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Recombinant protein 1/secretoglobin 1A1 participates in the actin polymerization of human platelets

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Abstract

Background: Human protein1 which was originally isolated from pathological urine is identified with clara cell 10 kDa protein and uteroglobin. It has immunomodulatory and anti-inflammatory effects in many vertebrates. Although there have been thorough studies on the structure, molecular biology and biochemical characteristics, the precise mechanism of its biological activities is unknown. The purpose of this research is to clarify the biochemical mechanism of protein1 through its effect on the platelet aggregation process. *Methods*: Changes in calcium mobilization, actin filament concentrations and functions of integrin $\alpha_{IIb}\beta_3$ resulting from platelet stimulation were measured in the presence or absence of recombinant protein1 (rP1). *Results*: Recombinant protein1 inhibited U46619-and thapsigargin-induced platelet aggregation by preventing store mediated calcium entry (SMCE). The binding of rP1 to resting platelets induced an increase in the actin filament that accompanied the structural changes of $\alpha_{IIb}\beta_3$. When rP1 pretreated platelets were activated by thrombin or thapsigargin, the alterations in the actin filament and $\alpha_{IIb}\beta_3$ resulting from the stimulation decreased. *Conclusion*: These results suggest that rP1 inhibits platelet aggregation by participation in the actin polymerization through which SMCE is mediated.

1. Introduction

Protein1 (P1) is an evolutionarily conserved multifunctional protein secreted by the mucosal epithelia of various organs. Based on the organ where it was initially found and its functions, the protein is variously named uteroglobin, clara cell 10kDa protein and PCB-binding protein. The official name was determined to be secretoglobin 1A1 [1]. It is expressed constitutively in the lung, while in the endometrium, the prostate and the seminal vesicles, its expression is regulated by the steroid hormones. P1 is a homodimer of a peptide with 70 amino acids that is connected in an anti-parallel fashion by two disulfide bonds [2]. It is present in various body fluids such as bronchoalveolar lavage fluid (760 ng/ml), seminal fluid (1030 ng/ml), blood (10ng/ml) and urine (10 ng/ml) [3].

In the research over the last 30 years or more, P1 has been reported to have immunomodulatory activity, tumor suppression and anti-inflammation effects [2-7]. However, the mechanisms that regulate these various functions are not fully understood. The anti-inflammation effect of P1 has been assumed to be due to the inhibition of the secretory phospholipase A_2 ($sPLA_2$) function [8] because the inflammatory mediators thromboxanA_2 (TxA_2), eicosanoides and leukotriens are metabolized from the arachidonic acid (AA) released by $sPLA_2$. Inhibition of thrombin-induced platelet aggregation by P1 has also been attributed to the $sPLA_2$ inhibition [9, 10]. However, it was confirmed that cytosolic phospholipase A_2, rather than $sPLA_2$ releases AA from the platelets activated by thrombin [11, 12].

Recently, high affinity P1 binding protein was found in several cell types, and a receptor like property has been suggested [2, 6, 13]. Because the platelet is a cell without a nucleus and the activation mechanism via the receptor is known in some detail, research on the influence of P1 on platelet function is suitable to elucidate of the mechanism of the effects of P1 on the cell functions.

2. Materials and Methods

2.1. Materials

ETYA was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). Apyrase, indomethacin, thapsigargin (TG) and FITC conjugated phalloidin were from Sigma (St Louis, MO USA). Rabbit anti human urine P1, rabbit immunoglobulin fraction negative control and rabbit anti human α-1-microglobulin were from DAKO (Glostrup, Denmark). PAC1 FITC was obtained from Becton Dickinson (Tokyo, Japan). Fura-2 acetoxymethyl ester (Fura-2 AM) was obtained from Dojin Chemicals (Fukuoka, Japan). Other reagents were purchased from Wako Chemicals Co Ltd (Osaka, Japan).

2.2. Preparation of platelets

Platelets were separated from the buffy coat which was provided from Asahikawa Red Cross Blood Center. The buffy coat was diluted one and half times with a wash buffer containing 20g/l bovine serum albumin (BSA), 5.5mmol/l dextrose, 128mmol/l NaCl, 4.26 mmol/l Na_2HPO_4, 7.46 mmol/l NaH_2PO_4 and 4.77 mmol/l trisodium citrate (pH6.5) and then treated in the same way as freshly drawn blood. For integrin and antigen analysis, the buffy coat was preincubated with 20μmol/l indomethacin. It was washed by differential centrifugation according to a slight modification of the method previously reported [14]. Briefly, the diluted buffy coat was centrifuged at 75G for 15min, and the supernatant was centrifuged at 220G for 5min to eliminate red blood cells. The supernatant was centrifuged again at 220G for 10min. Precipitated platelets were suspended in the wash buffer and centrifuged at 220G for 10 min. Washed platelets were suspended in modified calcium free HEPES -Tyrode solution containing 3.5g/l BSA, 136mmol/l NaCl, 5.5mmol/l dextrose, 1mmol/l $MgCl_2$, 0.47mmol/l NaH_2PO_4, 11.62mmol/l $NaHCO_3$, 2.7mmol/l KCl and 10mmol/l HEPES (pH 7.3). This was used as the reaction buffer throughout the experiments. Within 30 hours after blood collections, about 70% of platelets prepared from the buffy coat retained the same reactivity to U46619 as those

from freshly drawn blood. For intracellular calcium analysis, platelet rich plasma or the supernatant from diluted buffy coat was preincubated with 5 μmol/l Fura-2 AM for 30min at room temperature and then washed in a manner similar to that described above. Platelet counts and the mean platelet volume were measured by ADVIA -120 (Bayer-Medical, Dublin, Ireland).

2.3. Measurement of calcium mobilization and aggregation

Aggregation of 2.5-3.0 x 10^8/ml platelets in the reaction buffer was measured by light transmission using PAM -6C aggregometer (SSR engineering Co.Ltd, Kanagawa, Japan) at 37°C. Fluorescence measurements of 3.0 x10^8/ml Fura 2-AM loaded platelets for simultaneous calcium mobilization and aggregation studies were performed using a Hitachi F-4500 fluorescence spectrophotometer with an F4500 IC program. For measurement of cytosolic calcium concentration, excitation was performed at 340 and 380 nm, and emission was at 510 nm. To investigating aggregation with shape change, the decrease in light scattering was monitored at excitation 380 nm and emission 400 nm. Except where otherwise stated, the data shown in figures are representative of 4-5 experiments using different samples. All results produced using buffy coat were reproduced with fleshly drawn blood.

2.4. Flow cytometry

The binding of the antibody and the actin filament concentration were analyzed by a flow cytometer EPICS XL-MCL (Coulter, Hialeah, FL, USA). The method used in the previously reported experiments was partially modified [15-17]. To assess the P1 binding to platelets, rabbit polyclonal antibody to human urine P1, anti α-1 microglobulin or rabbit immunoglobulin were incubated in the reaction buffer with washed platelets (5-8 x10^8/ml) for 20 min at room temperature. Samples were washed twice with saline by centrifugation at 220G for 7 min. Platelets were suspended in the 200μl reaction buffer and incubated with 1μg/ml FITC conjugated monoclonal mouse anti rabbit antibody for 20 min at room temperature. Platelets were washed once with saline. To study the effects of rP1 on fibrinogen mimetic PAC1 binding to platelets, platelets in the reaction buffer were preincubated with

20μg/ml of rP1 in PBS or an equal volume of PBS for 10min at room temperature. Platelets were activated in the presence of 0.25μg/ml FITC labeled PAC1 by 0.4U/ml thrombin for 1 min or 1μmol/l TG for 5 min. The activation was stopped by fixing with 1ml of 1% paraformaldehyde for 10min, followed by centrifugation at 220G for 7min. Pellets were washed once with PBS. In order to evaluate the polymerization of actin, platelets were fixed as above after preactivation with 0.4U/ml thrombin or 1μmol/l TG in the presence or absence of 20μg/ml rP1 and then permeabilized by incubation with 0.025% Nonidet P-40 for 10min at room temperature. Platelets were collected by centrifugation and suspended in the 200μl reaction buffer, then incubated with 1μmol/l FITC labeled phalloidin for 30 min at 37°C. Platelets were washed once with PBS. The FITC fluorescence of individual platelets was measured by a flow cytometer. The mean fluorescence intensity of 10 $x10^4$ cells was considered to be the index of the reaction.

2.5. Preparation of Recombinant P1

Recombinant P1 was kindly donated by Eiken Chemical Ltd (Tokyo, Japan). It was prepared according to the previously reported methods [18]. Lyophilized rP1 was dissolved in PBS, divided into small aliquots and stored at –80°C.

2.6. Statistical analysis

Data are presented as mean ± S.D. Student's paired t-test was used to determine the difference between samples with and without rP1.

3. Results

3.1. Effects of rP1 on platelet aggregation

The inhibition of platelet aggregation by uteroglobin has been re-examined using rP1. The influence of rP1 on the process of platelet aggregation was analyzed by means of receptor stimulation, PKC activation and intracellular calcium increases. The aggregation induced by TxA_2 mimetic U46619 was inhibited by rP1 in a concentration dependent manner (Fig 1). No effect on PMA induced aggregation was seen (data not shown). The effects of rP1 on the aggregation by a Ca^{2+}-ATPase inhibitor, TG, depended on the extra cellular calcium concentration. A small but distinct delay in the initial phase of aggregation was demonstrated in the solution containing 2mmol/l calcium (Fig2 A), and the efficacy decreased with decreasing calcium concentration (Fig2 B). However, when the AA metabolism was prevented by 10μmol/l indomethasin and ETYA [19], the effect of rP1 was more extensive (Fig 2 C). These results suggest that the inhibition by rP1 is not affected by AA metabolism and that it is related closely to the calcium entry process. The serum concentration of P1 is 5-25 ng/ml [3]; at such concentrations, synthesized P1 has no effect on the platelet aggregation.

3.2. Inhibition of store mediated calcium entry by rP1

After depletion of the stored calcium by 1μmol /l TG in the reaction buffer containing 10μmol/l indomethasin, 10μmol/l ETYA and 1U/ml apyrase, SMCE took place with the addition of calcium. When the intracellular calcium increase was 1300 nmol/l or less, rP1 inhibited the calcium influx in a concentration dependent manner (Fig 3A). The irreversible aggregation was inhibited at the same time (Fig 3B). Although rP1 inhibits SMCE in a concentration dependent manner, the inhibition was impaired as the calcium entry exceeded 1000nmol/l. These results suggest that rP1 does not directly block the calcium channels on the platelet membrane. Since mobilization of calcium from the calcium stores by TG was not altered by rP1 (data not shown), the inhibition was not due to prevention of

Ca^{2+}-ATPase. Progesterone (10μmol/l), which is reported to inhibit platelet aggregation [9], had no effect on the SMCE (data not shown).

3.3. Flow cytometric detection of bound P1 on the platelet membrane

Next, whether natural P1 in the blood is associated with the platelet membrane was investigated. Rabbit polyclonal antibody to human urine P1 bound specifically to the platelets from a healthy volunteer, and the binding was saturated (Fig 4 A). Recombinant P1 at the level of 40μg/ml or more, which is the concentration that has the great effect on platelet function, greatly increased the antibody binding (Fig 4 B). Vasanthakumar et al. observed that rP1 binding was reversible in the case of neutrophil and monocytes and was flushed out in the washing process [7]. The binding of anti P1 antibody to the platelets scarcely increased when platelets were washed twice with saline after preincubation even with 100 μg/ml rP1 (data not shown). The fluorescence intensity at rP1 =0, which indicates the binding of natural P1, was variable among samples. Some samples showed fluorescence above the level of the non-specific even after washing twice. Collectively, these results imply that the binding of rP1 to platelets is reversible, and that there are differences between natural P1 and synthesized P1 with regard to affinity to the platelets.

3.4. Changes in actin polymerization and function of $\alpha_{IIb}\beta_3$

Recently, a model for SMCE in which actin polymerization plays a pivotal role was proposed and supported by some evidence [17, 20]. On the other hand, close interaction of actin with the platelet membrane and integrin has been reported [16, 20-22]. Thus, the effects of rP1 binding on the actin polymerization and on the function of integrin on the membrane were examined. The addition of 20μg/ml rP1 to the resting platelets increased actin filaments by about 19 % (1.19±0.16-fold of the control , $P<0.001$, n= 16). In contrast, actin polymerization induced by 0.4U/ml thrombin or 1μmol/l

TG deteriorated in the presence of 20μg/ml rP1 (Fig 5). It was also found that rP1 induced binding of PAC1 to the integrin $\alpha_{IIb}\beta_3$ in 9 of 12 samples of resting platelets by 2-9 % ($P < 0.025$); nevertheless, it decreased the binding in the activated state (Fig 6).

4. Discussion

The results reported here demonstrate that rP1 inhibits platelet aggregation through prevention of SMCE. Reconstitution of the actin filament plays a crucial role in SMCE [17, 20, 23]. While rP1 binding to the resting platelets increases in actin filaments, it impairs a thrombin or TG induced increase of the actin filaments (Fig 5). These phenomena corresponded to the small but statistically significant activation of $\alpha_{IIb}\beta_3$ in resting platelets and the impairment of further activation of the integrin by thrombin or TG (Fig 6). The actin polymerization due to 1μmol/l TG was measured before the SMCE took place (Fig 3A and legend for fig 5). Therefore, it is clear that rP1 prevents SMCE through participation in actin reorganization. Actin reorganization is a basic requirement for various cell functions such as neutrophil chemotaxis and invasion of cells, including cancer cells [24-26]. Several experiments have demonstrated that uteroglobin or rP1 modulates these functions [2, 5-7, 13].

It has been reported that recombinant uteroglobin modulates the activity of $sPLA_2$ by sequestration of the cofactor Ca^{2+} [27], and that it showed Ca^{2+} dependent binding to negatively charged phospholipid liposomes [28]. According to Andersson et al, rP1 has two calcium binding sites for 1 molecule and the Kd is the lower millimolar range [27]. Because rP1 of 50μg/ml is about 3μmol/l, to sequestrate the calcium of 0.3mmol/l, 100 molecules of calcium should bind to one molecule of rP1. There is an argument that calcium can be bound to rP1 only when the protein is tagged by the histidine. Recombinant uteroglobin without tagged histidine, natural rabbit and human uteroglobin do not bind calcium [2, 29]. Recombinant P1 used here doesn't have such histidine residues [18]. For the following reasons as well, it is hardly conceivable that the inhibition of the SMCE by rP1 is induced through the interaction of rP1 with calcium. First, Nord and colleagues reported that although recombinant uteroglobin bound to negatively charged phospholipid liposomes in the presence of 5mmol/l calcium, no binding was detected in the presence of μmol/l orders of calcium [28]. As has stated before, rP1 exerts its influence upstream of SMCE. The experiments were carried out in the

Ca^{2+} free reaction buffer, although there was a μmol/l concentration of calcium, probably from added BSA. Second, in resting platelets, negatively charged phospholipids (PtdSer and PtdIns) are within the inner side of the membrane.

Specific binding of P1 monomer to microsomes and the cell membrane and the existence of uteroglobin binding proteins have been reported. Uteroglobin or rP1 fails to affect the cells that have no such protein [6, 13, 30]. Hence, it has been proposed that P1 has a receptor through which it regulates cellular functions. In platelets, a close relationship between actin filament reorganization and the function of receptors, e.g., $\alpha_{IIb}\beta_3$ has been demonstrated [16, 21, 31]. The results of this study also showed that activation of $\alpha_{IIb}\beta_3$ and actin polymerization are closely connected to each other. The exact relationship between the effect of rP1 and the actin reorganization is uncertain from the present study, and a P1 binding protein in platelets has not been found yet. If it exists, it is probably closely related to the actin cytoskeleton. The role of actin reorganization in the cell function is different in different cell types. How the reconstitution of the actin filament contributes to the cell function seems to decide the physiological effects of P1 on each cell reaction.

Natural P1 in blood specifically binds to the circulating platelets. It is bound more firmly to a platelet membrane than synthesized P1 because many samples binding of the anti P1 antibody above the non-specific binding after washing. Preincubation even with 100 μg/ml of synthesized P1 did not give an increase in the fluorescence intensity after washing. Recombinant P1 was also observed to have less reactivity to a peripheral membrane protein compared to natural P1 [32]. The low affinity might be one of the reasons a comparatively large amount of rP1 is necessary to influence platelet function. The reason why synthesized P1 shows a weak affinity is not known. Endogenous binding materials, such as phospholipids, progesterone or retinol might be related [2]. At the normal blood concentration of P1, the binding has no effect on platelet function. The effective concentration of rP1 to inhibit calcium influx varied an order of one among samples. The heterogeneity in filamentous actin

contents may cause the difference in part [15]. However, if rP1 interacts at the same sites or with the receptor protein of the natural P1, a relatively high concentration of natural P1 binding may reduce the quantity of rP1 necessary for the inhibition.

In summary, it was demonstrated that rP1 inhibits the SMCE leading to impaired platelet aggregation, and that the inhibition of the SMCE was the consequence of the interference of actin reorganization. It is premature to come to a conclusion about the basic role of P1 from results using rP1 and platelets, but it is probable that participation in actin reconstitution is one of the basic and common functions of the P1.

5. List of abbreviations

ETYA: 5, 8, 11, 14-eicosatetraynoic acid.

SMCE: store mediated calcium entry.

TG: thapsigargin

FITC: fluorescein isothiocyanate

PCB: polychlorinated biphenyl

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

PtdSer: phosphatidylserine

PtdIns: phosphatidylinositol

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Legends for illustrations

Figure 1 Effects of rP1 on platelet aggregation induced by U46619

$3x10^8$/ml washed platelets separated from buffy coat was preincubated in the reaction buffer with various concentrations of rP1 for 2min and stimulated by 1μmol/l U46619 in the presence of 1mmol/l calcium chloride. Tracings were superimposed on the same time scale.

Figure 2. Effects of rP1 on platelet aggregation induced by TG

$3x10^8$/ml washed platelets separated from buffy coat was preincubated with 48μg/ml rP1 in the reaction buffer containing 2mmol/l calcium chloride (A), 0.1mmol/l calcium chloride (B) and 0.1mmol/l calcium chloride with 10μmol/l ETYA and indomethacin (C) for 2min and then stimulated by 1μmol/l TG.

Figure 3. Inhibition of store mediated calcium entry by rP1

A. Concentration dependent inhibition of SMCE

$3x10^8$/ml of Fura-2 loaded platelets was preincubated with indicated concentrations of rP1 and 1μmol/l TG for 5min at 37°C. Then 0.3mmol/l calcium chloride was added (arrowhead). Traces were representative of 10 samples except for concentrations of added calcium and rP1. The optimum concentration varied among samples.

B. Simultaneous inhibition of aggregation

Simultaneous measurement of the light scattering of the same samples was performed as described in the Materials and Methods section. The first small decrease shows a shape change and reversible aggregation, and the following large decrease shows irreversible aggregation.

Figure 4. Detection of natural P1 on platelet membrane

A. Binding of rabbit polyclonal antibody to human urine P1 to platelets.

Fluorescence intensities of bound antibody were determined by flow cytometry as described in the Materials and Methods section. Anti α1-micro globulin and rabbit IgG were used to indicate nonspecific binding. 150μg/ml of IgG produced the same fluorescence intensity as at 15μg/ml. A representative pattern of 10 different samples is shown.

Mean fluorescence intensity at the indicated concentration:○, anti α1-micro globulin; □,anti P1 antibody; Δ, rabbit IgG.

B. Effects of rP1 on the antibody binding

Platelets were pre-incubated with 3μg/ml anti P1 antibody and indicated concentrations of rP1, then treated as described in the Materials and Methods section.

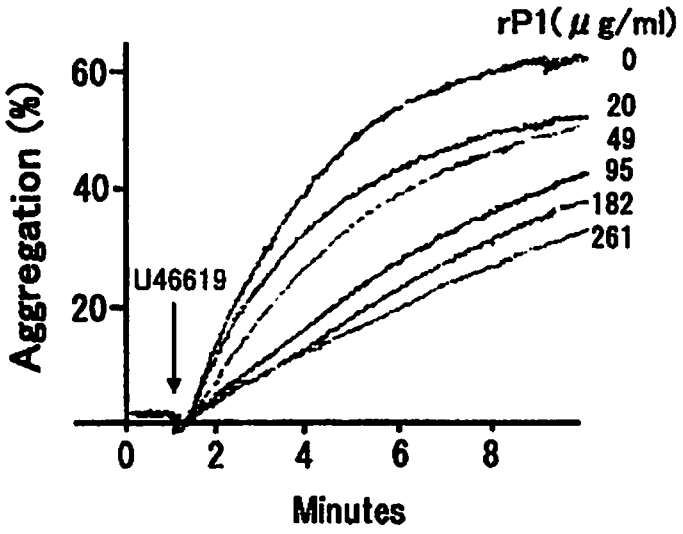
Mean fluorescence intensity at the indicated concentration: ○, anti α1-microglobulin; □, anti P1 antibody; Δ, FITC labeled anti rabbit antibody without the first antibody.

Figure 5. Effect of rP1 on actin filament content of TG-or thrombin-stimulated platelets

Platelets pretreated with 20μmol/l indomethacin were activated by 1μmol/l TG for 5min or 0.4U/ml thrombin for 1min in the virtually calcium free reaction buffer and fixed with 1% paraformaldehyde. The actin filament concentration of the platelets was determined as described in the Materials and Methods section. The changes of the mean fluorescence intensity of FITC conjugated phalloidin are expressed as a percentage of those of resting platelets without rP1. Results of Student's paired t-test of significant difference due to rP1 treatment were as follows: for resting platelets, $P<0.001$ (n=16); for 1μmol/l TG activation, $P< 0.005$ (n=12); for 0.4U/ml thrombin activation, $P< 0.005$ (n=6). Grey column: control. Black column: pretreated with 20μg/ml rP1.

Figure 6. Effect of rP1 on PAC1 binding

PAC1 binding to platelets was determined as described in the Materials and Methods section. The mean fluorescence intensity of FITC conjugated PAC1 on the platelets was considered an indication of activation of $\alpha_{IIb}\beta_3$. The changes of mean fluorescence intensity are expressed and analyzed as described under the legend of figure 5. The results of Student's paired t-test of significant difference due to rP1 treatment were as follows: for resting platelets, $P<0.025$ (n=12); for 1μm/l TG activation $P< 0.05$ (n=12); for 0.4U/ml thrombin activation, $P< 0.05$ (n=6). Grey column: control. Black column: Pre-treated with 20μg/ml rP1.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Aggregation (%)
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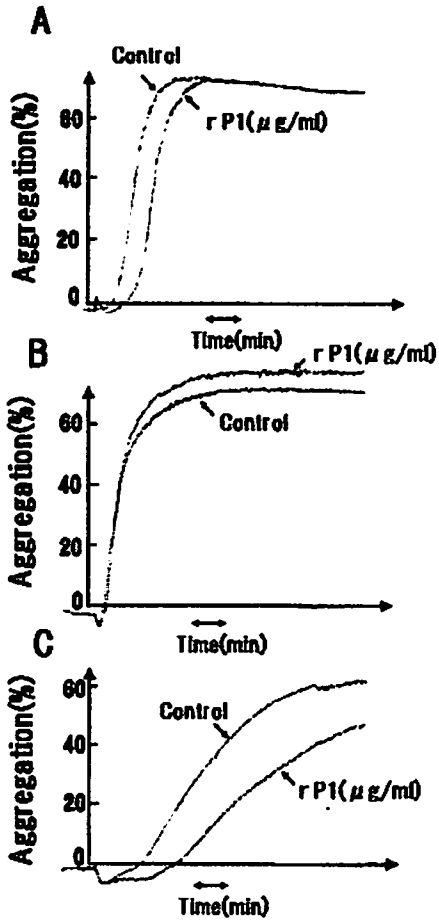
Fig 1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Time(min)

Fig 2

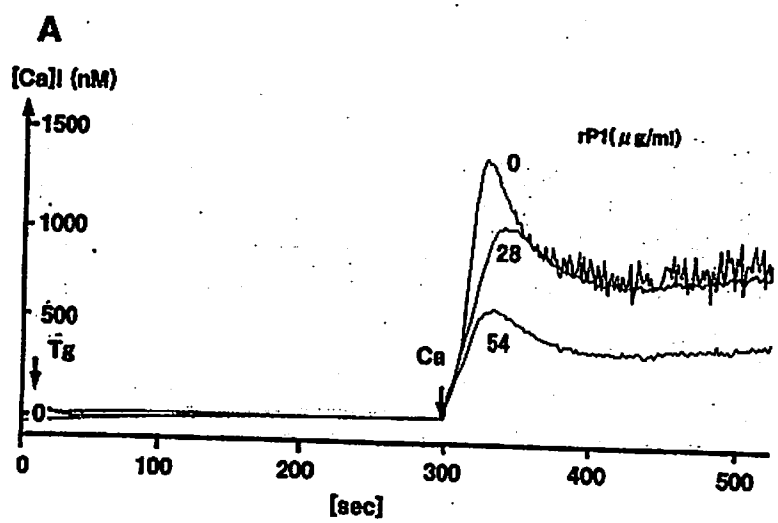
A
[Ca]I (nM)
1500
1000
500
0
Tg
Ca
rP1(μg/ml)
0
28
54
0
100
200
300
400
500
[sec]

Fig 3 A

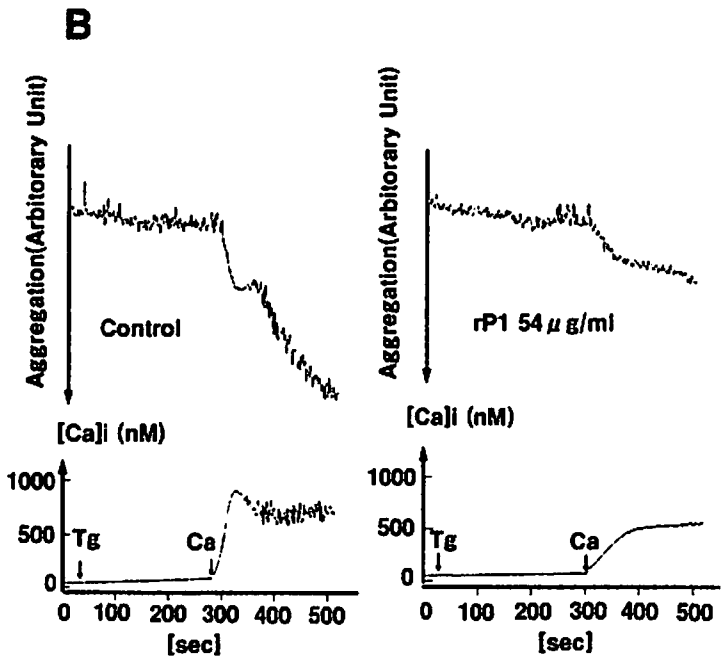
B
Aggregation(Arbitorary Unit)
Control
[Ca]i (nM)
1000
500
0
Tg
Ca
0 100 200 300 400 500
[sec]
Aggregation(Arbitorary Unit)
rP1 54 μg/ml
[Ca]i (nM)
1000
500
0
Tg
Ca
0 100 200 300 400 500
[sec]

Fig 3 B

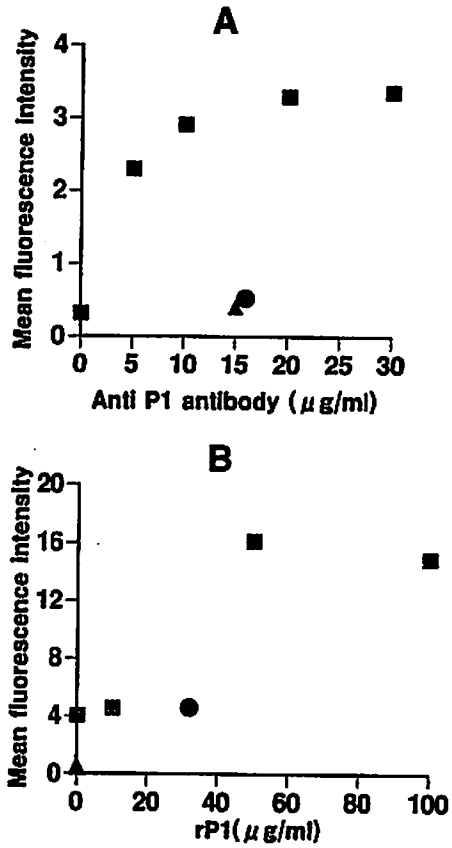
A
Mean fluorescence intensity
0
1
2
3
4
0 5 10 15 20 25 30
Anti P1 antibody (μg/ml)
B
Mean fluorescence intensity
0
4
8
12
16
20
0 20 40 60 80 100
rP1(μg/ml)

Fig 4

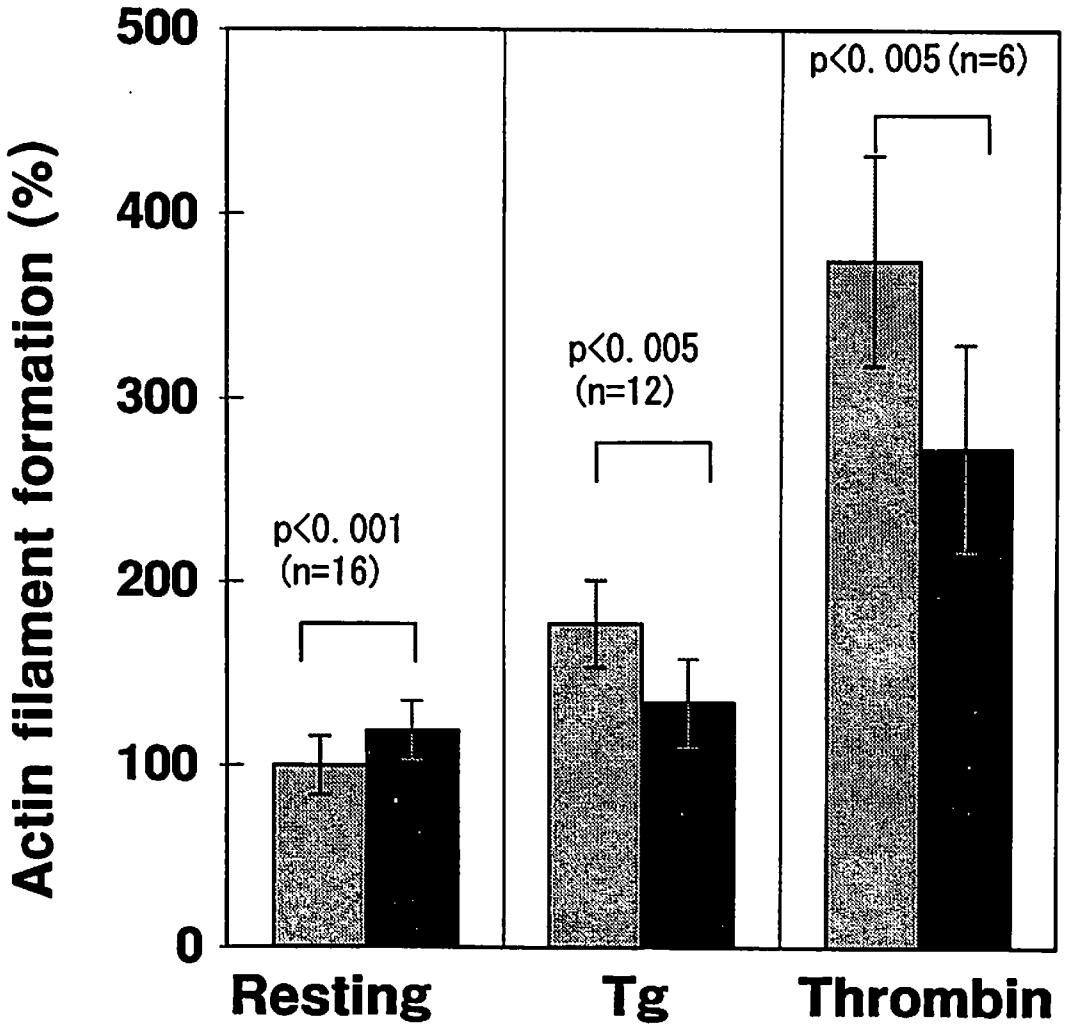
Actin filament formation (%)
500
400
300
200
100
0
p<0. 001
(n=16)
p<0. 005
(n=12)
p<0. 005 (n=6)
Resting
Tg
Thrombin

Fig 5

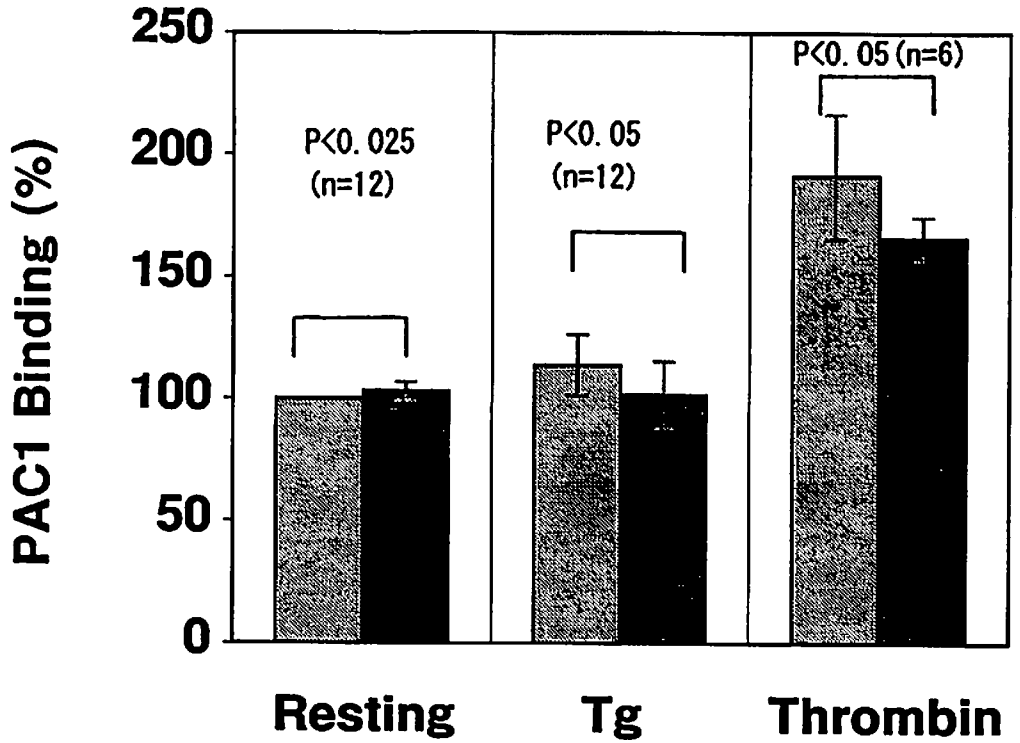
PAC1 Binding (%)
250
200
150
100
50
0
P<0. 025
(n=12)
P<0. 05
(n=12)
P<0. 05 (n=6)
Resting
Tg
Thrombin

Fig 6