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INTRACELLULAR IONIZED CALCIUM MOBILIZATION OF CD 9 MONOCLONAL ANTIBODY-ACTIVATED HUMAN PLATELETS

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Cytoplasmic Ca^{2+} mobilization in human platelets triggered by a CD 9 monoclonal antibody, TP82, was monitored by the Ca^{2+}-sensitive photoprotein, aequorin and Ca^{2+}-sensitive fluorophores, fura 2 and quin 2. Aequorin-indicated Ca^{2+} values were proportional to the concentration of TP82, which was in inverse proportion to the lag time before the onset of platelet aggregation and serotonin release. When fura 2 was used as a Ca^{2+} indicator, above a threshold concentration, the TP82-induced intracellular Ca^{2+} value remained unchanged even with increasing concentration. The findings obtained with quin 2 were compatible with the fact that the TP82-induced intracellular Ca^{2+} increase was largely dependent on the secondary effect of thromboxane A_2. These findings may be clues to explain the marked difference in the Ca^{2+}-response characteristics between the fluorescent indicators and aequorin as well as the properties of TP82-induced platelet activation.

One of the leukocyte differentiation antigens, CD 9 antigen, has been reported to show restricted expression within the hematopoietic lineage(1). Although this antigen was initially known as the platelet-common acute lymphoblastic leukemia associated antigen(2-4), it has been shown to be more widely distributed(1, 5). Although the precise role of CD 9 antigen remains to be elucidated, it is well known that monoclonal antibodies which recognize this antigen cause direct activation of platelets(6-9).

We previously reported a monoclonal antibody, designated TP82, which was raised against normal human platelets and caused irreversible platelet aggregation and the release of dense bodies

Abbreviations: TxA_2, thromboxane A_2; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; aspirin, acetylsalicylic acid; PRP, platelet-rich plasma.

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(ATP and serotonin) accompanied by thromboxane A_2(TxA_2) formation(9). TP82 is considered to be included in a CD 9 cluster in terms of molecular weight of an epitope, tissue specificity, and the antigen blocking study(9, 10). Recent studies have revealed that a CD 9 antibody induces increase in intracellular Ca^{2+} concentration($[Ca^{2+}]i$) monitored by aequorin(11). This is consistent with our previous finding that TP82 induces phosphorylation of the 20K protein(myosin light chain) as well as that of 47K protein(12), since myosin light chain kinase is Ca^{2+}-calmodulin dependent(13).

In this study, we examined TP82-induced intracellular calcium mobilization with the Ca^{2+}-sensitive photoprotein, aequorin and Ca^{2+}-sensitive fluorophores, fura 2 and quin 2. Interestingly, TP 82-triggered $[Ca^{2+}]i$ transients vary with the Ca^{2+} indicators used.

MATERIALS AND METHODS

Materials---The following materials were purchased from the indicated suppliers: aequorin(Baxter-Travenol, Tokyo, Japan); [^{14}C] serotonin(Amersham International, Bucks, U.K.); fura 2-AM(Molecular Probes, Inc., OR, USA); Quin 2-AM(Dojindo laboratories, Kumamoto, Japan); acetylsalicylic acid(aspirin)(Sigma, MO, USA); ionomycin (Calbiochem, CA, USA); fibrinogen(Kabi, Stockholm, Sweden). All other reagents were of analytical grade. TP82 was produced and purified as previously described(9).

Platelet Preparation---Citrate anti-coagulated blood was obtained by venepuncture from healthy human donors who denied medicine for 2 weeks preceding the experiment. The blood was centrifuged at 100 g for 10 min to obtain platelet-rich plasma(PRP). The procedure for washing platelets was as described previously(9). The final platelet pellet was resuspended in Hepes-Tyrode's buffer(129mM NaCl, 8.9mM $NaHCO_3$, 2.8mM KCl, 0.8mM KH_2PO_4, 0.8mM $MgCl_2$, 1mg/ml Glucose, 10mM Hepes, pH7.4) at a concentration of $3x10^8$/ml followed by supplementation with 1 mM Ca^{2+}. All experiments were performed at 37 ^oC under continuous stirring at 1000 rpm. Purified human fibrinogen(500 ug/ml) was added to all samples shortly before the addition of the agonist.

$[Ca^{2+}]i$ Measurement with Aequorin---Aequorin was introduced into platelets by incubation with 10 mM EGTA and 5 mM ATP as described by Johnson et al(14). After the loading, platelets were separated from unloaded aequorin by Sepharose 2B gel filtration using Hepes-Tyrode's buffer as eluant. Aequorin luminescence was measured in a Platelet Ionized Calcium Aggregometer(Chrono-Log,PA,USA). Calibration of aequorin signals from a standard curve was done as described elsewhere(14).

$[Ca^{2+}]i$ Measurement with Fura 2---PRP was incubated at 37^oC with 3 uM fura 2-AM for 45 min. After incubation, the platelets were washed twice and resuspended in Hepes-Tyrode's buffer. $[Ca^{2+}]i$ indicated by fura 2 signals were measured in a CAF-100(Japan Spectroscopic Co., Ltd., Tokyo, Japan) by the double-wavelength fluorometric method with excitation of 340/380 nm and emission of 500 nm. $[Ca^{2+}]i$ values were calculated from fura 2 ratios(R) by the equation(15): $[Ca^{2+}]i=Kd(Fo/Fs)(R-Rmin.)/(Rmax.-R)$ where

Rmin. and Rmax. are the ratios(340 nM/380 nM) obtained in zero or saturating calcium concentration, Kd is the effective dissociation constant(224 nM), Fo is the 380 nm excitation signal in the absence of calcium, and Fs is the 380 nm excitation signal at saturating calcium concentration.

$[Ca^{2+}]i$ Measurement with Quin 2---PRP was incubated at 37 °C with 10 uM or 100 uM quin 2-AM for 45 min. After incubation, the platelets were washed twice and resuspended in Hepes-Tyrode's buffer. $[Ca^{2+}]i$ indicated by quin-2 signals were measured in a CAF-100(Japan Spectroscopic Co., Ltd., Tokyo, Japan). The excitation wavelength was 340 nm and emission wavelength 500 nm.

Platelet Aggregation---Platelet aggregation was measured in a Platelet Ionized Calcium Aggregometer or a CAF-100 simultaneously with $[Ca^{2+}]i$ measurement. The instruments were calibrated for zero light transmission with a platelet suspension and with Hepes-Tyrode's buffer for 100% transmission.

Platelet Serotonin Release---PRP was incubated with $[^{14}C]$ serotonin(100 nCi/ml of PRP) at 37°C for 45 min. The platelets were washed twice and resuspended in Hepes-Tyrode's buffer. The platelet suspensions were stimulated in an aggregometer cuvettes under continuous stirring at 37 °C. The release reaction was stopped with 0.1% glutaraldehyde. After centrifugation for 10 min at 2000 g, aliquots of the supernatant were removed for determination of radioactivity by liquid scintillation counting. The results were expressed as the percent release of the total $[^{14}C]$serotonin radioactivity incorporated into platelets.

Treatment of Platelets with Aspirin---When indicated, the platelet suspension was pretreated with 1 mM aspirin at 37°C for 5 min before stimulation. This concentration of aspirin completely blocked 10 uM arachidonate or 1 ug/ml collagen-induced platelet activation(data not shown).

Miscellaneous---In some experiments, $[^{14}C]$serotonin release, aggregation, and $[Ca^{2+}]i$ measurement with aequorin were observed with the same sample as previously described(16), and similar results were obtained. Data presented are representatives of at least three experiments that gave similar results.

RESULTS

Aequorin-indicated $[Ca^{2+}]i$ Increase of TP82-activated Platelets

As already reported(9), TP82-induced platelet aggregation and release reaction were associated with the lag period that was prolonged in inverse proportion to the antibody concentration(Fig. 1). The elevation of $[Ca^{2+}]i$ occurred coincidentally with the onset of aggregation or serotonin release, not upon the addition of TP82 nor during the lag phase(Fig. 1A). The rise in $[Ca^{2+}]i$ became larger with increasing concentration of TP82(Fig. 1B). While pretreatment with 500 uM aspirin, a cyclooxygenase inhibitor, partially inhibited TP82-induced platelet aggregation(Fig. 2B), the increase in $[Ca^{2+}]i$ was completely suppressed(Fig. 1B). Aequorin-indicated $[Ca^{2+}]i$ increases are considered to be fully dependent on the TxA_2 produced.

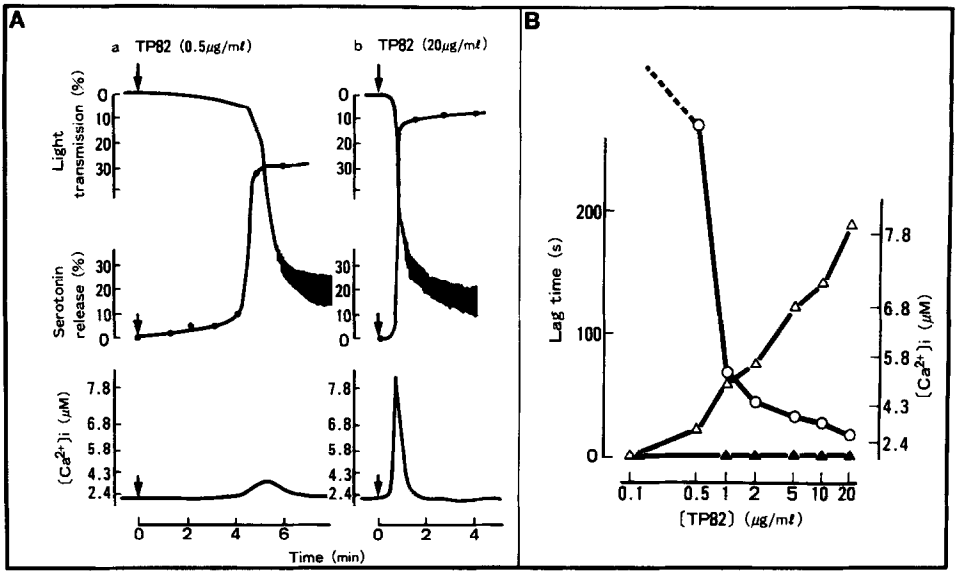

Fig.1. TP82-induced platelet activation and $[Ca^{2+}]i$ rise detected with aequorin.
[A]Time courses of platelet aggregation(upper panel), serotonin release(middle panel), and $[Ca^{2+}]i$ change (lower panel) triggered by 0.5 ug/ml(a) or 20 ug/ml(b) of TP82.
[B]Peak $[Ca^{2+}]i$(▲) and the lag time before the onset of aggregation (o) at various concentrations of TP82. Peak $[Ca^{2+}]i$ of TP82-stimulated platelets pretreated with aspirin is also plotted(▲).

Fura 2-indicated $[Ca^{2+}]i$ Increase of TP82-activated Platelets

When fura 2 was used as a Ca^{2+} indicator, a biphasic pattern of $[Ca^{2+}]i$ increase was observed(Fig. 2A). While $[Ca^{2+}]i$

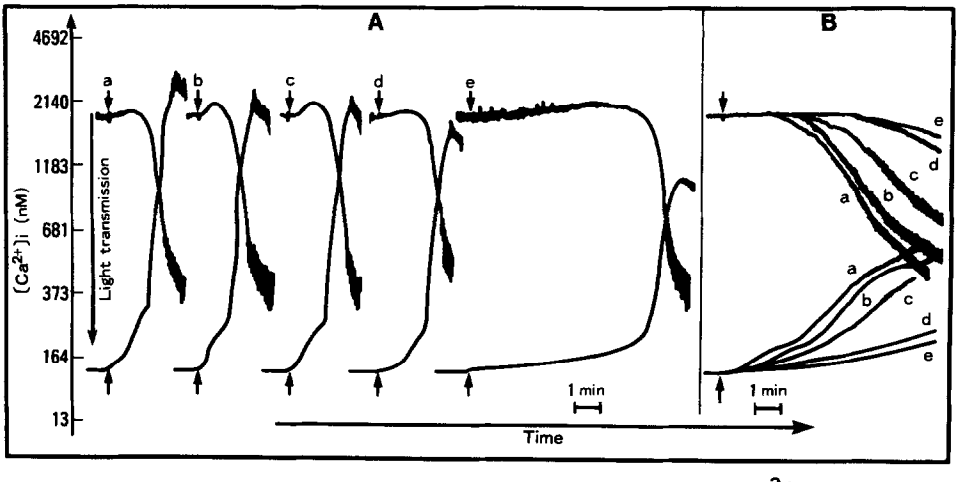

Fig.2. TP82-induced platelet aggregation and $[Ca^{2+}]i$ change detected with fura 2.
Platelet aggregation and $[Ca^{2+}]i$ change at various concentrations of TP82 without[A] or with[B] 500 uM aspirin. Concentrations of TP82 used are 10 ug/ml(a), 5 ug/ml(b), 2 ug/ml(c), 1 ug/ml(d), and 0.5 ug/ml(e), respectively.

increased gradually during the lag phase, it revealed a sharp peak coincidentally with the onset of aggregation. The peak $[Ca^{2+}]i$ value was almost unchanged by the increase in the concentration of TP82 used. TP82-induced platelet aggregation traces were of a single wave profile at various concentrations despite variation in tha lag time(Fig. 1A, 2A). At this point, the fura 2-indicated $[Ca^{2+}]i$ rise resembled the aggregation response. Pretreatment with 500 uM aspirin abolished the latter sharp peak, but not the initial gradual increase(Fig. 2B).

Quin 2-indicated $[Ca^{2+}]i$ Increase of TP82-activated Platelets

When 100 uM of quin 2-AM was preincubated with PRP for the loading, TP82-induced increase in $[Ca^{2+}]i$ as well as platelet aggregation was completely inhibited(Fig. 3B). Under the same condition, no effect on 100 nM ionomycin was observed(Fig. 3B). We previously reported that no measurable change in $[Ca^{2+}]i$ was observed using quin 2 as a calcium indicator in platelets activated by TP82 under the condition thrombin induced marked $[Ca^{2+}]i$ increase(12). When a lower concentration of quin 2-AM was used for its loading, the TP82-induced $[Ca^{2+}]i$ increase was barely detected(Fig. 3A). We considered that TP82-induced platelet activation is subject to inhibition by quin 2 loading.

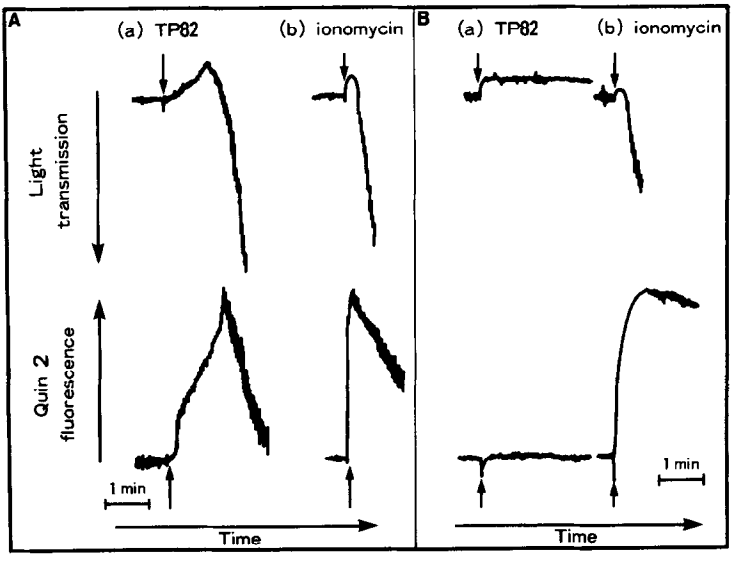

Fig.3. TP82-induced platelet aggregation and $[Ca^{2+}]i$ change detected with quin 2.
Concentrations of loaded quin 2-AM are 10 uM[A] and 100 uM[B], respectively. Platelet aggregation(upper panel) and $[Ca^{2+}]i$ change(lower panel) triggered by 5 ug/ml TP 82(a) or 500 uM ionomycin(b).

DISCUSSION

As described previously(11), CD 9(TP82)-induced platelet activation is accompanied by $[Ca^{2+}]i$ elevation. Interestingly, the $[Ca^{2+}]i$ values varied greatly with the Ca^{2+} indicator used. While $[Ca^{2+}]i$ transients detected with fura 2 were of a single profile, aequorin-indicated $[Ca^{2+}]i$ changes varied with the concentration of TP 82 which is inversely proportional to the length of the lag phase.

The use of fluorescent indicators, i.e. fura 2 and quin 2, and/or Ca^{2+}-sensitive photoprotein, i.e. aequorin, has made possible the direct estimation of agonist-induced changes in $[Ca^{2+}]i$(17). However, these fluorescent and bioluminescent indicators differ markedly in the Ca^{2+}-response characteristics(14, 17-19). One possible explanation for these discrepancies is that aequorin detects a localized pool of relatively high $[Ca^{2+}]i$, changes in which are not reflected in the spatial average $[Ca^{2+}]i$ that is registered by fluorescent indicators(17). CD 9-induced $[Ca^{2+}]i$ transients which we report herein may be clues to the entire explanation of the marked difference in the Ca^{2+}-response characteristics between the fluorescent indicators and aequorin.

Owing to its high Ca^{2+} buffering capacity, quin 2 is often used as a potent intracellular Ca^{2+} chelator(20). Consequently, phospholipase A_2, which is Ca^{2+}-dependent(21, 22), has been reported to be subject to inhibition by quin 2 loading(23, 24). In platelets, phospholipase A_2 is a key enzyme for the production of TxA_2(22, 23). Our study that quin 2 loading inhibits both $[Ca^{2+}]i$ increase and aggregation by TP 82 is explained by the inhibition of phospholipase A_2 by quin 2, since pretreatment with aspirin, the inhibitor of cyclooxygenase, blocked the aequorin-indicated $[Ca^{2+}]i$ increase and larger peak detected by fura 2. This means that the $[Ca^{2+}]i$ increase caused by CD 9 depends mostly on the TxA_2 produced. The physiological significance of the initial gradual increase detected with fura 2, not aequorin, remains to be clarified.

In summary, $[Ca^{2+}]i$ transients detected with fluorescent and bioluminescent Ca^{2+} indicators differed markedly in platelets stimulated with CD 9. These findings may be important for the entire explanation of the marked difference in the Ca^{2+}-response characteristics between these indicators as well as the properties of CD 9-induced platelet activation.

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