Protein kinase C- and calcium-regulated pathways independently synergize with G_i pathways in agonist-induced fibrinogen receptor activation

Todd M. QUINTON*†, Soochong KIM*, Carol DANGELMAIER‡, Robert T. DORSAM‡, Jianguo JIN*, James L. DANIEL†‡ and Satya P. KUNAPULI*†‡[1]

*Department of Physiology, Temple University Medical School, 3420 N. Broad Street, Philadelphia, PA 19140, U.S.A., †Sol Sherry Thrombosis Research Center, Temple University Medical School, Philadelphia, PA 19140, U.S.A., and ‡Department of Pharmacology, Temple University Medical School, Philadelphia, PA 19140, U.S.A.

Platelet fibrinogen receptor activation is a critical step in platelet plug formation. The fibrinogen receptor (integrin αIIbβ3) is activated by agonist-mediated G_q stimulation and resultant phospholipase C activation. We investigated the role of downstream signalling events from phospholipase C, namely the activation of protein kinase C (PKC) and rise in intracellular calcium, in agonist-induced fibrinogen receptor activation using Ro 31-8220 (a PKC inhibitor) or dimethyl BAPTA [5,5′-dimethyl-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid], a high-affinity calcium chelator. All the experiments were performed with human platelets treated with aspirin, to avoid positive feedback from thromboxane A2. In the presence of Ro 31-8220, platelet aggregation caused by U46619 was completely inhibited while no effect or partial inhibition was seen with ADP and the thrombin-receptor-activating peptide SFLLRN, respectively. In the presence of intracellular dimethyl BAPTA, ADP- and U46619-induced aggregation and anti-αIIbβ3 antibody PAC-1 binding were completely abolished. However, similar to the effects of Ro 31-8220, dimethyl BAPTA only partially inhibited SFLLRN-induced aggregation, and was accompanied by diminished dense-granule secretion. When either PKC activation or intracellular calcium release was abrogated, aggregation and fibrinogen receptor activation with U46619 or SFLLRN was partially restored by additional selective activation of the G_i signalling pathway. In contrast, when both PKC activity and intracellular calcium increase were simultaneously inhibited, the complete inhibition of aggregation that occurred in response to either U46619 or SFLLRN could not be restored with concomitant G_i signalling. We conclude that, while the PKC- and calcium-regulated signalling pathways are capable of inducing activating fibrinogen receptor independently and that each can synergize with G_i signalling to cause irreversible fibrinogen receptor activation, both pathways act synergistically to effect irreversible fibrinogen receptor activation.

Key words: aggregation, platelet, protease-activated receptor (PAR), thrombin receptor, thromboxane.

INTRODUCTION

Platelet activation plays an important role in haemostasis and thrombosis [1]. When platelets are stimulated with agonists such as ADP, thromboxane A_2, thrombin or collagen, they aggregate, release their granule contents and generate thromboxane A_2. Platelet aggregation is achieved by fibrinogen-dependent intercellular adhesion through activation of fibrinogen receptors on the platelet surface. Activation of phospholipase C (PLC) β2 or γ2 is essential for agonist-induced physiological responses in platelets. Upon activation of PLC, PtdIns(4,5)P_2 in the plasma membrane's inner leaflet is hydrolysed to diacylglycerol and Ins(1,4,5)P_3. While diacylglycerol serves as a stimulatory cofactor for the activation of protein kinase C (PKC), InsP_3 allows the release of calcium from the platelet dense tubular system, resulting in a rapid rise in intracellular calcium. Various PKC- and calcium-dependent events ensue, two of which are the secretion of contents stored in the platelet's dense granules and the phosphorylation of pleckstrin, a 47 kDa substrate of PKC [2]. Among the secreted contents, adrenaline ('epinephrine') and ADP activate the inhibitory G-protein (G_i) pathway. Based on studies of patients with defective PLCβ2 [3–5] and examination of P2 receptors in platelets [6,7], stimulation of both the PLC and G_i pathways in platelets leads to activation of the fibrinogen receptor (also known as integrin αIIbβ3). The activated αIIbβ3 receptors from other platelets then bind fibrinogen between them, leading to the formation of platelet aggregates.

Three of the most physiologically relevant agonists capable of causing platelet aggregation, ADP, thromboxane A_2 and thrombin [8], utilize G-protein-coupled receptors (GPCRs) to transduce their signals to the platelet cytoplasm. Two GPCRs for ADP, the P2Y1 and P2Y12 receptors, stimulate the G_q and G_i signalling pathways, respectively, and their co-stimulation is needed for ADP-induced primary aggregation [9–12]. The thromboxane A_2 receptor also couples to G_q, as well as G_{12} and G_{13} [13], but requires agonists secreted from platelets for activation of G_i pathways and aggregation [7]. Thrombin activates a family of G-protein-coupled protease-activated receptors (PARs). These receptors are activated by a unique mechanism in which the protease creates a new extracellular N-terminus that functions as a tethered ligand, resulting in intramolecular activation [14]. Three of the four known PARs, PAR-1, PAR-3 and PAR-4, are activated by thrombin. PAR-1, the predominant receptor on human platelets for thrombin, couples to G_q [15] and has a major role in activation of human platelets by thrombin, but plays no role in mouse platelets [14].

We have previously shown that ADP- and thromboxane A_2-induced platelet aggregation is accomplished by concomitant signalling from both G_q and G_i pathways [6,7]. ADP activates G_q through stimulation of the P2Y1 receptor [6], while activating

Abbreviations used: A3P5P, adenosine 3′-phosphate 5′-phosphate; dimethyl BAPTA, 5,5′-dimethyl-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; GPCR, G-protein-coupled receptor; PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C; PRP, platelet-rich plasma.

[1] To whom correspondence should be addressed, at the Department of Physiology (e-mail kunapuli@nimbus.temple.edu).

G_i through stimulation of the P2Y12 receptor [6,11]. Activation of the G_q pathway leads to PLC stimulation, which ultimately leads to activation of PKC and an increase in intracellular calcium. In the absence of G_q stimulation, as in G_q-deficient mice [16] or in human patients with a dysfunctional G_q [17,18], agonist-induced platelet secretion and aggregation are affected. Similarly, events downstream of signalling, i.e. PLCβ2 activation (namely, activation of PKC and mobilization of calcium ions from intracellular stores through diacylglycerol and InsP_3, respectively), are also essential for agonist-induced fibrinogen receptor activation, as demonstrated in patients with reduced PLCβ2 levels [3–5]. PKC activation has long been proposed to be involved in agonist-mediated fibrinogen receptor activation. Other groups have demonstrated that PKC inhibition by staurosporine blocks αIIbβ3 activation [19,20]. Twelve PKC isoforms have been identified to date [21] and human platelets have been shown to express at least six PKC isoforms, namely α, β, δ, θ, η' and ζ [22–25]. Staurosporine inhibits all the PKC isoforms with varying potency [26], but also inhibits other kinases, including tyrosine kinases [27], whereas Ro 31-8220 (bisindolylmaleimide IX) selectively inhibits PKC isoforms α, β, γ and ϵ [28].

Non-physiological platelet-activating agents such as calcium ionophores and phorbol esters have been shown to cause platelet fibrinogen receptor activation through calcium and PKC pathways, respectively [2,26,29–31]. However, the relevance of these pathways in agonist-induced fibrinogen receptor activation has not been studied in detail. In this study, we investigated the role of these downstream signalling events from PLC and demonstrate that platelets contain a calcium-regulated pathway and a PKC-regulated pathway independently regulating agonist–fibrinogen receptor activation. In the absence of both PKC activation and intracellular calcium mobilization, fibrinogen receptor is not activated.

EXPERIMENTAL

Materials

Whole blood was drawn from informed healthy human volunteers at the Thrombosis Research Center of Temple University, Philadelphia, PA, U.S.A. The thromboxane analogue U46619 [15(*S*)-hydroxy-9,11-epoxymethanoprosta-5*Z*,13*E*-dienoic acid] and PKC inhibitor Ro 31-8220 (bisindolylmaleimide IX) were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). Thrombin-receptor-activating peptide (SFLLRN) was purchased from Research Genetics (Huntsville, AL, U.S.A.). Calcium fluorophore fura PE-3-acetoxymethyl ester was purchased from Teflabs (Austin, TX, U.S.A.). The acetoxymethyl ester of the calcium chelator 5,5′-dimethyl-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (dimethyl BAPTA) was purchased from Molecular Probes (Eugene, OR, U.S.A.). [^{3}H]5-Hydroxytryptamine ('serotonin') was purchased from NEN (Boston, MA, U.S.A.). The FITC-conjugated anti-αIIbβ3 antibody PAC-1 was purchased from Becton-Dickinson (San Jose, CA, U.S.A.). Imipramine was purchased from ICN (Costa Mesa, CA, U.S.A.). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Platelet isolation

Whole human blood was collected into tube containing the anticoagulant ACD (85 mM sodium citrate, 71 mM citric acid and 110 mM glucose) such that the volume of ACD was one-sixth the total volume of blood collected. Platelet-rich plasma (PRP) was isolated by centrifuging diluted blood at 230 ***g*** for 20 min and further incubated with 1 mM acetylsalicylic acid at 37 °C for 30 min, followed by 15 min at ambient temperature. Platelets were then isolated by centrifuging PRP at 980 ***g*** for 10 min and washed by resuspending the platelet pellet in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 5 mM glucose, 10 mM Hepes, pH 7.4, and 0.2 % BSA) containing 0.05 unit/ml apyrase. Platelets were counted using a Coulter model Z particle counter (Hialeah, FL, U.S.A.), after which cell densities were adjusted to 3.5×10^8 cells/ml. All experiments were repeated at least three times using platelets from different donors.

Platelet aggregation and pleckstrin phosphorylation analysis

Agonist-induced platelet aggregation was analysed using a Chrono-Log model 440-VS aggregometer (Havertown, PA, U.S.A.) with sample volumes of 0.5 ml in a thermostat-controlled cuvette holder at 37 °C with constant stirring. Fibrinogen (1 mg/ml) was added to those samples with which ADP and U46619 were used as agonists to facilitate their respective aggregations. Aggregometer output was recorded using a Kipp & Zonen type BD 12E flatbed chart recorder (SCI-TEC, Saskatoon, Canada) set at 0.2 mm/s. Experiments involving PKC inhibition required the samples to be incubated with 10 μM Ro 31-8220 at 37 °C for 5 min prior to addition of agonist. Pleckstrin phosphorylation was detected by gel electrophoresis and autoradiography as described earlier [14].

Dimethyl BAPTA loading of platelets and calcium-mobilization measurements

PRP was incubated with 5 μM fura PE-3, a leakage-resistant form of the calcium fluorophore fura-2, or an equal volume of DMSO (solvent for fura PE-3) and incubated simultaneously with acetylsalicylic acid. Platelets were then isolated and washed as described above. Platelets were loaded with 10 μM dimethyl BAPTA by incubating the washed platelets with the acetoxymethyl ester form or DMSO (control) for 10 min. Changes in fluorescence were measured using an Aminco-Bowman Series 2 luminescence spectrometer with a water-jacketed thermostat-controlled cuvette holder at 37 °C and set at constant stirring. Sample volumes of 0.5 ml were analysed with an excitation wavelength of 340 nm and emission wavelength of 510 nm. Fluorescence measurements were converted to calcium concentrations using the equation reported by Grynkiewicz et al. [32], where F_{min} and F_{max} were determined with each respective platelet preparation.

Platelet secretion measurement

PRP was incubated with 1 μCi/ml [^{3}H]5-hydroxytryptamine simultaneously with acetylsalicylic acid. Platelets were then isolated and washed as described above, with the addition of 1 μM imipramine to the Tyrode's buffer to prevent re-uptake of secreted 5-hydroxytryptamine. Secretion experiments were performed concurrently with aggregation measurements such that 2 min after addition of agonist for aggregation, a final concentration of 3 % formaldehyde was added. Samples were then centrifuged at 5000 ***g*** for 1 min, and the supernatant was collected and counted using a Beckman LS 4700 liquid scintillation counter. Secretion was expressed as the percentage of total [^{3}H]5-hydroxytryptamine present in platelets without activation.

PAC-1-binding measurements

Platelets were isolated and washed as described above. Platelets were diluted in Tyrode's buffer without BSA to a concentration

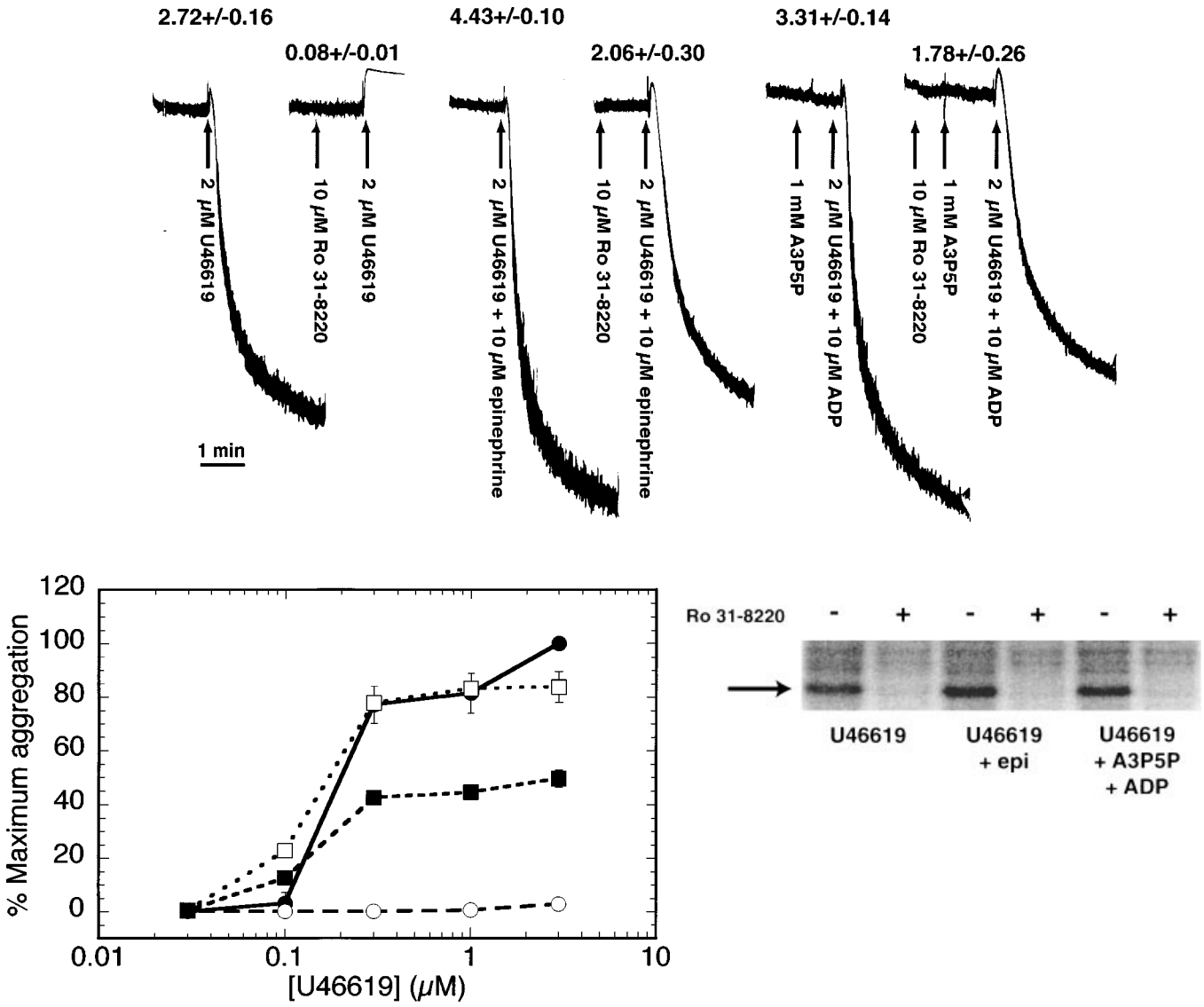

Figure 1 Effect of G_i signalling on U46619-induced aggregation, fibrinogen receptor activation and pleckstrin phosphorylation in platelets with PKC inhibition

Adrenaline (epinephrine, 10 μM), or ADP in the presence of 1 mM A3P5P, was added simultaneously with U46619 to aspirin-treated washed human platelets, in the absence or presence of 10 μM Ro 31-8220, to stimulate P2Y12-mediated G_i signalling. Top panel: representative aggregation tracings. Values above each tracing represent the corresponding mean fluorescence of PAC-1 binding. Bar indicates 1 min along the *x* axis. Tracing with agonist alone represents full-scale aggregation and was normalized to 100%. Bottom left panel: dose–response relationship between U46619 concentration and platelet aggregation during PKC inhibition. Platelets were stimulated with various concentrations of U46619 in the absence (●) or presence (○) of Ro 31-8220. In addition, ADP was added to final concentrations of 1 μM (■) or 10 μM (□) simultaneously with U46619 in the presence of A3P5P. Bottom right panel: pleckstrin phosphorylation, with the arrow denoting the position of the 47 kDa phosphorylated pleckstrin. Numerical data are expressed as means ± S.D., and all data are representative of results from three independent experiments.

of 2.5×10^8 cells/ml. Various agonists were then added to platelets with a mixing period of approx. 1 s. After allowing a specific duration for agonist stimulation, the reaction was stopped by the addition of paraformaldehyde to a final concentration of 1%. Platelets were centrifuged and washed with a 3-fold volume of Tyrode's buffer and then resuspended at 2.5×10^8 cells/ml containing 60 μg/ml FITC-conjugated PAC-1 antibody. The antibody was allowed at least 15 min for binding, whereupon the samples were diluted 7-fold with Tyrode's buffer and analysed with a Coulter EPICSXL flow cytometer at the Flow Cytometry Facility of the Wistar Institute (University of Pennsylvania, Philadelphia, PA, U.S.A.).

RESULTS

Evaluation of the role of PKC in agonist-mediated fibrinogen receptor activation

To begin investigating the contribution of the signalling pathways downstream of PLC activation to agonist-induced fibrinogen receptor activation, we first evaluated the role of PKC with Ro 31-8220, an inhibitor of conventional and novel PKC isoforms. Platelets were stimulated with different agonists in the presence of Ro 31-8220 to determine its effect on platelet aggregation and αIIbβ3 activation. Stimulation of Ro 31-8220-treated platelets with thrombin resulted in slightly decreased aggregation in

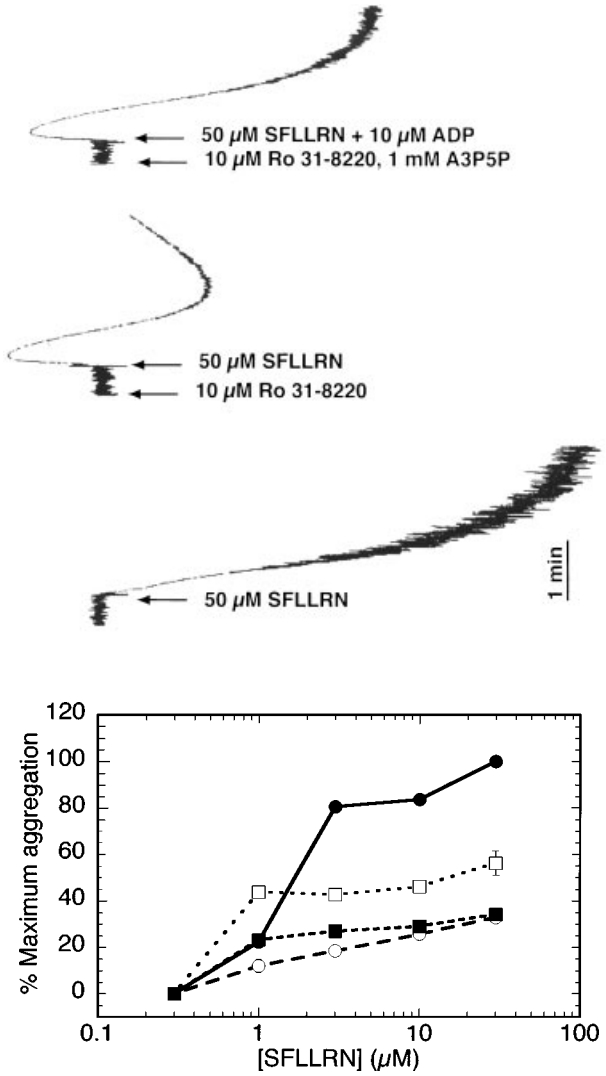

Figure 2 Effect of G_i signalling on SFLLRN-induced aggregation in platelets with PKC inhibition

Adrenaline (10 μM), or ADP in the presence of 1 mM A3P5P, was added simultaneously with SFLLRN to aspirin-treated, washed human platelets, in the absence or presence of 10 μM Ro 31-8220, to stimulate P2Y12-mediated G_i signalling. Top panel: representative aggregation tracings. Bar indicates 1 min along the *x* axis. Tracing with agonist alone represents full-scale aggregation and was normalized to 100%. Bottom panel: dose–response relationship between SFLLRN concentration and aggregation during PKC inhibition. Platelets were stimulated with various concentrations of SFLLRN in the absence (●) or presence (○) of Ro 31-8220. In addition, ADP was added to final concentrations of 1 μM (■) or 10 μM (□) simultaneously with SFLLRN in the presence of A3P5P. Numerical data are expressed as means ± S.D., and all data are representative of results from three independent experiments.

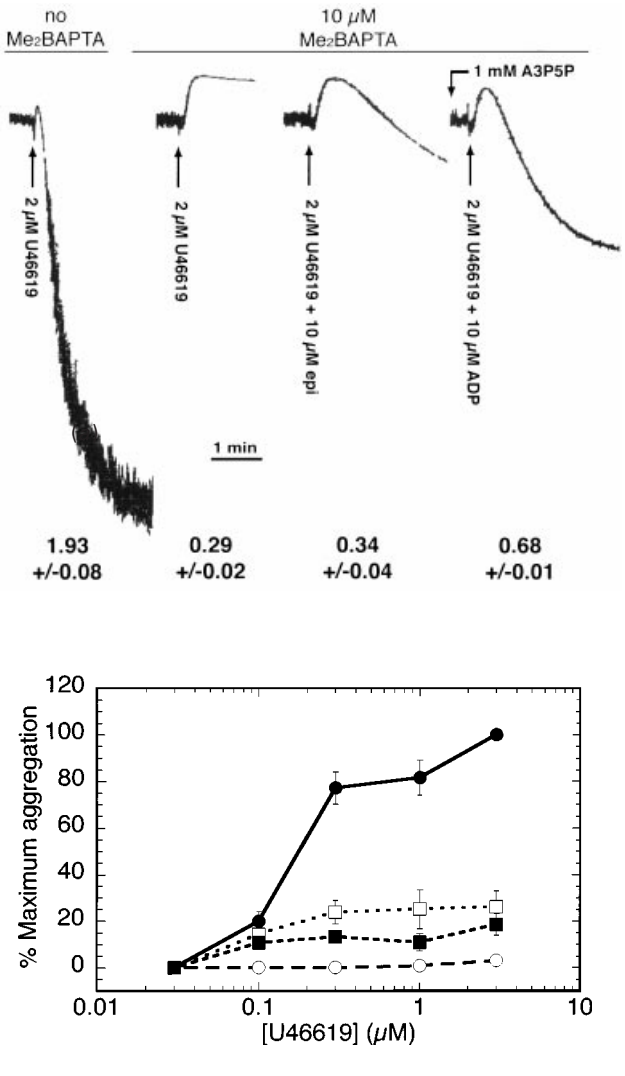

Figure 3 Effect of G_i signalling on U46619-induced platelet aggregation and fibrinogen receptor activation in calcium-impaired platelets

Adrenaline (epi, 10 μM), or ADP in the presence of 1 mM A3P5P, were added simultaneously with U46619 to platelets loaded with vehicle or 10 μM dimethyl BAPTA (Me_2BAPTA). Top panel: representative aggregation tracings. Values below each tracing represent the corresponding mean fluorescence of PAC-1 binding. Bar indicates 1 min along the *x* axis. Tracing with agonist alone represents full-scale aggregation and was normalized to 100%. Bottom panel: dose–response relationship between U46619 concentration and aggregation in the absence of intracellular calcium. Platelets were stimulated with various concentrations of U46619 in the absence (●) or presence (○) of dimethyl BAPTA. In addition, ADP was added to final concentrations of 1 μM (■) or 10 μM (□) simultaneously with U46619 in the presence of A3P5P. Numerical data are expressed as means ± S.D., and all data are representative of results from three independent experiments.

comparison with non-treated platelets, whereas ADP stimulation of Ro 31-8220-treated platelets had no significant inhibition (results not shown).

To confirm that Ro 31-8220 fully inhibited PKC in our system, pleckstrin phosphorylation was measured. Stimulation of untreated platelets with thrombin resulted in pleckstrin phosphorylation, which was blocked by pretreating platelets with Ro 31-8220 (results not shown). The presence of Ro 31-8220 also inhibited what little ADP-induced pleckstrin phosphorylation occurred (results not shown). To confirm the participation of ADP, this phosphorylation was completely inhibited by adenosine 3′-phosphate 5′-phosphate (A3P5P), a selective antagonist at the P2Y1 receptor (results not shown). AR-C66096, a selective P2Y12 receptor antagonist, however, did not affect the ADP-induced phosphorylation of pleckstrin, suggesting the lack of a role for P2Y12 receptor in this event (results not shown). The fact that ADP-mediated aggregation occurs with little or no induced pleckstrin phosphorylation indicates that platelet aggregation can occur independently of PKC activation.

U46619-induced platelet aggregation, but not shape change, was completely abolished by Ro 31-8220 (Figure 1, top panel). U46619-induced platelet aggregation depends on activation of

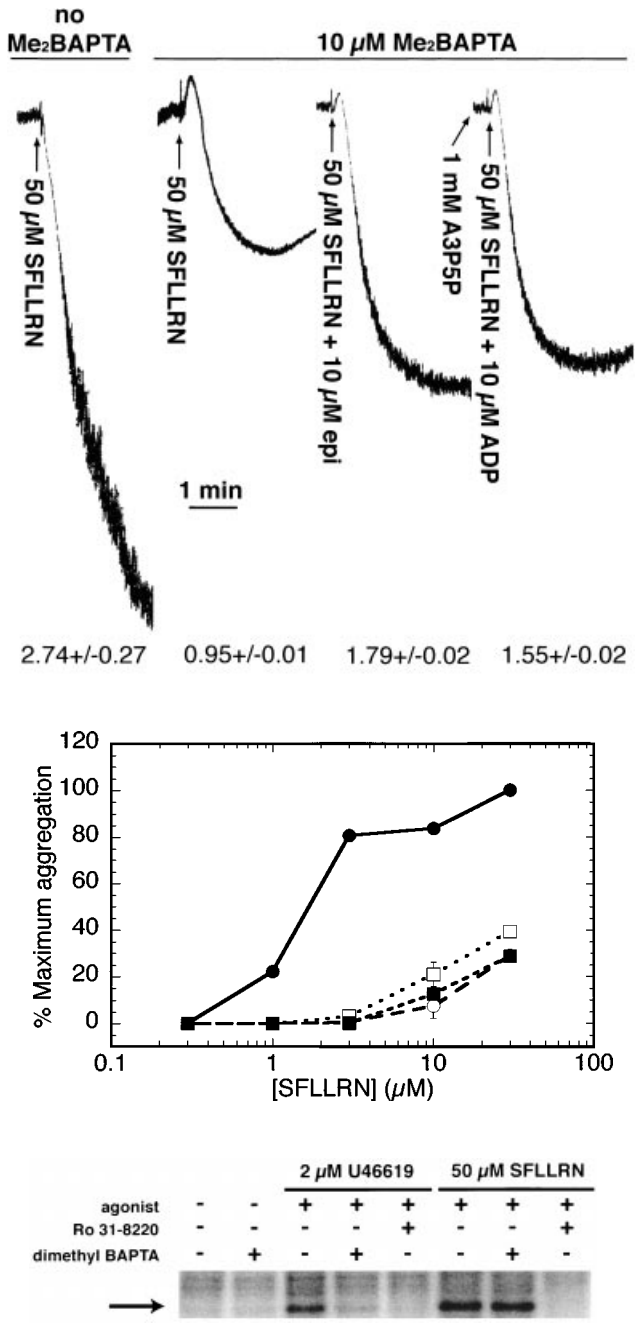

Figure 4 Effect of G_i signalling on SFLLRN-induced platelet aggregation and fibrinogen receptor activation in calcium-impaired platelets

Adrenaline (epi, 10 μM), or ADP in the presence of 1 mM A3P5P, was added simultaneously with SFLLRN to platelets loaded with 10 μM dimethyl BAPTA (Me_2BAPTA). Top panel: representative aggregation tracings. Values below each tracing represent the corresponding mean fluorescence of PAC-1 binding. Bar indicates 1 min along the *x* axis. Tracing with agonist alone represents full-scale aggregation and was normalized to 100%. Middle panel: dose–response relationship between SFLLRN and platelet aggregation in the absence of intracellular calcium. Platelets were stimulated with various concentrations of SFLLRN in the absence (●) or presence (○) of dimethyl BAPTA. In addition, ADP was added to final concentrations of 1 μM (■) or 10 μM (□) simultaneously with SFLLRN in the presence of A3P5P. Bottom panel: pleckstrin phosphorylation, with the arrow denoting the position of the 47 kDa phosphorylated pleckstrin. Platelets were stimulated with either U46619 (2 μM) or SFLLRN (50 μM) in the presence or absence of Ro 31-8220 (10 μM) or dimethyl BAPTA (10 μM) as indicated above. Numerical data are expressed as means ± S.D., and all data are representative of results from three independent experiments.

the G_i signalling cascade by secreted ADP [7]. PKC has been shown to be involved in the platelet secretion process [33], and the effects of Ro 31-8220 could thus be mediated by blocking secretion of ADP, which could activate G_i through P2Y12 receptors. We investigated whether U46619-induced platelet aggregation blocked by Ro 31-8220 could be restored by co-stimulation of G_i signalling cascade. Either adrenaline alone or ADP in the presence of a P2Y1 antagonist, A3P5P [6,7], was used to stimulate the G_i-dependent signalling in the presence of Ro 31-8220. U46619- or SFLLRN-induced platelet dense-granule release was completely blocked by pre-treatment of platelets with Ro 31-8220 (results not shown). As shown in Figure 1 (top panel), either α_{2A} receptor stimulation (by adrenaline) or selective activation of the P2Y12 receptor (by ADP in the presence of A3P5P) restored Ro 31-8220-blocked aggregation induced by U46619; the selective activation of the P2Y12 receptor by ADP by itself does not cause any aggregation [6,7]. This restoration of aggregation by G_i signalling occurred at various doses of U46619 (Figure 1, bottom left panel). At higher U46619 concentrations, full restoration of aggregation occurred when G_i signalling was stimulated with a maximal ADP concentration (10 μM), but only 40% restoration occurred with a sub-maximal concentration of ADP (1 μM; Figure 1, bottom left panel). However, restoration of aggregation is not accompanied by restoration of pleckstrin phosphorylation (Figure 1, bottom right panel). This result indicates that U46619 can cause platelet aggregation in the presence of G_i signalling without PKC activation.

As shown in Figure 2 (top panel), SFLLRN-induced irreversible aggregation is converted into reversible aggregation in the presence of Ro 31-8220. Consistent with other findings, selective G_i signalling potentiates aggregation in Ro 31-8220-treated platelets stimulated with SFLLRN and converts reversible aggregation into irreversible aggregation (Figure 2, top panel). This potentiation occurred at all concentrations of SFLLRN tested, with more potentiation occurring due to G_i stimulation with 10 μM ADP than with 1 μM ADP (Figure 2, bottom panel).

Examination of the role of calcium in U46619- and SFLLRN-mediated signalling pathways

In order to examine the role of calcium in agonist-mediated intracellular signalling, the high-affinity intracellular calcium chelator dimethyl BAPTA was used to block intracellular calcium increases and thereby interfere with those downstream signalling pathways dependent upon increased intracellular calcium. Measurements of intracellular calcium made in the presence of dimethyl BAPTA confirmed that agonist-induced calcium increases were completely blocked (results not shown; please see [34]). The presence of dimethyl BAPTA completely abolishes aggregation in response to ADP [34] and U46619 (Figure 3, top panel), leaving only a shape-change response.

A possible explanation for the effect of dimethyl BAPTA is that it blocks platelet secretion. U46619- or SFLLRN-induced dense-granule release was completely blocked in dimethyl BAPTA-treated platelets (results not shown). As thromboxane A_2-induced platelet aggregation is dependent upon secreted ADP to activate inhibitory G-proteins [7,35,36], dimethyl BAPTA might interfere with secretion rather than downstream signalling events in U46619-induced platelet fibrinogen receptor activation. If a lack of calcium signalling is responsible for the reduction of U46619-mediated aggregation by impairing secretion, then the inclusion of selectively activating G_i signalling components, added simultaneously with agonist to platelets, should restore partial or full aggregation [7,37]. Upon simultaneous addition of

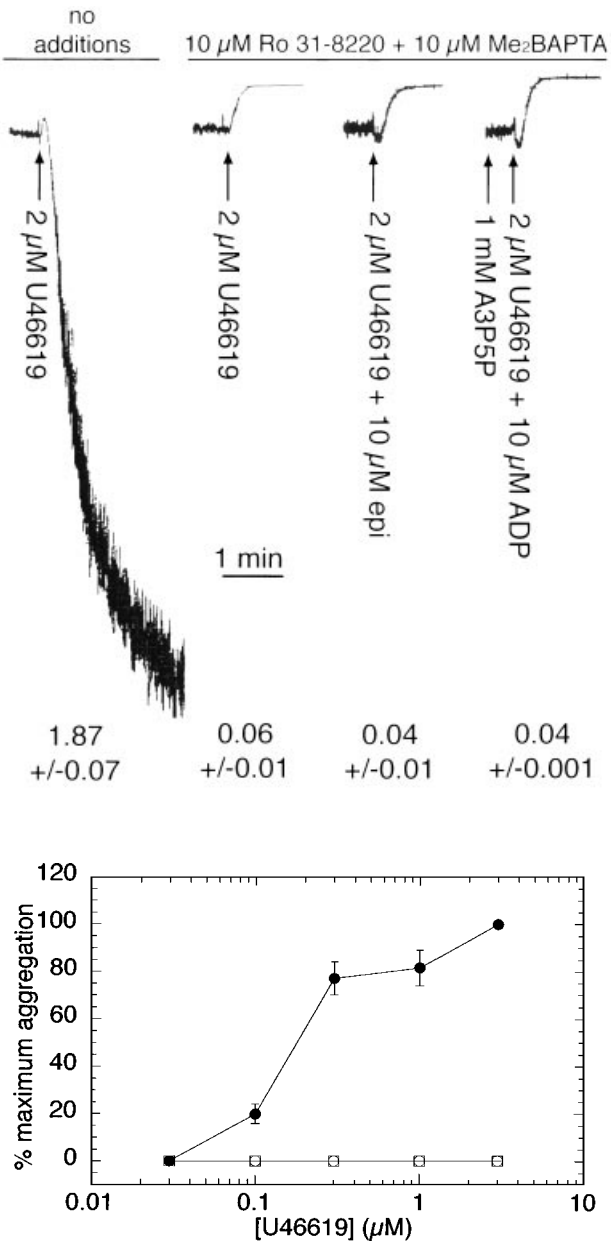

Figure 5 Effect of G_i signalling on U46619-induced platelet aggregation and fibrinogen receptor activation in PKC-inhibited calcium-impaired platelets

Ro 31-8220 (10 μM) was added prior to further additions. Adrenaline (epi, 10 μM), or 10 μM ADP in the presence of 1 mM A3P5P, was added simultaneously with U46619 to platelets loaded with 10 μM dimethyl BAPTA (Me_2BAPTA). Top panel: representative aggregation tracings. Values below each tracing represent the corresponding mean fluorescence of PAC-1 binding. Bar indicates 1 min along the *x* axis. Tracing with agonist alone represents full-scale aggregation and was normalized to 100%. Bottom panel: dose–response relationship between U46619 and aggregation in the absence of PKC activity and intracellular calcium. Platelets were stimulated with various concentrations of U46619 in the absence (●) or combined presence (○) of Ro 31-8220 and dimethyl BAPTA. In addition, ADP was added simultaneously with U46619 in the presence of A3P5P (□). Numerical data are expressed as mean ± S.D., and all data are representative of results from three independent experiments.

U46619 and adrenaline to dimethyl BAPTA-treated platelets, there was a slight stimulation of aggregation in comparison with U46619 alone (Figure 3, top panel). When U46619 was added to dimethyl BAPTA-loaded platelets together with ADP in the presence of A3P5P, a more pronounced aggregation was observed. These results were also reflected in the fibrinogen receptor activation as measured by PAC-1 binding. Furthermore, at all concentrations of U46619, the extent of platelet aggregation was abolished by dimethyl BAPTA and was partially restored by supplementing the G_i pathways (Figure 3, bottom panel). Thus in the presence of G_i signalling, U46619 can cause platelet fibrinogen receptor activation without depending on calcium-sensitive pathways.

Contrary to results with U46619, SFLLRN-induced aggregation was not abolished in the absence of measurable calcium, but full irreversible aggregation was converted into reversible aggregation (Figure 4, top panel). These results were similar to that achieved with SFLLRN in the presence of Ro 31-8220 (compare the top panels of Figures 4 and 2) and are consistent with a measurable decrease in fibrinogen receptor activation using PAC-1 binding (Figure 4, top panel). Furthermore, augmenting SFLLRN with selective G_i stimulation in dimethyl BAPTA-treated platelets resulted in partial restoration of irreversible aggregation and fibrinogen receptor activation (Figure 4, top panel). Similar aggregation patterns with G_i signalling were also obtained with various concentrations of SFLLRN (Figure 4, middle panel). We also measured pleckstrin phosphorylation mediated by U46619 and SFLLRN in the presence of dimethyl BAPTA. As Figure 4 (bottom panel) shows, there is no difference in SFLLRN-mediated pleckstrin phosphorylation in the absence or presence of dimethyl BAPTA, indicating that dimethyl BAPTA treatment was not affecting PKC activity induced by PAR-1 activation. However, dimethyl BAPTA treatment did significantly reduce U46619-mediated pleckstrin phosphorylation, indicating some reliance of PKC activation upon calcium signalling.

Effect of dimethyl BAPTA and Ro 31-8220 on U46619- and SFLLRN-induced platelet aggregation

We investigated the effect of combined blockade of both PKC and calcium pathways on U46619- and SFLLRN-induced platelet aggregation. As seen previously with either Ro 31-8220 or dimethyl BAPTA treatment alone, the combined treatment with both agents prevents U46619-mediated aggregation (Figure 5). However, in contrast with either treatment alone, supplemental G_i signalling failed to restore aggregation. Similarly, the presence of both Ro 31-8220 and dimethyl BAPTA totally abolished SFLLRN-mediated platelet aggregation as compared with the control, and was insensitive to restoration efforts with selective activation of either the adrenaline receptor (α_{2A}) or the G_i-coupled P2Y12 receptor (Figure 6).

DISCUSSION

Fibrinogen receptor (integrin αIIbβ3) activation occurs through the stimulation of platelets by a number of agonists such as ADP, thromboxane and thrombin. In turn, aggregation results when platelets are cross-linked by fibrinogen molecules bound to activated αIIbβ3 receptors on the platelet surface. Previous studies have shown that phorbol esters, activating PKC and calcium ionophores, causing increases in intracellular calcium, can cause platelet aggregation [2,26,29–31]. However, these agents are not physiological agonists, and the physiological relevance of these pathways is not clear. Recent studies demonstrated that these pathways are important for glycoprotein VI-mediated platelet fibrinogen receptor activation [38,39]. Using a combination of agonists, antagonists and inhibitors with established effectiveness [6,7,10,34,40], this paper demonstrates

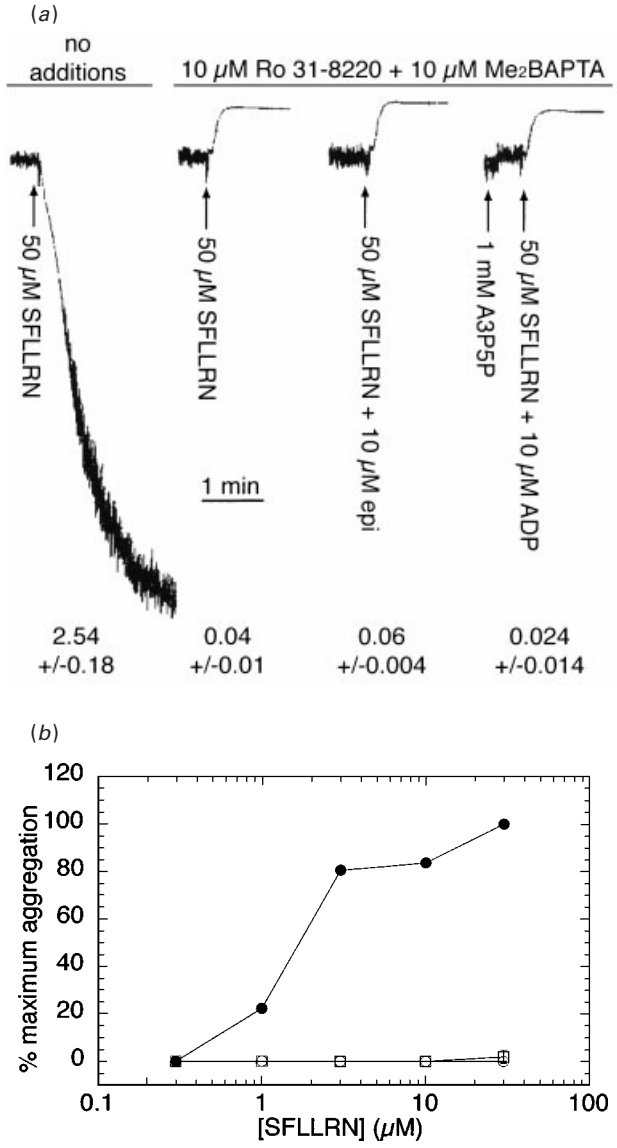

Figure 6 Effect of G_i signalling on SFLLRN-induced platelet aggregation and fibrinogen receptor activation in PKC-inhibited calcium-impaired platelets

Ro 31-8220 (10 μM) was added prior to further additions. Adrenaline (epi, 10 μM), or 10 μM ADP in the presence of 1 mM A3P5P, was added simultaneously with SFLLRN to platelets loaded with 10 μM dimethyl BAPTA (Me_2BAPTA). Top panel: representative aggregation tracings. Values below each tracing represent the corresponding mean fluorescence of PAC-1 binding. Bar indicates 1 min along the *x* axis. Tracing with agonist alone represents full-scale aggregation and was normalized to 100%. Bottom panel: dose–response relationship between SFLLRN and aggregation in the absence of PKC activity and intracellular calcium. Platelets were stimulated with various concentrations of SFLLRN in the absence (●) or combined presence (○) of Ro 31-8220 and dimethyl BAPTA. In addition, ADP was added simultaneously with SFLLRN in the presence of A3P5P (□). Numerical data are expressed as means ± S.D., and all data are representative of results from three independent experiments.

that platelets contain a calcium-regulated pathway and a PKC-regulated pathway, downstream of PLC activation, that independently regulate agonist-induced fibrinogen receptor activation. Furthermore, in the absence of both PKC activation and intracellular calcium mobilization, physiological agonists fail to activate fibrinogen receptor. Activated platelets generate thromboxane A2 that could enhance the aggregation responses. Some of the reagents used in this study might interfere with thromboxane A2 generation and hence might lead to misinterpretation of the results. To avoid such complexities in the interpretation of results, we have used aspirin-treated platelets, wherein thromboxane A2 generation is impaired.

It is well established that the PKC inhibitor Ro 31-8220 blocks agonist-induced platelet secretion. This drug has been used to show that secreted ADP can potentiate aggregation by other agonists such as SFLLRN or thromboxane mimetics, but it does not inhibit aggregation induced by direct addition of ADP. Furthermore, in the presence of Ro 31-8220, SFLLRN-induced secretion is completely blocked [33], thereby converting irreversible aggregation to reversible aggregation. In the case of the thromboxane mimetic U46619, platelet aggregation is completely blocked by the PKC inhibitor. This inhibition of U46619-induced aggregation by Ro 31-8220 could be reversed by supplementing with activation of the P2Y12 or adrenergic a2A receptor activation (Figure 1 [7]).

Previously, several investigators have used different PKC inhibitors to investigate the role of PKC in platelet fibrinogen receptor activation [26,29–31,41,42]. However, these results have some inconsistencies. Watson et al. [31] treated platelets with staurosporine (1 μM) for relatively shorter time periods and observed that thrombin-induced secretion is abolished with some effect on platelet aggregation. Likewise, bisindolylmaleimide I was shown to block thrombin- and platelet-activating-factor-induced, but not ADP-induced, fibrinogen binding [29]. These data are similar to our observations in that Ro 31-8220 inhibited thrombin- and SFLLRN-induced platelet aggregation to comparable levels, and that ADP-induced fibrinogen receptor activation is unaffected by Ro 31-8220. It has been shown repeatedly that platelet secretion is blocked under conditions of PKC inhibition, during which the contribution of secreted ADP to fibrinogen receptor stimulation is eliminated. The significant inhibition of thrombin-induced platelet fibrinogen receptor activation by bisindolylmaleimide I might arise from a combination of secretion blockade (thereby eliminating the synergy of secreted ADP) and inhibition of other PKC isoforms that might play a role in thrombin-induced platelet aggregation. Furthermore, the absence of thrombin-induced secretion from staurosporine treatment for short time periods could reflect the fact that specific PKC isoforms are inhibited, since higher staurosporine concentrations or prolonged exposure to low concentrations result in additional effects on thrombin-mediated platelet aggregation [31]. Although Ro 31-8220 has only a small inhibitory effect on the aggregation at high concentrations of thrombin, we predict that this compound would have a more dramatic effect on aggregation induced by very low concentrations of thrombin.

Since the PKC-regulated pathway is not solely essential for agonist-induced fibrinogen receptor activation, we investigated the role of the calcium pathway in this event. Blockade of increased intracellular calcium with chelators such as dimethyl BAPTA abolishes ADP- or U46619-induced, but not SFLLRN-induced, platelet fibrinogen receptor activation. Part of the role for calcium in agonist-induced fibrinogen receptor stimulation appears to involve its influence on the PKC-regulated pathway, as shown by the partial loss of pleckstrin phosphorylation in response to U46619 stimulation in dimethyl BAPTA-treated platelets (Figure 4, bottom panel). This result most likely reflects the participation of calcium-dependent (i.e. conventional) PKC isoforms in U46619-mediated platelet activation. Furthermore, the fact that pleckstrin phosphorylation is not totally abolished with dimethyl BAPTA treatment indicates that one or more calcium-independent isoforms of PKC are activated upon agonist stimulation in the absence of a calcium-regulated pathway.

However, similar to PKC activation, intracellular calcium increases also have a role in platelet secretion. Inhibition of either the PKC or calcium signalling pathways not only abolishes U46619-mediated platelet aggregation and fibrinogen receptor activation (Figures 1 and 3) but also blocks agonist-induced platelet secretion through which secreted ADP activates G_i signalling contributing to fibrinogen receptor activation. Hence, U46619-induced fibrinogen receptor activation in dimethyl BAPTA-treated platelets was partially restored when supplemented with selective G_i signalling either with P2Y12 receptor activation or with α_{2A} receptor activation. Therefore, the calcium-independent PKC isoforms that are active during dimethyl BAPTA treatment are potentiated with selective G_i signalling.

We have shown elsewhere that concomitant G_q and G_i signalling is important for agonist-induced aggregation [9]. It should be emphasized that the technique of preferentially stimulating the P2Y12 receptor with ADP has been used in this study simply as a method to induce G_i signalling concomitantly with other G_q-stimulating agonists. Under conditions where either PKC activation or intracellular calcium release are blocked, G_i signalling with a sub-maximal concentration of ADP (1 μM) resulted in lower degrees of potentiated aggregation than with the maximal ADP concentration of 10 μM. The achievement of different levels of restored aggregation from two separate ADP concentrations further illustrates the synergy of G_i signalling with the PKC- and calcium-regulated pathways.

Resembling the outcome with U46619, SFLLRN-induced platelet secretion is also inhibited by impairment of the calcium signalling pathway (results not shown). However, unlike U46619 where platelet aggregation was totally abolished with the inhibition of either PKC or calcium action, SFLLRN-induced aggregation becomes reversible under each condition (Figures 2 and 4) and thus appears to have both secretion-dependent and -independent requirements. In addition, this primary aggregation could be greatly potentiated with G_i signalling to a similar extent whether treated with dimethyl BAPTA or Ro 31-8220. SFLLRN-mediated aggregation could be potentiated with G_i signalling to a greater extent than with U46619 in the functional absence of calcium; this implies that SFLLRN has greater potency than U46619 in stimulating a signalling pathway where an intracellular calcium increase is not detectible (compare Figures 4 and 5).

Fibrinogen receptor activation occurs in dimethyl BAPTA-treated platelets in response to SFLLRN, or to U46619 when supplemented with G_i signalling, but not with ADP, even in the presence of G_i signalling. Hence, intracellular calcium increases are an essential part of ADP-induced fibrinogen receptor activation, but alternate pathways appear to mediate SFLLRN- or U46619-induced fibrinogen receptor activation in the absence of intracellular calcium increases. Similar to this action, phorbol esters cause fibrinogen receptor activation without increases in intracellular calcium. Furthermore, phorbol ester-mediated platelet aggregation is unaffected in platelets from mice deficient in G_q [16], suggesting that phorbol esters can stimulate fibrinogen receptor through PKC without a role for calcium.

These data lead to two possible scenarios in agonist-induced fibrinogen receptor activation. First, an alternate pathway downstream of receptor activation can mediate fibrinogen receptor activation in the cases of SFLLRN and U46619, but not in the case of ADP. This is conceivable, since thrombin and U46619, but not ADP, can activate alternative pathways leading to shape change in G_q-deficient platelets. The P2Y1 receptor couples only to G_q, whereas PAR-1 and thromboxane A_2 receptors couple to other G-proteins, including G_{12}, G_{13} and G_{16} [43]. Furthermore, the P2Y1, PAR-1 and thromboxane A_2 receptors can activate the RhoA-p160ROCK pathway in addition to the PLC-induced pathway [34,44]. The other possibility is that both PKC- and calcium-regulated pathways activate the fibrinogen receptor and, when either pathway is blocked, the other pathway independently contributes to fibrinogen receptor activation. We evaluated these two scenarios with a single experiment in which both PKC and intracellular calcium were blocked. In the case of SFLLRN and U46619, fibrinogen receptor activation was abolished when both PKC- and calcium-regulated pathways were blocked, even when supplemented with G_i signalling (Figures 5 and 6). This system has recently been proposed in non-GPCR-mediated platelet activation where PKC activation and increased calcium are required for full aggregation induced by the glycoprotein VI agonist convulxin [38,39]. ADP appears to utilize only the calcium-regulated pathway; this is not unexpected, since ADP minimally activates PKC and its activity is much less compared with that for U46619 and SFLLRN, as shown by pleckstrin phosphorylation. On the other hand, phorbol ester, which activates PKC isofoms [21], conceivably induces platelet aggregation solely through the PKC-regulated pathway without utilizing the calcium-regulated pathway.

In conclusion, we have shown that platelets have both calcium-regulated and PKC-regulated pathways that can independently mediate agonist-induced fibrinogen receptor activation. In addition, G_i signalling can synergize with either PKC-regulated pathway or calcium-regulated pathway, but by itself cannot cause fibrinogen receptor activation. Furthermore, both pathways act synergistically with one another to activate the fibrinogen receptor.

This work was supported by research grants HL60683 and HL64943 from the National Institutes of Health (to S.P.K.), and a Grant-in-Aid from the Pennsylvania-Delaware affiliate of the American Heart Association (to J.L.D). T.M.Q. and R.T.D. are supported by training grant T32 HL07777. T.M.Q. is the recipient of a Post-Doctoral Fellowship from the Pennsylvania-Delaware affiliate of the American Heart Association.

REFERENCES

1 Packham, M. A. (1994) Role of platelets in thrombosis and hemostasis. Can. J. Physiol. Pharmacol. **72**, 278–284
2 Shattil, S. J., Kashiwagi, H. and Pampori, N. (1998) Integrin signaling: the platelet paradigm. Blood **91**, 2645–2657
3 Rao, A. K., Kowalska, M. A. and Disa, J. (1989) Impaired cytoplasmic ionized calcium mobilization in inherited platelet secretion defects. Blood **74**, 664–672
4 Lee, S. B., Rao, A. K., Lee, K. H., Yang, X., Bae, Y. S. and Rhee, S. G. (1996) Decreased expression of phospholipase C-beta 2 isozyme in human platelets with impaired function. Blood **88**, 1684–1691
5 Yang, X., Sun, L., Ghosh, S. and Rao, A. K. (1996) Human platelet signaling defect characterized by impaired production of inositol-1,4,5-triphosphate and phosphatidic acid and diminished Pleckstrin phosphorylation: evidence for defective phospholipase C activation. Blood **88**, 1676–1683
6 Jin, J. and Kunapuli, S. P. (1998) Co-activation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc. Natl. Acad. Sci. U.S.A. **95**, 8070–8074
7 Paul, B. Z., Jin, J. and Kunapuli, S. P. (1999) Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. J. Biol. Chem. **274**, 29108–29114
8 Hourani, S. M. and Cusack, N. J. (1991) Pharmacological receptors on blood platelets. Pharmacol. Rev. **43**, 243–298
9 Daniel, J. L., Dangelmaier, C., Jin, J., Ashby, B., Smith, J. B. and Kunapuli, S. P. (1998) Molecular basis for ADP-induced platelet activation I: evidence for three distinct ADP receptors on platelets. J. Biol. Chem. **273**, 2024–2029
10 Jin, J., Daniel, J. L. and Kunapuli, S. P. (1998) Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J. Biol. Chem. **273**, 2030–2034
11 Hollopeter, J., Jantzen, H.-M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.-B., Nurden, P., Nurden, A., Julius, D. J. and Conley, P. B. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature (London) **409**, 202–207

12 Foster, C. J., Prosser, D. M., Agans, J. M., Zhai, Y., Smith, M. D., Lachowicz, J. E., Zhang, F. L., Gustafson, E., Monsma, Jr, F. J. et al. (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J. Clin. Invest. **107**, 1591–1598

13 Djellas, Y., Manganello, J. M., Antonakis, K. and Le Breton, G. C. (1999) Identification of Galpha13 as one of the G-proteins that couple to human platelet thromboxane A2 receptors. J. Biol. Chem. **274**, 14325–14330

14 Coughlin, S. R. (1999) How the protease thrombin talks to cells. Proc. Natl. Acad. Sci. U.S.A. **96**, 11023–11027

15 Benka, M. L., Lee, M., Wang, G. R., Buckman, S., Burlacu, A., Cole, L., DePina, A., Dias, P., Granger, A., Grant, B. et al. (1995) The thrombin receptor in human platelets is coupled to a GTP binding protein of the G alpha q family. FEBS Lett. **363**, 49–52

16 Offermanns, S., Toombs, C. F., Hu, Y.-H. and Simon, M. I. (1997) Defective platelet activation in Gaq-deficient mice. Nature (London) **389**, 183–186

17 Rao, A. K., Koike, K., Willis, J., Daniel, J. L., Beckett, C., Hassel, B., Day, H. J., Smith, J. B. and Holmsen, H. (1984) Platelet secretion defect associated with impaired liberation of arachidonic acid and normal myosin light chain phosphorylation. Blood **64**, 914–921

18 Gabbeta, J., Yang, X., Kowalska, M. A., Sun, L., Dhanasekaran, N. and Rao, A. K. (1997) Platelet signal transduction defect with Gα subunit dysfunction and diminished Gαq in a patient with abnormal platelet responses. Proc. Natl. Acad. Sci. U.S.A. **94**, 8750–8755

19 Shattil, S. J., Cunningham, M., Wiedmer, T., Zhao, J., Sims, P. J. and Brass, L. F. (1992) Regulation of glycoprotein IIb-IIIa receptor function studied with platelets permeabilized by the pore-forming complement proteins C5b-9. J. Biol. Chem. **267**, 18424–18431

20 Trybulec, M., Kowalska, M. A., McLane, M. A., Silver, L., Lu, W. and Niewiarowski, S. (1993) Exposure of platelet fibrinogen receptors by zinc ions: role of protein kinase C. Proc. Soc. Exp. Biol. Med. **203**, 108–116

21 Mellor, H. and Parker, P. J. (1998) The extended protein kinase C superfamily. Biochem. J. **332**, 281–292

22 Grabarek, J., Raychowdhury, M., Ravid, K., Kent, K. C., Newman, P. J. and Ware, J. A. (1992) Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. J. Biol. Chem. **267**, 10011–10017

23 Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C. and Fisher, G. J. (1992) Translocation of protein kinase C isozymes in thrombin-stimulated human platelets. Correlation with 1,2-diacylglycerol levels. J. Biol. Chem. **267**, 15585–15590

24 Crabos, M., Imber, R., Woodtli, T., Fabbro, D. and Erne, P. (1991) Different translocation of three distinct PKC isoforms with tumor-promoting phorbol ester in human platelets. Biochem. Biophys. Res. Commun. **178**, 878–883

25 Wang, F., Naik, U. P., Ehrlich, Y. H., Freyberg, Z., Osada, S., Ohno, S., Kuroki, T., Suzuki, K. and Kornecki, E. (1993) A new protein kinase C, nPKC eta′, and nPKC theta are expressed in human platelets: involvement of nPKC eta′ and nPKC theta in signal transduction stimulated by PAF. Biochem. Biophys. Res. Commun. **191**, 240–246

26 van Willigen, G. and Akkerman, J. W. (1991) Protein kinase C and cyclic AMP regulate reversible exposure of binding sites for fibrinogen on the glycoprotein IIB-IIIA complex of human platelets. Biochem. J. **273**, 115–120

27 Daniel, J. L., Dangelmaier, C. and Smith, J. B. (1994) Evidence for a role for tyrosine phosphorylation of phospholipase Cγ2 in collagen-induced platelet cytosolic calcium mobilization. Biochem. J. **302**, 617–622

28 Wilkinson, S. E., Parker, P. J. and Nixon, J. S. (1993) Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. Biochem. J. **294**, 335–337

29 Hers, I., Donath, J., van Willigen, G. and Akkerman, J. W. (1998) Differential involvement of tyrosine and serine/threonine kinases in platelet integrin αIIbβ3 exposure. Arterioscler. Thromb. Vasc. Biol. **18**, 404–414

30 van Willigen, G. and Akkerman, J. W. (1992) Regulation of glycoprotein IIB/IIIA exposure on platelets stimulated with α-thrombin. Blood **79**, 82–90

31 Watson, S. P., McNally, J., Shipman, L. J. and Godfrey, P. P. (1988) The action of the protein kinase C inhibitor, staurosporine, on human platelets. Evidence against a regulatory role for protein kinase C in the formation of inositol trisphosphate by thrombin. Biochem. J. **249**, 345–350

32 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260**, 3440–3450

33 Hashimoto, Y., Togo, M., Tsukamoto, K., Horie, Y., Watanabe, T. and Kurokawa, K. (1994) Protein kinase C-dependent and -independent mechanisms of dense granule exocytosis by human platelets. Biochim. Biophys. Acta **1222**, 56–62

34 Paul, B. Z., Daniel, J. L. and Kunapuli, S. P. (1999) Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. J. Biol. Chem. **274**, 28293–28300

35 Pulcinelli, F. M., Ashby, B., Gazzaniga, P. P. and Daniel, J. L. (1995) Protein kinase C activation is not a key step in ADP-mediated exposure of fibrinogen receptors on human platelets. FEBS Lett. **364**, 87–90

36 Pulcinelli, F. M., Ciampa, M. T., Favilla, M., Pignatelli, P., Riondino, S. and Gazzaniga, P. P. (1999) Concomitant activation of Gi protein-coupled receptor and protein kinase C or phospholipase C is required for platelet aggregation. FEBS Lett. **460**, 37–40

37 Pulcinelli, F. M., Pesciotti, M., Pignatelli, P., Riondino, S. and Gazzaniga, P. P. (1998) Concomitant activation of Gi and Gq protein-coupled receptors does not require an increase in cytosolic calcium for platelet aggregation. FEBS Lett. **435**, 115–118

38 Atkinson, B. T., Stafford, M. J., Pears, C. J. and Watson, S. P. (2001) Signalling events underlying platelet aggregation induced by the glycoprotein VI agonist convulxin. Eur. J. Biochem. **268**, 5242–5248

39 Quinton, T. M., Ozdener, F., Dangelmaier, C., Daniel, J. L. and Kunapuli, S. P. (2002) Glycoprotein VI-mediated platelet fibrinogen receptor activation occurs through calcium-sensitive and PKC-sensitive pathways without a requirement for secreted ADP. Blood **99**, 3228–3234

40 Jin, J., Quinton, T. M., Zhang, J., Rittenhouse, S. E. and Kunapuli, S. P. (2002) Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin α(IIb)β(3) and ADP receptors. Blood **99**, 193–198

41 Saitoh, M., Salzman, E. W., Smith, M. and Ware, J. A. (1989) Activation of protein kinase C in platelets by epinephrine and A23187: correlation with fibrinogen binding. Blood **74**, 2001–2006

42 Gabbeta, J., Yang, X., Sun, L., McLane, M. A., Niewiarowski, S. and Rao, A. K. (1996) Abnormal inside-out signal transduction-dependent activation of glycoprotein IIb-IIIa in a patient with impaired pleckstrin phosphorylation. Blood **87**, 1368–1376

43 Brass, L. F., Manning, D. R., Cichowski, K. and Abrams, C. S. (1997) Signaling through G proteins in platelets: to the integrins and beyond. Thromb. Haemost. **78**, 581–589

44 Bauer, M., Retzer, M., Wilde, J. I., Maschberger, P., Essler, M., Aepfelbacher, M., Watson, S. P. and Siess, W. (1999) Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. Blood **94**, 1665–1672

Received 5 February 2002/18 July 2002; accepted 5 September 2002
Published as BJ Immediate Publication 5 September 2002, DOI 10.1042/BJ20020226