Protein kinase C activation is not a key step in ADP-mediated exposure of fibrinogen receptors on human platelets

Fabio M. Pulcinelli[a], Barrie Ashby[b], Pier Paolo Gazzaniga[a,*], James L. Daniel[b]

[a]*Department of Experimental Medicine and Pathology, University La Sapienza, Viale Regina Elena 324, 00161 Rome, Italy*
[b]*Department of Pharmacology, School of Medicine, Temple University, Philadelphia, PA, USA*

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Abstract A selective inhibitor of protein kinase C (PKC), Ro 31-8220, blocks pleckstrin (P47) phosphorylation in platelets activated with either ADP, ADP plus synthetic thromboxane agonist U46619 and ADP plus U46619 plus epinephrine, while inducing a weak inhibition of platelet aggregation, and no significant effect on the fibrinogen binding. In platelets activated by U46619 alone, P47 phosphorylation, platelet aggregation, fibrinogen binding and serotonin release are all inhibited by Ro 31-8220. In the presence of an ADP scavenger system, U46619 induces pleckstrin phosphorylation, serotonin release and calcium mobilization but not platelet aggregation and fibrinogen binding, unless epinephrine is added. In conclusion: (1) PKC activation is required for ADP secretion; (2) ADP or epinephrine are essential for fibrinogen receptor exposure induced by U46619; (3) fibrinogen receptor exposure induced by ADP is independent of activation of PKC.

Key words: Platelet aggregation; Protein kinase C; ADP; Thromboxane A2

1. Introduction

Platelet aggregation involves exposure of fibrinogen receptors on the platelet surface induced by agonists such as ADP or thrombin acting through distinct receptors. Little is known about the signalling pathway that results in exposure of the fibrinogen receptor GpIIb-IIIa [1–3]. It is clear that protein kinase C (PKC) is important in secretion of ADP and PKC has been proposed to be directly involved in the process of fibrinogen receptor exposure as well [4]. However, in a previous study we demonstrated that in suspensions of thrombin-degranulated platelets the exposure of GpIIb-IIIa is dissociated from phospholipase C activation, and because PLC activation produces diacylglycerol, a PKC activator, we suggested that the activation of PKC is not important in inducing fibrinogen binding [3]. Other studies using complement-permeabilized platelets demonstrated a role for G proteins and protein tyrosine phosphorylation, which may be involved in fibrinogen receptor exposure by an undetermined mechanism [5]. Moreover, other authors demonstrated that the inhibition of the small GTP-binding protein rho-A with Botulinum C3 partially inhibits thrombin induced aggregation [6].

Ro 31-8220 is a more specific inhibitor of PKC activation than other agents such as staurosporine [7,8].

*Corresponding author. Dipartimento di Medicina Sperimentale, Università degli Studi di Roma 'La Sapienza', Viale Regina Elena 324, 00161 Roma, Italia. Fax: (39) (6) 445 4820.

The present study examines the separate effects of Ro 31-8220 on agonist-induced calcium response, ADP secretion and subsequent exposure of fibrinogen receptors and aggregation. The results support the idea that while protein kinase C activation is important for ADP secretion it is not important for exposure of fibrinogen receptors and aggregation.

2. Materials and methods

Blood samples, using acid/citrate/dextrose (ACD) [9] as anticoagulant (1:7 v/v), were obtained from healthy volunteers who denied having taken any drugs in the two weeks before blood sampling.

Platelet Rich Plasma (PRP) was obtained after centrifugation ($180 \times g$ for 15 min), then was centrifuged ($800 \times g$ for 20 min) to concentrate the platelets (6×10^8 cells/ml). The concentrated platelets were incubated with [^{32}P]orthophosphoric acid (ICN, Costa Mesa, CA, USA) (0.25 mCi/ml of the platelet suspension) or with [^{14}C]5-hydroxytryptamine ([^{14}C]5HT, 1 μM) (ICN) for 1 h at 37°C, or with 3 μM Fura-2-AM (Moleculare Probes) for 30 min at 37°C and 15 min at room temperature. Excess [^{32}P]phosphate, [^{14}C]5HT and Fura-2-AM were separated from the platelets by gel-filtration (gel-filtered-platelets, GFP) on Sepharose 2B-CL (Pharmacia, Uppsala, Sweden) using Ca^{2+}-free Tyrode's buffer containing 0.2% albumin (Bovine serum albumin fraction V – BSA), 0.1% glucose and 10 mM HEPES (pH 7.35) (Sigma, Chemicals Co., St. Louis, MO, USA).

In some experiments platelets were aspirinated by treatment of the concentrated PRP with aspirin (1 mM at 37°C for 15 min).

In all experiments to inhibit PKC activity the platelets were incubated for 3 min with 10 μM Ro 31-8220 (generous gift of Dr. G. Lawton, Roche Research Centre, Welwyn Garden City, Herts, UK); to scavenger ADP released from the platelets creatine phosphate (20–40 mM) and creatine kinase (50–100 U/ml) (CP/CPK) were used; both inhibitors were used before the addition of agonists.

2.1. Platelet aggregation

In vitro platelet aggregation was evaluated according to Born [10], in a four sample Menarini (Florence, Italy) 3210 Aggrecorder, using siliconized glass cuvets, at 37°C and under continuous stirring at 1000 rpm. ADP (10 μM), Epinephrine (10 μM) (both from Menarini) and U46619 (1–10 μM) (Sigma), either alone or in combination, were used as aggregation inducers. Platelets were resuspended at the concentration of 2.5×10^8/ml and fibrinogen (1 mg/ml) was added before the agonists.

2.2. Fibrinogen binding

Immediately after agonist activation GFP (2.5×10^8/ml) were incubated with 0.18 mM [^{125}I]fibrinogen (ICN). To measure nonspecific ^{125}I binding, parallel samples were incubated with 15 mM of unlabelled human fibrinogen in the presence of [^{125}I]fibrinogen at the same concentration as above.

To remove the unbound [^{125}I]fibrinogen the platelet suspension was layered over a mixture of silicon oils (Dow-Corning 550:200; 5:1) in binding tubes and centrifuged in a microfuge for 1 min at $12{,}000 \times g$. The tips of the tube were cut off and the radioactivities of the pellet and supernatant were counted using a γ counter (Cobra, Camberra Packard, Meriden, USA) [3].

The data are expressed as the mean ± S.E.M. of the difference in cpm

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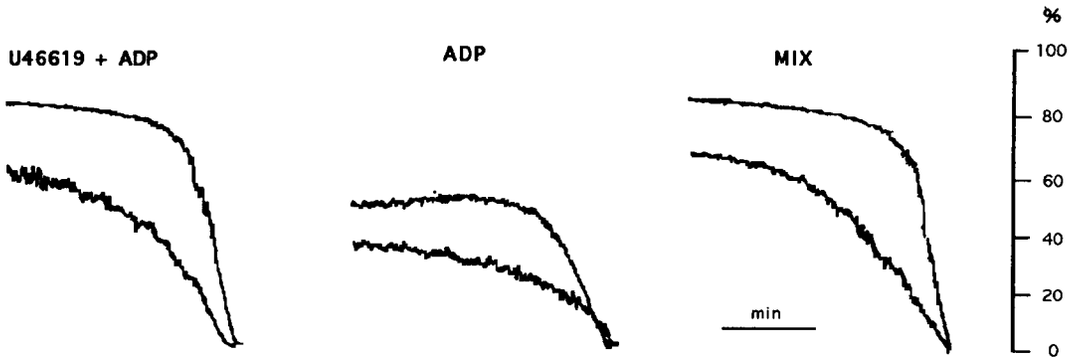

Fig. 1. Platelet aggregation patterns in response to ADP alone (20 μM), ADP plus U46619 (10 μM and 1 μM, respectively) and MIX (ADP 10 μM, epinephrine 10 μM and U46619 1 μM), in control GFP (upper curves) and after Ro 31-8220 treatment (lower curves). The figure is representative of four experiments.

between the platelet suspension incubated with [^{125}I]fibrinogen alone and that incubated with [^{125}I]fibrinogen plus unlabelled fibrinogen.

2.3. PKC activation

PKC activation was evaluated by analyzing the phosphorylation of pleckstrin (P47), the major PKC substrate in platelets, using 10 or 12.5% acrylamide in SDS-PAGE [11] of ^{32}P-labelled platelets sampled 10 min after activation.

2.4. Platelet secretion

Platelet secretion was evaluated by measuring the release of [^{14}C]5HT and expressed as the percentage of the total [^{14}C]5HT content.

The activation of labelled [^{14}C]5HT platelets was stopped after two min with formaldehyde/EDTA according to the method of Costa and Murphy [12]; after centrifugation (5000 × *g* for 1 min) the radioactivity of the supernatant was measured using a LKB (Pharmacia, Uppsala, Sweden) liquid scintillation counter.

2.5. Changes in intracellular calcium concentrations

The changes in intracellular calcium concentrations were monitored by using the fluorescence dye Fura-2. The agonists were added to Fura-2-loaded platelets into cuvettes thermostatically regulated at 37°C and stirred continuously. Then the fluorescence changes were monitored with a Kontron SFM 25 fluorimeter, set at 340 nM excitation and 510 nM emission. In order to convert fluorescence measurements into Ca^{2+} concentrations, F_{min} was determined after the addition of digitonin (50 μM) (Sigma) in the presence of EGTA (2 mM) and Tris base (20 mM); F_{max} was measured by the addition of excess $CaCl_2$ (10 mM). Differences in free calcium concentrations between the unstimulated and stimulated platelets were calculated using the measured values and a K_d of 224 nM after correction for extracellular dye [13], and expressed as ΔnM.

3. Results and discussion

The selective protein kinase C inhibitor Ro 31-8220 (10 μM) completely inhibited phosphorylation of pleckstrin (P47), the major PKC substrate in platelets, induced by ADP (20 μM) added alone or in combination with the synthetic thromboxane receptor agonist U46619 (1 μM). Ro 31-8220 (10 μM) also inhibited P47 phosphorylation induced by a mixture of ADP, epinephrine and U46619 (10, 10 and 1 μM, respectively) (MIX) (data not shown).

The data can be compared with aggregation studies shown in Fig. 1. A weak inhibition of platelet aggregation was present after adding Ro 31-8220 when the platelets were stimulated with ADP (20 μM) (42.7 ± 10.5% versus 55.8 ± 11.7%), or ADP plus U46619 (58.9 ± 8.9 versus 72.7 ± 11.3) or with U46619 plus epinephrine (66.0 ± 10.1 versus 76.5 ± 7.8). Using aspirinated platelets aggregation was similar either in control platelets or in Ro 31-8220 treated platelets (data not shown), with all the agonists used. Moreover Ro 31-8220 did not induce any statistically significant inhibition of the fibrinogen binding after three min of platelet activation with MIX, U46619 plus ADP and ADP alone (Fig. 2).

These results are in agreement with those of Walker and Watson [7] who also showed that Ro 31-8220 completely inhibited thrombin-induced (1 U/ml) phosphorylation of pleckstrin while aggregation was only slowed slightly.

Ro 31-8220 did not change the intraplatelet calcium response to all the agonists used, as shown in Fig. 3. Conversely, Ro 31-8220 inhibited the serotonin release induced by all the agonists used (data not shown).

In contrast, Ro 31-8220 inhibited platelet P47 phosphorylation (data not shown) as well as platelet aggregation (Fig. 4) and fibrinogen binding (Fig. 5) induced by the thromboxane analog, U46619. This result may be explained if one assumes that secreted ADP is essential for thromboxane-induced exposure of fibrinogen receptors. Since Ro 31-8220 inhibits the release of

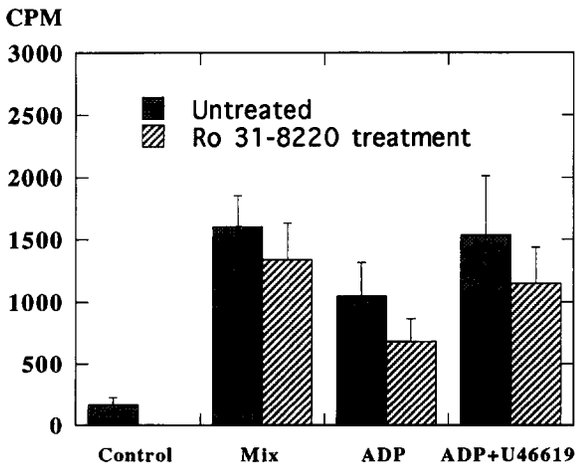

Fig. 2. Specific [^{125}I]fibrinogen binding in control GFP and in platelets treated with Ro 31-8220 after activation with agonists. The results are expressed as mean ± S.E.M. of the cpm measured in the platelet pellet from six different experiments. The agonist concentrations used were the same as in Fig. 1.

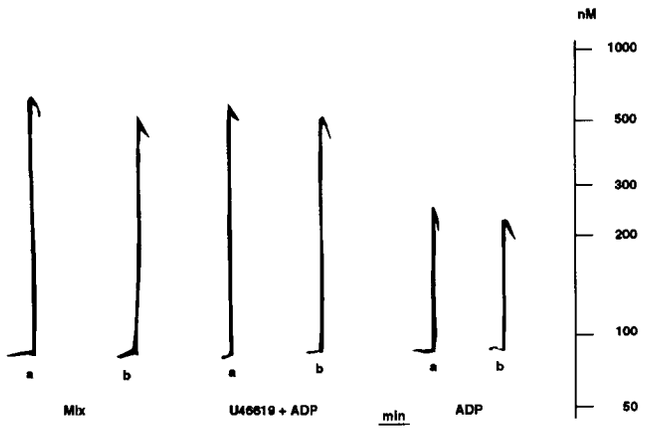

Fig. 3. A representative pattern of four experiments performed of the changes in intracellular calcium concentration in Ro 31-8220 treated (b) and control (a) Fura-2 loaded GFP in response to different agonists. The agonist concentrations used were the same as in Fig. 1.

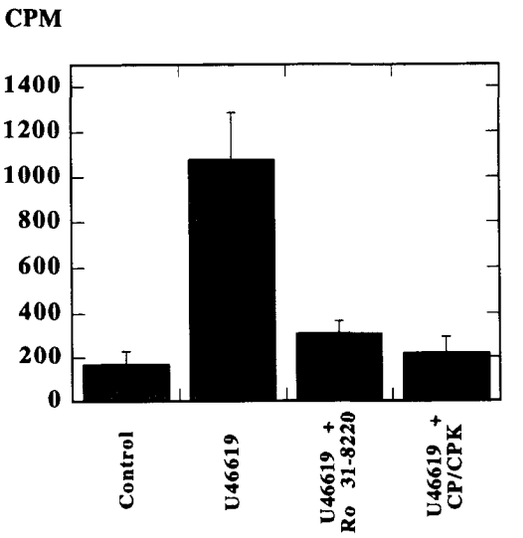

Fig. 5. Specific [^{125}I]fibrinogen binding in control GFP, in U46619-stimulated platelets, and in U46619 stimulated platelets after treatment with Ro 31-8220 or with the ADP scavenger system CP/CPK. The results are expressed as mean ± S.E.M. of the cpm measured in the platelet pellet from six different experiments.

endogenous ADP, as demonstrated by the reduction of [^{14}C]5HT release (8.3 ± 1.1% versus 78.1 ± 11.4%), it would inhibit if neither ADP nor epinephrine were added. This hypothesis is supported by experiments demonstrating that the addition of the ADP scavenger system creatine phosphate and creatine kinase (CP/CPK), inhibits thromboxane-induced platelet aggregation in normal platelets [14]; moreover, we previously found that in thrombin-degranulated platelets, U46619 does not induce platelet aggregation unless ADP or epinephrine was present [15]. Furthermore, we have determined whether PKC was activated in gel-filtered platelets stimulated with U46619 in the presence of CP/CPK (20–40 mM and 50–100 U/ml). The presence of CP/CPK did not inhibit U46619 (1 mM) induced P47 phosphorylation (data not shown), serotonin release (72.5 ± 15.7% versus 78.1 ± 11.4) or intracellular calcium mobilization (Fig. 4) while completely inhibiting aggregation (Fig. 5) and fibrinogen binding (Fig. 6). Similar results were obtained if the concentration of U46619 was increased to 10 mM. When epinephrine 20 μM was added with U46619 in CP/CPK treated platelets the aggregation was restored (43 ± 11 maximum percent). Thus epinephrine can substitute for ADP in producing fibrinogen receptor activation.

In support of these results, Savi et al. [16] in studies of platelet activation by thrombin receptor activating peptide (TRAP) found that TRAP-induced platelet aggregation was abolished when CP/CPK (10 mM and 80 U/ml respectively) were added to the suspension. The only difference between the behaviour of U46619 and TRAP was if higher concentration of TRAP was added the inhibitory effect of ADP removal was reduced.

In conclusion, our results support the idea that while activation of protein kinase C in platelets is important for secretion, it is not required for platelet aggregation. Exposure of the fibrinogen receptor seems to be independent of an increase in intracellular Ca^{2+} since U46619 which induces an increase in

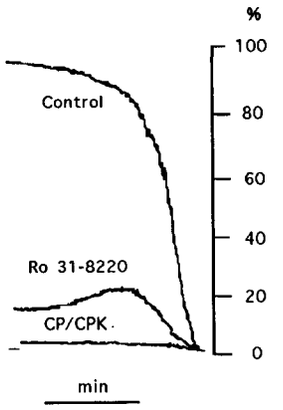

Fig. 4. Platelet aggregation patterns in response to U46619 (1 μM) in control platelets, in Ro 31-8220 treated platelets and in ADP scavenger system CP/CPK-treated platelets. The figure is representative of five experiments.

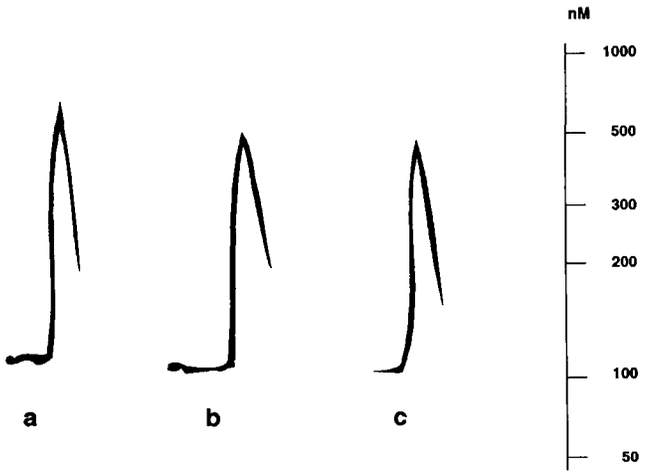

Fig. 6. Changes in intracellular calcium concentration in control (a), Ro 31-8220 treated (b) and CP/CPK treated (c) Fura-2 loaded GFP in response to U46619 (1 μM). The figure is representative of five experiments.

intracellular Ca^{2+} independently of ADP or epinephrine does not induce a concomitant platelet aggregation.

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