Biochem. J. (1990) 266, 527–535 (Printed in Great Britain)

Stimulus–response coupling in human platelets activated by monoclonal antibodies to the CD9 antigen, a 24 kDa surface-membrane glycoprotein

Roger C. CARROLL,* Ronald E. WORTHINGTON* and Claude BOUCHEIX†

*Department of Medical Biology, University of Tennessee Medical Center at Knoxville, 1924 Alcoa Highway, Knoxville, TN 37920, U.S.A. and †INSERM U 268, Oncogenese Appliquée, Groupe Hospitalier Paul-Brousse, 14 & 16, avenue Paul Vaillant-Couturier, 94800, Villejuif, France

The CD9 molecule is a 24 kDa surface-membrane glycoprotein present on platelets and a variety of haematopoetic and non-haematopoetic tissues. In the present study we utilized specific inhibitors of thromboxane A_2 (TxA_2) formation (aspirin), protein kinase C {H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine]} and autocrine stimulation by secreted ADP (apyrase) to modify platelet activation by a monoclonal antibody ALB-6 to the CD9 antigen. This activation is only partially inhibited by aspirin alone but, in combination with either H-7 or apyrase, more than 50% inhibition of platelet aggregation and secretion was observed. This combination of inhibitors was also required to inhibit effectively the phosphorylation of myosin light chain and the 47 kDa substrate of protein kinase C. Intracellular Ca^{2+} flux monitored by the fluorescent dye fura-2 showed that this was almost completely mediated by the aspirin-sensitive TxA_2 pathway. We suggest that the aspirin-insensitive pathway is primarily mediated by phospholipase C formation of diacylglycerol to activate protein kinase C. The inhibition by apyrase suggests a strong dependency on autocrine stimulation by secreted ADP to fully activate both phospholipase C and express fibrinogen-binding sites mediating platelet aggregation. This alternate pathway of phospholipase C activation by ALB-6 may be mediated by cytoplasmic alkalinization {monitored by SNARF-1 (5′(6′)-carboxy-10-bismethylamino-3-hydroxy-*spiro*-{7*H*-benzo[*c*]xanthine-1′,7(3*H*)-isobenzofuran}-3′-one) fluorescence of the dye}. Both activation pathways are dependent on intact antibodies, since $F(ab')_2$ fragments of SYB-1, a monoclonal antibody against the CD9 antigen with activation characteristics identical with those of ALB-6, do not elicit activation. Besides thrombin, collagen is another physiological agonist shown to induce aspirin-insensitive activation. Similarities to ALB-6 in collagen sensitivity to apyrase in combination with aspirin inhibitors were noted with respect to aggregation and secretion, as well as a complete block of Ca^{2+} flux by aspirin. However, it is unlikely that collagen activation is mediated by the CD9 antigen, since SYB-1 $F(ab')_2$ fragments had no effect on collagen activation and aspirin also completely blocked the alkalinization response to collagen, in contrast with ALB-6.

INTRODUCTION

Monoclonal antibodies (MAbs) to various epitopes of the CD9 antigen (designation of the First International Conference on Human Leukocyte Antigens), a non-transmembranous 24 kDa surface glycoprotein (Newman *et al.*, 1982), trigger human platelet activation (Boucheix *et al.*, 1983). One MAb (ALB-6) described in the present study was raised by immunizing mice with human acute-lymphoblastic-leukaemia cells. The CD9 antigen recognized by this and several related MAbs is widely distributed in both haematopoetic and non-haematopoetic tissues. The physiological role of the CD9 antigen in platelet function, or in any cell, is as yet unknown. On platelets it appears to be unrelated to any of the well-characterized surface glycoproteins such as Ib, IIb/IIIa or IX, which either contain disulphide-linked subunits or are proteins of similar molecular mass (Boucheix *et al.*, 1983; Benoit *et al.*, 1987).

Bivalent interaction may be required for platelet activation leading to aggregation and granule secretion, since these responses are not elicited by univalent Fab fragments of ALB-6 (Boucheix *et al.*, 1983), Ba2 (Brown *et al.*, 1984) and FMC8 and FMC56 MAbs (Gorman *et al.*, 1985). Another MAb (PMA2) against the CD9 antigen has recently been shown to trigger fibrinogen-receptor expression and platelet aggregation solely through platelet activation (Hato *et al.*, 1988). The present study confirms the report by Rendu *et al.* (1987) that both an aspirin-sensitive thromboxane A_2 (TxA_2) pathway and an aspirin-insensitive pathway for secretion of ADP are responsible for platelet aggregation induced by MAbs to the CD9 antigen (ALB-6 and SYB-1). Rendu *et al.* (1987) suggested that this activation was coupled to both phospholipase A_2 formation of arachidonic acid, with subsequent formation of TxA_2, and to polyphosphoinositide metabolism by phospholipase C to generate inositol trisphosphate and diacylglycerol. Both

Abbreviations used: TxA_2, thromboxane A_2; MAbs monoclonal antibodies; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; 5HT, 5-hydroxytryptamine; aspirin, acetylsalicylic acid; PBS, phosphate buffered saline; PRP, platelet-rich plasma; TxB_2, thromboxane B_2; r.i.a., radioimmunoassay; MLC, myosin light chain; $[Ca]_i$, internal Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate. SNARF 1, 5′(6′)-carboxy-10-bismethylamino-3-hydroxy-*spiro*-{7*H*-benzo[*c*]xanthine-1′,7(3′*H*)-isobenzofuran}-3′-one; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine.

the aspirin-sensitive formation of TxA_2 and aspirin-insensitive formation of inositol trisphosphate should mobilize Ca^{2+} from intracellular stores (Rybicki *et al.*, 1983; O'Rourke *et al.*, 1985) to mediate myosin-light-chain (MLC) phosphorylation (Hallam *et al.*, 1985). Diacylglycerol, perhaps augmented by the cytosolic Ca^{2+} elevation, could activate protein kinase C (Kishimoto *et al.*, 1980; Siess & Lapetina, 1988). The major substrate of this kinase in platelets is a 47 kDa protein of unknown function, but which has been closely linked to platelet granule secretion (Sano *et al.*, 1983). Secreted ADP, in addition to directly causing expression of fibrinogen receptor leading to aggregation, could serve, along with TxA_2 to further accelerate phospholipase C (Rittenhouse, 1984).

Since the CD9 molecule does not span the platelet membrane, signal transduction via this pathway was suggested by Rendu *et al.* (1987) to be mediated by either a change in membrane fluidity or an unidentified transmembrane component associated with the CD9 molecule. Cytoplasmic alkalinization, suggested to be involved in platelet signal transduction by agonists such as adrenaline (epinephrine) and thrombin, has not been examined as a possible coupling mechanism for activation by CD9 MAbs. Adrenaline-induced alkalinization was suggested to be coupled to phospholipase A_2 activation through localized Ca^{2+} flux (Banga *et al.*, 1986). Another model has been proposed by Siffert & Akkerman (1988), who suggested that thrombin-induced cytoplasmic alkalinization was a consequence of protein kinase C activation and augmented Ca^{2+} mobilization from intracellular stores. Other reports dispute the view that Na^+/H^+ exchange is obligatory for either thrombin- (Zavoico & Cragoe, 1988) or ADP- (Funder *et al.*, 1988) induced platelet activation.

The role of external Ca^{2+} in the activation by CD9 MAbs is also unclear. Higashihara *et al.* (1985) observed inhibition of platelet secretion by a CD9 MAb (TP82) with the bivalent-cation chelator EDTA. However, Rendu *et al.* (1987) indicated no external Ca^{2+} requirement for platelet secretion and protein phosphorylation induced by ALB-6.

It is tempting to speculate that the CD9 antigen serves as a physiological agonist receptor that is turned on by antibody interaction. Collagen is another agonist eliciting activation mediated by both aspirin-sensitive and -insensitive pathways (Connellan *et al.*, 1986; Pollock *et al.*, 1986). It was initially reported that the Fab fragments of ALB-6 blocked collagen activation (Boucheix *et al.*, 1983). However, Fab fragments of two different CD9 MAbs were reported to augment platelet activation by collagen and ADP (Gorman *et al.*, 1985). Several putative collagen-platelet receptors have been isolated (Chiang *et al.*, 1987; Santoro *et al.*, 1988; Tandon *et al.*, 1989), none of which have been suggested to have a 24 kDa component.

In the present study we have examined ALB-6-induced platelet activation, as measured by aggregation, dense-granule secretion and protein phosphorylation mediated by specific protein kinase pathways in terms of: (1) a requirement for autocrine stimulation by ADP; (2) cytosolic Ca^{2+} flux and the influence of external Ca^{2+}; (3) TxA_2 generation; and (4) the effect of the protein kinase C inhibitor H-7 on the aspirin-insensitive pathway. We have also examined the role that cytoplasmic alkalinization plays in transduction of the CD9 MAb activation signal. These activation characteristics and sensitivity to inhibitors are compared with the collagen pathway. In addition, we compared the effects of non-activating CD9 MAb $F(ab')_2$ fragments on collagen and CD9 MAb activation.

MATERIALS AND METHODS

Materials

Electrophoresis reagents were acquired from Bio-Rad (Richmond, CA, U.S.A.); monensin, H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine], fura-2-AM, and carboxy-SNARF-1-AM were purchased from Molecular Probes (Eugene, OR, U.S.A.). TxB_2 radioimmunoassay (r.i.a.) kits, ^{14}C-labelled 5-hydroxytryptamine ([^{14}C]5HT) at 50 μCi/ml in ethanol, and [^{32}P]orthophosphate (at 50 mCi/ml of 0.02 M-HCl) were acquired from New England Nuclear (Boston, MA, U.S.A.). Apyrase, thrombin, acetylsalicylic acid (aspirin) and buffer reagents were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Collagen was obtained from Hormon-Chemie (Munich, Germany) and suspended, with the aid of sonication, at 0.5 mg/ml in phosphate-buffered saline (PBS), pH 7.4. The CD9 MAb, ALB-6, of the IgG_1 subclass, was produced and purified to homogeneity as previously described (Boucheix *et al.*, 1983). It was added to the platelets at the indicated concentration from a 100-fold-concentrated solution in PBS. Attempts at producing $F(ab')_2$ fragments of ALB-6 were unsuccessful. However, SYB-1, a CD9 MAb, also of the IgG_1 subclass and with similar activation characteristics to ALB-6 (Rendu *et al.*, 1987), was readily converted into $F(ab')_2$ fragments by utilizing agarose-immobilized papain (Sigma). Antibody at a concentration of 4–5 mg/ml in 100 mM-sodium citrate, pH 3.5, was incubated overnight with 0.025 mg of papain–agarose/mg of antibody at room temperature with constant agitation. The papain–agarose beads were removed by brief centrifugation. SYB-1 $F(ab')_2$ fragments were separated from intact IgG and Fc fragments by passage through a protein A–agarose column (Pierce Chemical, Rockford, IL, U.S.A.) equilibrated in PBS. $F(ab')_2$ fragments were at least 95% pure on the basis of Coomassie Blue staining of SDS/PAGE reduced and non-reduced gels and were devoid of intact IgG. Thrombin and apyrase were also made up in PBS at 1000-fold-concentrated stock solutions. H-7, fura-2-AM and carboxy-SNARF-1-AM were freshly dissolved at 1000-fold concentration in anhydrous dimethyl sulphoxide. Monensin and aspirin were freshly made as 1000-fold-concentrated stock solutions in 95% ethanol. These final concentrations of solvents had no effect on platelet function.

Blood collection and preparation of platelet-rich plasma

Blood was collected from volunteers giving informed consent who denied taking any medication for the previous 2 weeks. The blood was drawn from the antecubital vein into syringes containing 0.1 vol. of 100 mM-sodium citrate and 130 mM-glucose, pH 6.5. The blood was centrifuged for 3 min at 1000 *g* at room temperature to separate the platelet-rich plasma (PRP).

Aggregometry

Platelet aggregation in PRP at (2–4) $\times 10^8$ platelets/ml was determined at 37 °C with constant stirring on a

Payton dual-channel aggregometer calibrated to 100% light transmittance with platelet-poor plasma. Percentage aggregation was calculated by taking 100% light transmittance as 100% aggregation and unstimulated PRP light transmittance as 0% aggregation. Lag time was measured from the point of activator addition to the point that increased light transmission due to platelet aggregation caused the trace to cross the median of the baseline.

Thromboxane quantification

PRP was prepared and platelet aggregation was performed as described above. Samples for thromboxane B_2 (TxB_2), a stable metabolite of TxA_2, were taken before or after a complete aggregation response to ALB-6 (15 μg/ml) with or without pretreatment with 100 μM-aspirin or other platelet inhibitors. In either case, PRP (0.5 ml) was diluted with an equal volume of ice-cold indomethacin (20 μg/ml) dissolved in water. Samples were sonicated for three 5 s on–off cycles, and the cellular debris was removed by centrifugation at 15000 *g* for 10 min. The supernatant fluids from the above centrifugation were assayed for TxB_2 using a commercial r.i.a. kit (New England Nuclear) according to the manufacturer's instructions.

5HT secretion

PRP was allowed to incorporate a tracer amount of [^{14}C]5HT (0.05 μCi/ml; added from 1000-fold stock solution) for at least 30 min before experiments. PRP aliquots were monitored by aggregometry as described above. Before addition of agonists and at indicated time intervals, 0.1 ml aliquots were removed and immediately mixed in 1.5 ml microfuge tubes with 0.9 ml of ice-cold PBS plus 10 mM-EGTA and 0.2% glutaraldehyde to stop release. The platelets were removed by centrifugation for 2 min at 10000 *g* before taking 0.5 ml aliquots of the supernatant for liquid-scintillation counting of released [^{14}C]5HT. Similarly diluted aliquots which were not centrifuged were counted for total radioactive label. Zero-time radioactivity, which was less than 10% of the total, was subtracted from both the time-course samples and total radioactivity before dividing the corrected time-course sample radioactivity by total radioactivity and multiplying by 100 to calculate the percentage released.

Fura-2 loading, carboxy-SNARF-1 loading and fluorescence measurements

Platelets were loaded wth fura-2 by incubation of PRP with 2 μM-fura-2-AM for 30 min at 37 °C. The carboxy-SNARF-1-AM dye was similarly loaded at a final concentration of 15 μM after pelleting the platelets from the PRP and resuspending them in 0.2 vol. of Tangen/Hepes/BSA buffer [a Hepes-buffered pH 7.4 balanced-salts solution with BSA (1 mg/ml) developed by Tangen & Berman (1972) for platelet isolation, modified as previously described (Cox *et al.*, 1984)]. The plasma proteins and unincorporated dyes were removed by gel filtration at room temperature on a 5-times bed vol. of Sepharose 2B equilibrated with Tangen/Hepes/BSA buffer, pH 7.4, as previously described (Cox & Carroll, 1986).

Fura-2 fluorescence monitoring at 510 nm was performed in a Perkin–Elmer fluorimeter at 335 nm excitation in plastic cuvettes thermostatically controlled at 37 °C. Samples at (1–2) $\times 10^8$ platelets/ml were unstirred, except for a brief mixing after addition of agonists or inhibitors. Maximum fluorescence was measured after platelet lysis with 0.02% SDS and the addition of 1 mM-$CaCl_2$. Minimum fluorescence was determined by addition of 5 mM-EGTA to lysed samples. Calculation of

Table 1. Effect of inhibitors on platelet aggregation and secretion in PRP activated by ALB-6 or collagen

Pretreatment with aspirin was for 10 min; H-7 pretreatment was for 5 min at 37 °C. Addition of apyrase or EGTA was within 1 min of adding the activator. The lag time was determined from the aggregometry trace as the point from the addition of the activator until the trace crossed the median of the baseline of the sample before activation. The percentage aggregation was also determined from the aggregometry trace, taking the median light transmission of unactivated samples as 0% aggregation and the corresponding PRP as 100% aggregation. Secretion of [^{14}C]5HT was determined as described in the Materials and methods section. The number of determinations is indicated by the *n* value. Experiments were done with at least two different subjects and values given are means ± S.D. Abbreviation: ND, not determined.

Activator	Pretreatment	*n*	Lag time (s)	Aggregation (%)	Secretion (%)
(a) ALB-6 (15 μg/ml)	None	4	87±10	88±7	87±6
	5 mM-EGTA	3	ND	< 5	69±6
	100 μM-Aspirin	3	152±34	58±6	31±6
	100 μM-H-7	3	114±22	63±12	74±17
	Apyrase (10 units/ml)	3	190±41	72±4	71±8
	Aspirin + H-7	4	276±89	< 5	7±3
	Aspirin + apyrase	5	193±76	15±10	25±7
	Aspirin + apyrase + EGTA	3	ND	< 5	35±4
(*b*) Collagen (5 μg/ml)	None	7	25±5	91±3	67±5
	5 mM-EGTA	5	ND	< 5	43±6
	100 μM-Aspirin	8	33±10	74±10	26±6
	100 μM-H-7	4	23±6	80±11	63±3
	Apyrase (10 units/ml)	3	32±3	81±6	59±7
	Aspirin + H-7	6	26±6	71±9	26±4
	Aspirin + apyrase	3	52±11	29±12	21±3
	Aspirin + apyrase + EGTA	2	ND	< 5	14±6

internal Ca^{2+} concentration ($[Ca]_i$) was done as described by Grynkiewicz *et al.* (1985), using a K_d of 224 nM for the fura-2 and Ca^{2+} complex.

SNARF-1 fluorescence emission was similarly monitored at 574 nm with 488 nm excitation. At these wavelengths alkalinization of the cytosol results in a decreased fluorescence. Calibration with monensin, nigericin or NH_4Cl, as described by Siffert & Akkerman (1988), was attempted, as well as calibration by lysis with digitonin into various pH buffers. Although these treatments gave fluorescence shifts in the appropriate direction they did not equal the fluorescence shifts obtained with the various activators.

Platelet protein phosphorylation

Platelet isolation, labelling the metabolic pools with [^{32}P]orthophosphate (0.5–1 mCi/ml), and gel filtering to obtain ^{32}P-labelled platelets at 4×10^8/ml free of plasma proteins and non-incorporated label, were carried out as previously described (Cox *et al.*, 1984). Platelet samples (0.06 ml) were taken at indicated times, immediately denatured with 0.03 ml of 6% (w/v) SDS/6% (v/v) β-mercaptoethanol/15 mM-EGTA/30% (v/v) glycerol/0.03% Bromophenol Blue/150 mM-Tris/HCl, pH 6.8, electrophoresed and autoradiographed to reveal ^{32}P-labelled peptides as previously described (Cox *et al.*, 1984). Label associated with the 20 kDa MLC and a 47 kDa cytosolic phosphoprotein of unknown function (40P) was quantified by a Zeineh laser densitometer with adjustable slit width set to cover the entire width of the gel lane. The peak area associated with the MLC or 40P bands was quantified on an interfaced Shimadzu CR3-A recording integrator. All X-ray film from a labelling experiment was simultaneously processed after being submaximally exposed with linearity of exposure determined from different-length exposure times.

RESULTS

Aggregation and secretion of 5HT

The effects of various inhibitors on ALB-6-induced aggregation and secretion in PRP are summarized in Table 1. Measurements ($n = 3$) of the TxA_{2P} metabolite, TxB_2, gave values of 300 ± 28 ng/ml for platelets activated by ALB-6 versus 2 ± 1 ng/ml for unactivated platelets at 2×10^8 platelets/ml. Pretreatment with 100 μM-aspirin for 10 min at 37 °C gave greater than 99% inhibition of TxA_2 synthesis in ALB-6-activated platelets. Pretreatment with H-7 at 100 μM for 5 min was used to inhibit protein kinase C (Tohmatsu *et al.*, 1986). Pre-added apyrase was utilized at 10 units/ml to block the autocrine stimulation by secreted ADP. At this concentration, secretion and aggregation induced by adding 5 μM-ADP was completely blocked (result not shown). Neither H-7 nor apyrase had any effect on the extent of ALB-6-induced TxA_2 synthesis.

Individually these inhibitors principally increased the lag time without substantially affecting the extent of aggregation. Significant partial inhibition of 5HT secretion by aspirin, but not by H-7 or apyrase, was reproducibly observed. Whereas EGTA chelation of external Ca^{2+} could completely block the aggregation response to ALB-6, there was only a slight decrease in 5HT secretion. The combination of blocking the TxA_2 pathway with aspirin, and inhibiting protein kinase C directly with H-7, almost completely inhibited both aggregation and secretion. Apyrase in combination with aspirin substantially inhibited the aggregation response, although the inhibition of secretion was no greater than with aspirin alone. This residual 26 ± 9% 5HT secretion in the presence of aspirin and apyrase was also unaffected by the further addition of EGTA.

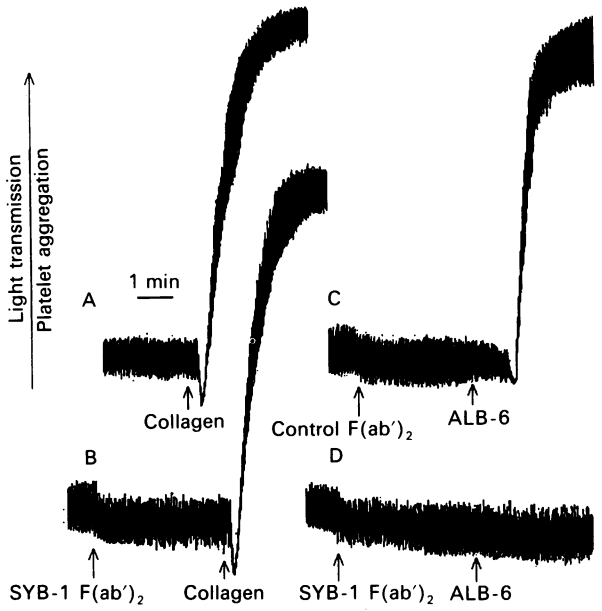

Fig. 1. Effect of CD9 MAb SYB-1 F(ab')$_2$ fragments on aggregation of platelets by ALB-6 or collagen

PRP was stirred in a Payton aggregometer at 37 °C with constant stirring at 1000 rev./min. Samples B and D were premixed for 3 or 4 min with SYB-1 F(ab')$_2$ fragments (45 μg/ml) added from a 100-fold-concentrated stock solution in PBS. Control samples were primarily pretreated with mouse IgG_1-F(ab')$_2$ fragments (trace C). Agonists were added at the arrow as indicated. Trace A, activated with collagen (1 μg/ml) alone; B, pretreated with SYB-1 F(ab')$_2$ fragments and activated with collagen (1 μg/ml); C, pretreated with control mouse IgG_1-F(ab')$_2$ fragments before addition of ALB-6 (15 μg/ml) and D, pretreated with SYB-1 F(ab')$_2$ before addition of ALB-6 (15 μg/ml). Similar results were obtained in three separate experiments with different donors. Aggregation is indicated by increased light transmission as indicated by the vertical arrow. All the aggregation traces are scaled to the same light-transmission settings for 0 and 100% aggregation as described in the Materials and methods section. The baselines of traces A and C are offset for presentation in this Figure.

Collagen-induced generation of TxA_2 has been shown to be inhibited by aspirin (Connellan *et al.*, 1986). Similarly to the ALB-6 results, collagen-induced platelet aggregation and secretion was only partially blocked by aspirin at higher (5 μg/ml) doses of collagen (Table 1). Similarly to the ALB-6 results the combination of apyrase (but not H-7) and aspirin was more effective at inhibiting collagen-induced aggregation. However neither apyrase nor H-7 had any effect alone on secretion, nor did they increase the inhibition of 5HT secretion by aspirin. The collagen-induced secretion was slightly inhibited by chelation of external Ca^{2+} with EGTA, and this inhibition, in contrast with the ALB-6 results, was seen in combination with aspirin and apyrase.

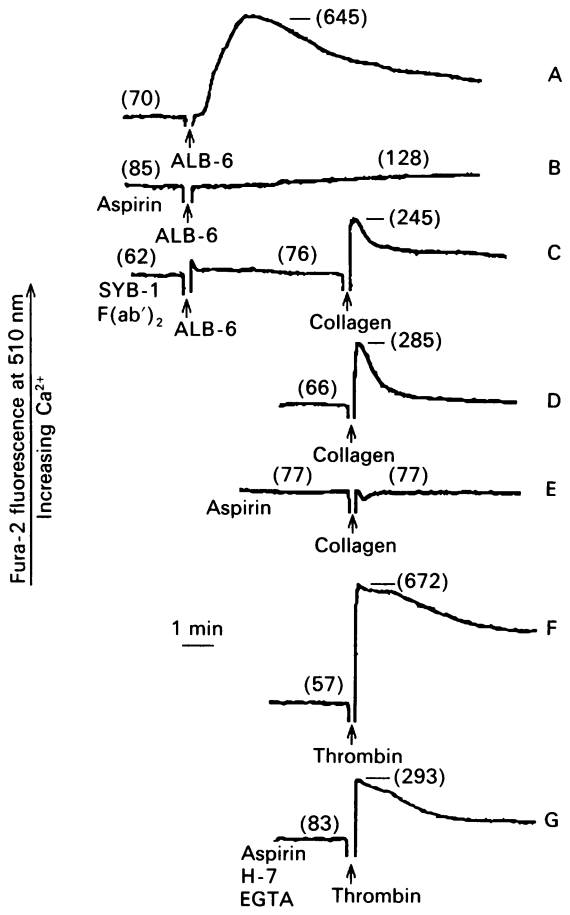

Fig. 2. Effect of aspirin pretreatment on Ca^{2+} flux induced by ALB-6, collagen or thrombin

Fura-2-loaded platelets were monitored with a Perkin–Elmer fluorimeter at 510 nm emission with 335 nm excitation in plastic cuvettes thermostatically controlled at 37 °C. Samples were not stirred, except briefly, after addition of ALB-6 (15 μg/ml) (traces A, B and C) or collagen (1 μg/ml) (trace C), collagen (5 μg/ml) (traces D and E) or thrombin (0.2 unit/ml) (traces F and G). Samples shown in traces B, E and G were pretreated for 10 min at 37 °C with 100 μM-aspirin. The sample in trace G was additionally pretreated with 100 μM-H-7 for 5 min and 5 mM-EGTA for 2 min before thrombin addition. Values in parentheses are the calculated $[Ca]_i$ values (in nM) for basal and peak stimulated levels respectively. Breaks in the traces represent periods during which the shutter was closed during addition and mixing of agonists. SYB-1 $F(ab')_2$ fragments were pre-added 4 min before ALB-6 (5 μg/ml) addition (trace C).

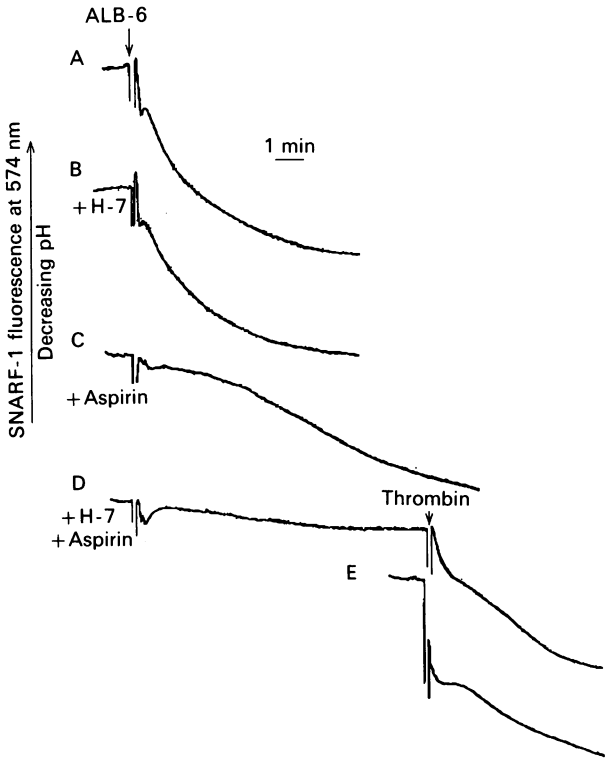

Fig. 3. Effect of H-7 and aspirin on ALB-6-induced cytoplasmic alkalinization as monitored by SNARF-1 fluorescence

Platelets were preloaded with SNARF-1 and gel-filtered in Tangen/Hepes/BSA buffer, pH 7.4., as described in the Materials and methods section. Platelets were equilibrated at 37 °C in plastic fluorimeter cuvettes at 2×10^8 cells/ml. Fluorescence was monitored at 574 nm in a Perkin–Elmer fluorimeter with excitation at 488 nm. Samples were not stirred, except briefly with a pipetter, after agonist addition. Breaks in the trace are during these additions and mixing. Samples B and D were pretreated for 5 min at 37 °C with 100 μM-H-7, whereas samples C and D were pretreated for 10 min with 100 μM-aspirin. ALB-6 (15 μg/ml) was added to all the samples except E, at the first break in the trace as indicated. Thrombin (0.2 unit/ml) was added to samples D and E at the indicated point. SNARF-1 fluorescence at these wavelengths increases as the pH decreases, as indicated by the arrow, and decreases upon cytoplasmic alkalinization.

Effect of CD9 MAb $F(ab')_2$ fragments on platelet aggregation

ALB-6 proved difficult to convert into $F(ab')_2$ fragments with pepsin digestion, so SYB-1 MAbs were utilized. This MAb interacts with a different epitope on the CD9 antigen (Benoit *et al.*, 1987), but also induces platelet activation with characteristics identical with those of ALB-6 (Rendu *et al.*, 1987). The effect of SYB-1 $F(ab')_2$ on platelet aggregation in PRP is shown in Fig. 1. The SYB-1 $F(ab')_2$ fragments did not induce a detectable platelet response at 45 μg/ml, but prevented activation by either the intact SYB-1 (results not shown) or ALB-6 at 15 μg/ml (trace D). Irrelevant mouse IgG_1 $F(ab')_2$ fragments had no effect on ALB-6 activation at this dose (trace C). This same dose of SYB-1 $F(ab')_2$ had no effect on collagen (1 μg/ml)-induced aggregation (traces A and B). These results were reproducible with three different donors as well as with gel-filtered platelets.

Cytosolic Ca^{2+} flux

We next utilized the fluorescent dye fura-2 to measure cytosolic Ca^{2+} in gel-filtered platelets under conditions of activation and inhibition similar to those used in the experiments of Table 1 and Fig. 1. As Fig. 2 (trace A) shows, fura-2-loaded platelets exposed to ALB-6 show a delayed, but substantial, flux as compared with those exposed to thrombin (Fig. 2, trace F). As expected from the aggregation and 5HT-secretion studies, chelation of

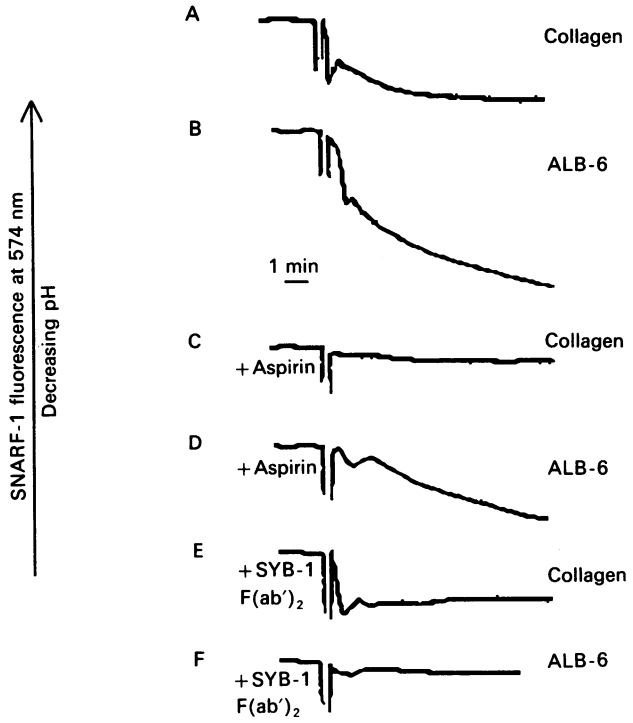

Fig. 4. Comparison of ALB-6- and collagen-induced cytoplasmic alkalinization as monitored by SNARF-1 fluorescence

Samples were preloaded with SNARF-1, gel-filtered, and SNARF-1 fluorescence was monitored as in Fig. 3 and the Materials and methods section. The pretreatments are indicated at the beginning of each trace, whereas activator additions are indicated to the right of each trace. Samples C and D were pretreated with aspirin, as in Fig. 3. Samples E and F were pretreated for 4 min with SYB-1 $F(ab')_2$ fragments (45 μg/ml). Cytoplasmic alkalinization results in diminished fluorescence and, as indicated by the arrow, decreases in pH augment fluorescence of SNARF-1 at these wavelengths. ALB-6 was added as indicated at 15 μg/ml, whereas collagen (1 μg/ml) was used in traces A and E. The aspirin-pretreated sample (trace C) was activated with collagen (5 μg/ml).

external Ca^{2+} or addition of H-7 or apyrase alone had little effect on Ca^{2+} mobilization induced by ALB-6 (results not shown). An unexpected finding was that aspirin pretreatment almost completely blocked ALB-6-induced Ca^{2+} flux detectably by fura-2 fluorescence in the absence of other inhibitors (Fig. 2, trace B). By contrast, even the combination of aspirin, H-7 and chelation of external Ca^{2+} decreased thrombin (0.2 unit/ml)-stimulated Ca^{2+} levels by only about 50% (trace G). These results suggest that TxA_2 generation is the major pathway for sustained elevation of cytosolic Ca^{2+} by ALB-6.

A different time course of Ca^{2+} flux was elicited by collagen (5 μg/ml), as shown in Fig. 2 (trace D) in relation to ALB-6 (15 μg/ml; trace A). As with ALB-6, the Ca^{2+} flux elicited by collagen was blocked by aspirin (trace E). A similar finding has been reported by Pollock *et al.* (1986) for collagen, where indomethacin was used to block TxA_2 synthesis. As with aggregation, the SYB-1 $F(ab')_2$ fragments were effective in blocking the reaction to ALB-6, but had little effect on the Ca^{2+} flux in response to collagen (1 μg/ml; trace C).

Role of cytoplasmic alkalinization in ALB-6 activation

Studies with platelets loaded with the fluorescent pH indicator SNARF-1 showed an alkalinization response composed of a rapid initial phase followed by a slower phase which gradually levelled off (Fig. 3, trace A). Occasionally a brief acidification could be observed just after the addition of an agonist, but since the traces were interrupted for these additions, this is not always evident. No major effect of H-7 alone was observed (trace B). Aspirin alone inhibited the initial rapid response to ALB-6, but the subsequent slow phase, although partially inhibited, was still apparent (Fig. 3, trace C). The combination of H-7 and aspirin almost completely inhibited both the initial and this later slow response (trace D). However, this inhibition could be overcome by thrombin (0.2 unit/ml; added at the end of trace D). The response to thrombin at 0.2 unit/ml in the absence of inhibitors is shown in trace E.

Although these results were reproducible in three separate experiments, we were unable to calibrate these pH shifts, since none of the reagents used to equilibrate the internal pH with the buffer gave fluorescence changes as large as those obtained with the intact activated platelets.

A comparison of the alkalinization response with collagen is shown in Fig. 4. Collagen (1 μg/ml) (trace A) elicited a rapid initial alkalinization response similar to that elicited by ALB-6. However, with either 5 μg/ml (result not shown) or 1 μg/ml, the response levelled off in contrast with the further slow alkalinization observed with ALB-6 (trace B). To ensure good collagen activation, samples were more thoroughly mixed, which prevented us observing the initial acidification response in these experiments. In contrast with ALB-6-activated aspirin-treated platelets (trace D), collagen-induced alkalinization (5 μg/ml) was almost completely blocked by aspirin alone (trace C). Pre-added SYB-1 $F(ab')_2$ fragments, which completely blocked the ALB-6 response (trace F), did not affect the collagen response (trace E). These results were reproducible with different donors in three separate experiments.

Protein phosphorylation

To evaluate stimulus–response coupling at the level of protein kinase activation, we examined phosphorylation of platelet MLC and a cytosolic 47 kDa protein of unknown function (40P). In agreement with the aggregation and secretion studies above, we found that the peak time and extent of phosphorylation of both proteins in ^{32}P-labelled gel-filtered platelets (shown in Table 2) in response to ALB-6 was only delayed or slightly diminished by aspirin. Despite reports that H-7 is an effective protein kinase C inhibitor (Hidaka *et al.*, 1984; Kawamoto & Hidaka, 1984), no significant inhibition of phosphorylation induced by ALB-6 was observed with H-7 or apyrase pretreatment alone. However, the combination of aspirin with either apyrase or H-7 markedly inhibited phosphorylation of both MLC and 40P.

The phosphorylation response to thrombin (0.2 unit/ml) was only slightly diminished by aspirin alone (Table 2). Combinations of H-7 or apyrase with aspirin gave a small, but insignificant, increase in the inhibition. A lack of significant inhibition of thrombin-induced 40P phosphorylation by H-7 pretreatment had been previously reported by Tohmatsu *et al.* (1986).

Table 2. Effects of inhibitors on ALB-6 and thrombin-induced phosphorylation of platelet 40P and MLC

^{32}P-labelled platelets were pretreated at 37 °C with 100 μM-H-7 for 5 min, 100 μM-aspirin for 10 min, either separately or in combination, as indicated. Apyrase was added to give a final concentration of 10 units/ml within 60 s of ALB-6 addition. Samples were placed in a Payton aggregometer thermostatically controlled at 37 °C with constant stirring at 1000 rev./min. Zero-time samples (0.06 ml) were removed just before the addition of the activator and at 30 s (for ALB-6) for 15 s (for thrombin) time intervals after addition. These samples were immediately denatured by mixing with 0.03 ml of SDS denaturing buffer as described in the Materials and methods section. Time-course samples were subsequently electrophoresed on SDS/polyacrylamide slab gels, and phosphorylation was quantified by laser densitometer of autoradiograms obtained from these gels. Maximum MLC and 40P phosphorylation levels in arbitrary area integration units were determined on a Shimadzu CR3-A recording integrator interfaced to the Zeineh laser densitometer. Repeat scans or duplicate autoradiograms give values within $\pm$10%. The peak phosphorylation times were determined as the time interval between agonist addition and the initial sample time in which 40P or MLC phosphorylation was within 10% of the maximum phosphorylation level for that time-course series. The peak phosphorylation responses of 40P or MLC were corrected for the zero-time basal level (less than 10% for 40P, and between 20 and 30% for MLC in maximally stimulated platelets). Percentage inhibition was derived by dividing the peak response in the presence of the indicated inhibitors by the peak response of the same preparation of platelets identically activated in the absence of inhibitors. Results shown are for three experiments with different donor platelets.

Activator	Pretreatment	*n*	Inhibition of 40P phosphorylation (%)	Peak time (s)	Inhibition of MLC phosphorylation (%)	Peak time (s)
ALB-6	None	8	–	62±19	–	53±20
(15 μg/ml)	Apyrase (10 units/ml)	4	0±8	83±29	0±2	56±16
	100 μM-H-7	3	7±10	85±9	14±1	55±9
	100 μM-Aspirin	3	16±2	157±33	10±9	117±35
	Aspirin + apyrase	4	57±19	203±38	59±16	195±30
	Aspirin + H-7	3	62±9	340±139	59±13	220±92
Thrombin	None	3	–	30±16	–	30±12
(0.2 unit/ml)	Aspirin	3	5±8	30±7	10±7	30±16
	Aspirin + H-7	3	16±9	30±7	10±7	30±6
	Aspirin + apyrase	3	23±12	30±13	17±5	30±15

DISCUSSION

These studies with ALB-6 further confirm that the platelet stimulus–response coupling triggered through MAb interaction with the CD9 antigen involves both an aspirin-sensitive TxA_2 pathway for cytosolic Ca^{2+} elevation as well as a potent alternative pathway. It would appear that this alternate pathway requires cytosolic alkalinization, is dependent on secreted ADP autocrine stimulation to obtain full platelet aggregation and MLC phosphorylation, and is sensitive to the protein kinase C inhibitor H-7.

These data would support the suggestion of Rendu *et al.* (1987) that this aspirin-insensitive pathway is coupled to phospholipase C metabolism. However, our results additionally suggest that this coupling leads to diacylglycerol formation and, ultimately, protein kinase C activation in the absence of substantial $[Ca]_i$ elevation. MLC phosphorylation in the absence of a major increase over basal levels of cytosolic Ca^{2+} has been reported for low-dose-thrombin- and platelet-activating-factor-stimulated platelets (Hallam *et al.*, 1985). These studies demonstrated MLC phosphorylation and dense-granule secretion in the absence of substantial increases in cytosolic Ca^{2+}, as monitored by quin-2 fluorescence. However, the possibility of localized Ca^{2+} flux or sensitization to a minor increase in cytosolic Ca^{2+} is not excluded in either this or previous studies. It is apparent from these studies that influx of Ca^{2+} from the external media plays only a minor role in these activation pathways. We propose that secreted ADP would serve to initiate MLC phosphorylation independent of TxA_2 synthesis (Daniel *et al.*, 1984), as well as to cause direct expression of fibrinogen receptors supporting platelet aggregation (Hato *et al.*, 1988). Thus, in the presence of both aspirin and apyrase, these activation pathways are blocked.

H-7 has been shown to be a somewhat selective protein kinase C inhibitor *in vitro*, with a K_i of 6 μM (Hidaka *et al.*, 1984). It is a competitive inhibitor reversible by ATP and has a similar K_i for cyclic AMP and cyclic GMP-dependent protein kinases, but a K_i of 97 μM for MLC kinase. At 50 μM, H-7 was an effective inhibitor *in situ* of phorbol 12-myristate 13-acetate (PMA)-stimulated 40P phosphorylation in rabbit platelets (Kawamoto & Hidaka, 1984). It was selective, since MLC phosphorylation by the ionophore A23187 was not appreciably inhibited at that concentration of H-7. In human platelets, 50 μM-H-7 only delayed 40P phosphorylation induced by PMA (Inagaki *et al.*, 1984). In another study with human platelets, Tohmatsu *et al.* (1986) utilized 50 μM-H-7 to block the inhibiting effects of PMA on subsequent thrombin (1 unit/ml) stimulation of Ca^{2+} flux and polyphosphoinositide metabolism. In agreement with these studies we did not observe significant inhibition by H-7 alone, or in combination with aspirin, of thrombin-stimulated 40P phosphorylation. We also did not observe significant inhibition of ALB-6-induced 40P phosphorylation by H-7 alone. However, platelet activation by ALB-6, including 40P phosphorylation, was significantly inhibited by the combination of H-7 with aspirin. This inhibition is possibly more effective with

aspirin because of the lack of extra augmentation of phospholipase C activity by TxA_2 and, presumably, production of diacylglycerol to stimulate protein kinase C (Rittenhouse, 1984). The combination of H-7 and aspirin was also effective at preventing cytoplasmic alkalinization. This may reflect inhibition of protein kinase C, which has been suggested by Siffert & Akkerman (1988) to stimulate alkalinization through Na^+/H^+ exchange.

We would suggest that alkalinization might be part of the coupling mechanism of platelet activation by CD9 MAbs that initiates the phospholipase C breakdown of phospholipids to produce sufficient diacylglycerol to activate protein kinase C. A similar transduction model has been suggested for vascular-smooth-muscle cells (Griendling *et al.*, 1988). This activation of protein kinase C would occur even in the absence of substantial Ca^{2+} mobilization or additional phospholipase C stimulation mediated by TxA_2. Alternatively, or as a feedback mechanism, protein kinase C activation may induce or augment cytosolic alkalinization, as suggested by Siffert & Akkerman (1988).

From these studies, we would propose some refinements to the model for CD9 MAb-initiated platelet activation. In this new scheme, the CD9 MAb signal transduction is coupled to both phospholipase A_2 and phospholipase C, as suggested by Rendu *et al.* (1987). The TxA_2 resulting from phospholipase A_2 activation would be a potent transducer of both Ca^{2+} mobilization from the dense tubular system and phospholipase C activation to form diacylglycerol. The resulting activation of myosin kinase and protein kinase C would trigger granule secretion and ADP-independent fibrinogen-receptor expression (Shattil & Brass, 1987). In the absence of TxA_2, as in aspirin-treated platelets, the activation becomes completely dependent on both cytoplasmic alkalinization and autocrine stimulation by secreted ADP to activate the phospholipase C pathway as well as trigger fibrinogen-receptor expression and MLC phosphorylation. The exact role that cytoplasmic alkalinization plays remains to be elucidated. One possibility is that it may augment the secretion of dense granules (Pollard *et al.*, 1977). Alternatively, as suggested by the studies on vascular smooth muscle, it may be more directly linked to phospholipase C activation (Griendling *et al.*, 1988). With these additional links added to the model for CD9 MAb-initiated platelet activation, we hope to narrow the possible mechanisms for the nature of the stimulus triggered by these MAbs against the CD9 molecule.

In some respects platelet activation by CD9 antibodies is similar to collagen-induced activation. Both agonists have a characteristic lag time before activation is apparent and have an aspirin-insensitive pathway of activation. However, in the present study it would appear that collagen does not activate through an identical mechanism. First, $F(ab')_2$ fragments of a CD9 MAb (SYB-1), which completely blocked ALB-6-induced platelet aggregation as well as cytosolic alkalinization and Ca^{2+} flux, had little effect on collagen activation in the same platelet preparations. Another key difference is that the collagen-induced alkalinization response seems to be predominantly the aspirin-sensitive rapid component, as opposed to ALB-6 induction of a rapid aspirin-sensitive and slower aspirin-insensitive alkalinization.

The apparent requirement for the Fc portion of a CD9 MAb in order to trigger activation is noteworthy. Previous studies with CD9 MAbs, as well as activating antibodies to GPIIb/IIIa (Lecompte *et al.*, 1989; Modderman *et al.*, 1988), have also suggested a requirement for the Fc region. Platelets do possess Fc receptors which may be involved in platelet activating by aggregated IgG (Rosenfeld *et al.*, 1985). The present studies suggest a major role for the Fc receptor in platelet activation by CD9 MAbs.

This work was funded in part by a Tennessee Affiliate American Heart Association grant-in-aid to R.C.C., National Institutes of Health grants HL-34445 awarded to R.C.C. and CA-42902 to R.E.W., an INSERM (France) grant to C.B. and an NCI–INSERM Cancer Research Programme grant to R.E.W. and C.B.

Note added in proof (received 18 January 1990)

The role of the Fc receptor in CD9 MAb platelet activation was further elucidated by utilizing a MAb (IV-3, donated by Dr. C. Anderson, Columbus, OH, U.S.A.) to the platelet Fc receptor ($F_{c\gamma}RII$), which blocks platelet activation by aggregated IgG. This $F_{c\gamma}RII$ MAb completely blocks activation by either ALB-6 or SYB-1. Once this $F_{c\gamma}RII$ MAb binds to the platelet, secondary antibody cross-linking causes platelet activation in the absence of any other stimulus. Comparison of the properties of this activation pathway and its sensitivity to various inhibitors indicates that it is identical to platelet activation elicited by the ALB-6 or SYB-1 MAbs. These studies are in the process of being published. (Worthington *et al.*, 1990).

REFERENCES

Banga, H. S., Simons, E. R., Brass, L. F. & Rittenhouse, S. E. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9197–9201

Benoit, P., Perrot, J. Y., Krief, P., Billard, M., Boucheix, C. & Jasmin, C. (1987) Biochem. (Life Sci. Adv.) **6**, 111–116

Boucheix, C., Soria, C., Mirshahi, M., Soria, J., Perrot, J. Y., Fournier, N., Billard, M. & Rosenfeld, C. (1983) FEBS Lett. **161**, 289–295

Brown, J. E., White, J. G., Hockett, R. D., Hagert, K. R. & Kersey, J. H. (1984) in Leucocyte Typing (Bernard, A., Boumsell, L., Dausset, J. & Milstein, C., eds.), pp. 542–550, Springer-Verlag, Berlin

Chiang, T. M., Jin, A. & Kang, A. H. (1987) H. Immunol. **139**, 887–892

Connellan, J. M., Thurlow, P. J., Barlow, B., Lowe, M. & McKenzie, I. F. C. (1986) Thromb. Haemostasis **55**, 153–157

Cox, A. C. & Carroll, R. C. (1986) Biochim. Biophys. Acta **886**, 390–398

Cox, A. C., Carroll, R. C., White, J. G. & Rao, G. H. R. (1984) J. Cell Biol. **98**, 8–15

Daniel, J. L., Molish, I. R., Rigmaiden, M. & Stewart, G. (1984) J. Biol. Chem. **259**, 9826–9831

Funder, J., Hersco, L., Rothstein, A. & Livne, A. (1988) Biochim. Biophys. Acta **938**, 425–433

Gorman, D. J., Castaldi, P. A., Zola, H. & Berndt, M. C. (1985) Nouv. Rev. Fr. Hematol. **27**, 255–259

Griendling, K. K., Berk, B. C. & Alexander, R. W. (1988) J. Biol. Chem. **263**, 10620–10624

Grynkiewicz, G., Poeni, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450

Hallam, T. J., Daniel, J. L., Kendrick-Jones, J. & Rink, T. J. (1985) Biochem. J. **232**, 373–377

Hato, T., Ikeda, K., Yasukawa, M., Watanabe, A. & Kobayashi, Y. (1988) Blood **72**, 224–229
Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) Biochemistry **23**, 5036–5041
Higashihara, M., Maeda, H., Yatomi, Y., Takahata, K., Oka, H. & Kume, S. (1985) Biochem. Biophys. Res. Commun. **133**, 306–313
Inagaki, M., Kawamoto, S. & Hidaka, H. (1984) J. Biol. Chem. **259**, 14321–14323
Kawamoto, S. & Hidaka, H. (1984) Biochem. Biophys. Res. Commun. **125**, 258–264
Kishimoto, A., Takai, Y., Moro, T., Kikkawa, U. & Nishizuka, Y. (1980) J. Biol. Chem. **255**, 2273–2276
Lecompte, T., Morel, M. C., Champeix, P., Favier, R., Potevin, F., Samana, M., Salmon, C. & Kaplan, C. (1989) Br. J. Haematol. **71**, 57–63
Modderman, P. W., Huisman, H. G., Van Mourik, J. A. & Von dem Borne, A. E. G. K. (1988) Thromb. Haemostasis **60**, 68–74
Newman, R. A., Sutherland, D. R., Lebien, T. W., Kersey, J. M. & Greaves, M. F. (1982) Biochim. Biophys. Acta **701**, 318–327
O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) J. Biol. Chem. **260**, 956–962
Pollard, H. B., Tack-Goldman, K., Pazoles, C. J., Creutz, C. E. & Shulman, N. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5295–5299
Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–887
Rendu, F., Boucheix, C., Lebret, M., Bourdeau, N., Benoit, P., Maclouf, J., Soria, C. & Levy-Toledano, S. (1987) Biochem. Biophys. Res. Commun. **146**, 1397–1404
Rittenhouse, S. E. (1984) Biochem. J. **222**, 103–110
Rosenfeld, S. I., Looney, R. J., Leddy, J. P., Phipps, D. C., Abraham, G. N. & Anderson, C. L. (1985) J. Clin. Invest. **76**, 2317–2322
Rybicki, J. P., Venton, D. L. & Le Breton, G. C. (1983) Biochim. Biophys. Acta **751**, 66–73
Sano, K., Takai, Y., Yamanishi, J. & Nishizuka, Y. (1983) J. Biol. Chem. **258**, 2010–2013
Santoro, S. A., Rajpapa, S. M., Staatz, W. D. & Woods, V. L., Jr. (1988) Biochem. Biophys. Res. Commun. **153**, 217–223
Shattil, S. J. & Brass, L. F. (1987) J. Biol. Chem. **262**, 992–1000
Siess, W. & Lapetina, E. G. (1988) Biochem. J. **255**, 309–318
Siffert, W. S. & Akkerman, J. W. N. (1988) J. Biol. Chem. **263**, 4223–4227
Tandon, N. N., Kralisz, U. & Jamieson, G. A. (1989) J. Biol. Chem. **264**, 7576–7583
Tangen, O. & Berman, H. J. (1972) in Platelet Function and Thrombosis (Mannucci, P. M. & Gorini, S., eds.), pp. 235–248, Plenum Press, New York
Tohmatsu, T., Hattori, H., Nagao, S., Ohki, K. & Nozawa, Y. (1986) Biochem. Biophys. Res. Commun. **134**, 868–875
Worthington, R. E., Carroll, R. C. & Boucheix, C. (1990) Br. J. Haematol., in the press
Zavoico, G. B. & Cragoe, E. J. (1988) J. Biol. Chem. **263**, 9635–9639

Received 14 March 1989/8 September 1989; accepted 2 October 1989