博士論文

Studies on mechanism underlying insufficient aggregation of platelets from cattle affected with Chediak-Higashi syndrome

Chediak-Higashi 症候群を呈するウシにおける血小板凝集不全の原因解明に関する研究

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I Introduction

1) Chediak-Higashi syndrome

Chediak-Higashi syndrome (CHS) is an autosomal recessive genetic disease, which is manifested of partial oculocutaneous albinism, increased susceptibility to infections, and a bleeding tendency. The disease was initially described in humans by Beguez-Cesar in 1943 and named as CHS after the works by Chediak (1952), who described the hematological characteristics of the disorder, and Higashi (1954), who reported giant peroxidase-containing granules within cells from the disease-affected patients. In most humans affected with CHS, death often occurs in a first decade of the life from infection, bleeding, or development of the 'accelerated phase' which is characterized by a lymphoproliferative syndrome with lymphohistiocytic infiltration. A disease attributable to CHS is found in cattle, minks, cats, mice, rats and fox (Padgett *et al.*, 1964; Leader *et al.*, 1963; Kramer *et al.*, 1977; Lutzner *et al.*, 1967; Nishimura *et al.*, 1989; Sjaastad *et al.*, 1990).

CHS in cattle is found in Hereford (Padgett *et al.*, 1964), Japanese Black (Umemura *et al.*, 1983) and Brangus breeds (Ayers *et al.*, 1988). In Japan, Japanese Black cattle affected with CHS are frequently seen in South Kyusyu area (Miyazaki and Kagoshima Prefectures, Ogawa *et al.*, 1997) and infrequently in Tottori and Iwate Prefectures. The average age at the time of death in Hereford cattle with CHS has been reported to be 12.4 months (Padgett *et al.*, 1968). In contrast to Hereford cattle or humans affected with CHS, Japanese Black cattle affected with CHS were not lethal in most cases (Ogawa *et al.*, 1997). Manifestations of CHS seen in each animal species are listed in Table 1. In the following two sections, I review reported hematological abnormalities which are associated with recurrent infection and bleeding diathesis in CHS.

Table 1. *Clinical signs of Chediak Higashi syndrome*

	Human	Her/Cattle	JB/Cattle
Infection	Dermatitis Rhinopharyngitis Otitis Oral ulcer Abscess‡ Pneumonia Nephritis Hepatitis Peritonitis	Abscess‡ Pneumonia Endocarditis Nephritis Dermatitis Diarrhaea Upper respiratory infection Peritonitis	Abscess‡ Pneumonia Endocarditis Nephritis Dermatitis
Bleeding	Easy bruisability Mucosal bleeding Epitaxis	Bleeding follwing surgical procedures Hematoma Mucosal bleeding	Bleeding follwing surgical procedures Hematoma Epitaxis Melena Umbilical bleeding
Ocular findings	Pale iris Photophobia Nystagmus Abnormal fundic reflection	Pale iris Photophobia Abnormal fundic reflection	Pale iris Abnormal fundic reflection
Hypopigmentation of skin, and hair	Present	Present	Present
Neurologic findings*	Present	Absent	Absent
Accelerated phase†	Present	Absent	Absent

Her, Hereford; JB, Japanese Black. *Peripheral and cranial neuropathy, autonomic dysfunction, weakness, sensory deficits, hyporeflexia, clumsiness, and seizures. †Fever, anemia, neutropenia, thrombocytopenia, hepatosplenomegaly, lymphadenopathy and jaundice. ‡Muscle, lung, skin and liver.

2) Abnormalities in leukocytes

The most important clinical manifestations in humans affected with CHS are recurrent severe pyogenic infections, which may be related to decreased functions of leukocytes. Abnormally giant granules are frequently found in neutrophils, eosinophils, lymphocytes, monocytes and their precursor cells (Padgett *et al.*, 1967;

Padgett *et al.*, 1968; Blume *et al.*, 1969). Appearance of giant granules, which are probably lysosomes (Jones *et al.*, 1992), is regarded as a hallmark of CHS in all animal species. Giant cytoplasmic granules are also present in other granule-containing cells such as melanocyte, neurocyte and hepatocyte (Padgett, 1968; White & Clawson, 1980). Although the leukocyte count is normal in CHS-affected Hereford and Japanese Black cattle (Renshaw *et al.*, 1974; Ogawa *et al.*, 1997), all eosinophils and basophils have giant granules (Padgett *et al.*, 1968; Ogawa *et al.*, 1997).

Abnormal granule-containing cells show a decreased activity of lysosomal enzymes (Holcombe *et al.*, 1994). Levels of cathepsin G and elastase appear to be decreased in humans and mice with CHS (Holcombe *et al.*, 1994). Chemotactic activity of neutrophils from humans affected with CHS is decreased to approximately 40% of the normal level (Clark & Kimball, 1971). Mononuclear leukocytes in CHS-affected humans, minks and Hereford cattle also exhibit a decreased chemotactic response (Gallin *et al.*, 1975). Chemotactic defect may be because giant cytoplasmic granules inhibit cell deformability (Clark & Kimball, 1971; Clawson *et al.*, 1978). Renshaw *et al.* (1974) revealed that a defect in bactericidal activity was associated with a decrease in intracellular killing activity of polymorphonuclear leukocytes in Hereford cattle affected with CHS. Impaired cytotoxic function in T cells and natural killer (NK) cells were also reported in CHS-affected humans and mice (Abo *et al.*, 1982; Haliotis *et al.*, 1980; Klein *et al.*, 1980; Targan & Oseas, 1983; Baetz *et al.*, 1995). This abnormality probably contributes to increased susceptibility to infection and development of the accelerated phase.

3) Platelets

Most human and animal patients affected with CHS show bleeding diathesis. Prolonged bleeding time has been reported in most affected animal species (Buchanan

& Handin, 1976; Ogawa *et al.*, 1997; Bell *et al.*, 1976; Ayers *et al.*, 1988; Cowles *et al.*, 1992; Meyers *et al.*, 1981; Bell *et al.*, 1980; Sjaastad *et al.*, 1990). On the other hand, coagulation system is normal in humans, Japanese Black cattle, and cats affected with CHS (Buchanan & Handin., 1976; Meyer *et al.*, 1981; Ogawa *et al.*, 1997). Platelet count is normal in most CHS cases of humans, Japanese Black, Hereford cattle, and mink (Bell *et al.*, 1976; Bell *et al.*, 1980; Ogawa *et al.*, 1997). These findings imply that the bleeding diathesis of CHS may result from impairment of platelet functions but not from thrombocytopenia or coagulopathy. However, severe gastrointestinal hemorrhage which is observed in an accelerated phase may be occurred by thrombocytopenia.

Platelets are small discoid cell fragments produced from megakaryocytes of approximately 0.5 x 3.0 μm long. Under a normal condition, platelets are not adhesive to a vessel wall and do not adhere each other. When a blood vessel is damaged at the luminal side, however, platelets adhere to a collagen matrix in the subendothelial layer and become activated. Activated platelets change the shape from a discoid to a spheroid form, accompanied by formation of filopodia-like structures. This is followed by secretion of granule contents, production of thromboxane A_2 (TXA_2) and platelet aggregation. Rapid formation of a platelet plug at a site of injury is a main mechanism for primary hemostasis. Next, this primary thrombus is intensified by formation of fibrin fibrils through a coagulation cascade. In addition, platelets help hemostasis by participating in coagulation mechanisms, since several enzymatic reactions involved in blood coagulation occur on the membrane surface of platelets (Heemskerk *et al.*, 2000).

(1) Abnormalities in platelet function

Table 2 lists data from *in vitro* aggregation experiments using platelets from CHS patients. Decreased platelet aggregation in response to collagen is a common feature in almost all patients affected with CHS. In humans with CHS, impairment

of epinephrine-induced aggregation was also observed (Buchanan & Handin, 1976; Bell *et al.*, 1976; Apitz-Castro *et al.*, 1985). Platelet aggregation by ADP is normal or sometimes slightly decreased with a tendency to disaggregate in CHS platelets (Table 2). Phorbol-12-myristate 13-acetate (PMA)-induced aggregation was normal in Japanese Black cattle with CHS, while it was depressed in mouse with CHS (Suzuki *et al.*, 1996; Pratt *et al.*, 1991).

TXA_2 is released from activated platelets as a result of a sequential cascade consisted of phospholipase A_2, cyclooxygenase-1 and thromboxane synthase and acts as a secondary agonist on platelets (Nakano *et al.*, 1989; Pollock *et al.*, 1986; Siess *et al.*, 1983). Aggregation to exogenous arachidonic acid, which is converted to TXA_2, was reported to decrease in two human CHS cases (Weening *et al.*, 1981; Apitz-Castro *et al.*, 1985), although another patient showed normal aggregation response (Rendu *et al.*, 1983). Suzuki *et al.* (1996) suggested that impairment of production of TXA_2 is responsible for a depression of aggregation to collagen in CHS platelets since indomethacin, a cyclooxygenase inhibitor, inhibited the collagen-induced aggregation in normal platelets while it exerted a smaller effect on the aggregation in CHS platelets from Japanese Black cattle. However, in minks affected with CHS, decreased aggregation of platelets was apparent even after influence of endogenous TXA_2 was removed by pretreatment with aspirin (Bell *et al.*, 1980). Thus, it is controversial whether a decrease in TXA_2 production is important for a decreased response to collagen in CHS platelets.

Table 2. *Platelet aggregation induced by various agonists in animals affected with Chediak-Higashi syndrome.*

Animal	Coll	ADP	Thr	Epi	A23187	PMA	Ser	AA	Author
Human	A	N	-	A	-	-	-	-	Buchanan & Handin (1976)
	A	A	-	A	-	-	-	-	Bell *et al.* (1976)
	V	V	-	V	-	-	-	-	Boxer *et al.* (1977)
	N	N	-	-	-	-	A	-	Parmley *et al.* (1979)
	A	A*	-	-	-	-	-	A	Weening *et al.* (1981)
	-	N	A	-	-	-	-	N	Rendu *et al.* (1983)
	A	N	-	-	-	-	-	-	Legrand & Nurden (1985)
	A*	A*	-	A	V	-	-	A	Apitz-Castro *et al.* (1985)
Cattle/Her	A	A	-	0	-	-	-	-	Bell *et al.* (1976)
Cattle/JB	A	A*	N	-	-	N	-	-	Suzuki *et al.* (1996)
	A	A*	-	-	-	-	-	-	Ogawa *et al.* (1997)
Mink	A	N	-	-	-	-	-	-	Bell *et al.* (1980)
Cat	A	A	-	0	-	-	A	-	Meyers *et al.* (1981)
Fox	A	A*	-	0	-	-	V	A	Sjaastad, *et al.* (1990)
Mice	A†	-	A†	-	A†	A	-	-	Pratt *et al.* (1991)
Rat	A	A*	-	-	-	-	-	-	Ozaki *et al.* (1998)

Her, Hereford; JB, Japanese Black. Coll, collagen; Thr, thrombin; Epi, epinephrine; Ser, serotonin; AA, arachidonic acid. A, abnormal; N, normal; 0, no aggregation; V, variable. *, tend to disaggregation; †, only at low concentration.

(2) Abnormalities in platelet structure

The ultrastructural examination of platelets revealed that there is no difference in a shape and size of platelets from CHS-affected Hereford (Prieur *et al.*, 1976), Japanese Black cattle (Ogawa *et al.*, 1997) and humans (Rendu *et al.*, 1983). A platelet has at least three types of secretary granule; alpha granules, lysosomes, and dense granules (otherwise called δ-granules). An abnormality in lysosomes was not observed in platelets from cattle (Prieur *et al.*, 1976; Meyer *et al.*, 1982; Menard *et al.*, 1990), cats (Meyer *et al.*, 1982), minks (Meyer *et al.*, 1982) and most humans affected with CHS (Rendu *et al.*, 1983; Boxer *et al.*, 1977). Some studies have showed that platelets from CHS patients exhibit δ-storage pool deficiency (δ-SPD), which is

characterized by reduced and irregular dense granules (McNicol & Israels, 1999). Dense granules contain ADP, ATP, serotonin and Ca^{2+}. ADP is released when platelets are activated by various kinds of stimulation, and acts as a secondary agonist, thus, playing a crucial role in thrombosis as well as TXA_2 in human platelets. The number of dense granules in platelets from Hereford and Japanese Black cattle (Prieur *et al.*, 1976; Ogawa *et al.*, 1997; Menard & Meyers, 1988; Meyer *et al.*, 1982; Meyers *et al.*, 1979), humans (Rendu *et al.*, 1983), and rats (Ozaki *et al.*, 1998) was fewer in patients than in normal controls. An absence of dense granule precursors in megakaryocytes has been reported in CHS-affected Hereford cattle (Menard & Meyers, 1988). Alpha granules in CHS platelets were similar in structure and quantity to those in normal platelets (Prieur *et al.*, 1976; Meyer *et al.*, 1982; Meyers *et al.*, 1979; Rendu *et al.*, 1983). In accord with these observations, contents in dense granules (ADP, ATP, serotonin and Ca^{2+}) were greatly decreased in humans and animals affected with CHS (Boxer *et al.*, 1977; Meyers *et al.*, 1979; Ogawa *et al.*, 1997; Bell *et al.*, 1980; Meyers *et al.*, 1981; Sjaastad *et al.*, 1990; Pratt *et al.*, 1991; Ozaki *et al.*, 1998). It has been suggested that the structural and functional defects in CHS partly result from an impaired microtubule assembly (Oliver *et al.*, 1975; Oliver & Zurier, 1976; Hinds & Danes, 1976). In CHS platelets, however, abnormal structure or function of microtubule has not been reported in all species.

4) A gene responsible for the disease

Recently, the gene responsible for CHS has been cloned in humans, mice and Japanese Black cattle (Barbosa *et al.*, 1996; Nagle *et al.*, 1996, Kunieda *et al.*, 1999). A gene associated with CHS in these species encodes a novel -430-kDa protein, LYST, which stands for lysosome trafficking regulator (Barbosa *et al.*, 1996; Nagel *et al.*, 1996). Nagle *et al.* (1996) found that the LYST protein contains a series of hydrophobic helices resembling HEAT and ARM domain, and seven consecutive

WD40 motifs at the C-terminal region. These motifs are similar to a yeast serine/threonine protein kinase, Vps15, which plays a role in vacuolar protein sorting (Klionsky & Emr, 1990). These findings suggest that the LYST protein may function in trafficking of subcellular organelle proteins. However, the biological function of the LYST protein remains to be clarified.

LYST is expressed in many types of cell and is localized to the cytosol (Perou *et al.*, 1997), although association with microtubules has also been reported (Faigle *et al.*, 1998). In humans and mice with CHS, all the LYST gene alterations published so far result in a truncated protein because of a nonsense or a frame shift mutation (Nagle *et al.*, 1996; Barbosa *et al.*, 1997; Karim *et al.*, 1997; Certain *et al.*, 2000). In Japanese Black cattle affected with CHS, a single base is substituted, resulting in substitution of histidine at a residue 2015 to arginine. This site is highly conserved among humans, mice, and Japanese Black cattle (Kunieda *et al.*, 1999).

5) Aim of this study

As reviewed above, the mechanisms underlying the platelet disorder in CHS-affected patients have not been fully clarified. In this study, I aimed to clarify the mechanism of insufficient aggregation of CHS platelets. I focused my attention on alterations in Ca^{2+} signaling of CHS platelets, since Ca^{2+} is an important intracellular messenger within many cells (Berridge, 1993). In platelets, it is agreed that an increase in $[Ca^{2+}]_i$ is an initial event required for activation by almost all agonists (Rink & Sage, 1990; Smith *et al.*, 1992a). Such Ca^{2+} is supplied by release of Ca^{2+} from the dense tubular system, intracellular Ca^{2+} stores in platelets (Ebbeling *et al.*, 1992), and by Ca^{2+} entry from the extracellular fluid. However, it has not yet been determined whether abnormal handling of cytosolic Ca^{2+} is involved in the impairment of platelet aggregation in patients affected with CHS.

As aforementioned, the response to collagen is impaired in CHS platelets.

The mechanism by which collagen induces Ca^{2+} mobilization has not been clarified in details, especially no report about the mechanism of Ca^{2+} mobilization in bovine platelets has been published. Therefore, at first I tried to delineate the characteristics of collagen-induced Ca^{2+} mobilization in bovine platelets. Then, I examined whether the impaired aggregation of platelets from Japanese Black cattle with CHS was attributable to alterations in Ca^{2+} handling.

Platelet activation due to collagen induces granule secretion and arachidonic acid liberation, which in turn enhance an increase in $[Ca^{2+}]_i$ and aggregation (Emms & Lewis, 1986). Some groups proposed that a decrease in release of ADP as a result of δ-SPD is a major cause for defective aggregation in CHS platelets (Boxer *et al.*, 1977; Meyers *et al.*, 1979; Rendu *et al.*, 1983). Nieuwenhuis *et al.* (1987) reported 106 patients with confirmed δ-SPD, of whom 23% had normal aggregation responses to ADP, epinephrine and collagen. In platelets from normal Hereford cattle, ADP content is only about 30% of that of human platelets (Bell *et al.*, 1976; Meyer *et al.*, 1982). This suggests that ADP plays a minor role in aggregation of bovine platelets as compared with human ones. On the other hand, Suzuki *et al.* (1996) postulated that a deficit in arachidonic acid cascade is a main cause for insufficient aggregation of CHS platelets in Japanese Black cattle. However, aggregation of bovine platelets induced by collagen, ADP or platelet-activating factor was virtually insensitive to cyclooxygenase inhibitors (Bondy & Gentry, 1989; Gentry *et al.*, 1989). Therefore, a role of TXA_2 in the collagen-induced response is expected to be small in these platelets. Taken together, it is doubtful that neither a decrease in secondary endogenous agonists resulting from δ-SPD nor abnormalities in arachidonic acid metabolism is responsible for the defective activation induced by collagen in CHS platelets. Consequently, I tested whether endogenous ADP or arachidonic acid products were involved in the impaired Ca^{2+} handling.

Glycoprotein (GP) Ia/IIa (also termed integrin α2β1) and GPVI are predominant receptors for collagen in platelets (Nieuwenhuis *et al.*, 1985; Sugiyama

et al., 1987; Watson, 1999). Although GPIa/IIa is suggested to be responsible for the adhesion to collagen and GPVI for the platelet activation, the precise role of each receptor in Ca^{2+} mobilization has not been fully understood. Recently, several proteins that preferentially act on GPIa/IIa or GPVI have been identified in snake venoms. Rhodocytin, isolated from the *Calloselasma rhodostoma* venom, and convulxin, isolated from the *Crotalus durissus terrificus* venom, have been introduced to activate platelets by interacting with GPIa/IIa and GPVI, respectively (Jandrot-Perrus *et al.*, 1997; Polgár *et al.*, 1997; Shin & Morita, 1998; Inoue *et al.*, 1999; Suzuki-Inoue *et al.*, *in press*). Besides, collagen-related peptide (CRP), which consists of a glycine-proline-hydroxyproline repeat and is cross-linked via cysteine residues at its C- and N-terminals, activates platelets by interacting with GPVI (Morton *et al.*, 1995; Kehrel *et al.*, 1998; Verkleij *et al.*, 1998). These substances have become tools to investigate roles of GPIa/IIa and GPVI in collagen-induced platelet activation. To address which collagen receptor was impaired in CHS platelets, I observed the Ca^{2+} signaling produced by collagen, convulxin or rhodocytin in platelets from normal or CHS-affected cattle.

II Materials and Methods

1) Materials

Fura-PE3/AM (TEFLAB, Austin, TX, USA), aspirin (Takeda Chemical Industries, Osaka, Japan), U73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), ionomycin (Calbiochem-Novabiochem Co., La Jolla, CA, USA), thapsigargin (RBI, Natick, MA, USA), cytochalasin D (Sigma, St. Louis, MO, USA), and ONO-RS-082 (2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid, Ono Pharmaceutical Co., Osaka, Japan) were dissolved in DMSO. Adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS), mouse IgG1 κ (MOPC-21), RGDS (Arg-Gly-Asp-Ser, Sigma, St. Louis, MO, USA), ARL66096 (2-propylthio-D-β,γ-difluoromethylene ATP, a gift of Dr. R. G. Humphires of AstraZeneca R&D Charnwood, Loughborough, UK), U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_2\alpha$, Cayman Chemical, Ann Arbor, MI, USA), and thrombin (Mochida Pharmaceutical Co., Tokyo, Japan) were dissolved in distilled water. Prostaglandin E_1 (PGE_1, BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) was dissolved in ethanol. Acid soluble type I collagen from calf skin was from Sigma (St. Louis, MO, USA). Monoclonal antibody CD49b (Gi9) was purchased 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). Rhodocytin was kindly provided by Dr T. Morita (Department of Biochemistry, Meiji College of Pharmacy, Kiyose, Tokyo, Japan). Convulxin and CRP were gifted from Dr M. Moroi (Department of Protein Biochemistry, Institute of Life Science, Kurume University, Kurume, Fukuoka, Japan). Bovine serum albumin (BSA), Fraction V, was purchased from Sigma (St. Louis, MO, USA). Native collagen fibrils from equine tendons were purchased from Nycomed (Munich, Germany). The pH of vehicle to suspend collagen was 3.0. The vehicle did not alter the pH of Hepes

buffer suspending platelets and had no effect on cytosolic Ca^{2+} concentration in platelets.

2) Animals

Healthy Japanese Black cattle have been maintained in Sumiyoshi Ranch of Miyazaki University. CHS-affected Japanese Black cattle used were total 4 and brought from farms in our area to Veterinary Hospital of Miyazaki University. None of the cattle had clinical signs of acute infection at the time of the study. Human blood was collected from healthy volunteers who had not received any medication in the previous two weeks. For all cattle and humans, platelet counts were within the normal range.

3) Measurement of platelet aggregation

Blood was collected by puncture of the jugular vein of Japanese Black cattle and anticoagulated with 0.1 volume of 4.6% trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifuging blood at 160 x g for 10 min at 16°C and collecting the supernatant. Platelet-poor plasma (PPP) was obtained by recentrifuging the sediment at 1,800 x g for 10 min at 16°C and collecting the supernatant. For evaluation of platelet aggregation, PRP, to which PPP had been added to become 3 x 10^8 platelets/ml, was put in a cuvette and stirred continuously at 1,000 rpm with a magnetic stirrer. Aggregation responses were determined in the presence of $CaCl_2$ (1 mM) by measuring light transmission with an aggregometer (HEMA TRACER, Niko Bioscience Co., Tokyo, Japan). For each experiment, the aggregometer was calibrated with PPP (100% transmission) and non-aggregated PRP (0% transmission). When needed to exclude the influence of arachidonic acid metabolites, PRP was pretreated with indomethacin (10 μM) or aspirin (1 mM) for 30 min at 37°C. When

used, ONO-RS-082 (5 μM) or ARL66096 (3 μM) were applied for 4 or 2 min before addition of an agonist, respectively.

4) Measurement of $[Ca^{2+}]_i$

PGE_1 (1 μM) was added to PRP to prevent platelet activation, and the PRP was centrifuged at 800 x g for 10 min at 16°C. The supernatant was discarded, and platelets in the pellet were suspended 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) BSA, pH 7.4) at a concentration of 1 x 10^9 platelets/ml. Platelet suspension was incubated with 2 μM fura-PE3/AM for 30 min at 37°C. PGE_1 (1 μM) was then added, and the suspension was centrifuged at 700 x g for 10 min at 16°C. The supernatant was discarded, and platelets in the pellet were resuspended in Hepes buffer at a concentration of 3 x 10^8 platelets/ml. After fura-PE3-loaded platelets were put in a cuvette, 1 mM Ca^{2+} was added. When needed to exclude the influence of arachidonic acid metabolites, indomethacin (10 μM) or aspirin (1 mM) was added during incubation with fura-PE3/AM. To further exclude the influence of endogenous ADP, ARL66096 (100 nM) and/or A3P5PS (100 μM) were applied to aspirin-pretreated platelets for 90 and 30 sec, respectively, before addition of agonists. Fluorescences at 500 nm emission after alternative excitation at 340 nm (F_{340}) and 380 nm (F_{380}) were measured in a fluorimeter (CAF-100, JASCO, Tokyo, Japan) at 37°C. For calibration, platelets were lysed with 0.25% Triton X-100, and fluorescences at minimum Ca^{2+} concentration (F_{min}) and at saturated Ca^{2+} concentration (F_{max}) were obtained by adding EGTA (5 mM) and subsequently adding $CaCl_2$ (10 mM) (Thomas & Delaville, 1991). $[Ca^{2+}]_i$ was calculated by use of the ratio method (Grynkiewicz *et al.*, 1985) with a K_D value of 290 nM for fura-PE3 (Vorndran *et al.*, 1995).

Human blood was collected by venipuncture and anticoagulated with 0.1 volume of 3.8% trisodium citrate. Human platelets were loaded with fura-PE3/AM

in a way similar to bovine ones.

5) Measurement of Mn^{2+} entry

Entry of Mn^{2+} into platelets was measured by the fura-PE3 fluorescence quenching technique (Alonso, *et al.*, 1989; Sargent *et al.*, 1992). In the presence of $CaCl_2$ (1 mM), $MnCl_2$ (0.5 mM) was added 1 min before the application of an agonist. Fluorescence was monitored at an emission of 500 nm following excitation at 360 nm (F_{360}), an isosbestic point where fura-PE3 is insensitive to a change in Ca^{2+} concentration (Vorndran *et al.*, 1995).

6) Measurement of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)

PRP was centrifuged and suspended in Hepes buffer to a concentration of 1 x 10^9 platelets/ml. Platelet suspension was incubated with aspirin (1 mM) for 30 min at 37°C, then centrifuged and resuspended as described above. Platelets were resuspended in Hepes buffer to make a concentration of 2 x 10^9 platelets/ml. The suspension (200 µl) was incubated with drugs at 37°C while being stirred with a magnetic stirrer in the presence of 1 mM $CaCl_2$. At the indicated time, the reaction was terminated by addition of 20% ice-cold trichloroacetic acid (TCA, 70 µl). After leaving on ice for 15 min, samples were centrifuged at 1, 000 x 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 competitive binding of $[^3H]Ins(1,4,5)P_3$ to the receptor, which was obtained from calf cerebellum, using an $Ins(1,4,5)P_3$ assay kit (NEN Life Science Products, Boston, MA, USA) according to the manufacturer's protocol.

7) Adhesion experiment

Adhesion of platelets to collagen-coated microtiter wells was measured by a modified method originally described by Moroi *et al.* (1992). 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 with citrate buffer (6.85 mM citrate, 130 mM NaCl, 4 mM KCl, pH 6.0) containing 0.3% BSA and resuspended in Mg^{2+}-free Hepes buffer containing 1% BSA at a concentration of 2 x 10^8 cells/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).

Acid soluble type I collagen was dissolved in 0.05% acetic acid at a concentration of 300 μg/ml unless otherwise stated. An aliquot of collagen solution (50 μl) was added to each well of a microtiter plate (96 wells) and allowed to settle for 2 hr at room temperature. Collagen-coated wells were also incubated with Mg^{2+}-free Hepes buffer containing 1% BSA (250 μl) for 1 hr at room temperature to block non-specific adhesion of platelets. An aliquot (50 μl) of ^{51}Cr-labeled platelets suspended in Hepes buffer containing 1% BSA and EGTA (2 mM) or $MgCl_2$ (2 mM) was added to each well and allowed to settle for the indicated time at room temperature. After each well was washed 4 times with Hepes buffer with or without $MgCl_2$, adhered platelets were solubilized with 2% SDS (100 μl) for 30 min and the radioactivity of ^{51}Cr was measured in a gamma scintillation counter (ARC-300, Aloka Co. Ltd, Tokyo, Japan). 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.

8) 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.

III Results

1) Characteristics of collagen-induced aggregation and Ca^{2+} mobilization in bovine platelets

(1) Collagen- and ADP-induced aggregation in bovine platelets

Collagen or ADP was applied to PRP from normal cattle 3 min after addition of $CaCl_2$ (1 mM). Collagen (3-15 μg/ml) or ADP (1-10 μM) induced platelet aggregation in a concentration-dependent manner (see Fig. 10 and 14). A shape change, which is distinguished as a downward deflection of the trace, preceded the aggregation in platelets from normal cattle. Collagen (3-15 μg/ml) induced sustained aggregation. On the other hand, 1 or 3 μM ADP induced reversible aggregation whereas at 10 μM the aggregation was sustained in bovine platelets.

(2) Collagen- and ADP-induced increase Ca^{2+} mobilization

Collagen (1-10 μg/ml) or ADP (1-10 μM) was applied to fura-PE3-loaded bovine platelets 2 min after addition of $CaCl_2$ (1 mM) (Fig. 1). The basal level of $[Ca^{2+}]_i$ after addition of $CaCl_2$ was 83.1 ± 6.1 nM (n = 10). ADP (1 μM) rapidly increased $[Ca^{2+}]_i$, which attained the peak at 11.4 ± 0.7 sec (n = 4), and then declined to a steady state level at about 2 min. In contrast, collagen (10 μg/ml) gradually and sustainedly increased $[Ca^{2+}]_i$ (Fig. 1A). At this concentration, it took 53.0 ± 3.1 sec (n = 4) to attain the sustained phase. At lower concentrations of collagen, the rate of rise in $[Ca^{2+}]_i$ was slower (Fig. 1A). An increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was calculated by subtracting the basal $[Ca^{2+}]_i$ from the maximum $[Ca^{2+}]_i$ during 4min after the addition of agonists. $\Delta[Ca^{2+}]_i$ induced by collagen or ADP was concentration-dependent between 1-10 μg/ml or 1-10 μM, respectively (Fig. 1B).

(3) Effects of arachidonic acid cascade inhibitors or ADP receptor

antagonist on the collagen-induced aggregation

To determine whether arachidonic acid metabolites or endogenous ADP were involved in the collagen-induced aggregation of bovine platelets, effects of pretreatment with arachidonic acid cascade inhibitors or ADP receptor antagonists were investigated. After PRP from normal cattle was preincubated with indomethacin (10 μM), a cyclooxygenase inhibitor, or vehicle (0.1% DMSO) for 30 min, platelet aggregation in response to collagen was tested (Fig. 2). Indomethacin had no effect on the magnitude of collagen (15 μg/ml)-induced aggregation, although the onset of aggregation was delayed slightly. Similarly, pretreatment with aspirin (1 mM), another cyclooxygenase inhibitor, for 30 min did not have any effect on the collagen-induced aggregation (data not shown).

To evaluate involvement of endogenous ADP in the collagen-induced aggregation, effects of ARL66096, a selective antagonist of $P2T_{AC}$ purinoceptors which is functionally coupled to aggregation mechanism due to ADP (Humphries *et al.*, 1994), on the collagen-induced responses were examined (Fig. 3). Incubation of platelets with ARL66096 (3 μM) for 3 min inhibited ADP (3 μM)-induced aggregation in PRP from normal cattle by 83.2 ± 2.8% (n = 5). ARL66096 (3 μM) partially inhibited the collagen (15 μg/ml)-induced aggregation of platelets from normal cattle (inhibition; 14.1 ± 3.6%, n = 6).

(4) Effects of arachidonic acid cascade inhibitors or ADP receptor antagonist on the collagen-induced Ca^{2+} mobilization

The effect of pretreatment with aspirin or ADP receptor antagonist on Ca^{2+} mobilization induced by collagen in bovine platelets was compared with that in human platelets. In human platelets (Fig. 4A), collagen (10 μg/ml) caused a transient increase in $[Ca^{2+}]_i$, which was strongly depressed when pretreated with aspirin (1 mM). In bovine platelets (Fig. 4B), in contrast, pretreatment with aspirin only slightly inhibited the collagen-induced increase in $[Ca^{2+}]_i$ (inhibition; 14.9 ±

4.7%, n = 4). Pretreatment with indomethacin (10 μM) for 30 min and or pretreatment with ONO-RS-082 (5 μM), a phospholipase A_2 inhibitor (Banga *et al.*, 1986), for 5 min also did not affect the collagen-induced increase in $[Ca^{2+}]_i$ (data not shown). Involvement of endogenous ADP in the collagen-induced Ca^{2+} mobilization in bovine platelets was checked by the effect of A3P5PS, an antagonist to $P2Y_1$ receptor, which is functionally coupled to Ca^{2+} mobilization due to ADP (Boyer *et al.*, 1996). Pretreatment with A3P5PS (100 μM) for 30 sec inhibited the ADP (1 μM)-induced increase in $[Ca^{2+}]_i$ by 96.6 ± 0.4% (n = 4) in aspirin-treated bovine platelets. However, A3P5PS at this concentration showed only a partial inhibition on the collagen-induced increase in $[Ca^{2+}]_i$ in aspirin-pretreated platelets (inhibition; 21.5 ± 5.6%, n = 4) or non-pretreated ones (inhibition; 33.2 ± 4.8%, n = 4) (Fig. 4B).

The above results suggest that arachidonic acid metabolites, mainly TXA_2, are not involved in the collagen-induced Ca^{2+} mobilization in bovine platelets. To see whether bovine platelets are insensitive to TXA_2, I observed the effect of U46619, a TXA_2 mimetic, on aggregation or Ca^{2+} mobilization in normal platelets. Addition of 10 μM U46619 caused a shape change in normal platelets but did not cause aggregation (data not shown). The increase in $[Ca^{2+}]_i$ induced by U46619 was pretty small, compared with that induced by ADP or collagen (see Fig. 12). In the later experiments, I used aspirin-treated bovine platelets to exclude the effect of arachidonic acid metabolites.

(5) Effects of U73122 on the collagen-induced Ca^{2+} mobilization

In order to address the mechanism responsible for the collagen-induced Ca^{2+} mobilization, the effect of U73122, a phospholipase C (PLC) inhibitor (Bleasdale *et al.*, 1990; Vickers, 1993), on the collagen-induced increase in $[Ca^{2+}]_i$ was observed in the presence of extracellular Ca^{2+}. U73122 (0.3-5 μM), which was applied 3 min before collagen (10 μg/ml), inhibited the collagen-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 5A). At 5 μM, U73122 inhibited the

collagen-induced increase in $[Ca^{2+}]_i$ by more than 90%. U73122 similarly inhibited the thrombin (0.1 U/ml)- or ADP (1 μM)-induced increase in $[Ca^{2+}]_i$, as about 90% of the increase in $[Ca^{2+}]_i$ due to each agonist was inhibited by 5 μM U73122 (Fig. 5B).

Thapsigargin (10 nM)-induced increase in $[Ca^{2+}]_i$ consisted of an initial peak and a sustained phase (Fig. 6). The peak $[Ca^{2+}]_i$ due to thapsigargin was sensitive to U73122, but the inhibition was less than in the case of collagen, ADP or thrombin since the inhibition by 5 μM U73122 of the peak $[Ca^{2+}]_i$ was 49.2 ± 7.3% (n = 4). U73122 (5 μM) did not significantly decrease the sustained phase of $[Ca^{2+}]_i$ due to thapsigargin (Fig. 6). When the platelets had been pretreated with A3P5PS (100 μM), the peak $[Ca^{2+}]_i$ reached by thapsigargin was decreased by 61.2 ± 10.4% (n = 4). In the presence of A3P5PS, U73122 (5 μM) attenuated the peak $[Ca^{2+}]_i$ due to thapsigargin by only 17.5 ± 11.5% (n = 4). In contrast, ionomycin (1 μM) increased $[Ca^{2+}]_i$ to 271.0 ± 31.7 nM (n = 4), but the response was insensitive to U73122 (Fig. 5). These data suggest that thapsigargin stimulates secretion of ADP and then activates PLC, and this is partially responsible for the Ca^{2+} mobilization.

(6) Dependence of the collagen-induced Ca^{2+} mobilization on $Ins(1,4,5)P_3$ production

The data with U73122 suggest that inositol phospholipid breakdown through PLC is involved in the collagen-induced Ca^{2+} mobilization. To ensure this, I measured the production of $Ins(1,4,5)P_3$ in bovine platelets stimulated with collagen using a competitive radioligand binding assay. Collagen- or thrombin-induced production of $Ins(1,4,5)P_3$ was measured at the time when $[Ca^{2+}]_i$ attained the peak in aspirin-treated platelets. With this assay, I confirmed that in $Ins(1,4,5)P_3$ was increased by 2-fold at 0.5 min after addition of thrombin (0.1 U/ml), which is known to stimulate PLC (Tarver *et al.*, 1987) (Fig. 7). At 1 min after challenging with collagen (10 μg/ml), $Ins(1,4,5)P_3$ increased to 3.5-fold over the resting level (Fig. 7). The collagen-induced increase in $Ins(1,4,5)P_3$ was blocked by pretreatment with

U73122 (5 μM).

Next, I measured the collagen-induced $[Ca^{2+}]_i$ mobilization in the absence of external Ca^{2+} to observe if Ins(1,4,5)P3 produced by collagen induced Ca^{2+} release from Ca^{2+} stores. EGTA (3 mM) was added 1 min after incubating with $CaCl_2$ (1 mM) to chelate external Ca^{2+}. In the presence of external EGTA, thrombin (0.1 U/ml) increased $[Ca^{2+}]_i$ to 43.6 ± 7.5% (n = 4) of the response obtained in the presence of external Ca^{2+}. Although collagen (10 μg/ml) could increase $[Ca^{2+}]_i$ in the presence of EGTA, this increase was very small as compared with that in the absence of EGTA (7.3 ± 2.6% of the response in the absence of EGTA, n = 4). In contrast to collagen, ADP (1-10 μM) induced sizeable Ca^{2+} mobilization in Ca^{2+}-free medium, which was about 60% of that observed in normal medium (see Fig. 15 and 16).

(7) Collagen-induced Mn^{2+} entry and quenching of fura-PE3 fluorescence

In non-excitable cells, Mn^{2+} enters cells through a receptor-operated Ca^{2+} entry pathway by competing with Ca^{2+} and quenches fura-2 fluorescence inside cells (Alonso *et al.*, 1989; Sargeant *et al.*, 1992). Therefore, Mn^{2+} quenching can be used as an index of Ca^{2+} entry through receptor-operated Ca^{2+} entry. Using this property, I observed quenching of cytosolic fura-PE3 fluorescence by Mn^{2+} in order to know the extent of several agonists-induced Ca^{2+} entry in platelets. To see the quenching, I set the excitation filter at 360 nm, an isosbestic wavelength at which Ca^{2+} does not affect the fluorescence of fura-PE3 (Vorndran *et al.*, 1995). In the presence of $CaCl_2$ (1 mM), Mn^{2+} slowly quenched the fluorescence excited at 360 nm. This means that there is a small amount of Ca^{2+} entry (or leak) in the resting state. Addition of collagen (10 μg/ml), ADP (1 μM), thrombin (0.1 U/ml) or thapsigargin (10 nM) accelerated the quenching of fluorescence, indicating that these agonists stimulated Mn^{2+} entry (Fig. 8). The onset of quenching due to collagen was delayed as compared with ADP or thrombin. In contrast to other agonists, however, collagen

continuously accelerated the Mn^{2+} quenching, which was indicated by a faster decline of fluorescence than the control (no collagen) over the entire time course. Collagen increased the maximal slope of Mn^{2+} entry to 13-fold of that in the absence of an agonist (Table 3). Pretreatment with U73122 (5 μM) greatly decreased the rate of Mn^{2+} entry after addition of collagen, ADP or thrombin (inhibition of the slope; 86.8 ± 1.9, 92.1 ± 0.7 or 86.9 ± 0.7%, n = 4, respectively). On the other hand, thapsigargin increased the maximal slope of Mn^{2+} entry by about 5-fold, which was hardly affected by U73122 (Fig. 8 and Table 3).

Table 3. *Maximal slope of Mn^{2+}-quenching of fura-PE3 by collagen, ADP, thrombin and thapsigargin, and the effect of U73122.*

	Collagen	ADP	Thrombin	Thapsigargin
Vehicle	13.12 ± 0.28	20.65 ± 4.08	10.44 ± 1.55	4.94 ± 0.62
U73122	1.73 ± 0.25*	1.62 ± 0.15*	1.37 ± 0.07*	4.73 ± 0.72

Summarized data from the experiments shown in Fig 8. The slope of fluorescence decline before the addition of an agonist is defined as 1. The results shown are means ± S.E. of 4 experiments. *Significantly different from vehicle at $p < 0.01$.

(8) Sensitivity to drugs of the response to collagen in bovine platelets: comparison with that in human platelets

In this section, I compared the nature of collagen-induced Ca^{2+} mobilization in bovine and human platelets. In aspirin-treated human platelets, collagen at 50 μg/ml significantly increased $[Ca^{2+}]_i$ but not at 10 μg/ml (Fig. 4 and 9). The increase in $[Ca^{2+}]_i$ induced by 50 μg/ml collagen was not affected by treatment with A3P5PS (100 μM), whereas this concentration of A3P5PS inhibited the ADP (10 μM)-induced increase in $[Ca^{2+}]_i$ by 85.6 ± 5.0% (n = 5) in human platelets (Fig. 9). In the presence of A3P5PS, increase in $[Ca^{2+}]_i$ induced by 50 μg/ml collagen was also not affected by pretreatment with PGE_1 (2.5 μM) which increases cyclic AMP. Pretreatment with

U73122 (5 μM) significantly inhibited an increase in $[Ca^{2+}]_i$ due to 50 μg/ml collagen, but about 35% of $[Ca^{2+}]_i$ elevation was resistant to U73122.

In bovine platelets pretreated with both aspirin and A3P5PS, a large fraction of the increase in $[Ca^{2+}]_i$ induced by 10 μg/ml collagen was largely sensitive to PGE_1 (inhibition; 74.5 ± 4.4%, n = 5, Fig. 9). At 50 μg/ml, however, the collagen-induced increase in $[Ca^{2+}]_i$ in bovine platelets pretreated with aspirin and A3P5PS was partially inhibited by PGE_1 (inhibition; 49.9 ± 7.0% in aspirin and A3P5PS pretreated platelets, n = 6). U73122 (5 μM) suppressed an increase in $[Ca^{2+}]_i$ induced by 50 μg/ml collagen in bovine platelets (inhibition: 94.4 ± 0.9%, n = 6) (Fig. 9).

2) Collagen- or ADP-induced platelet aggregation and Ca^{2+} mobilization in platelets from normal or CHS-affected cattle

(1) Collagen-induced aggregation and Ca^{2+} mobilization

Collagen was applied to PRP from normal or CHS-affected cattle 3 min after addition of $CaCl_2$ (1 mM). Collagen (3-15 μg/ml) induced sustained aggregation of platelets from normal cattle (Fig. 10). With a low concentration of collagen (3 μg/ml), the onset of aggregation was slow. The onset became faster as the concentration of collagen was increased. In platelets from cattle affected with CHS, in contrast, aggregation in response to collagen was markedly depressed. At 15 μg/ml, the collagen-induced aggregation in CHS platelets was only 12 ± 1.4% of that in normal ones. A shape change preceded the aggregation in normal platelets, but the change was not apparent in CHS platelets.

Collagen (1-10 μg/ml) was applied to fura-PE3-loaded platelets 2 min after the addition of 1 mM $CaCl_2$. Collagen gradually increased $[Ca^{2+}]_i$ in CHS platelets, while it more rapidly increased $[Ca^{2+}]_i$ in normal platelets (Fig. 11A). In CHS platelets, the maximum increase in $[Ca^{2+}]_i$ induced by collagen at each concentration was significantly lower than that in normal platelets (Fig. 11B).

(2) Effects of arachidonic acid cascade inhibitors and ADP receptor antagonists on the collagen-induced Ca^{2+} mobilization

I already demonstrated that arachidonic acid metabolites and ADP play only a minor role in the collagen-induced response of bovine platelets (Fig. 2-4). I also compared effect of U46619 on platelet aggregation and Ca^{2+} mobilization between normal and CHS platelets. U46619 (10 μM) caused a shape change in both platelets but did not cause aggregation (data not shown). The increase in $[Ca^{2+}]_i$ induced by U46619 in CHS platelets was 69.0 ± 10.2 nM, n = 5. The increase was similar to that in normal platelets (69.0 ± 10.2 nM, n = 5) (Fig. 12). Hence, I think that a main cause for the defective activation by collagen in CHS platelets is due to an impairment of a collagen receptor-related pathway underlying Ca^{2+} signaling rather than a decreased release of ADP or abnormality in arachidonic acid cascade. For this purpose, I particularly focused my attention on the Ca^{2+} signaling due to the direct action of collagen under a condition to exclude the influences of endogenous agonists. In order to exclude the effects of secondary agonists on the collagen-produced Ca^{2+} signaling in normal and CHS platelets, the collagen-induced increase in $[Ca^{2+}]_i$ was estimated in platelets pretreated with aspirin (1 mM), ARL66096 (100 nM) and A3P5PS (100 μM). Pretreatment with ARL66096 (100 nM) for 90 sec completely inhibited ADP (10 μM)-induced aggregation in washed platelets form normal cattle (data not shown).

When these inhibitors were not used, the maximum increase in $[Ca^{2+}]_i$ due to collagen (10 μg/ml) in CHS platelets was 60.2 ± 2.5% (n = 5) of that in normal platelets (Fig. 13). Pretreatment with aspirin, ARL66096 and A3P5PS decreased the $[Ca^{2+}]_i$ response to collagen in normal platelets to 62.2 ± 6.6% (n = 6) of that in their absence, and in CHS platelets to 15.1 ± 2.5% (n = 5). As a result, in platelets treated with inhibitors, the maximum increase in $[Ca^{2+}]_i$ by collagen in CHS platelets was only 14.6 ± 2.5% (n = 5) of that in normal ones.

(3) ADP-induced aggregation and Ca^{2+} mobilization

Aggregation in response to various concentrations of ADP (1 to 10 μM) was slightly depressed in platelets from cattle with CHS, compared with the response in normal platelets (Fig. 14). At the highest concentration (10 μM), ADP induced an irreversible aggregation in normal platelets. On the other hand, although ADP (10 μM) induced aggregation in CHS platelets by 83 ± 5.3% of that in normal ones, the platelets tended to disaggregate after 5 min.

As aforementioned, the ADP-induced increase in $[Ca^{2+}]_i$ composed of an initial peak and the following sustained phase (Fig. 15). The sustained phase was lower in CHS platelets than in normal platelets, but the initial peak was not different between two groups (Fig. 15). I observed the ADP-induced Ca^{2+} mobilization in Ca^{2+}-free medium in order to know whether Ca^{2+} stores in CHS platelets retain an amount of Ca^{2+} similar to that in normal platelets. The response to ADP (1-10 μM) in Ca^{2+}-free medium was only slightly depressed in CHS platelets as compared with normal ones (Fig. 16). The extent of the depression in CHS platelets was similar to that seen in normal medium (Fig. 15 and 16).

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

As already shown (Fig. 6 and 8), PLC is involved in the collagen-induced Ca^{2+} mobilization in bovine platelets. In this section, I measured whether the production of Ins(1,4,5)P_3 due to collagen or thrombin was altered in CHS platelets. Ins(1,4,5)P_3 was measured when $[Ca^{2+}]_i$ attained the peak after application of collagen (10 μg/ml) or thrombin (0.1 U/ml) to aspirin-treated platelets. The basal level of Ins(1,4,5)P_3 was not different between normal and CHS platelets (0.88 ± 0.2 or 0.93 ± 0.15 pmol/10^9 platelets, n = 5 or 6, respectively, Fig. 17). Thrombin increased Ins(1,4,5)P_3 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 at 0.5 min after the addition of

thrombin, respectively). When challenged with collagen, Ins(1,4,5)P_3 increased to 255.5 ± 11.0% (n = 4) of the resting level at 1 min in normal platelets. On the other hand, collagen did not significantly increase Ins(1,4,5)P_3 in CHS platelets (Fig. 17).

3) Identification of collagen receptor subtype responsible for impaired Ca^{2+} signaling in CHS platelets

As reviewed in Introduction, two major receptors for collagen in platelets are GPIa/IIa and GPVI. To clarify which receptor-signal transduction system of GPVI or GPIa/IIa was impaired in CHS platelets, I compared Ca^{2+} mobilization induced by convulxin, a GPVI-specific agonist (Polgár *et al.*, 1997), or rhodocytin, a GPIa/IIa agonist (Inoue *et al.*, 1999; Suzuki-Inoue *et al.*, in press), in normal and CHS platelets.

(1) Response to convulxin

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. 18A left). Convulxin-induced increase in $[Ca^{2+}]_i$ was concentration-dependent between 1 and 10 ng/ml (Fig. 18B left). In this dose-effect study, the peak $[Ca^{2+}]_i$ in normal platelets seemed to be slightly higher than that seen in CHS platelets, although the difference was not statistically significant. A similar pattern of increase in $[Ca^{2+}]_i$ was observed in platelets activated by 30-200 ng/ml CRP and no difference was found between normal and CHS platelets (data not shown). Next, the response to convulxin in platelets that had been pretreated with a cyclooxygenase inhibitor and ADP receptor antagonists was observed (Fig. 18B right). In this series of experiments, the control response to 3 ng/ml convulxin was significantly greater in normal platelets than in CHS ones (656.6 ± 27.7 vs. 446.8 ± 39.8 nM, n = 7 and 5, respectively, $p < 0.05$, Fig. 18B 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 aspirin, ARL66096 and A3P5PS was not different between normal and CHS platelets (487.2 ± 34.6 vs. 428.1 ± 27.0 nM, n = 7 and 5, respectively, Fig. 18B right).

(2) Response to rhodocytin

In contrast to convulxin, rhodocytin gradually increased $[Ca^{2+}]_i$ with a lag of about 4 min in platelets from normal cattle (Fig. 19A left). In CHS platelets, the upstroke phase of $[Ca^{2+}]_i$ from the basal level could not be clearly distinguished. In Fig. 19B, 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, and the response was much less in CHS platelets with 10 and 25 nM rhodocytin than in normal ones (Fig. 19B left). When inhibitors were absent, rhodocytin (10 nM) increased $[Ca^{2+}]_i$ by 403.5 ± 64.7 nM (n = 5) in normal platelets and by only 44.2 ± 6.0 nM in CHS platelets for 8 min (11.0 ± 1.5% of the response in normal platelets, n = 5, Fig. 19A right). Pretreatment with a cyclooxygenase inhibitor and ADP receptor antagonists slowed the development of rhodocytin-induced increase in $[Ca^{2+}]_i$ in normal platelets and made the response in CHS platelets undetectable (Fig. 19A right). In this case, since the response was very slow, $[Ca^{2+}]_i$ was observed for 16 min after the addition of rhodocytin. When the participation of endogenous substances was excluded, rhodocytin increased $[Ca^{2+}]_i$ by 253.3 ± 58.8 nM (n = 5) in normal platelets and only by 45.9 ± 2.7 nM in CHS platelets (18.1 ± 1.1% of the response in normal platelets, n = 5, Fig. 19B right).

(3) Effects of cytochalasin D on collagen-, convulxin- or rhodocytin-induced Ca^{2+} mobilization

It has been reported that rhodocytin-induced activation of human platelets was inhibited by cytochalasin D, an inhibitor of actin polymerization (Inoue *et al.*, 1999; Suzuki-Inoue *et al.*, in press). In order to see if the response of bovine platelets was similarly sensitive to this agent, cytochalasin D (10 μM) was applied 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 aspirin, ARL66096 and A3P5PS had been treated (inhibition of the peak $[Ca^{2+}]_i$: 75.5 ± 3.9% in platelets treated with vehicle or 50.3 ± 9.7% in platelets treated with inhibitors against secondary agonists, n = 5 or 7, respectively, Fig. 20C). However, the response to rhodocytin in CHS platelets was so small that the effect of cytochalasin D could not be detected. Cytochalasin D inhibited the collagen (10 μg/ml)-induced increase in $[Ca^{2+}]_i$ in normal platelets treated with either vehicle or inhibitors against secondary agonists (inhibition of the peak $[Ca^{2+}]_i$: 40.3 ± 8.4 or 44.5 ± 5.7%, n = 5 or 6, respectively, Fig. 20A). Similarly, the agent inhibited the collagen-induced increase in $[Ca^{2+}]_i$ in CHS platelets when aspirin, ARL66096 and A3P5PS were absent (inhibition of the peak $[Ca^{2+}]_i$: 60.1 ± 10.9%, n = 6, Fig. 20A) but not when platelets were treated with the inhibitors, probably because the response was too small. On the other hand, convulxin (3 ng/ml)-induced increase in $[Ca^{2+}]_i$ was insensitive to cytochalasin D in both platelets (Fig. 20B). ADP (1 μM)-induced increase in $[Ca^{2+}]_i$ was also insensitive to cytochalasin D in normal (616.4 ± 67.3 vs. 633.6 ± 53.1 nM, n = 5) and CHS (685.6 ± 16.5 vs. 647.9 ± 34.7 nM, n = 5) platelets.

(4) Adhesion of platelets to type I collagen

In order to know 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, which had been coated on a well of microtiter plate, was observed. When wells had been coated with different concentrations (30-1,000 μg/ml) of collagen, the

adhesion increased depending on the concentration (Fig. 21A), although adhesion of CHS platelets seems to be slightly larger than that of normal platelets at all concentrations. In the buffer containing 2 mM Mg^{2+}, platelets adhered to collagen-coated wells in a time-dependent manner (Fig. 21B). There was no significant difference in the extent and rate of adhesion to 300 μg/ml collagen between normal and CHS platelets (Fig. 21B). 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. 22). 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. 22).

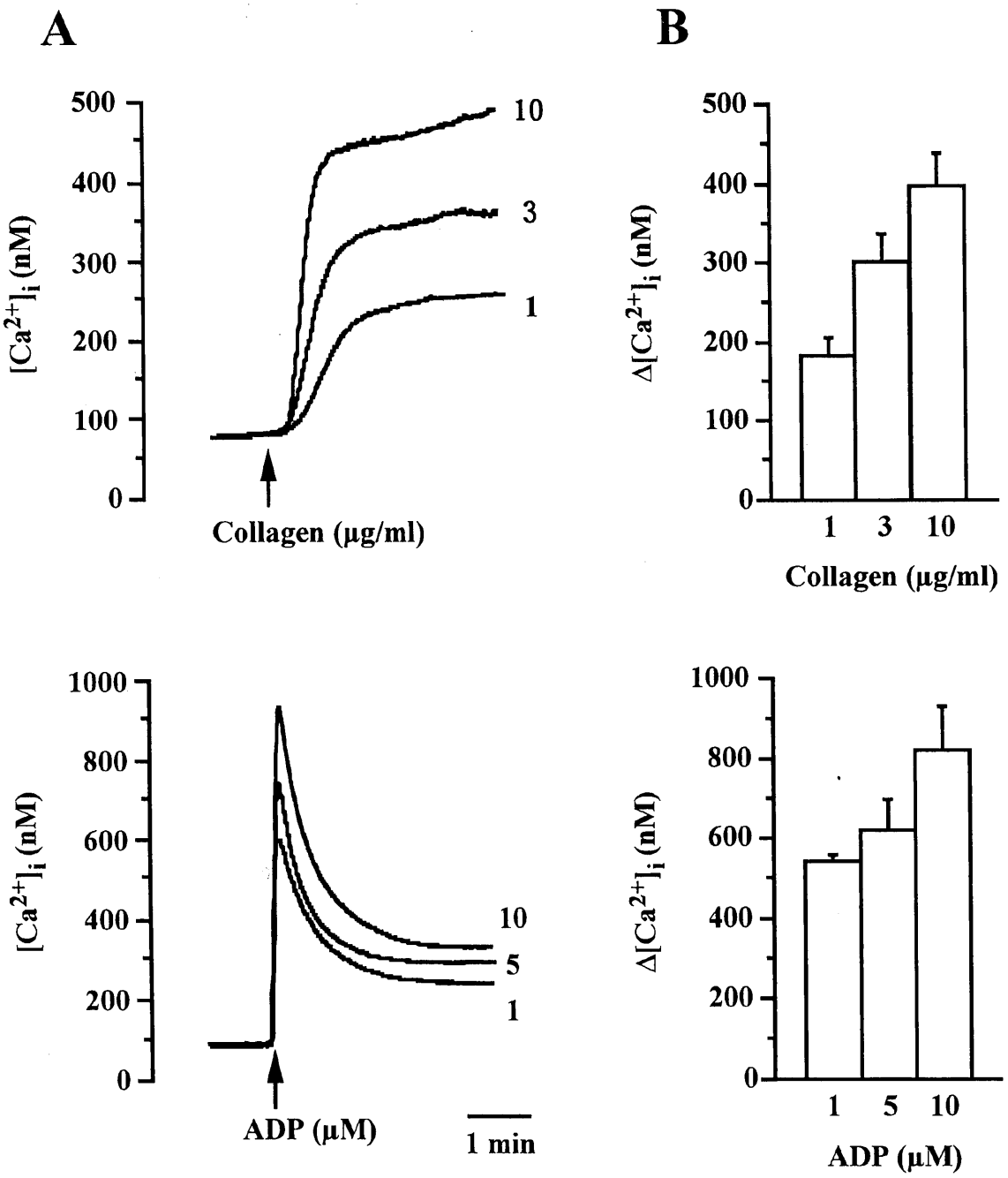

Fig. 1 **Collagen- or ADP-induced increase in $[Ca^{2+}]_i$ in bovine platele.** After fura-PE3-loaded platelets were incubated with $CaCl_2$ (1 mM) for 2 min, collagen or ADP was added. (A), a typical recording of increase in $[Ca^{2+}]_i$ induced by collagen (1-10 μg/ml) or ADP (1-5 μM). (B), an increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) induced by different concentrations of collagen or ADP. The results shown are means ± S.E. of 4 experiments.

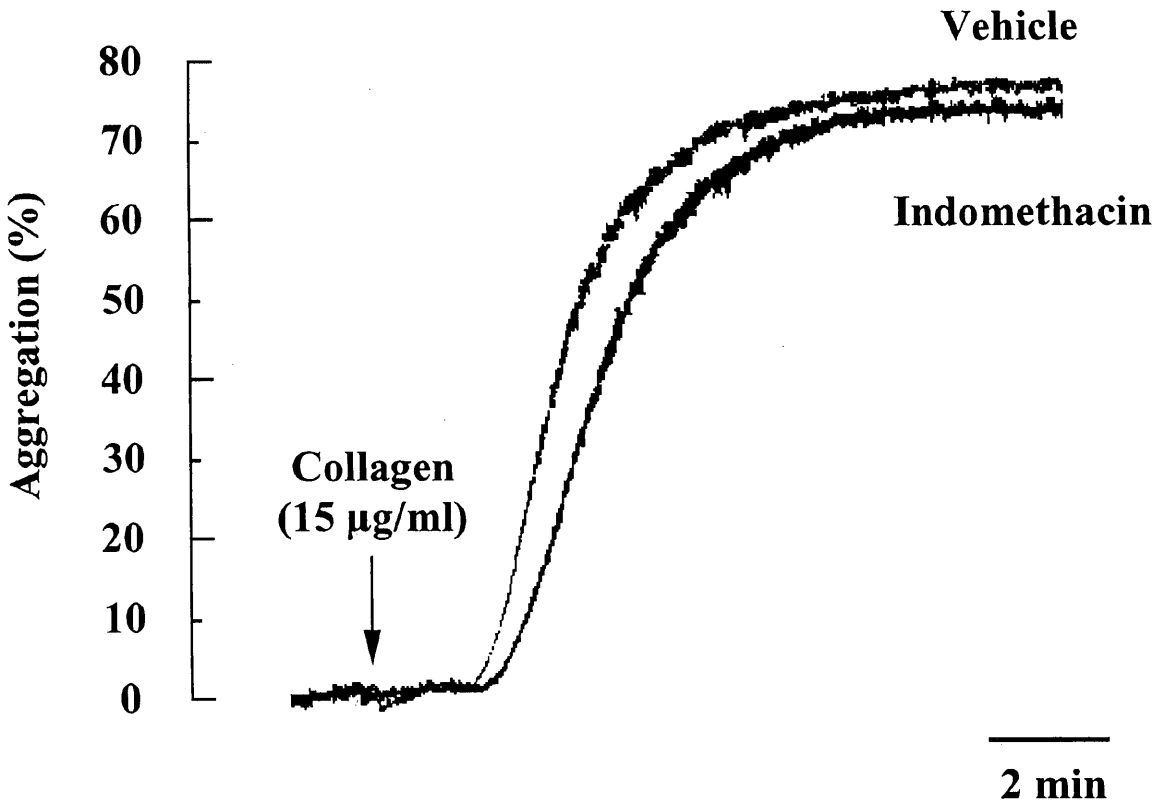

Fig. 2 **Effect of indomethacin on collagen-induced aggregation of bovine platelets.** Platelets were pretreated with indomethacin (10 µM) or vehicle (0.1% DMSO) for 30 min before addition of collagen (15 µg/ml). Each tracing is a representative of 6 experiments.

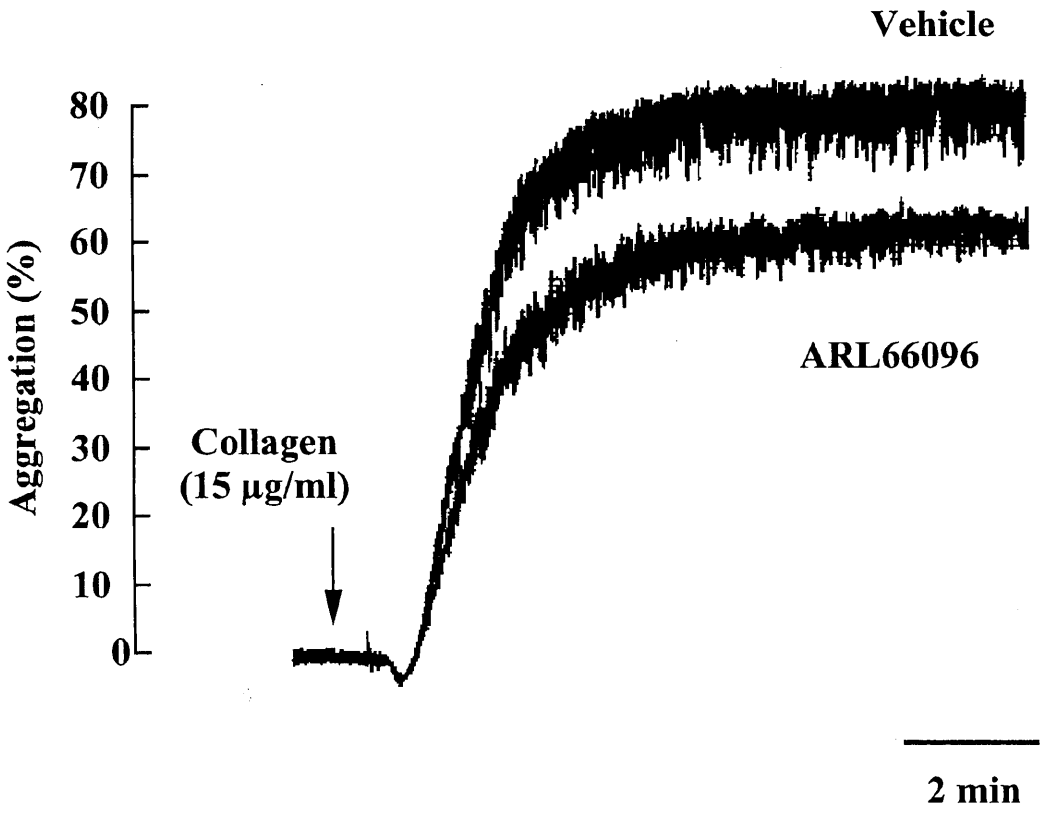

Fig. 3 **Effect of ARL 66096 on collagen-induced aggregation of bovine platelets.** Platelets were pretreated with ARL66096 (3 µM) for 2 min before addition of collagen (15 µg/ml). Each tracing is a representative of 6 experiments.

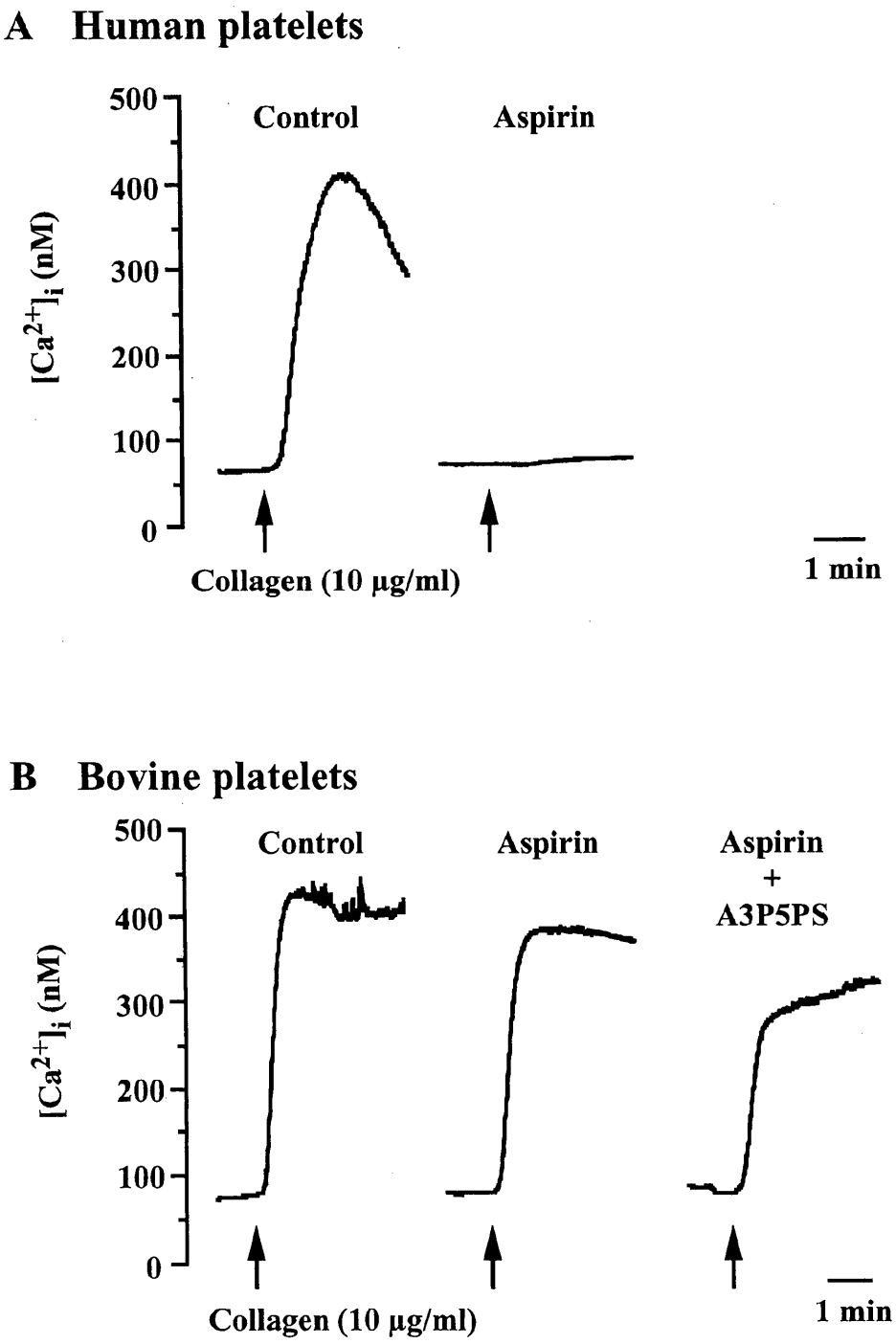

Fig. 4 **Effects of aspirin and A3P5PS on collagen-induced increase in $[Ca^{2+}]_i$ in human and bovine platelets.** Aspirin-treated platelets were prepared as described in "Materials and Methods". A3P5PS (100 μM) was treated 30 sec prior to the addition of collagen (10 μg/ml). An arrow indicates the addition of collagen. Each trace is a representative of 3 (A) or 4 experiments (B).

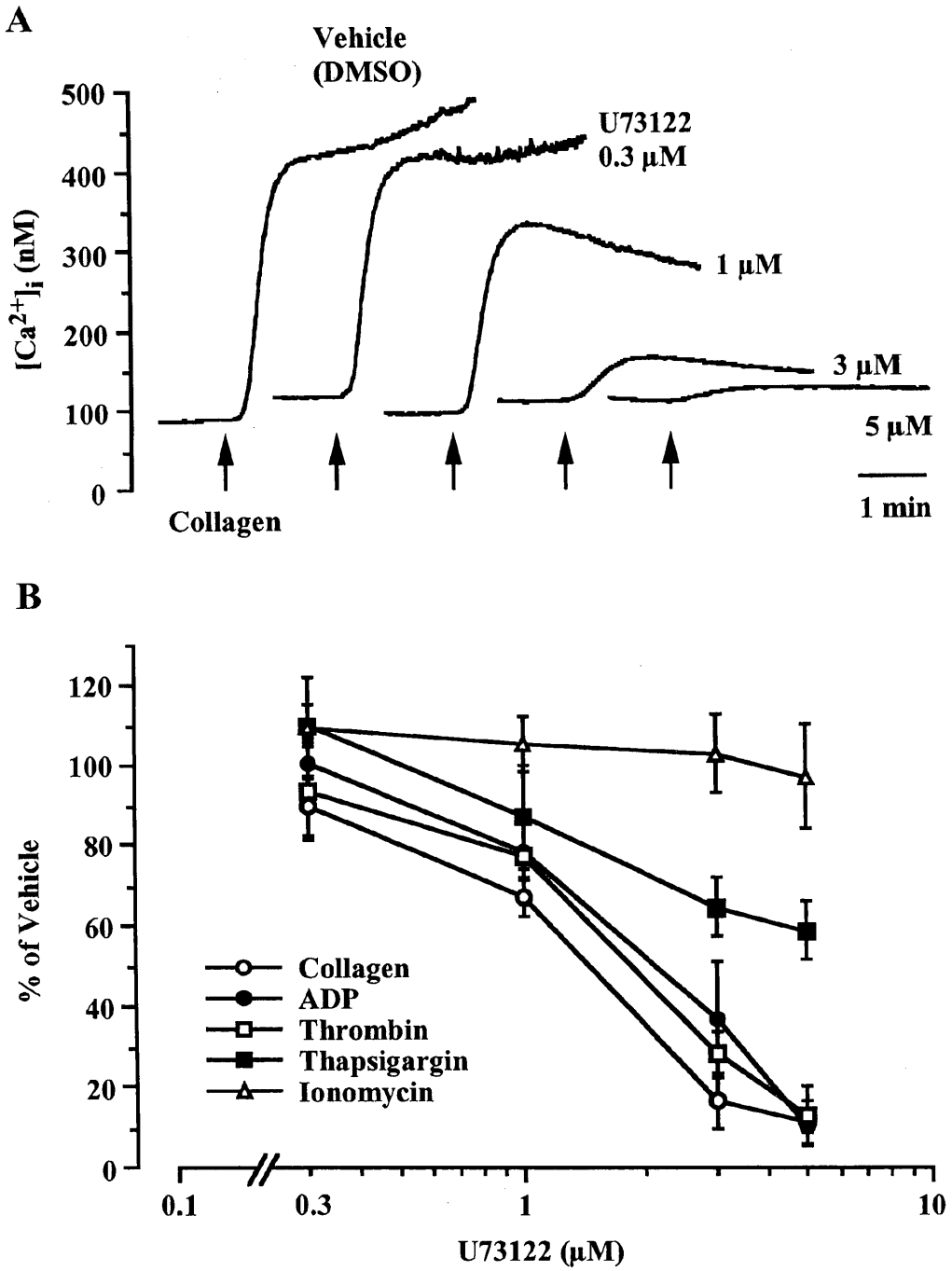

Fig. 5 **Effect of U73122 on agonists-induced increase in $[Ca^{2+}]_i$.** Aspirin-treated bovine platelets were incubated with vehicle (0.25% DMSO) or U73122 (0.3-5 μM) for 3 min prior to the addition of agonists. (A), a typical recording of increase in $[Ca^{2+}]_i$ induced by collagen (10 μg/ml) in platelets treated with vehicle or various concentrations of U73122. (B), dose-dependent effect of U73122 on the maximum increase in $[Ca^{2+}]_i$ induced by collagen (10 μg/ml), ADP (1 μM), thrombin (0.1 U/ml), thapsigargin (10 nM) or ionomycin (1 μM). The peak value due to each agonist in the absence of U73122 is defined as 100%. The results shown are means ± S.E. of 4 experiments.

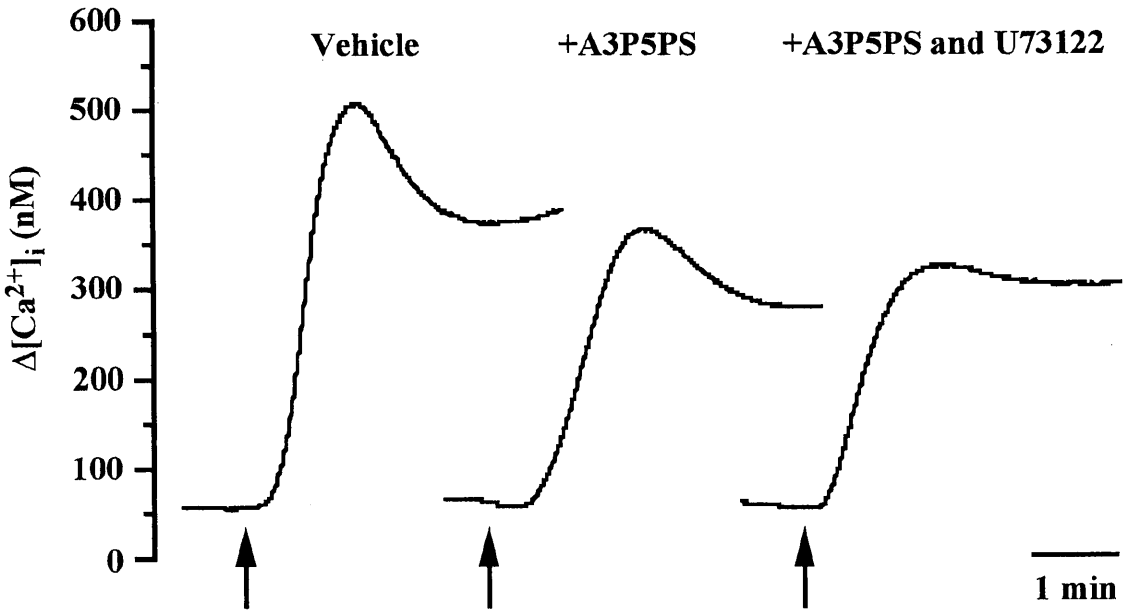

Fig. 6 **Effect of U73122 on thapsigargin-induced increase in $[Ca^{2+}]_i$ in the presence of A3P5PS.** A typical recording of increase in $[Ca^{2+}]_i$ induced by thapsigargin (10 nM). Aspirin-treated bovine platelets were incubated with vehicle (0.25% DMSO) or U73122 (5 μM) for 3 min prior to the addition of thapsigargin. A3P5PS (100 μM) was treated 30 sec prior to the addition of thapsigargin. An arrow indicates the addition of thapsigargin. Each trace is a representative of 4 experiments

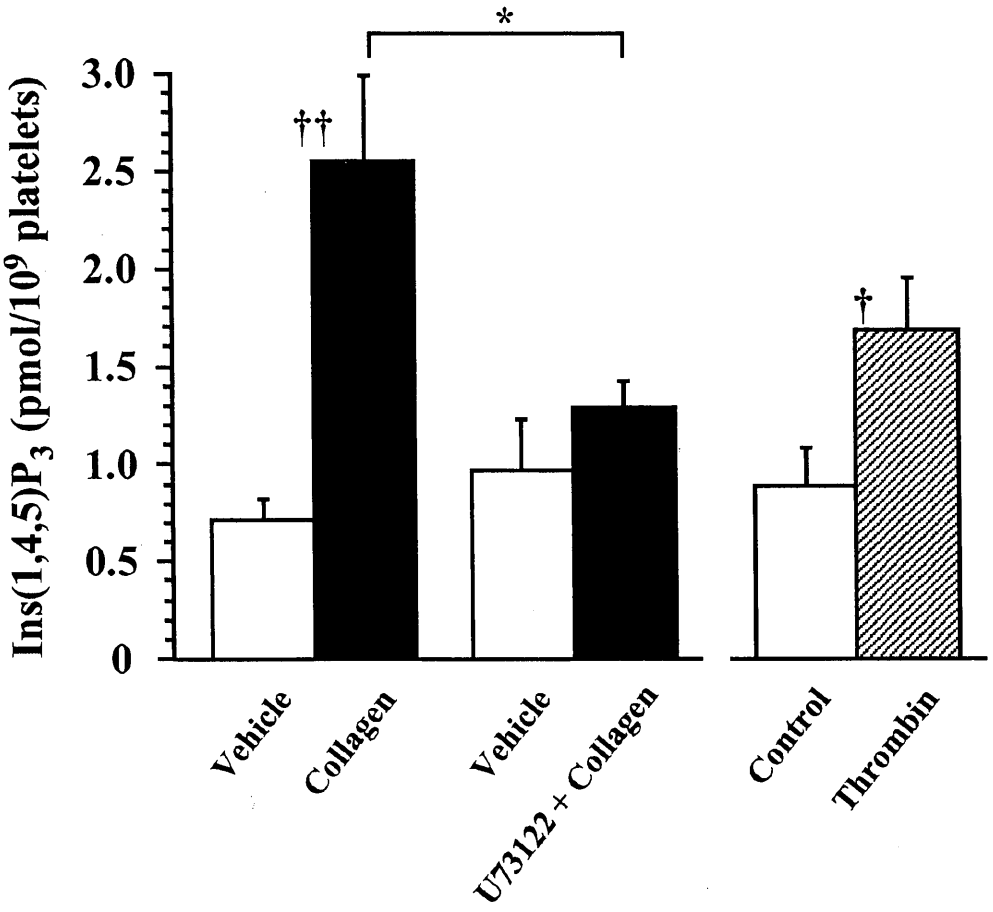

Fig. 7 **Collagen-induced production of Ins(1,4,5)P_3.** Aspirin-treated bovine platelets were incubated with vehicle (0.25% DMSO) or U73122 (5 μM) for 3 min prior to the addition of collagen or thrombin. Ins(1,4,5)P_3 production was determined 0.5 or 1 min after the addition of thrombin or collagen, respective. Results are expressed as means ± S. E. of 5-6 experiments. *Significantly different at $p < 0.05$. †, ††Significantly different from the resting level at $p < 0.05$ or $p < 0.01$, respectively.

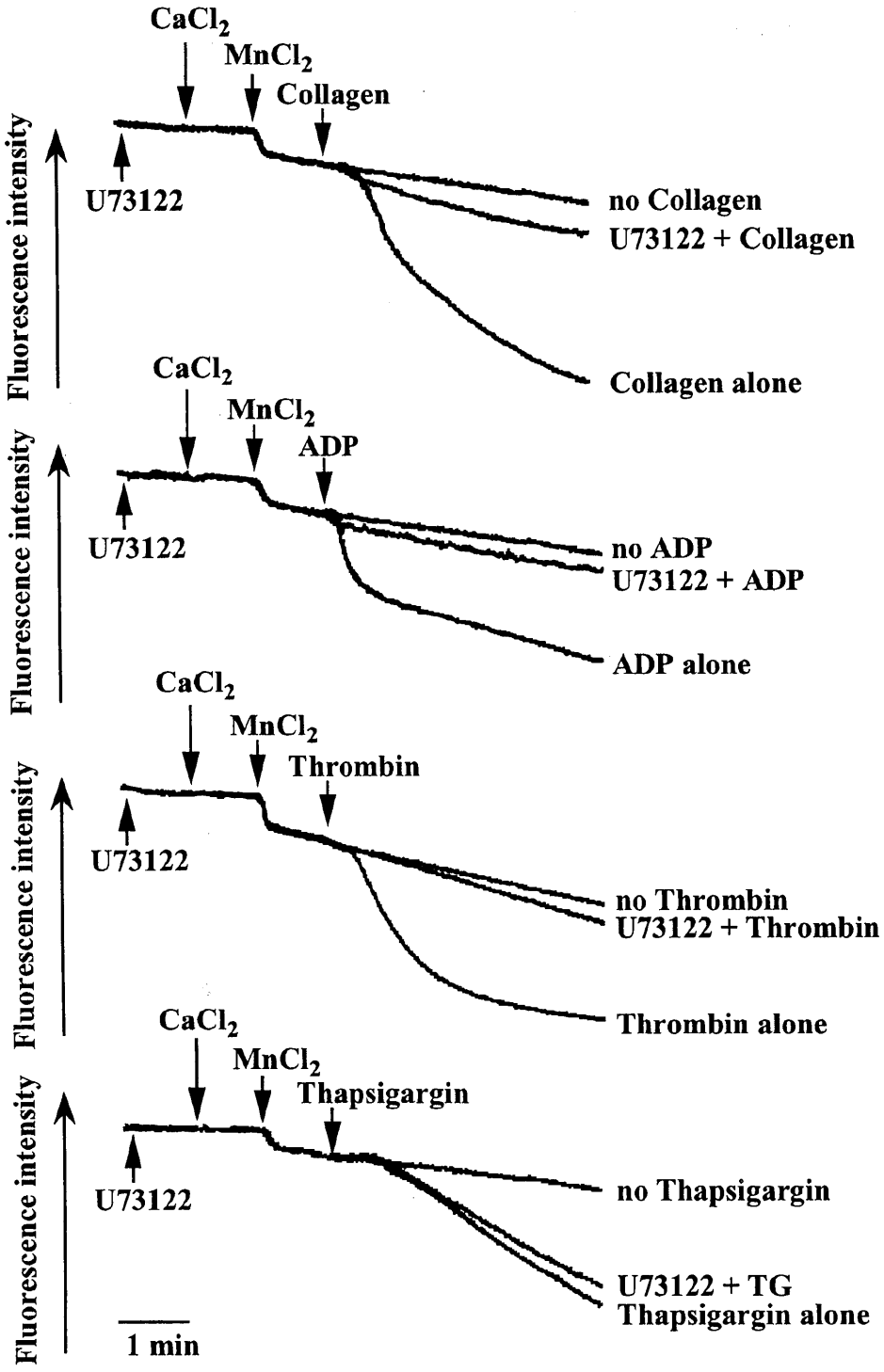

Fig. 8 **Agonists-induced acceleration of Mn^{2+} entry.** Agonists were added 1 min after addition of $MnCl_2$ (0.5 mM). When tested, U73122 (5 μM) was added 3 min before the application of collagen (10 μg/ml), ADP (1 μM), thrombin (0.1 U/ml) or thapsigargin (10 nM). As a control, vehicle (0.25% DMSO) was added instead of U73122. Each trace is a representative of 4 experiments.

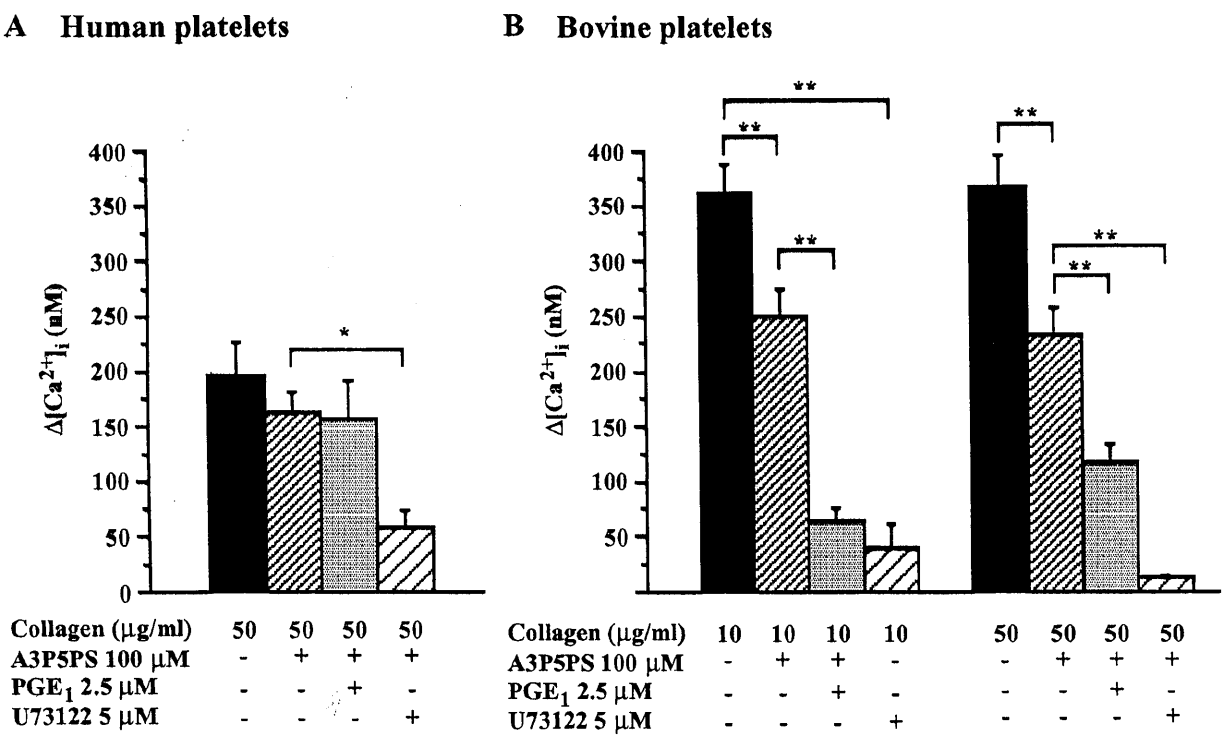

Fig. 9 **Sensitivity to drugs of $[Ca^{2+}]_i$-response to collagen in human and bovine platelets.** Aspirin-treated platelets were incubated with U73122 (5 μM), PGE_1 (2.5 μM) or A3P5PS (100 μM) for 3, 1 min or 30 sec prior to the addition of collagen, respectively. (A), human platelets, (B), bovine platelets. The results shown are means ± S.E. of 5-6 experiments. *,**Significantly different at $p < 0.05$ or 0.01, respectively.

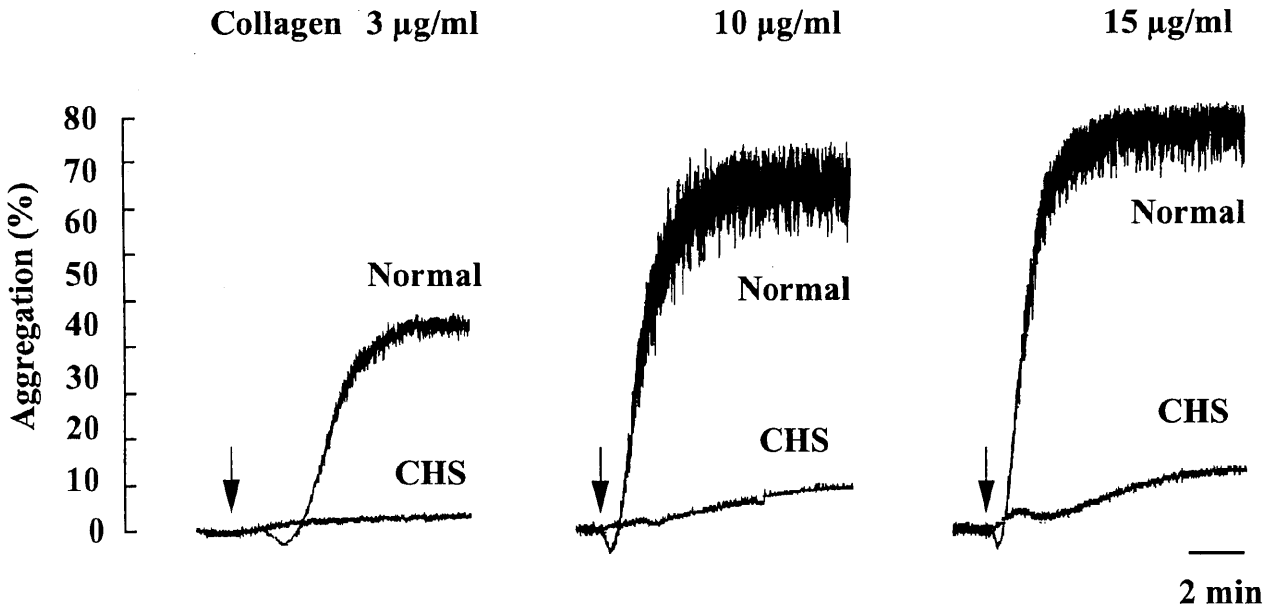

Fig. 10 **Typical recordings of collagen-induced aggregation of platelets from normal cattle and cattle affected with Chediak-Higashi syndrome (CHS).** Collagen was added 3 min after addition of $CaCl_2$ (1 mM). An arrow indicated the addition of collagen. Each tracing is a representative of 6 experiments.

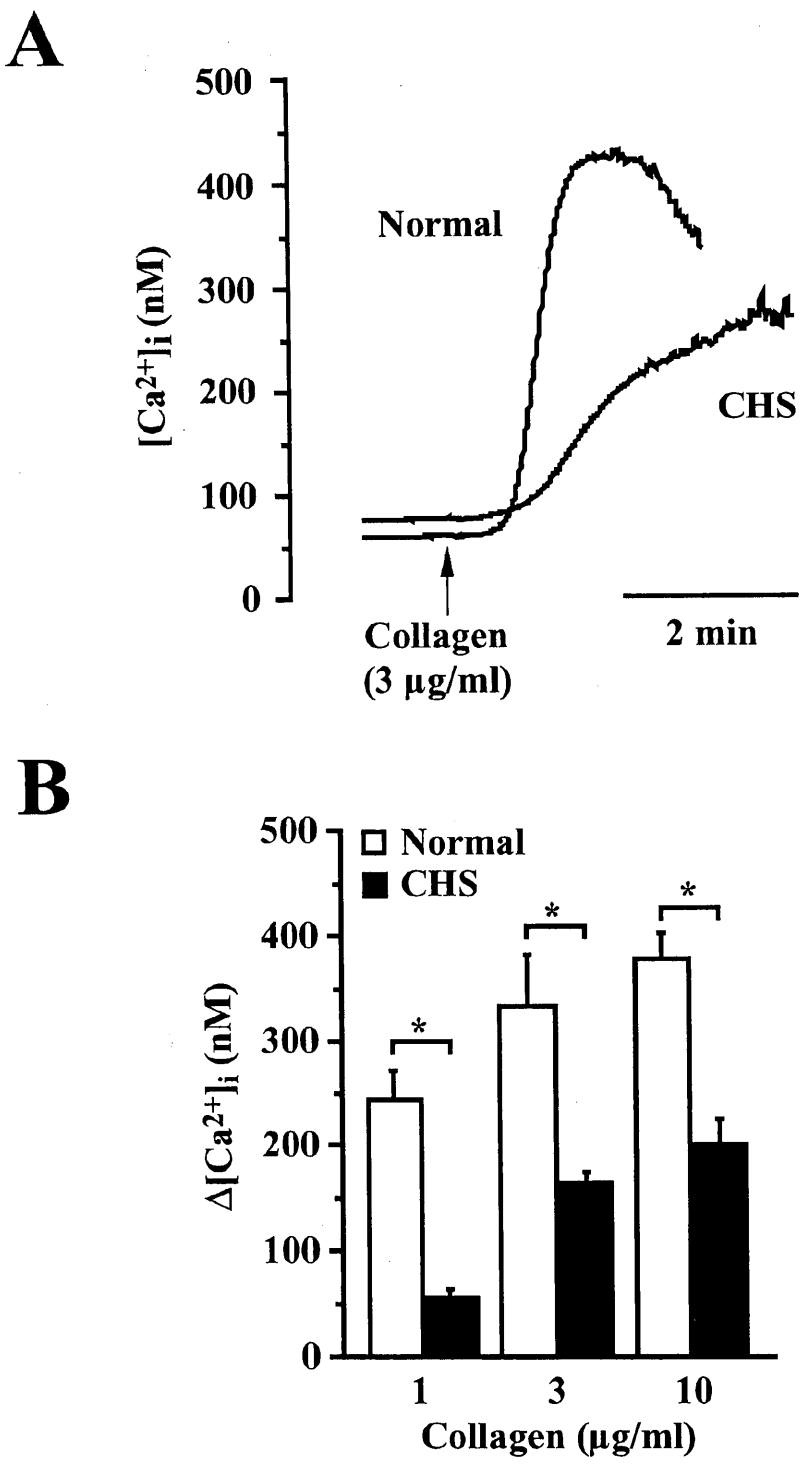

Fig. 11 **Collagen-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets.** (A), typical recordings of an increase in $[Ca^{2+}]_i$ induced by collagen (10 μg/ml). (B), $\Delta[Ca^{2+}]_i$ induced by collagen in normal (open column) or CHS platelets (filled column). $\Delta[Ca^{2+}]_i$ was calculated by subtracting the basal $[Ca^{2+}]_i$ from the maximum $[Ca^{2+}]_i$ during 4 min after addition of collagen. The data are mean ± S.E. of 5-6 experiments. *Significantly different at p <0.01.

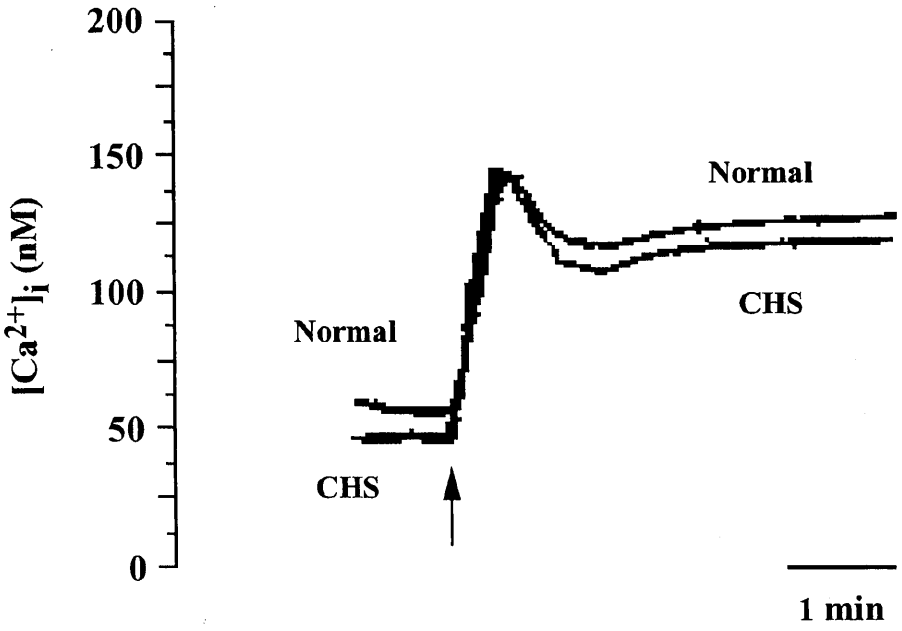

Fig. 12 **U46619-induced increase in $[Ca^{2+}]_i$ in bovine platele.** A typical recording of increase in $[Ca^{2+}]_i$ induced by U46619 (10 μM). An arrow indicates the addition of U46619. Each trace is a representative of 5 experiments.

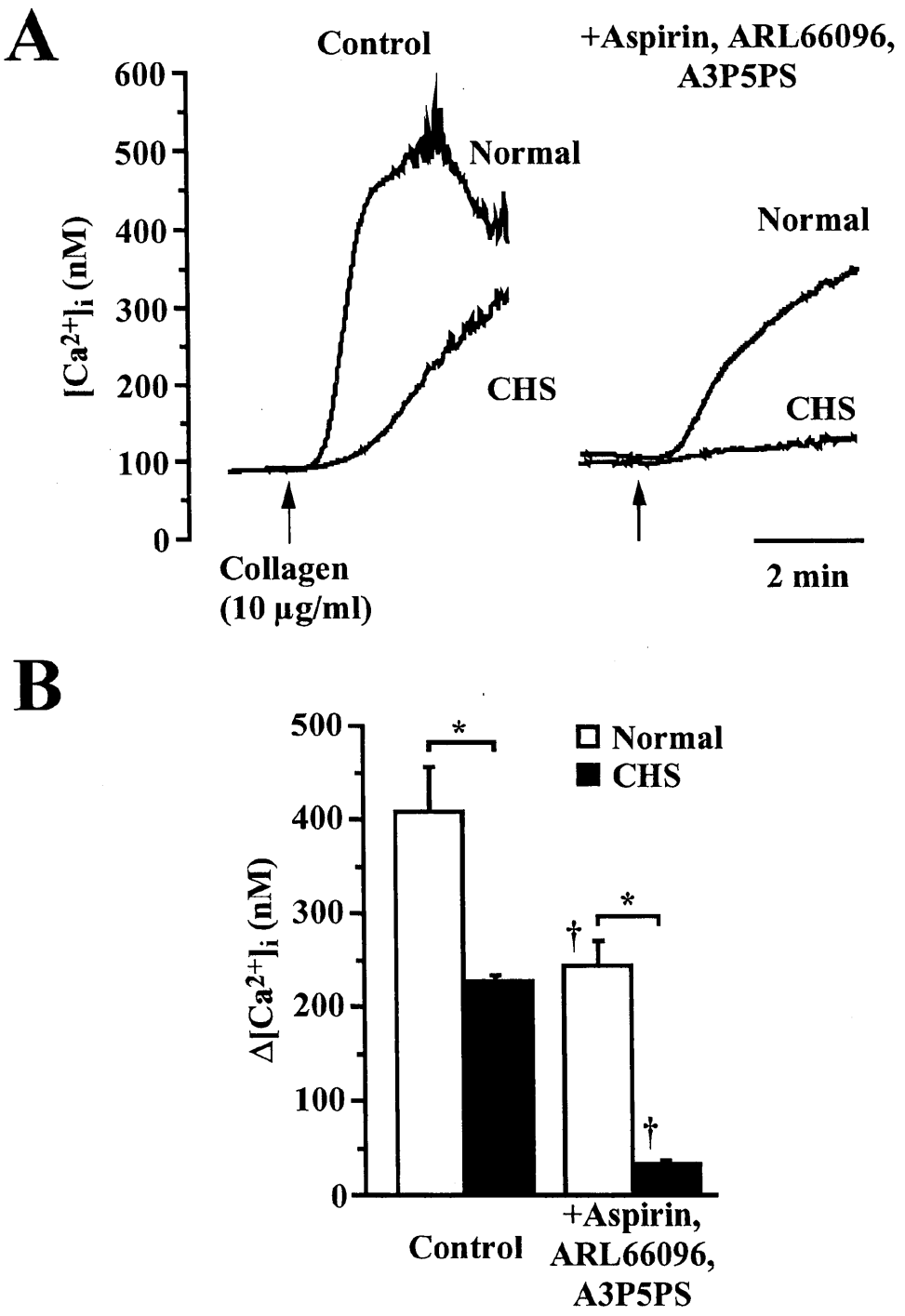

Fig. 13 **Effects of combination of a cyclooxygenase inhibitor and ADP receptor antagonists on collagen-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets.** Aspirin-treated platelets were prepared as described in "Materials and Methods". When used, ARL66096 (100 nM) and A3P5PS (100 µM) were applied for 90 and 30 sec, respectively, prior to the addition of 10 µg/ml collagen. (A), typical recordings of an increase in $[Ca^{2+}]_i$ induced by collagen in vehicle- (control, left), or inhibitors-treated (right) platelets. (B), $\Delta[Ca^{2+}]_i$ induced by collagen in normal (open column) or CHS platelets (filled column). The data are mean ± S.E. of 5-6 experiments. *Significantly different at p <0.01. †Significantly different from the control level at p <0.01.

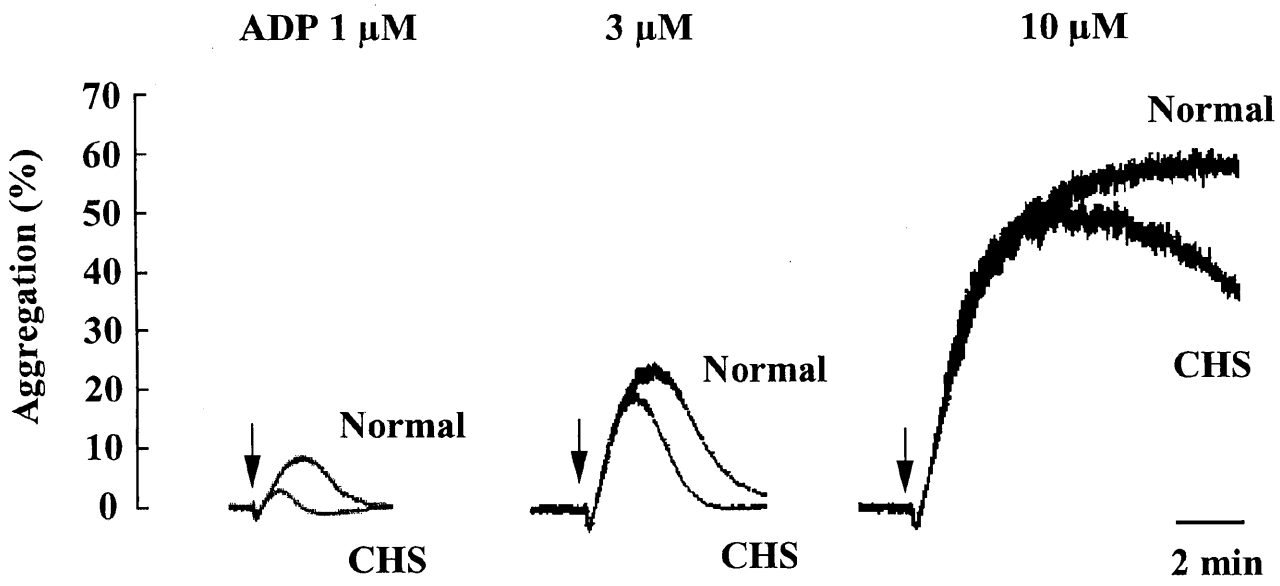

Fig. 14 **Typical recordings of ADP-induced aggregation of platelets from normal cattle and cattle affected with CHS.** ADP (1-10 μM) was added 3 min after addition of CaC_2 (1 mM). An arrow indicated the addition of ADP. Each tracing is representative of 6 experiments.

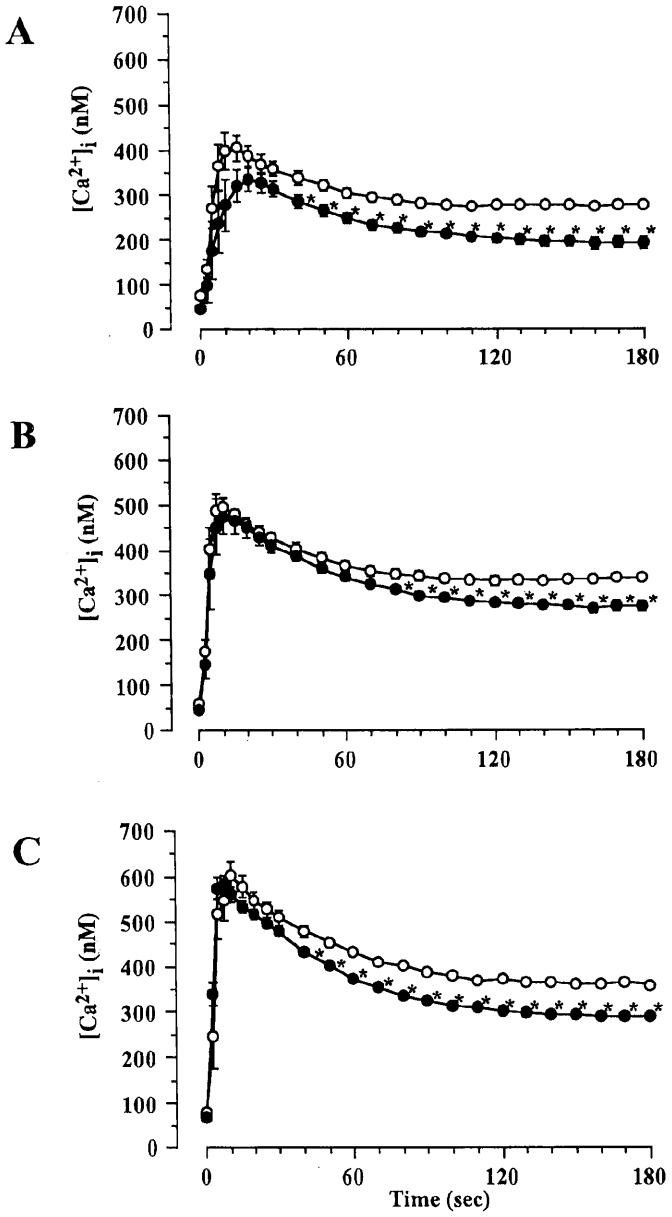

Fig. 15 **ADP-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets in the presence of external Ca^{2+}.** An increase in $[Ca^{2+}]_i$ induced by ADP at 1 μM (A), 3 μM (B), 10 μM (C) in normal (open circles) or CHS (filled circles) platelets. ADP was added at zero sec. The data are mean ± S.E. of 6 experiments. *Significantly different at p <0.05.

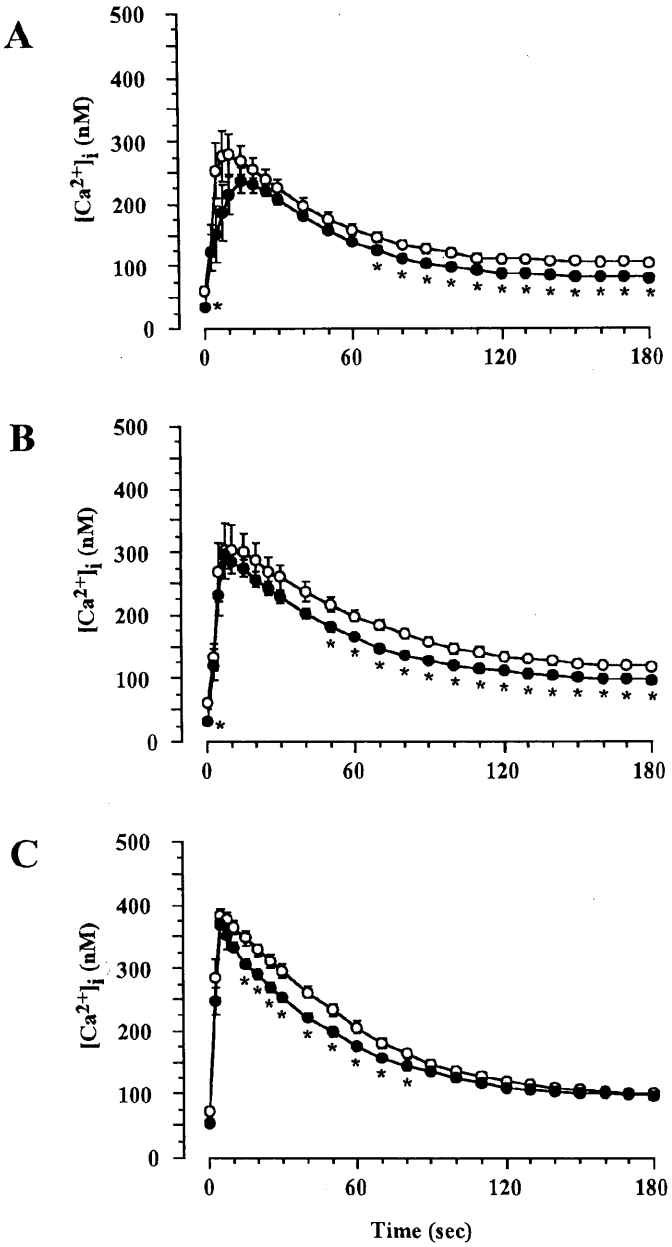

Fig. 16 **ADP-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets in the absence of external Ca^{2+}.** After loading platelets with 1 mM Ca^{2+} in the external medium, 4 mM EGTA was added to chelate external Ca^{2+}. ADP at 1 μM (A), 3 μM (B), 10 μM (C) was added to platelets from normal (open circles) or CHS (filled circles) cattle at zero sec. The data are mean ± S.E. of 6 experiments. *Significantly different at p <0.05.

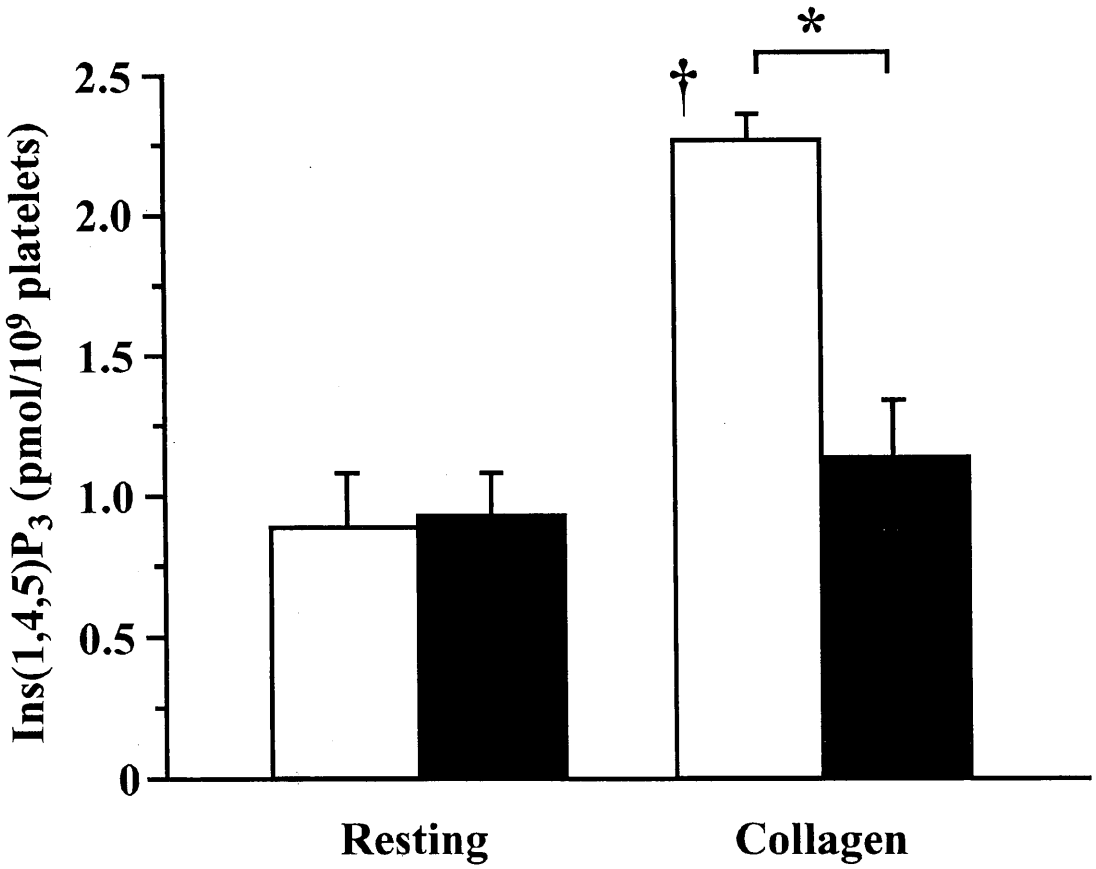

Fig. 17 **Collagen-induced $Ins(1,4,5)P_3$ production in normal and CHS platelets.** Aspirin-treated platelets were stimulated by collagen (10 μg/ml). The production of $Ins(1,4,5)P_3$ was determined in the resting state or 1 min after the addition of collagen in normal (open column) or CHS (filled column) platelets. Each column is expressed as a mean ± S.E. of 4-6 experiments. *Significantly different from normal platelets at $p < 0.01$. †Significantly different from the resting level at $p < 0.01$.

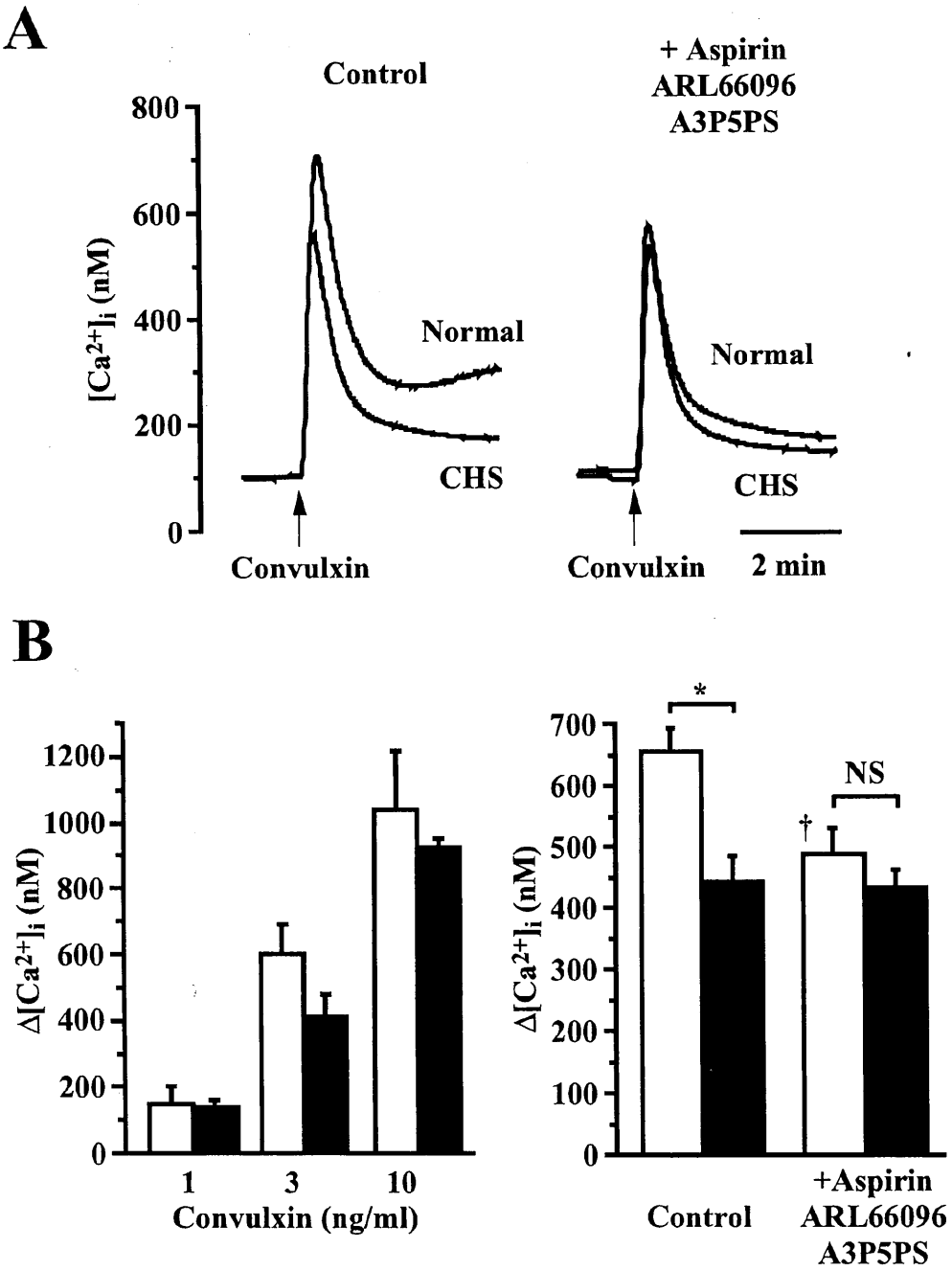

Fig. 18 **Convulxin-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets and the effect of pretreatment with a cyclooxygenase inhibitor and ADP receptor antagonists on the Ca^{2+} mobilization.** (A), typical recordings of the increase in $[Ca^{2+}]_i$ induced by 3 ng/ml convulxin in vehicle- (control, left) or aspirin (1 mM), ARL66096 (100 nM) and A3P5PS (100 μM)-treated (right) platelets. (B), $\Delta[Ca^{2+}]_i$ by various concentrations of convulxin (left), and the effects of inhibitors on the $\Delta[Ca^{2+}]_i$ due to 3 ng/ml convulxin (right) in normal (open column) or CHS (filled column) 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 convulxin. The data are means ± S.E. of 3 (B, left) or 5-6 (B, right) experiments. *Significantly different at p <0.01. †Significantly different from the control level at p <0.01. NS: not significant.

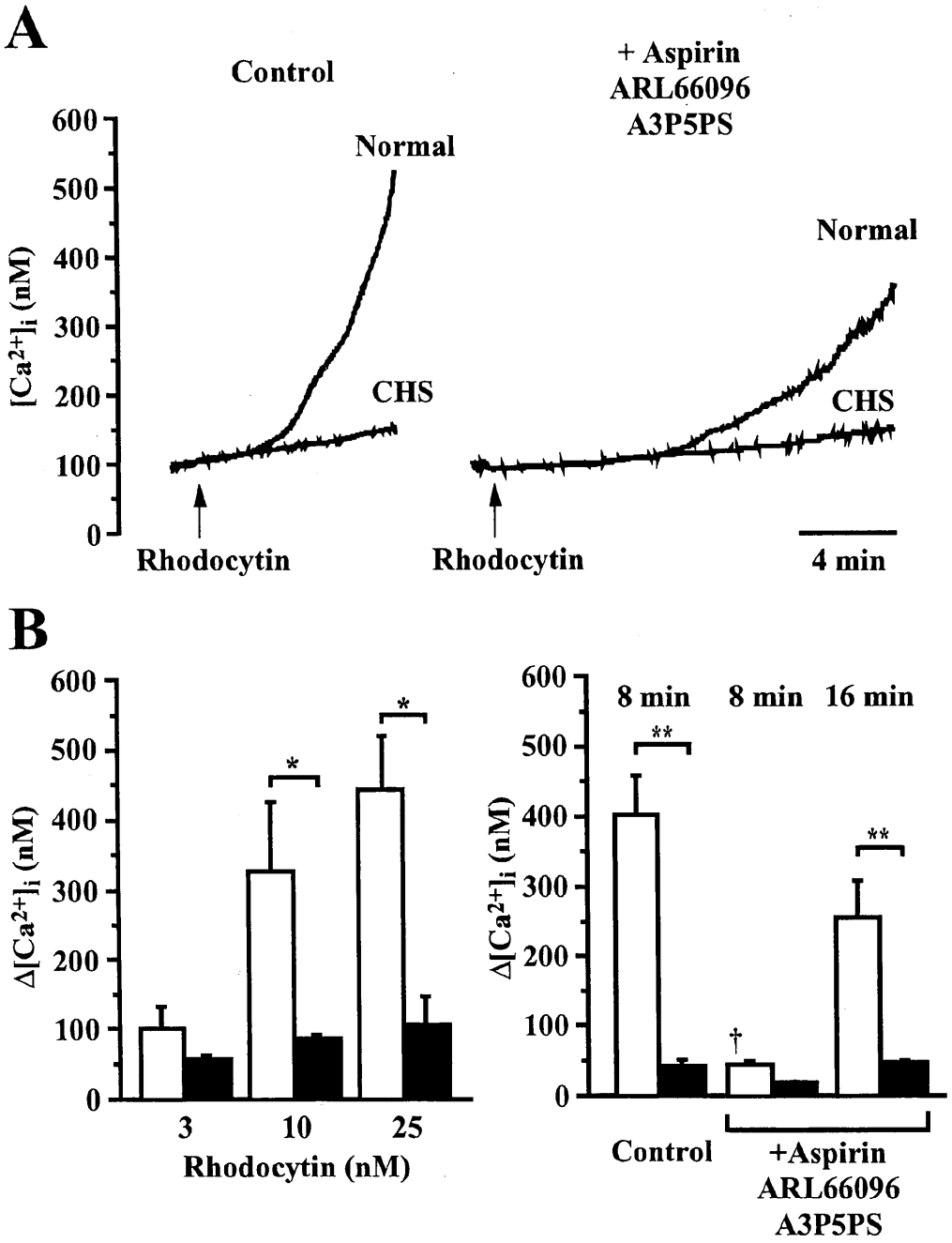

Fig. 19 **Rhodocytin-induced increase in $[Ca^{2+}]_i$ in normal and CHS platelets and the effect of pretreatment with a cyclooxygenase inhibitor and ADP receptor antagonists on the Ca^{2+} mobilization.** (A), typical recordings of the increase in $[Ca^{2+}]_i$ induced by 10 nM rhodocytin in vehicle- (control, left) or aspirin (1 mM), ARL66096 (100 nM) and A3P5PS (100 μM)-treated (right) platelets. (B), $\Delta[Ca^{2+}]_i$ induced by various concentrations of rhodocytin (left), and the effects of inhibitors on $\Delta[Ca^{2+}]_i$ due to 10 nM rhodocytin (right) in normal (open column) or CHS platelets (filled column). $\Delta[Ca^{2+}]_i$ 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 platelet treated with vehicle or inhibitors, respectively. The data are mean ± S.E. of 3 (B, left) or 5-6 (B, right) experiments. *,**Significantly different at p <0.05 or 0.01, respectively. †Significantly different from the control level at p <0.01.

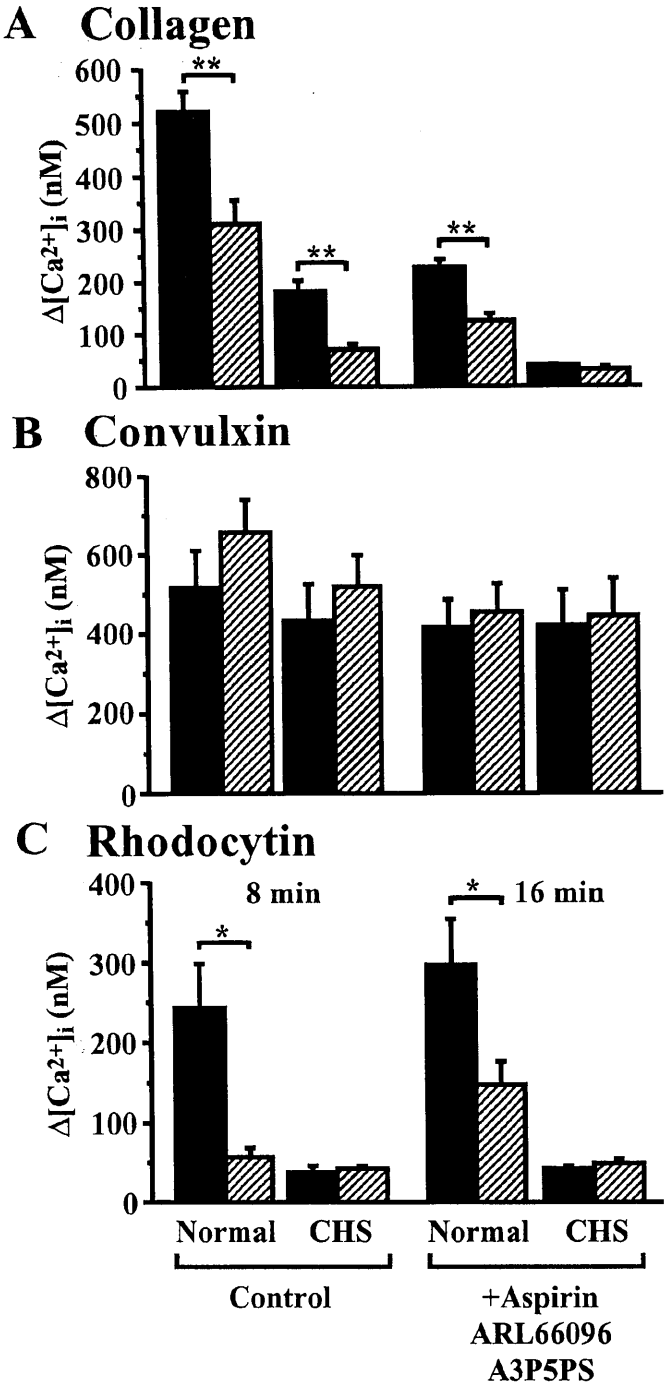

Fig. 20 **Effect of cytochalasin D on collagen-, convulxin- and rhodocytin-induced increase in $[Ca^{2+}]_i$.** Fura-PE3-loaded platelets were incubated with vehicle (0.25% DMSO) or cytochalasin D (10 μM) for 5 min prior to the addition of 10 μg/ml collagen (A), 3 ng/ml convulxin (B) or 10 nM rhodocytin (C). $\Delta[Ca^{2+}]_i$ was calculated by subtracting the basal $[Ca^{2+}]_i$ from the maximum $[Ca^{2+}]_i$ during 4 min after addition of collagen or convulxin, or during 8 or 16 min after the addition of rhodocytin in platelets treated with vehicle or inhibitors against secondary agonists, respectively. The data are means ± S.E. of 5-6 experiments. *,**Significantly different at p <0.05 or 0.01, respectively. Filled columns are without cytochalasin D (vehicle), while hatched columns are with cytochalasin D.

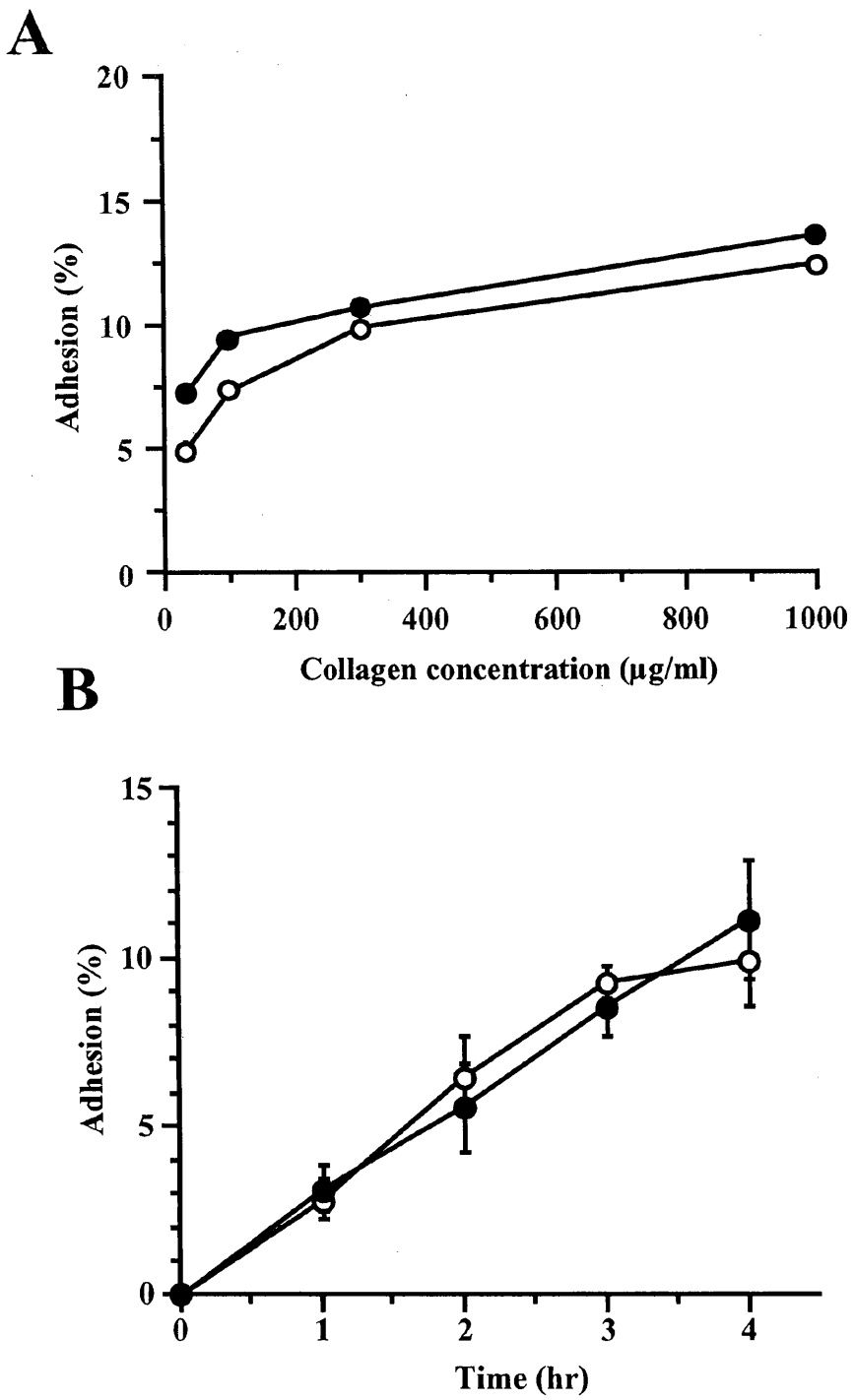

Fig. 21 **Adhesion of platelets to acid soluble type I collagen.** Adhesion of ^{51}Cr-labeled platelets to acid soluble type I collagen was determined in the presence of PGE_1 (1 μM) and RGDS (100 μg/ml) to prevent aggregation at room temperature. (A), concentration-dependent adhesion of normal (open circles) or CHS platelets (filled circles) in the presence of Mg^{2+} (2 mM). (B), time-dependent adhesion of normal (open circles) or CHS platelets (filled circles) to 300 μg/ml collagen in the presence of Mg^{2+} (2 mM). The data are means of 2-3 experiments (A) or means ± S.E. of 3 experiments (B).

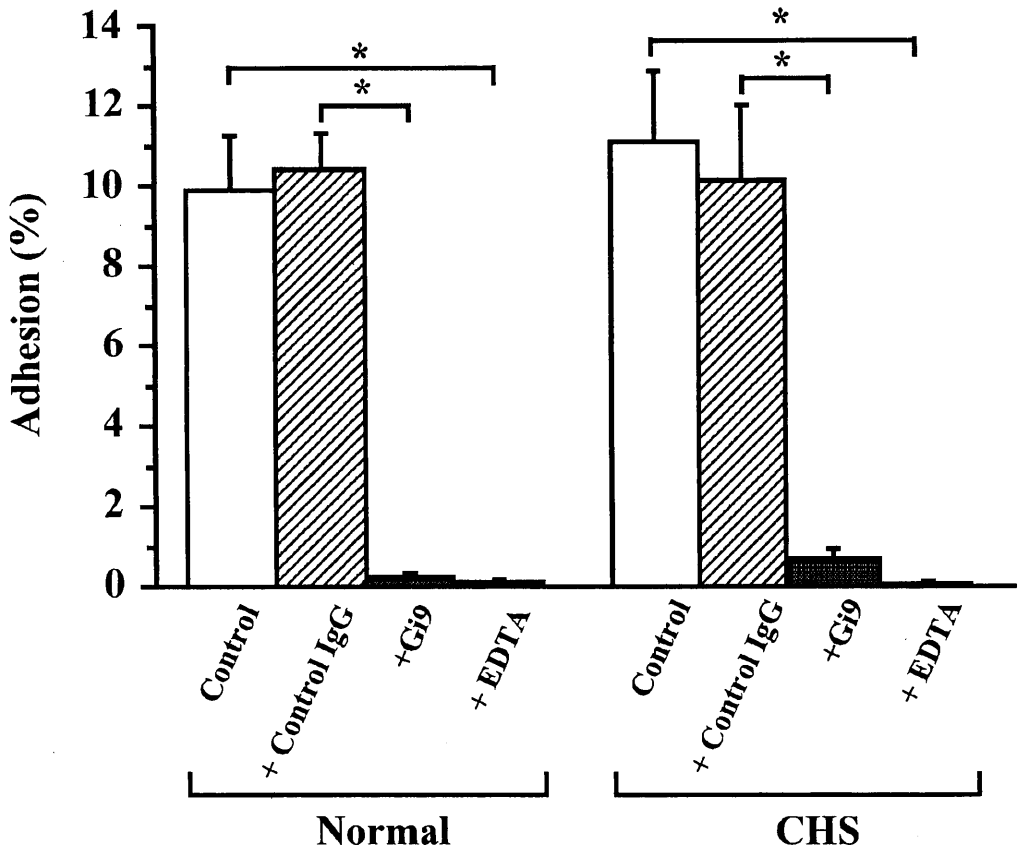

Fig. 22 **Effects of antiGPIa/IIa antibody or EDTA on adhesion.** Adhesion of ^{51}Cr-labeled platelets in the presence of control mouse IgG (10 μg/ml MOPC-21, hatched column), anti-GPIa/IIa antibody (10 μg/ml Gi9, shaded column) or EDTA (2 mM, filled column) on the adhesion. Open columns are the adhesion of platelets in the presence of Mg^{2+} (2 mM). The adhesion is expressed as percent of adhered platelets in the total platelets added. The data are means ± S.E. of 3 experiments. *Significantly different at p <0.01

IV Discussion

Characteristics of collagen-induced aggregation and Ca^{2+} mobilization in bovine platelets

An increase in $[Ca^{2+}]_i$ induced by collagen in human platelets was almost completely inhibited after treatment with aspirin, a cyclooxygenase inhibitor, suggests that arachidonic acid metabolites are essential for the Ca^{2+} mobilization. This agrees with the previous reports that TXA_2 plays a crucial role in the response to collagen in human platelets (Nakano *et al.*, 1989; Pollock *et al.*, 1986; Siess *et al.*, 1983). In bovine platelets, in contrast, pretreatment with indomethacin or aspirin exerted only a small inhibition on the collagen-induced aggregation and Ca^{2+} mobilization. I reconfirmed that the collagen-induced Ca^{2+} mobilization was also unaffected by pretreatment with the phospholipase A_2 inhibitor ONO-RS-082. Consistent with these data, it has been reported that in platelets from normal cattle, cyclooxygenase inhibitors had no effect on magnitude of aggregation response following stimulation with collagen, ADP, or platelet-activating factor (Bondy & Gentry, 1989; Gentry *et al.*, 1989). These results suggest that involvement of arachidonic acid metabolites in the collagen-induced response is very small in bovine platelets compared with that in human platelets. The observation that U46619, a TXA_2 mimetic, induced only slight Ca^{2+} mobilization and shape change but no aggregation indicates that bovine platelets are not so much responsive to TXA_2. So far, it is unclear whether bovine platelets stimulate arachidonic acid cascade in response to collagen.

It has been suggested that $P2T_{AC}$ purinoceptor mediates the ADP-induced aggregation in human platelets (Gordon, 1986), and ARL66096 has been reported to be a selective antagonist of $P2T_{AC}$ purinoceptor (Humphries *et al.*, 1994). On the other hand, $P2Y_1$ purinoceptor mediates Ca^{2+} signaling induced by ADP in human platelets, and A3P5PS has been reported to be a $P2Y_1$ purinoceptor antagonist (Boyer

et al., 1996). In this study, pretreatment with ARL66096 suppressed the ADP-induced aggregation while A3P5PS suppressed the increase in $[Ca^{2+}]_i$ in bovine platelets. These results suggest that $P2T_{AC}$ purinoceptor mainly mediates the ADP-induced aggregation, whereas $P2Y_1$ purinoceptor mediates the Ca^{2+} signaling due to ADP in bovine platelets in a manner similar to that in human platelets. In normal bovine platelets, pretreatment with ARL66096 exerted only about 20% inhibition on the collagen-induced aggregation. Similarly, A3P5PS inhibited the collagen-induced increase in $[Ca^{2+}]_i$ by about 30%. These results suggest that, in bovine platelets, a major fraction of collagen-induced aggregation and increase in $[Ca^{2+}]_i$ is not mediated by endogenous ADP or arachidonic acid metabolites but rather through the direct action of collagen.

Activation of platelets by several agonists causes a rise in $[Ca^{2+}]_i$, which consists of two components: Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} entry across the plasma membrane (Rink & Sage, 1990). Ca^{2+} release from intracellular Ca^{2+} stores is mediated by $Ins(1,4,5)P_3$ produced following activation of PLC (Berridge & Irvine, 1984). In order to elucidate the role of PLC in the collagen-induced Ca^{2+} mobilization in bovine platelets, I tested the effect of U73122, a PLC inhibitor (Bleasdale *et al.*, 1990; Vickers, 1993). U73122 inhibited an increase in $[Ca^{2+}]_i$ due to collagen with a potency similar to that due to ADP or thrombin. These data lead me to assume that the collagen-induced Ca^{2+} mobilization depends on the PLC activity. Unexpectedly, however, U73122 also inhibited the thapsigargin-induced Ca^{2+} mobilization, although the extent of inhibition was small as compared to the inhibition on collagen-, ADP- or thrombin-induced Ca^{2+} mobilization. This result raises a doubt about the selectivity of U73122. In fact, U73122 was reported to have a non-specific action such as inhibition of TXA_2 production (Heemskerk, 1997a) or inhibition of elevation of $[Ca^{2+}]_i$ induced by thapsigargin (Heemskerk *et al.*, 1997b), cyclopiazonic acid or ionomycin (Wang, 1996). In this study, since A3P5PS partially inhibited the thapsigargin-induced rise in $[Ca^{2+}]_i$, thapsigargin is likely to

liberate endogenous ADP. U73122 only slightly inhibited the increase in $[Ca^{2+}]_i$ caused by thapsigargin in the presence of A3P5PS. Hence, it is apparent that a major fraction of thapsigargin-induced rise in $[Ca^{2+}]_i$ that was sensitive to U73122 is attributable to endogenous ADP. Little effect of U73122 on the ionomycin-induced Ca^{2+} mobilization and on the thapsigargin-induced one in the presence of A3P5PS suggests that a non-specific action of U73122 (Heemskerk *et al.*, 1997a; Wang, 1996), if any, was negligible or very small in the present experiments.

Collagen increased cytosolic $Ins(1,4,5)P_3$, a product of inositol phospholipid breakdown by PLC, and U73122 prevented the production. These results strongly support the idea that PLC is involved in the Ca^{2+} mobilization caused by collagen. If so, collagen should increase Ca^{2+} release from Ca^{2+} stores through the action of $Ins(1,4,5)P_3$. In the presence of EGTA, however, collagen-induced increase in $[Ca^{2+}]_i$ was very small as compared to that in Ca^{2+}-containing medium. Although up to now I can not fully explain why the response to collagen was so small in the presence of high EGTA, one possible reason for this discrepancy is that activation of collagen receptor may be depressed in the presence of external EGTA, since it was suggested that adhesion of platelets to depends on the presence of divalent cations (Alonso *et al.*, 1989; Nakamura *et al.*, 1998). Further experiments are needed to confirm this possibility. Based on the data that an increase in $Ins(1,4,5)P_3$ production by collagen (10 μg/ml) was comparable to or slightly greater than that by thrombin (0.1 U/ml, Fig. 7), it is likely that collagen is capable of releasing a considerable amount of Ca^{2+} from the stores when platelets are incubated in a Ca^{2+}-containing medium.

Mn^{2+}-induced quenching of cytosolic fura-2 fluorescence has been shown to be useful to estimate the extent of Ca^{2+} entry (Alonso *et al.*, 1989; Sage *et al.*, 1990). Collagen increased the rate of quenching of fura-PE3 fluorescence by Mn^{2+}, indicating that collagen caused acceleration of divalent cation (Mn^{2+} or Ca^{2+}) entry. The acceleration of Mn^{2+} quenching by collagen was reversed when platelets had been

pretreated with U73122. A similar effect of U73122 was observed in the case of ADP- or thrombin-induced Mn^{2+} entry. In platelets and many non-excitable cells, Ca^{2+} entry dependent on depletion of intracellular Ca^{2+} stores is one of mechanisms for Ca^{2+} entry (Sargeant, *et al.*, 1992; Sage *et al.*, 1990; Putney & Bird, 1993). Thapsigargin is known to cause such entry as a result of inhibition of Ca^{2+}-ATPase (Sargeant *et al.*, 1992). U73122 had no effect on the thapsigargin-induced acceleration of Mn^{2+} entry, indicating that the store depletion-dependent Ca^{2+} entry is insensitive to U73122 in bovine platelets. Thus, it is evident that collagen stimulates Ca^{2+} entry in a PLC-dependent manner.

As discussed in the paragraph of page 52, the most important event after collagen receptor activation in bovine platelets is an increase in $[Ca^{2+}]_i$ whereas that in human platelets is liberation of arachidonic acid. Another species difference was observed in the sensitivity of Ca^{2+} mobilization to PGE_1, which increases cyclic AMP. The increase in $[Ca^{2+}]_i$ caused by a high concentration of collagen in the presence of aspirin and A3P5PS was insensitive to PGE_1 in human platelets, while that in bovine platelets was fairly sensitive to PGE_1. Consistent with the present data, the insensitivity to cyclic AMP-elevating agents of collagen-induced Ca^{2+} mobilization in human platelets has been reported (Smith *et al.*, 1992b). These differences suggest a possibility that some points of signal transduction pathway downstream of collagen receptors are different between bovine and human platelets. However, when a high concentration of collagen was used on bovine platelets, the sensitivity to PGE_1 became less as compared with the response to a low concentration of collagen. Hence, it is possible that depending on concentration human and bovine platelets share a common pathway which is associated with cyclic AMP-resistant Ca^{2+} mobilization. Two glycoproteins (GPIa/IIa and GPVI) are responsible for the collagen-induced platelet activation (Kehrel *et al.*, 1998; Moroi *et al.*, 1996; Nieuwenhuis *et al.*, 1985). Therefore, another possibility about the species difference is that the degree of contribution of each glycoprotein to collagen-induced

platelet activation is different between two species. However, I could not extend my study to address this point.

Mechanism responsible for insufficient platelet activation in platelets from cattle affected with CHS

In agreement with earlier studies (Table 1), platelet aggregation following stimulation with collagen or ADP was depressed in platelets from cattle with CHS, although the inhibition was greater in the case of collagen than that of ADP. Suzuki *et al.* (1996) reported that platelets from normal cattle exhibited significantly decreased aggregation response to collagen when pretreated with indomethacin, and suggested that a cause for the decreased response to collagen in CHS platelets was an impairment in a signal transduction process between the collagen receptor to arachidonic acid metabolism. In this study, however, pretreatment with indomethacin or aspirin exerted almost no effect on magnitude of platelet aggregation to collagen. The reason for this difference between my study and Suzuki group's study is so far unclear. I observed that U46619, a TXA_2 mimetic, caused only a slight increase in $[Ca^{2+}]_i$ without causing aggregation in platelets from normal and CHS affected cattle, consistent with data that arachidonic acid does not induce aggregation of normal bovine platelets (Liggitt *et al.*, 1984). Therefore, I think that arachidonic acid metabolites play, if any, only a minor role in effects of collagen on platelets.

Some groups have postulated that a decrease in ADP release as a result of δ-SPD could be a major cause for the insufficient aggregation of CHS platelets (Bell *et al.*, 1976; Rendu *et al.*, 1983). Platelet aggregation induced by collagen in platelets from normal cattle was partially inhibited by ARL66096. This suggests that endogenous ADP was partially involved in the collagen-induced aggregation of bovine platelets. ADP-dependent aggregation seems to be 20 to 30% of the total

aggregation response to collagen in platelets from normal cattle. Obviously, decreased release of endogenous ADP is not the sole mechanism for impaired aggregation to collagen in platelets from CHS-affected cattle, because the aggregation response to collagen in normal platelets after blockade of $P2T_{AC}$ purinoceptors was still much greater than that in CHS platelets (Fig. 3 and 10). Thus, I conclude that the primary cause for the impaired response to collagen in CHS platelets is not a decreased release of endogenous substances.

To examine whether impaired aggregation of CHS platelets was attributable to decreased Ca^{2+} mobilization, I compared the collagen-induced increase in $[Ca^{2+}]_i$ between normal and CHS platelets. Collagen-induced Ca^{2+} mobilization was depressed in CHS platelets, whereas ADP-induced Ca^{2+} mobilization was slightly inhibited in those platelets, in agreement with the data from platelet aggregation experiments. From the experiment with the cyclooxygenase inhibitor aspirin and the ADP receptor antagonists A3P5PS and ARL66096, the fraction of collagen-induced Ca^{2+} mobilization which was independent of both arachidonic acid metabolites and ADP in CHS platelets was only about 15% of that in normal platelets (Fig. 11). This suggests that a Ca^{2+} signaling mechanism which is a sequence of the direct action of collagen is defective in CHS platelets and that this defect is more important in the etiology of insufficient aggregation. I showed that PLC plays a crucial role in the Ca^{2+} signaling by collagen in bovine platelets. 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.

To address which of the collagen receptors GPIa/IIa and GPVI was impaired in CHS platelets, I observed Ca^{2+} mobilization induced by convulxin or rhodocytin in platelets from normal or CHS-affected cattle. The GPVI-specific activator convulxin-induced increase in $[Ca^{2+}]_i$ was normal or only slightly inhibited in CHS

platelets. Moreover, when platelets had been pretreated with aspirin and ADP receptor antagonists, no difference in the maximum increase in $[Ca^{2+}]_i$ by convulxin was observed between normal and CHS platelets. These results indicate that a signaling system downstream GPVI which leads to Ca^{2+} mobilization is nearly intact in CHS platelets. In contrast to the case of convulxin, rhodocytin-induced increase in $[Ca^{2+}]_i$ was greatly depressed in CHS platelets. The response to rhodocytin (10 nM) 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.

It has been reported that the activation of human platelets by rhodocytin was inhibited by anti-GPIa/IIa antibodies (Inoue *et al.*, 1999; Suzuki-Inoue *et al.*, in press), and rhodocytin-induced platelet activation was independent of GPIb, which is receptor for von Willebrand factor (Shin & Morita, 1998). Recently, Inoue group (Inoue *et al.*, 1999; Suzuki-Inoue *et al.*, in press) has provided evidence that rhodocytin binds to GPIa/IIa and then activates human platelets. In their study, the rhodocytin-induced Ca^{2+} mobilization of human platelets was sensitive to cytochalasin D whereas the CRP-induced platelet activation was not affected by this compound (Inoue *et al.*, 1999). *In vitro* studies have revealed that actin binds to $\alpha 2$ integrin chain (Fox, 1985; Kieffer *et al.*, 1995). Furthermore, it is generally accepted that the cytoplasmic domain of $\beta 1$ integrin chain binds to actin cytoskeleton via actin-binding proteins (Otey *et al.*, 1990; Clark & Brugge, 1995). These findings imply that actin plays a role in GPIa/IIa stabilization and/or its activation. Hence, the inhibition by cytochalasin D of the collagen-induced Ca^{2+} mobilization is ascribed to its inhibitory effect on GPIa/IIa-actin complex. 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 one was insensitive in bovine platelets. This

provides argument that rhodocytin is a GPIa/IIa agonist in bovine platelets. Therefore, I conclude that the GPIa/IIa-mediated Ca^{2+} signaling is impaired in platelets from CHS cattle.

Blunted response to rhodocytin in CHS platelets suggests that the insufficient collagen-produced Ca^{2+} signaling in CHS platelets could be due to a reduction in the number of GPIa/IIa, decreased affinity of GPIa/IIa for collagen, or a decrease in an intracellular signal generated following the binding of collagen to GPIa/IIa. In order to test the binding of collagen to platelets mediated by GPIa/IIa, I 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 (Santoro, 1986; Nakamura *et al.*, 1998). 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 like human platelets, the adhesion of bovine platelets to acid soluble collagen is mediated by GPIa/IIa. Adhesion of CHS platelets to this type of collagen was not different from that of normal platelets, suggesting that the GPIa/IIa receptor is expressed normally on CHS platelets and collagen can bind to it in a way similar to normal platelets under the experimental condition used here. Therefore, a deficit may exist in a downstream effector of GPIa/IIa, which contributes to activation of PLC, in CHS platelets.

A simplistic understanding of the roles of GPIa/IIa and GPVI is that GPIa/IIa functions as a receptor for the adhesion of platelets to collagen and GPVI for the platelet activation (Watson, 1999). Incompatible with this model, rhodocytin by itself can increase $[Ca^{2+}]_i$, suggesting that GPIa/IIa plays a role not only for the adhesion but also for the Ca^{2+} signaling. It is possible that GPIa/IIa generates a messenger for Ca^{2+} mobilization independently of GPVI. However, GPIa/IIa and GPVI are not independent of each other, but are likely to exchange signals, because upon stimulation of platelets by CRP, GPIa/IIa can be converted to a form with high affinity for soluble collagen (Jung & Moroi, 1998), and a topographic association

between GPIa/IIa and GPVI has been suggested (Kamiguti *et al.*, 2000). Probably a cooperation between GPIa/IIa and GPVI influences the adhesion and the platelet activation in the collagen-induced response. Therefore, an alternative scheme is possible that a signal from GPIa/IIa potentiates the GPVI-mediated activation of PLC. In this study, when the influence of endogenous agonists was excluded, collagen scarcely increased $[Ca^{2+}]_i$ in CHS platelets. Since the GPVI-mediated signaling (response to convulxin) was normal in CHS platelets (Fig. 18) and the binding of collagen to GPIa/IIa is normal (Fig. 21), collagen should have been able to increase $[Ca^{2+}]_i$ via a GPVI-mediated pathway in these platelets. The extremely depressed Ca^{2+} signaling as a result of direct action of collagen suggests that a signal from GPIa/IIa is necessary for collagen-induced Ca^{2+} mobilization.

Conclusion

The findings in this study can be summarized as follows; (i) in bovine platelets, major fractions of collagen-induced aggregation and increase in $[Ca^{2+}]_i$ are not due to an indirect action of endogenous ADP or arachidonic acid metabolites but rather due to a direct action of collagen, (ii) the collagen-induced Ca^{2+} mobilization in bovine platelets is mediated by PLC, (iii) the Ca^{2+} mobilization in bovine platelets is different from that in human ones as to the dependency on endogenous substances and the sensitivity to cyclic AMP, (iv) marked inhibition of collagen-induced Ca^{2+} mobilization is a primary cause for impaired responses to collagen of CHS platelets rather than decreased release of endogenous substances, and (v) CHS platelets have a deficit in a signal transduction system associated with GPIa/IIa which contributes to activation of PLC. Obviously more detailed study of a mechanism underlying insufficient Ca^{2+} signaling in CHS platelets is needed, especially to clarify a crosstalk between GPIa/IIa and GPVI. CHS platelets are a good model to explore the hemostasis mechanism under physiological and pathophysiological conditions.

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V Summary

Chediak-Higashi 症候群（CHS）は、各種動物で認められる劣性遺伝性疾患で、部分的アルビニズム、易感染性、出血傾向を主徴とする。CHS 罹患動物における出血傾向の原因は、コラーゲンに対する血小板凝集不全であるが、そのメカニズムは解明されていない。本研究では CHS を呈する黒毛和種牛の血小板を用い、コラーゲン凝集不全のメカニズムを解明することを第一の目的とした。その解明のために CHS ウシ血小板のコラーゲン凝集不全に Ca^{2+} 動員の異常が関係するかどうかを検討した。また、CHS 血小板において内因性 ADP 量の低下やトロンボキサン A_2（TXA_2）産生経路の異常が報告されていることから、これら内因性活性化物質が CHS 血小板における活性化不全に関与しているかどうかを検討した。さらに、血小板上のコラーゲンレセプターとして glycoprotein（GP）Ia/IIa と GPVI が重要であるので、このどちらのコラーゲンレセプターを介する経路が、CHS ウシ血小板における異常に関与しているのかを検討した。

ヒトの血小板では、血小板活性化に際し、内因性 ADP、TXA_2 が血小板より放出され、二次的アゴニストとして血小板活性化を増強する。また、血小板活性化には血小板内 Ca^{2+}濃度（$[Ca^{2+}]_i$）上昇が重要な役割を果たしているが、ウシ血小板における血小板活性化機序はほとんど未解明である。そこで、コラーゲンによるウシ血小板活性化の特性を合わせて検討した。

実験は、健康および CHS 罹患ウシあるいは健常ヒトより採血し、多血小板血漿（PRP)および洗滌血小板を作製し、PRP を用いて凝集の測定を行い、また、Ca^{2+}感受性蛍光色素 fura-PE3 を負荷した洗滌血小板を用いて $[Ca^{2+}]_i$ を測定した。

1. ウシ血小板のコラーゲンに対する反応の特性　ヒト血小板では、シクロオキシゲナーゼ阻害薬によりコラーゲンによる血小板凝集、$[Ca^{2+}]_i$ 上昇の大部分が抑制された。これに対し、ウシ血小板ではシクロオキシゲナーゼ

阻害薬は両パラメーターにほとんど影響しなかった。また、ADP レセプター拮抗薬を処置した血小板においても、コラーゲン凝集、$[Ca^{2+}]_i$ 上昇は部分的にしか抑制されなかったことから、ウシ血小板のコラーゲンに対する反応の大部分は、コラーゲンの直接作用によるもので、内因性アゴニストの関与の程度は小さいと考えられた。ウシ血小板のコラーゲンに対する$[Ca^{2+}]_i$ 上昇はホスホリパーゼC（PLC）阻害薬である U73122 によりほぼ完全に抑制された。またコラーゲンは血小板内 Ca^{2+}ストアからの Ca^{2+}遊離を起こすセカンドメッセンジャーであるイノシトール 3 燐酸（$Ins(1,4,5)P_3$）を増加し、この増加は U73122 により抑制された。細胞外に Ca^{2+}と、fura-PE3 蛍光を消光する Mn^{2+}を加えた条件下で、コラーゲンは Mn^{2+}流入を促進して蛍光の消光を増加した。以上の結果から、コラーゲンは Ca^{2+}ストアからの Ca^{2+}遊離と外液からの Ca^{2+}流入により$[Ca^{2+}]_i$ を増加することが明らかとなった。また、Mn^{2+}消光効果の増加は U73122 によって抑制されたことから、PLC 活性化がコラーゲンによる Ca^{2+}動員の中心的役割を果たすことが明らかとなった。

2. CHS 血小板の凝集不全の原因　CHS ウシ血小板のコラーゲンに対する凝集反応は、正常血小板に比べ顕著に抑制されていた。これは CHS 血小板ではコラーゲンが直接活性化する経路に異常があるためと考えられた。CHS ウシ血小板のコラーゲンによる $[Ca^{2+}]_i$ 上昇は有意に抑制されおり、$[Ca^{2+}]_i$ 上昇の異常は内因性アゴニストの影響を除いた条件下においても同様であった。このことからコラーゲンによる $[Ca^{2+}]_i$ 上昇の異常が、血小板凝集不全の直接の原因であることは明らかであった。また CHS 血小板では、コラーゲンによる $Ins(1,4,5)P_3$ の産生が有意に抑制されていたことから、コラーゲンとレセプターの結合から PLC 活性化までの経路に異常があると考えられた。そこで GPVI を特異的に活性化する convulxin および GPIa/IIa を活性化する rhodocytin を用いてどのレセプターに異常があるかを検討した。Convulxin による$[Ca^{2+}]_i$ 上昇は CHS 血小板でわずかに抑制されていたが、内因性アゴニストの影響を除いた条件下では差は認められなかった。これに対し rhodocytin による $[Ca^{2+}]_i$ 上昇は CHS 血小板で有意に抑制されており、その抑制は内因

性アゴニストの影響を除いた条件下においても認められた。一方で、GPIa/IIaに依存したコラーゲンと血小板の粘着に、両血小板で差は認められなかった。

以上の結果を総合して、CHS 罹患牛の血小板では GPIa/IIa の下流にあるシグナル伝達経路に異常があり、それが血小板凝集不全の原因となっていることが示唆された。

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