with 2 μM fura-PE3/AM for 30 min at 37° C. After fura-PE3-loaded platelets were put in a cuvette, 1 mM Ca^{2+} was added. For measurement of platelet activation by collagen, we used native collagen fibrils from equine tendons, a type commonly used for aggregation experiments. Collagen was applied 2 min after addition of Ca^{2+}. When needed to exclude the influence of arachidonic acid metabolites, aspirin (1 mM) was added during incubation with fura-PE3/AM. To further exclude the influence of endogenous ADP, AR-C66096 (100 nM), a $P2Y_{12}$ receptor antagonist (22, 23), and A3P5PS (100 μM), a $P2Y_1$ receptor antagonist (24), were applied to aspirin-pretreated platelets 90 and 30 sec, respectively, before addition of agonists. As reported previously (12) AR-C66096 and A3P5PS at these concentrations suppressed the $[Ca^{2+}]$i and the aggregation responses to exogenously applied ADP. When a response was observed in aspirin- or cytochalasin D-treated platelets, control platelets were pretreated with vehicle (0.1 or 0.25% DMSO, respectively). Fluorescence at 500 nm emission after alternative excitation at 340 nm (F340) or 380 nm (F380) was measured in a fluorimeter (CAF-100, JASCO, Tokyo, Japan) at 37° C. $[Ca^{2+}]_i$ was calculated by use of the ratio method (25) with a K_D value of 290 nM for fura-PE3 (26).

Measurement of Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]

Measurement of the production of Ins(1,4,5)P_3 was performed as described previously (12). In brief, washed platelet suspension at a concentration of 1×10^9 platelets/ml was incubated with aspirin (1 mM), then centrifuged and resuspended in Hepes buffer to make a concentration of 2×10^9 platelets/ml. The suspension (200 μl) was incubated with an agonist at 37˚ C in the presence of $CaCl_2$ (1 mM). At 0.5 or 1 min after addition of 0.1 unit/ml thrombin or 10 μg/ml collagen, respectively, the reaction was terminated by addition of 20% ice-cold trichloroacetic acid (TCA, 70 μl). Samples were centrifuged at 1,000 × g for 10 min at 4° C. The supernatant was transferred to a test tube and extracted 3 times with 10 volumes of water-saturated dietylether to remove TCA. Ins(1,4,5)P_3 in samples was quantitated by an Ins(1,4,5)P_3 assay kit according to the manufacturer's protocol.

Adhesion Experiment

Adhesion of platelets to collagen-coated microtiter wells was measured by a modified method originally described by Moroi et al. (27). Washed platelets in Mg^{2+}-free Hepes buffer containing 1% BSA were labeled with ^{51}Cr (50 μCi/ml) for 60 min at 37° C. ^{51}Cr-labeled platelets were washed twice and resuspended in Mg^{2+}-free Hepes buffer containing 1% BSA at a concentration of 2×10^8 platelets/ml. $MgCl_2$ (2 mM) or EDTA (2 mM) was added to the ^{51}Cr-labeled platelet suspension in the presence of PGE_1 (1 μM) and RGDS (100 μg/ml), which were used to prevent aggregation.

Wells were coated with acid soluble type I collagen (300 μg/ml unless otherwise stated) in 0.05% acetic acid for 2 h at room temperature. The reason for the use of this type of collagen was that it binds to platelets via GPIa/IIa (27). Collagen-coated wells were also incubated with Mg^{2+}-free Hepes buffer containing 1% BSA for 1 h at room temperature. An aliquot (50 μl) of ^{51}Cr-labeled platelets suspended in Hepes buffer containing 1% BSA and 2 mM EGTA or 2 mM $MgCl_2$ was added to each well and allowed to settle for the indicated time at room temperature. Adhered platelets were solubilized with 2% SDS for 30 min and the radioactivity of ^{51}Cr was measured in a gamma scintillation counter. The assay was done in duplicate for each experiment. Inhibition of adhesion by an anti-GPIa/IIa antibody (Gi9) or control IgG (MOPC-21) was tested by incubating the platelet suspension with the antibody in a test tube for 30 min at room temperature. The platelet plus antibody mixture was then added to a microtiter well, and the adhesion was measured as described above. Nonspecific adhesion, which was determined as radioactivity remaining on BSA-coated wells, was less than 0.5% of the total number of platelets added and was subtracted from all values.

Statistical Analysis

Results are expressed as means ± S.E. Multiple means were subjected to Tukey-Kramer test. Two means were compared by Student's t-test. A value of $p < 0.05$ was considered to be significant.

Results

Collagen-induced Increase in $[Ca^{2+}]_i$

We reported that collagen (3-15 μg/ml)-induced aggregation and increase in $[Ca^{2+}]_i$ were significantly depressed in CHS platelets (11). When a high concentration of collagen was used, a peak value of $[Ca^{2+}]_i$ could not be measured because aggregation, which occurred before $[Ca^{2+}]_i$ attained a peak, hampered the measurement. Therefore, an increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was evaluated by subtracting the basal $[Ca^{2+}]_i$ from maximum $[Ca^{2+}]_i$ during 4 min after addition of collagen. An increase in $[Ca^{2+}]_i$ induced by 10 μg/ml collagen in CHS platelets was 60.2 ± 2.5% (n = 5) of that in normal platelets (Fig. 1). Pretreatment with aspirin (1 mM) did not affect the collagen-induced increase in $[Ca^{2+}]_i$ in normal platelets but significantly decreased it in CHS platelets (Fig. 1B). When platelets were pretreated with the cyclooxygenase inhibitor (aspirin) and the ADP receptor antagonists (100 nM AR-C66096 and 100 μM A3P5PS) to exclude the influence of endogenous agonists, the $[Ca^{2+}]_i$ response to collagen decreased to a similar degree in both types of platelets (a decrease was about 200 nM from the control value), and the response in normal platelets changed to 62.2 ± 6.6% (n = 6) of the level in their absence, and in CHS platelets to 15.1 ± 2.5% (n = 5). As a result, the maximum increase in $[Ca^{2+}]_i$ due to the direct action of collagen in CHS platelets was only 14.6 ± 2.5% (n = 5) of that in normal ones.

Collagen-induced Production of Ins(1,4,5)P_3

We previously reported that phospholipase C (PLC) was involved in the collagen-induced Ca^{2+} mobilization in bovine platelets (12). Therefore, we measured whether the production of Ins(1,4,5)P_3 due to collagen was altered in CHS platelets. We also observed the Ins(1,4,5)P_3 production by thrombin, which is known to stimulate PLC

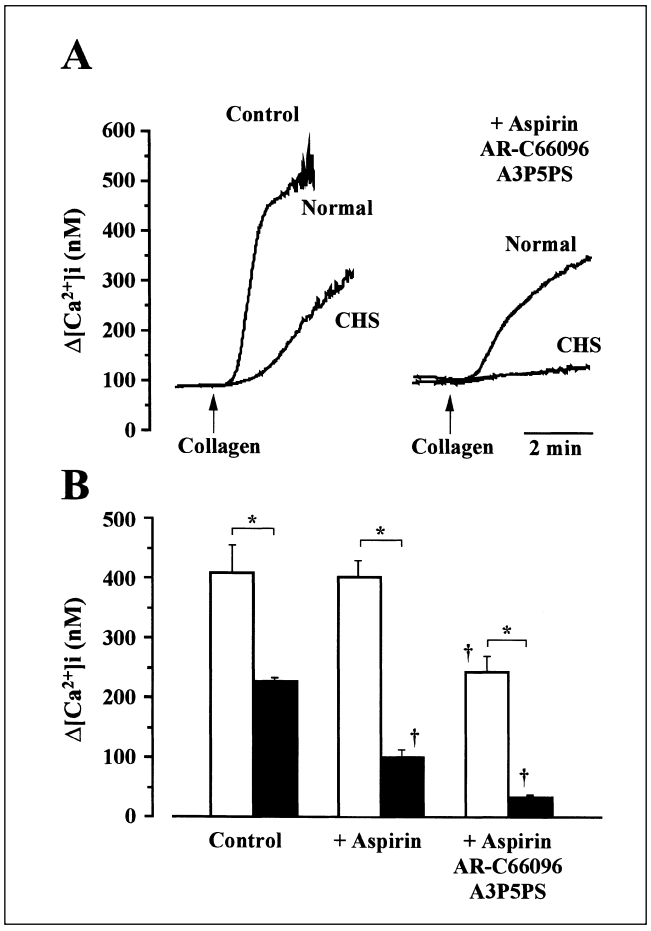

Fig. 1 Effects of a cyclooxygenase inhibitor and ADP receptor antagonists on the collagen-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets. A) Typical recordings of an increase in $[Ca^{2+}]_i$ induced by 10 μg/ml collagen in platelets pretreated with vehicle (control, left), or inhibitors against endogenous agonists (1 mM aspirin, 100 nM AR-C66096 and 100 μM A3P5PS, right). B) An increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) induced by 10 μg/ml collagen in normal (open column) or CHS platelets (filled column) in vehicle (control)-, aspirin- or combination of aspirin, AR-C66096 and A3P5PS-treated platelets. $\Delta[Ca^{2+}]_i$ was calculated by subtracting the basal $[Ca^{2+}]_i$ from the maximum $[Ca^{2+}]_i$ during 4 min after the addition of collagen. The data are mean ± S.E. of 5-8 experiments. *p <0.01. †p <0.01 (vs. control)

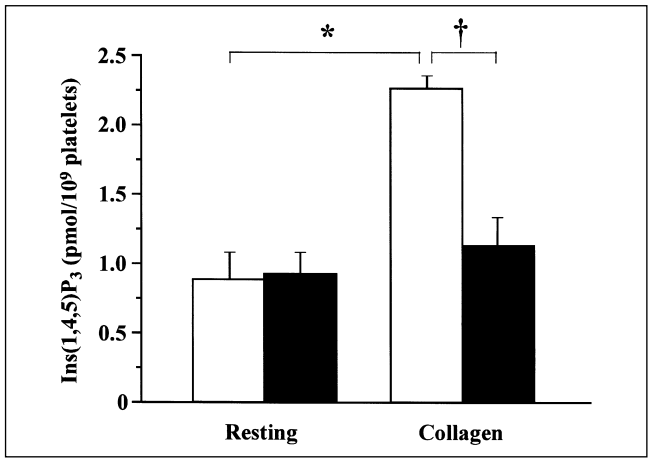

Fig. 2 Collagen-induced Ins(1,4,5)P_3 production. Aspirin-treated platelets were stimulated by 10 μg/ml collagen. Production of Ins(1,4,5)P_3 was measured 1 min after the addition of collagen in normal (open column) or CHS (filled column) platelets. Each column is expressed as a mean ± S.E. of 4-6 experiments. *p <0.01 (vs. resting). †p <0.01 (vs. normal platelets)

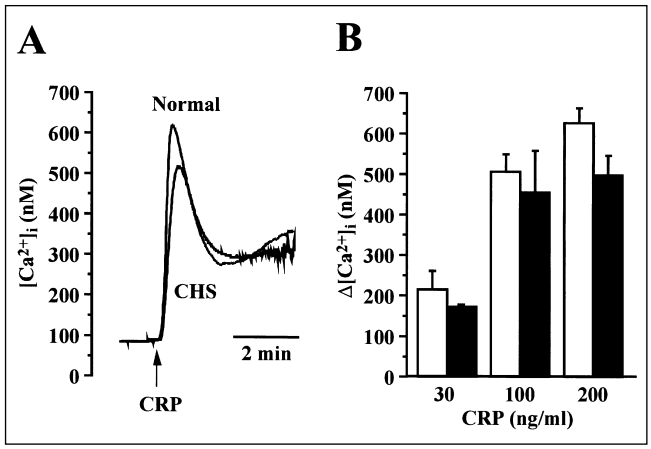

Fig. 3 CRP-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets. A) Typical recordings of an increase in $[Ca^{2+}]_i$ induced by 100 ng/ml CRP. B) $\Delta[Ca^{2+}]_i$ due to various concentrations of CRP (30-200 ng/ml) in normal (open column) or CHS (filled column) platelets. The data are means ± S.E. of 3 experiments

(28). Ins(1,4,5)P_3 was measured at the time when $[Ca^{2+}]_i$ attained a peak after the application of collagen (10 μg/ml) or thrombin (0.1 unit/ml) to aspirin-treated platelets. The basal level of Ins(1,4,5)P_3 was not different between normal and CHS platelets (Fig. 2). At 0.5 min after addition of thrombin, Ins(1,4,5)P_3 increased to a similar degree in normal and CHS platelets [190.3 ± 31.0% (n = 5) and 204.9 ± 38.0% (n = 5) of the pre-drug level, respectively]. When challenged with collagen, Ins(1,4,5)P_3 increased to 255.5 ± 11.0% (n = 4) at 1 min after the addition to normal platelets. On the other hand, collagen did not significantly increase Ins(1,4,5)P_3 in CHS platelets (Fig. 2).

Response to CRP or Convulxin

CRP or convulxin rapidly increased $[Ca^{2+}]_i$, which attained a peak within 1 min and then declined to a steady level at about 2 min in both platelets (Fig. 3A and Fig. 4A). A CRP- or convulxin-induced increase in $[Ca^{2+}]_i$ was concentration-dependent between 30 and 200 ng/ml, or 1 and 10 ng/ml, respectively (Fig. 3B and Fig. 4B, left). In this dose-effect study, the peak $[Ca^{2+}]_i$ attained by convulxin in normal platelets seemed to be slightly higher than that in CHS platelets, although we could not detect a significant difference between the two groups probably because of the small number of experiments (n = 3). Also, in the case of CRP, the response to 200 ng/ml CRP seemed to be slightly greater in normal platelets than in CHS ones but with no statistical difference (n = 3). Next, the response to convulxin in platelets that had been pretreated with inhibitors against endogenous agonists was observed. In this series of experiments, the control response to 3 ng/ml convulxin was significantly greater in normal platelets (n = 6) than in CHS ones (n = 5, Fig. 4B right). The response to convulxin in CHS platelets was not modified by these inhibitors, whereas it was significantly depressed in normal platelets. As a result, the maximum increase in $[Ca^{2+}]_i$ in platelets pretreated with inhibitors against endogenous agonists did not differ between normal and CHS platelets (Fig. 4B right).

Response to Rhodocytin

In contrast to convulxin or CRP, rhodocytin gradually increased $[Ca^{2+}]_i$ with a lag of about 4 min in platelets from normal cattle (Fig. 5A). In CHS platelets, the agonist-induced phase of $[Ca^{2+}]_i$ over the basal level could not be clearly distinguished. In Fig. 5B, an increase in $[Ca^{2+}]_i$ in response to rhodocytin (3-25 nM) was calculated by subtracting the level just before the addition of rhodocytin from the maximum $[Ca^{2+}]_i$ during 8 min after the addition of the agent. Rhodocytin increased $[Ca^{2+}]_i$ in a concentration-dependent manner in both platelets, the response being much less in CHS platelets with 10 and 25 nM rhodocytin than in normal ones (Fig. 5B left). When inhibitors were absent, rhodocytin (10 nM) increased $[Ca^{2+}]_i$ in CHS platelets by only 11.0 ± 1.5% (8 min after application) of the response in normal platelets (n = 5) (Fig. 5B right). Pretreatment with inhibitors slowed the development of rhodocytin-induced increase in $[Ca^{2+}]_i$ in normal platelets (Fig. 5A right). When endogenous substances were excluded, an increase in $[Ca^{2+}]_i$ at 16 min after application of 10 nM rhodocytin in CHS platelets was 18.1 ± 1.1% of the response in normal platelets (n = 5) (Fig. 5B right).

Effects of Cytochalasin D on Collagen-, Convulxin- or Rhodocytin-induced Increase in $[Ca^{2+}]_i$

It has been reported that collagen- or rhodocytin-induced activation of human platelets was inhibited by cytochalasin D, an inhibitor of actin polymerization (18, 19, 29). In order to see if the response of bovine platelets was similarly sensitive to this agent, cytochalasin D (10 μM) was added and 5 min later collagen (10 μg/ml), convulxin (3 ng/ml) or rhodocytin (10 nM) was added. In normal platelets, cytochalasin D inhibited the rhodocytin (10 nM)-induced increase in $[Ca^{2+}]_i$ whether or not inhibitors against endogenous agonists had been treated (Fig. 6C). However, the response to rhodocytin in CHS platelets was too small to detect the effect of cytochalasin D. Cytochalasin D inhibited the collagen (10 μg/ml)-induced increase in $[Ca^{2+}]_i$ in normal platelets treated with either vehicle or inhibitors against endogenous agonists (Fig. 6A). Similarly, the agent inhibited the collagen-induced increase in $[Ca^{2+}]_i$ in CHS platelets when inhibitors against endogenous agonists were absent (Fig. 6A) but not when platelets were treated with the inhibitors, possibly because the response was too small. On the other hand, a convulxin (3 ng/ml)-induced increase in $[Ca^{2+}]_i$ was

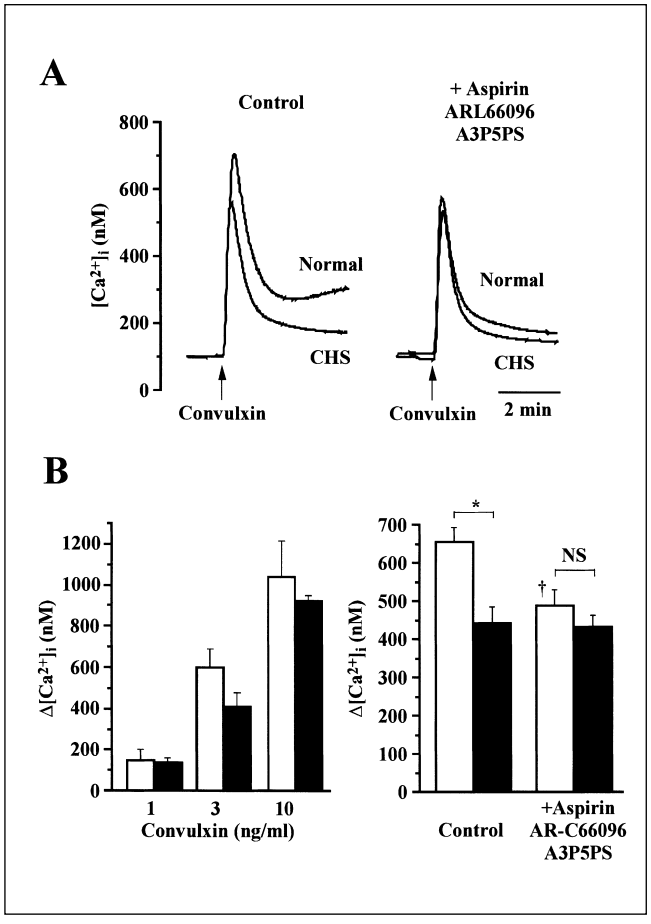

Fig. 4 Convulxin-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets and the effect of pretreatment with a cyclooxygenase inhibitor and ADP receptor antagonists on the Ca^{2+} mobilization. A) Typical recordings of the increase in $[Ca^{2+}]_i$ induced by 3 ng/ml convulxin in platelets pretreated with vehicle (control, left) or inhibitors against endogenous agonists (right). B) $\Delta[Ca^{2+}]_i$ by various concentrations of convulxin (left), and the effects of inhibitors on $\Delta[Ca^{2+}]_i$ due to 3 ng/ml convulxin (right) in normal (open column) or CHS (filled column) platelets. The data are means ± S.E. of 3 (B, left) or 5-6 (B, right) experiments. *p <0.01. †p <0.01 (vs. control). NS: not significant

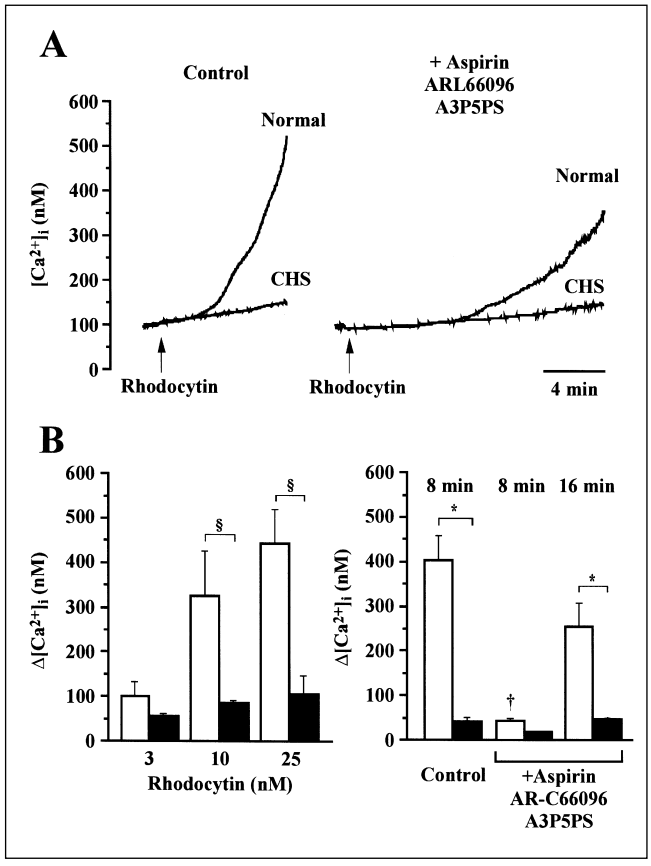

Fig. 5 Rhodocytin-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets and the effect of pretreatment with inhibitors against endogenous agonists on the Ca^{2+} mobilization. A) Typical recordings of the increase in $[Ca^{2+}]_i$ induced by 10 nM rhodocytin in platelets pretreated with vehicle (control, left) or inhibitors against endogenous agonists (right). B) $\Delta[Ca^{2+}]_i$ induced by various concentrations of rhodocytin (left), and the effect of inhibitors on Δ $[Ca^{2+}]_i$ due to 10 nM rhodocytin (right) in normal (open column) or CHS platelets (filled column). Δ $[Ca^{2+}]_i$ was calculated by subtracting the basal $[Ca^{2+}]_i$ from the maximum $[Ca^{2+}]_i$ during 8 or 16 min after the addition of rhodocytin in platelets treated with vehicle or inhibitors. The data are mean ± S.E. of 3 (B, left) or 5-6 (B, right) experiments. § p <0.05. * p <0.01. † p <0.01 (vs. control)

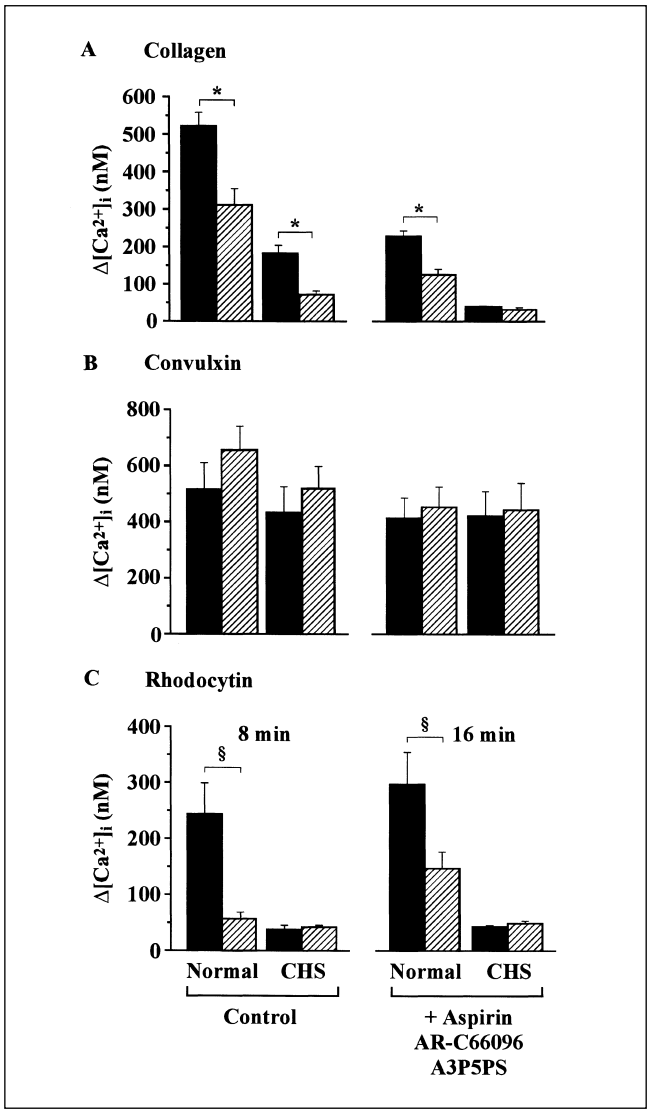

Fig. 6 Effect of cytochalasin D on the collagen-, convulxin- and rhodocytin-induced increase in $[Ca^{2+}]_i$. Fura-PE3-loaded platelets were incubated with vehicle (0.25% DMSO) or cytochalasin D (10 μM) 5 min prior to the addition of 10 μg/ml collagen (A), 3 ng/ml convulxin (B) or 10 nM rhodocytin (C). Filled columns are without cytochalasin D (vehicle), while hatched columns are with cytochalasin D. $\Delta[Ca^{2+}]_i$ was calculated by subtracting the basal $[Ca^{2+}]_i$ from the maximum $[Ca^{2+}]_i$ during 4 min after addition of collagen or convulxin. Because the response to rhodocytin was slow, $[Ca^{2+}]_i$ was measured for 8 or 16 min after the addition of rhodocytin in platelets treated with vehicle or inhibitors against endogenous agonists, respectively. The data are means ± S.E. of 5-6 experiments. § $p < 0.05$. * $p < 0.01$

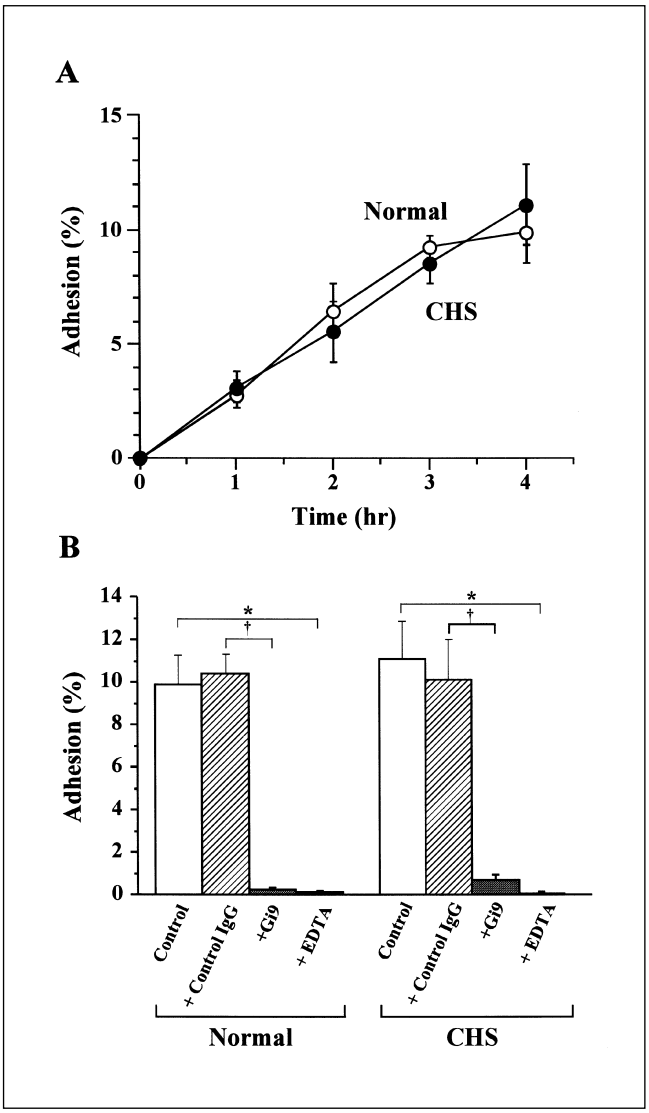

Fig. 7 Adhesion of platelets to acid-soluble type I collagen. A) Time-dependent adhesion of normal (open circles) or CHS platelets (filled circles) in the presence of 2 mM Mg^{2+}. B) Effects of control mouse IgG (10 μg/ml MOPC-21, hatched column), anti-GPIa/IIa antibody (10 μg/ml Gi9, shaded column) or EDTA (2 mM, filled column) on the adhesion. Open columns are the adhesion of platelets in the presence of 2 mM Mg^{2+}. The adhesion is expressed as percent of adhered platelets to total platelets added. The data are means ± S.E. of 3 experiments. *,† $p < 0.01$

insensitive to cytochalasin D in both platelets (Fig. 6B). ADP (1 μM)-induced increase in $[Ca^{2+}]_i$ was also insensitive to cytochalasin D in normal platelets (an increase in $[Ca^{2+}]_i$ in the absence or presence of cytochalasin D was 616.4 ± 67.3 or 633.6 ± 53.1 nM, respectively, n = 5) and CHS (685.6 ± 16.5 or 647.9 ± 34.7 nM, respectively, n = 5) platelets.

Adhesion of Platelets to Type I Collagen

In order to investigate whether the binding of platelets to collagen through GPIa/IIa was impaired in CHS platelets, adhesion of ^{51}Cr-labeled platelets to acid soluble type I collagen was observed. In the buffer containing 2 mM Mg^{2+}, platelets adhered to collagen-coated wells in a time-dependent manner (Fig. 7A). When wells had been coated with different concentrations (30-1,000 μg/ml) of collagen, the adhesion increased depending on the concentration (data not shown). The extent and rate of adhesion of CHS platelets was similar to that of normal platelets (Fig. 7A). When the buffer contained 2 mM EDTA but no Mg^{2+}, the adhesion was greatly inhibited, indicating that the adhesion was Mg^{2+}-dependent (Fig. 7B). Furthermore, when platelets were put into a well after they had been suspended together with anti-GPIa/IIa antibody (Gi9) or control mouse IgG (MOPC-21) in a test tube, the adhesion to a collagen-coated well was greatly inhibited by Gi9 but not by control IgG (Fig. 7B).

Discussion

Collagen releases endogenous substances such as arachidonic acid metabolites (mainly thromboxane A2) and ADP, and these substances in turn act as secondary agonists toward potentiation of the collagen effect. When a fraction attributable to endogenous ADP is calculated as the difference between the $[Ca^{2+}]_i$ response to collagen in the presence of aspirin and that in the presence of all inhibitors against endogenous agonists (aspirin, AR-C66096 and A3P5PS) (Fig. 1), it was smaller in CHS platelets than in normal platelets (about 60 nM and 150 nM, respectively), suggesting that ADP release was inhibited as a result of δ-SPD (7-9) in CHS platelets. Similarly to the previous paper (11), in which indomethacin was used, the cyclooxygenase inhibitor aspirin did not affect the Ca^{2+} mobilization due to collagen in normal platelets. In CHS platelets, however, aspirin significantly decreased the response (Fig. 1). Thus, it seems that a decrease in ADP release is compensated with increased contribution of arachidonic acid metabolites, although we have not further examined what is a mechanism for the compensation. From the results with use of all inhibitors, the extent of increased $[Ca^{2+}]_i$ levels dependent on endogenous agonists was similar in normal and CHS platelets (170 and 190 nM, respectively). On the other hand, the fraction of collagen-induced Ca^{2+} mobilization that was independent of endogenous agonists in CHS platelets was only about 15% of that in normal platelets. This suggests that a Ca^{2+} signaling mechanism that is a consequence of the direct action of collagen is defective in CHS platelets and that this defect is important in the etiology of insufficient aggregation. The collagen-induced increase in $Ins(1,4,5)P_3$ in CHS platelets was greatly inhibited compared with that in normal platelets. Therefore, a cause for the insufficient Ca^{2+} mobilization in CHS platelets is present in the pathway between the binding of collagen to receptors and the activation of PLC (PLCγ2).

Although a number of candidates have been proposed as collagen receptors to date, several lines of evidence have indicated that GPVI is a predominant receptor to activate platelets (30, 31). The GPVI-specific activator convulxin- or CRP-induced increase in $[Ca^{2+}]_i$ was normal or only slightly inhibited in CHS platelets. A slight inhibition of convulxin-induced Ca^{2+} mobilization in CHS platelets (Fig. 4B, right) may reflect a decreased release of ADP as discussed above, because when platelets had been pretreated with inhibitors against endogenous agonists, no difference was observed in the convulxin-induced response of normal and CHS platelets. These results indicate that a signaling system downstream of GPVI that leads to Ca^{2+} mobilization is nearly intact in CHS platelets. It appears that a deficit exists in a receptor-signaling pathway other than GPVI.

In contrast to the case of convulxin, the rhodocytin-induced increase in $[Ca^{2+}]_i$ was greatly depressed in CHS platelets. The response to rhodocytin of CHS platelets was still less than that of normal platelets when the influence of endogenous agonists was excluded. Thus, it is evident that a rhodocytin-sensitive mechanism is impaired in bovine CHS platelets. Recently, Inoue (or Suzuki-Inoue) group (18, 19) have shown that rhodocytin binds to GPIa/IIa and then activates human platelets. Furthermore, Navdaev et al. (32) have reported that aggretin, which is a polypeptide from the same snake *Calloselasma rhodostoma* and has the same N-terminal sequences as rhodocytin, also binds to GPIa/IIa and activates platelets. In contrast to these papers, Eble et al. (20) reported that a 29 kDa protein isolated from the same snake, which is presumably aggretin/rhodocytin, does not directly bind to recombinant GPIa/IIa. The same group also presented data showing that rhodocytin activated platelets which lacked GPIa/IIa (21). The cause for this discrepancy is unclear. Although we used rhodocytin that was isolated by Shin and Morita (17) and was shown to bind to recombinant GPIa/IIa (19), at the moment we can not assert that rhodocytin activates GPIa/IIa. In spite of such confusion, however, a similar depression in the collagen-induced response and in the rhodocytin-induced one of CHS platelets strongly suggests that a defect in the rhodocytin-sensitive mechanism is responsible for depression of the collagen-induced Ca^{2+} signaling.

In order to test the binding of collagen to platelets mediated by GPIa/IIa, we observed the adhesion of platelets to acid soluble type I collagen. It has been demonstrated that adhesion of human platelets to acid soluble type I collagen is Mg^{2+}-dependent and is mediated by GPIa/IIa (33). The adhesion of bovine platelets to acid soluble type I collagen was Mg^{2+}-dependent and was inhibited by the anti-GPIa/IIa antibody Gi9. Therefore, just as in human platelets, the adhesion of bovine platelets to this type of collagen was GPIa/IIa-dependent. Adhesion of CHS platelets to soluble collagen was not different from that of normal platelets, suggesting that the GPIa/IIa receptor is expressed normally on CHS platelets and that collagen can bind to it in a way similar to normal platelets at least under the experimental conditions used here. The question remains whether a signal downstream of GPIa/IIa is deficient in CHS platelets, so that clarification of the interaction between rhodocytin and GPIa/IIa must be awaited.

The rhodocytin-produced signals in human platelets were inhibited by cytochalasin D whereas this compound did not affect the CRP-induced activation (18, 19). In this study, cytochalasin D inhibited the collagen-induced Ca^{2+} mobilization, suggesting that a cytochalasin D-sensitive pathway is involved in the collagen-produced Ca^{2+} signaling in bovine platelets. Similarly, the rhodocytin-produced Ca^{2+} signaling was sensitive to cytochalasin D whereas the convulxin-produced Ca^{2+} signaling was insensitive to it. This implies that collagen and rhodocytin utilize a common pathway that is sensitive to cytochalasin D. There have been reports that actin cytoskeletal reorganization facilitates integrin clustering and activation of tyrosine kinases (34, 35). Hence, the inhibition by cytochalasin D of collagen-induced Ca^{2+} mobilization may result from its inhibitory effect on clustering of some integrin. The susceptibility of rhodocytin-induced Ca^{2+} signaling to cytochalasin D is an important clue for clarification of the rhodocytin-sensitive mechanism.

Rhodocytin by itself could increase $[Ca^{2+}]_i$ in human (18, 19) and bovine platelets (this study). Thus, the rhodocytin-sensitive pathway is related to a Ca^{2+} mobilizing mechanism. However, an increase in $[Ca^{2+}]_i$ due to rhodocytin was extremely slow when compared to that by collagen or convulxin. This slowness suggests that Ca^{2+} mobilization mediated through this mechanism does not play a significant role in the collagen-induced Ca^{2+} mobilization. If GPVI can be exclusively activated by collagen without any supportive signal from other receptors, collagen should have produced Ca^{2+} signaling in CHS platelets similarly to that in normal platelets, because the response to the GPVI activator convulxin or CRP was the same between normal and CHS platelets. However, the response to collagen was greatly depressed in CHS platelets. This suggests that a signal from the other pathway, namely from the rhodocytin-sensitive pathway, to GPVI is indispensable so that collagen produces a sufficient Ca^{2+} signaling. Therefore, a possible role of the rhodocytin-sensitive mechanism is delivery of a signal required for complete activation of GPVI-dependent Ca^{2+} signaling rather than replenishment of Ca^{2+}. There are several types of collagen receptors on platelets and they are not independent of each other. For example, GPIa/IIa can be converted to a form with high affinity for soluble collagen when platelets are stimulated with CRP (36, 37). In this context, the rhodocytin-sensitive mechanism may play a crucial role in the crosstalk between GPVI and other receptor(s) when platelets are exposed to collagen.

The present results demonstrate that the rhodocytin-sensitive mechanism is necessary for the collagen-produced Ca^{2+} signaling. CHS platelets are useful in clarifying the interrelationship between GPVI and the other receptor system not only in bovine platelets but also in human ones.

Addendum

The following investigators, all in Japan, also participated in this study: Dr. Hiroyuki Ogawa (Graduate School of Agriculture and Life Science, The University of Tokyo, Tokyo) for discussion and advice, Tsuyoshi Fujishiro and Kiichi Fukuyama (the Sumiyoshi Ranch University Farm, Faculty of Agriculture, Miyazaki University) for technical assistance.

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References

1. Padgett GA. The Chediak-Higashi syndrome. Advan Vet Sci 1968; 12: 239-84.
2. Barbosa MDFS, Nguyen QA, Tchernev VT, Ashley JA, Detter JC, Blaydes SM, Brandt SJ, Chotai D, Hodgman C, Solari RCE, Lovett M, Kingsmore SF. Identification of the homologous beige and Chediak-Higashi syndrome genes. Nature 1996; 382: 262-5.
3. Nagle DL, Karim MA, Woolf EA, Holmgren L, Bork P, Misumi DJ, McGrail SH, Dussault BJ Jr, Perou CM, Boissy RE, Duyk GM, Spritz RA, Moore KJ. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. Nat Genet 1996; 14: 307-11.
4. Kunieda T, Nakagiri M, Takami M, Ide H, Ogawa H. Clonig of bovine *LYST* gene and identification of a missense mutation associated with Chediak-Higashi syndrome of cattle. Mamm Genome 1999; 10: 1146-9.
5. Buchanan GR, Handin RI. Platelet function in the Chediak-Higashi syndrome. Blood 1976; 47: 941-8.
6. Ogawa H, Tu C-H, Kagamizono H, Soki K, Inoue Y, Akatsuka H, Nagata S, Wada T, Ikeya M, Makimura S, Uchida K, Yamaguchi R, Otsuka H. Clinical, morphologic, and biochemical characteristics of Chediak-Higashi syndrome in fifty-six Japanese Black cattle. Am J Vet Res 1997; 58: 1221-6.
7. Meyers KM, Holmsen H, Seachord CL, Hopkins GE, Borchard RE, Padgett GA. Storage pool deficiency in platelets from Chediak-Higashi cattle. Am J Physiol 1979; 237: R239-48
8. Rendu F, Breton-Gorius J, Lebret M, Klebanoff C, Buriot D, Griscelli C, Levy-Toledano S, Caen JP. Evidence that abnormal platelet functions in human Chédiak-Higashi syndrome are the result of a lack of dense bodies. Am J Pathol 1983; 111: 307-14.
9. Pratt HL, Carroll RC, Jones JB, Lothrop CD Jr. Platelet aggregation, storage pool deficiency, and protein phosphorylation in mice with Chediak-Higashi syndrome. Am J Vet Res 1991; 52: 945-50.
10. Suzuki T, Goryo M, Inanami O, Uetsuki J, Saito S, Kaketa K, Oshima T, Shimizu H, Okabe S, Tanaka T, Kamata R, Shuto F, Sato I, Tachikawa E, Sakaguchi M, Kobayashi H, Okada K. Inhibition of collagen-induced platelet aggregation in Japanese Black cattle with inherited platelet disorder, Chediak-Higashi syndrome. J Vet Med Sci 1996; 58: 647-54.
11. Shiraishi M, Ikeda M, Ogawa H, Tu C-H, Ito K. Impaired cytosolic calcium mobilization and aggregation in response to collagen in platelets from Japanese Black cattle with Chediak-Higashi syndrome. Am J Vet Res 1998; 59: 744-9.
12. Shiraishi M, Ikeda M, Fujishiro T, Fukuyama K, Ito K. Characteristics of collagen-induced Ca^{2+} mobilization in bovine platelets. Cell Calcium 2000; 27: 53-60.
13. Nieuwenhuis HK, Akkerman JWN, Houdijk WPM, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. Nature 1985; 318: 470-2.
14. Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. Blood 1987; 69: 1712-20.
15. Polgár J, Clemetson JM, Kehrel BE, Wiedemann M, Magnenat EM, Wells TNC, Clemetson KJ. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (tropical rattlesnake) venom via the p62/GPVI collagen receptor. J Biol Chem. 1997; 272: 13576-83.
16. Morton LG, Hargreaves PG, Farndale RW, Young RD, Barnes M. Integrin $\alpha2\beta1$-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for $\alpha2\beta1$-independent platelet reactivity. Biochem J 1995; 306: 337-44.
17. Shin Y, Morita T. Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin family, induces platelet aggregation independently of glycoprotein Ib. Biochem Biophys Res Commun 1998; 245: 741-5.
18. Inoue K, Ozaki Y, Satoh K, Wu Y, Yatomi Y, Shin Y, Morita T. Signal transduction pathways mediated by glycoprotein Ia/IIa in human platelets: Comparison with those of glycoprotein VI. Biochem Biophys Res Commun 1999; 256: 114-20.
19. Suzuki-Inoue K, Ozaki Y, Kainoh M, Shin Y, Wu Y, Yatomi Y, Ohmori T, Tanaka T, Satoh K, Morita T. Rhodocytin induced platelet aggregation, by interacting with glycoprotein Ia/IIa (GPIa/IIa, integrin $\alpha2\beta1$): involvement of GPIa/IIa-associated Src and protein tyrosine phosphorylation. J Biol Chem 2001; 276: 1643-52.
20. Eble JA, Beermann B, Hinz H-J, Schmidt-Hederich A. $\alpha2\beta1$ integrin is not recognized by rhodocytin, but is the specific, high-affinity target of rhodocetin, an RGD-independent disintegrin and potent inhibitor of cell adhesion to collagen. J Biol Chem 2001; 276: 12274-84.
21. Bergmeier W, Bouvard D, Eble JA, Mokhtari-Nejad R, Schulte V, Zimgibl H, Fässler R, Nieswandt B. Rhodocytin (aggretin) activates platelets lacking $\alpha2\beta1$ integrin, and the ligand binding domain of GP1bα. J Biol Chem 2001; 276: 25121-6.
22. Humphries RG, Tomlinson W, Ingall AH, Cage PA, Leff P. FPL 66096: a novel, highly potent and selective antagonist at human platelet P2T-purinoceptors. Br J Pharmacol 1994; 113: 1057-63.
23. Hollopeter G, Jantzen H-M, Vincent D, Li G, England L, Ramakrishnan V, Yang R-B, Nurden P, Nurden A, Julius D, Conley PB. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001, 409: 202-7.
24. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the P2Y1 receptor. Mol Pharmacol 1996; 50: 1323-9.
25. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260: 3440-50.
26. Vorndran C, Minta A, Poenie M. New fluorescent calcium indicators designed for cytosolic retention or measuring calcium near membranes. Biophys J 1995; 69: 2112-24.
27. Moroi M, Okuma M, Jung SM. Platelet adhesion to collagen-coated wells: analysis of this complex process and a comparison with the adhesion to matrigel-coated wells. Biochim Biophys Acta 1992; 1137: 1-9.
28. Tarver AP, King WG, Rittenhouse SE. Inositol 1,4,5-trisphosphate and inositol 1,2-cyclic 4,5-trisphosphate are minor components of total mass of inositol trisphosphate in thrombin-stimulated platelets. J Biol Chem 1987; 262: 17268-71.
29. Asazuma N, Yatomi Y, Ozaki Y, Qi R, Kuroda K, Satoh K, Kume S. Protein-tyrosine phosphorylation and $p72^{syk}$ activation in human platelets stimulated with collagen is dependent upon glycoprotein Ia/IIa and actin polymerization. Thromb Haemost 1996; 75: 648-54.

30. Watson SP. Collagen receptor signaling in platelets and megakaryocytes. Thromb Haemost 1999; 82: 365-76.
31. Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokkhtari-Nejad R, Lindhout T, Heemskerk JWM, Zirngibl H, Fässler R. Glycoprotein VI but not α2β1 integrin is essential for platelet interaction with collagen. EMBO J 2001; 20: 2120-30.
32. Navdaev A, Clemetson JM, Polgár J, Kehrel BE, Glauner M, Magnenat E, Wells TNC, Clemetson KC. Aggretin, a heterodimeric C-type lectin from *Calloselasma rhodostoma* (Malayan Pit Viper), stimulates platelets by binding to α2β1 integrin and GPIb, activating Syk and PLCγ2, but does not involve the GPVI/Fcγ collagen receptor. J Biol Chem 2001; 276: 20882-9.
33. Nakamura T, Jamieson GA, Okuma M, Kambayashi J, Tandon NN. Platelet adhesion to native type I collagen fibrils. J Biol Chem 1998; 273: 4338-44.
34. Fox JE. Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets. J Clin Invest 1985; 76: 1673-83.
35. Clark EA, Brugge JS. Integrins and signal transduction pathways: The road taken. Science 1995; 268: 233-9.
36. Jung SM, Moroi M. Platelets interact with soluble and insoluble collagen through characteristically different reactions. J Biol Chem 1998; 273: 14827-37.
37. Jung SM, Moroi M. Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin α2β1. J Biol Chem 2000; 275: 8016-26.

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