Protease-activated receptor 1 (PAR1) signalling desensitization is counteracted via PAR4 signalling in human platelets

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PARs (protease-activated receptors) 1 and 4 belong to the family of G-protein-coupled receptors which induce both Gα12/13 and Gαq signalling. By applying the specific PAR1- and PAR4-activating hexapeptides, SFLLRN and AYPGKF respectively, we found that aggregation of isolated human platelets mediated via PAR1, but not via PAR4, is abolished upon homologous receptor activation in a concentration- and time-dependent fashion. This effect was not due to receptor internalization, but to a decrease in Ca2+ mobilization, PKC (protein kinase C) signalling and α-granule secretion, as well as to a complete lack of dense granule secretion. Interestingly, subthreshold PAR4 activation rapidly abrogated these affected signalling events and functional responses, which was sufficient to re-establish aggregation. The lack of ADP release and P2Y12 receptor-induced Gαi signalling accounted for the loss of the aggregation response, as mimicking Gαi2 signalling with 2-MeS-ADP (2-methylthioadenosine-5′-O-diphosphate) or epinephrine (adrenaline) could substitute for intermediate PAR4 activation. Finally, we found that the re-sensitization of PAR1 signalling-induced aggregation via PAR4 relied on PKC-mediated release of both ADP from dense granules and fibrinogen from α-granules. The present study elucidates further differences in human platelet PAR signalling regulation and provides evidence for a cross-talk in which PAR4 signalling counteracts mechanisms involved in PAR1 signalling down-regulation.

Key words: ADP, desensitization, P2Y12 receptor, platelet, protease-activated receptor (PAR), protein kinase C (PKC).

INTRODUCTION

Human platelets express the two PAR (protease-activated receptor) subtypes 1 and 4 [1]. Thrombin, the main effector protease of the coagulation cascade, activates PARs by cleaving off the N-terminal exodomains, and the unmasked ‘new’ N-terminus serves as a tethered ligand that induces receptor activation. As the newly exposed termini comprise distinct amino acid sequences, peptides mimicking these termini are highly specific receptor agonists and useful tools to investigate receptor subtype signalling.

Both PAR1 and PAR4 belong to the family of GPCRs (G-protein-coupled receptors) and induce Gα12/13 and Gαq signalling [2]. The initial reorganization of the platelet cytoskeleton (‘shape change’) involves Gα12/13-mediated calcium/calmodulin and Rho/Rho-kinase signalling pathways [3,4], whereas Gαq signalling is required for platelet granule secretion [5] which is accomplished via PLCβ (phospholipase C β) signalling and subsequent Ca2+ - and DAG (diacylglycerol)-mediated activation of PKC (protein kinase C).

Platelet activation in general involves the generation and release of lipid mediators such as TXA2 (thromboxane A2), as well as of adenine nucleotides such as ADP from dense granules. Once released, these mediators initiate stimulatory loops by activating their respective platelet receptors, thereby representing important reinforcement mechanisms for platelet activation and aggregation.

For TXA2, the TPα (thromboxane/prostanoid receptor α) is the predominant isoform expressed on platelets, and couples, similarly to PAR1 and PAR4, to Gα12/13 as well as Gαq [6,7]. For ADP, the two GPCRs P2Y1 and P2Y12 have been identified on human platelets [8]. The P2Y1 receptor couples to Gαq, whereas the P2Y12 receptor couples to Gα12 [9], which, by negatively regulating adenylate cyclase and therefore cAMP levels, essentially contributes to full and sustained platelet aggregation and thrombus formation. Along this line, there are dissenting studies on whether PAR1 also contributes to platelet activation via Gαi [10,11].

However, although PAR1 and PAR4 couple to the same G-protein subtypes, they apparently differ in terms of affinity for thrombin and the duration of intracellular signalling. For a long time it has been thought that PAR1 is the main platelet thrombin receptor as it has, due to a hirudin-like sequence, a higher affinity towards thrombin than does PAR4 [12,13]. In contrast, more recent findings indicate that PAR4 is activated by thrombin likewise at low concentrations [14,15]. Furthermore, it has been found that PAR1 and PAR4 form heterodimers, and a model has been proposed in which thrombin binding to PAR1, besides inducing its respective receptor activation, subsequently facilitates PAR4 cleavage and signalling [14]. This biphasic model would serve to explain, for instance, the profile of intracellular Ca2+ mobilization provoked by thrombin, which can distinctly be separated into a rapid peak rise mediated via PAR1 and a delayed but sustained response caused by subsequent PAR4 signalling [12,16].

Nonetheless, little is known regarding the mechanisms which negatively regulate and shut off PAR1 and PAR4 signalling in human platelets. In an artificially created cell system, the rapid and transient signalling via PAR1 compared with the sustained
signalling via PAR4 has been attributed to differential receptor phosphorylation and velocity of receptor internalization [16].

Owing to the implied close relation of thrombin-induced platelet PAR1 and PAR4 activation, we aimed to investigate whether the signalling cascades induced by those receptors interfere with each other in terms of mutual signalling regulation. To exclude any interference of TXA₂-provoked TP₄⁻G⁻⁻⁴⁻ coupling, aspirinated platelets were used; and owing to the complex and ultimate nature of thrombin-induced PAR1 and PAR4 activation, the respective specific receptor-agonistic peptides were applied.

We found that PAR1-mediated platelet aggregation, but not that induced by PAR4, is abolished upon subthreshold homologous receptor activation. As this loss in responsiveness was not due to PAR1 internalization, but to a down-regulation of PAR1-mediated responses such as Ca²⁺ mobilization and PKC signalling, as well as to α-granule and dense granule secretion, we refer to our findings as PAR1 signalling desensitization rather than receptor desensitization.

Strikingly, we found that subthreshold PAR4 signalling counteracts PAR1 signalling desensitization, resulting in a partial reconstitution of Ca²⁺ mobilization and PKC-mediated granule secretion sufficient to re-establish full platelet aggregation. These findings suggest that PAR4 signalling might play a supportive if not limiting role on the duration of PAR1 signalling in vivo.

**EXPERIMENTAL**

**Reagents**
The specific PAR1- and PAR4-agonistic peptides, SFLLRN and AYPGKF-NH₂, respectively, were synthesized by the Biotechnology Centre of Oslo (Oslo, Norway). Acetysalicicylic acid (aspirin), apyrase (Grade III, from potato), TXA₂ mimetic U46619, LPA (α-lysophosphatidic acid; oleoyl-sn-glycero-3-phosphate), human AGP (α-1-acid glycoprotein), serotonin, 2-MeS-ADP (2-methylthiodenosine-5′-O-diphosphate), α,β-MeATP, epinephrine (adrenaline), fura 2/AM (fura 2 acetoxymethyl ester), Ro31–8220, Y27632 and yohimbine were obtained from Sigma–Aldrich. BAPTA/AM [1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid tetrakis(aceoyxymethyl ester)] and Ro31–8425 were from Calbiochem; collagen (type I, from equine tendons) and the luciferin/luciferase bioluminescence kit were from Chrono-Log. Cangrelor was provided by The Medicines Company. Human fibrinogen was from IMCO Corporation. All other reagents were of analytical grade.

The anti-phosphoserine PKC substrate antibody (#2261) was from Cell Signaling Technology, the antibody detecting unmodified pleckstrin (abi17020) was from Abcam, and secondary HRP (horseradish peroxidase)-conjugated goat anti-rabbit and rabbit anti-goat antibodies from Jackson ImmunoReserach and Pierce/Thermo Fisher Scientific respectively.

**Isolation of human platelets**
Heparinized blood (10 IU/ml) was obtained from Linköping University Hospital’s blood bank. Blood was transferred in a volumetric proportion of 5:1 to ACD (aged citric acid/sodium citrate/dextrose; 71 mM citric acid, 85 mM sodium citrate and 111 mM glucose) and centrifuged at 220 g for 20 min. The platelet-rich plasma thus obtained was incubated with 100 μM aspirin for 30 min, supplemented with 0.5 unit/ml apyrase, and platelets were collected by centrifugation at 520 g for 25 min. Platelet pellets were carefully washed three times with KRG (Krebs–Ringer glucose) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄ and 10 mM glucose, pH 7.3), and finally resuspended in KRG containing 1 unit/ml apyrase. The platelet count was determined using an automatic blood cell counter (ABX Diagnostics Micros 60) and was adjusted to a final assay concentration of 2.5 x 10⁵ platelets/ml. Suspensions were supplemented with 1 mM CaCl₂ 30 min prior to experimentation. All isolation steps were carried out at room temperature (21°C).

**Platelet aggregation and ATP release**
Measurements were performed at 37°C using a Chrono-Log Dual Channel lumi-aggregometer (Model 560) with stirring at 800 rev/min using a final volume of 0.5 ml of platelet suspension. Aggregation is expressed as percentage light transmission compared with KRG alone (= 100%). Extracellular ATP was assessed applying the luciferin/luciferase bioluminescence assay and calculated using an exogenously added ATP standard.

**PAR1 surface expression**
Measurements were performed as described previously in detail [17] using a Coulter Epics XL.MCL flow cytometer and Expo 32 ADC software (Beckman Coulter). For the experimental procedure, the Platelet Calibrator kit from Biocytex and the monoclonal anti-PAR1 antibody WEDE15 (Immunotech) were used. Experiments were performed according to the manufacturer’s protocol using diluted citrated whole blood. For every experiment, a calibration curve was created for calculating the number of WEDE15-binding sites. Owing to inter-individual variations in PAR1 expression [17], the number of exposed receptors assessed on resting platelets was set as 100%.

**Increase in cytosolic Ca²⁺**
Platelets were loaded with fura 2 by incubating platelet-rich plasma with 3 μM fura 2/AM for 45 min at room temperature and subsequently isolated as described above. Platelets were pre-incubated and stimulated as indicated at 37°C and fluorescence was recorded using a Hitachi F-7000 spectrofluorimeter at 510 nm with simultaneous excitation at 340 nm and 380 nm. Cytosolic calcium [Ca²⁺] was expressed as a fluorescence ratio (340/380 nm).

**PAI-1 (plasminogen activator inhibitor 1) release**
Platelets were stimulated as indicated at 37°C in a thermoshaker rotating at 600 rev/min in a total volume of 600 μl. Samples were subsequently centrifuged at 14000 g for 10 min at 4°C. Supernatants were analysed applying the tintELIZE PAI-1 kit (Biopool) according to the manufacturer’s protocol using a Multiskan Spectrum spectrophotometer (Thermo/ Electron Corporation, Thermo Fisher Scientific). Experiments were carried out in duplicate and mean values were used to calculate the amount of PAI-1 in ng released from 10⁶ platelets.

**Western blotting**
Stimulation of platelets was carried out at 37°C in a total volume of 200 μl in 2 ml round-bottom tubes in a thermoshaker rotating.
Figure 1  Effects of homologous subthreshold PAR1 and PAR4 activation on platelet aggregation in response to SFLLRN and AYPGKF respectively

Aggregation of aspirinated isolated human platelets was monitored as described in the Experimental section. (A, B, E and F) Original traces of platelet aggregation induced by SFLLRN or AYPGKF at the indicated concentrations, which are representative of ⩾3 independent experiments. (C and D) Histograms of maximal platelet aggregation under the indicated conditions, shown as means ± S.E.M. (A) Platelet aggregation in response to 3, 10 and 30 μM SFLLRN. (B) Upper trace, platelet aggregation provoked by cumulatively added concentrations of 0.1–30 μM SFLLRN as indicated at 3 min intervals. Lower trace, aggregation of platelets exposed to 3 μM SFLLRN for 10 min prior to the addition of 30 μM SFLLRN, followed by 300 μM AYPGKF after another 5 min. (C) Platelets were pre-treated with increasing subthreshold SFLLRN concentrations (0.1–3 μM) for 10 min and subsequently stimulated with 30 μM SFLLRN (n = 3). (D) Platelets were pre-treated with 3 μM SFLLRN for increasing periods of time (2–90 min) as indicated and subsequently challenged with 30 μM SFLLRN (n = 3). (E) Platelet aggregation induced by 30, 100 and 300 μM AYPGKF. (F) Upper trace, platelet aggregation evoked by cumulatively added concentrations of 1–300 μM AYPGKF at 3 min intervals as indicated. Lower trace, platelet aggregation induced by 300 μM AYPGKF after pre-treatment with 30 μM AYPGKF for 10 min.

at 900 rev./min; pre-incubations at 500 rev./min. Reactions were stopped by the addition of 50 μl of 5 × SDS sample buffer, and proteins were denatured at 95°C for 5 min. Proteins were separated on 4–12 % NuPAGE® Novex Bis-Tris gels with Mops running buffer (Invitrogen). To determine apparent molecular protein masses, MagicMark™ XP Western Protein Standard (Invitrogen) was used. Proteins were blotted on to ImmunBlot™ PVDF membranes (0.2 μm) (Bio-Rad Laboratories). For further steps, TBS-T (10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.1 % Tween-20) was used. Membranes were incubated with the anti-phosphoserine PKC substrate antibody followed by HRP-conjugated goat anti-rabbit IgG (both diluted 1:3000). Membranes were stripped and re-probed with the anti-pleckstrin antibody followed by HRP-coupled rabbit anti-goat IgG (both diluted 1:4000). Protein bands were visualized by the use of Immobilon™ Western Chemiluminescent HRP Substrate solution from Millipore, and chemiluminescence was recorded by a Fuji LAS 1000 system (Fuji Photo Film).
Statistical analysis

Data analysis was performed using GraphPad Prism 4 software. Results are presented as means ± S.E.M. and statistical significance was calculated as indicated.

RESULTS

PAR1-mediated platelet aggregation is abolished by homologous receptor activation in a concentration- and time-dependent fashion

Under the conditions used, as shown in Figures 1(A) and 1(E), SFLLRN at 3 μM and AYPGKF at 30 μM induced transient or sustained platelet shape change respectively, whereas 10 and 30 μM SFLLRN as well as 100 and 300 μM AYPGKF induced platelet aggregation.

The susceptibility of PAR1 and PAR4 to undergo desensitization upon successive homologous receptor activation was initially examined assessing platelet aggregation. As shown in Figure 1(B, upper trace) platelets exposed to SFLLRN cumulatively from 0.1 μM up to 30 μM at 3 min intervals lose their ability to form aggregates. Although SFLLRN at 0.1 μM had no effect, the additions of 0.3 and 1 μM provoked a stepwise increase in platelet shape change, indicated as a decrease in light transmission; and SFLLRN at 3 μM induced a slight but reversible shape change. When platelets were subsequently exposed to 10 and 30 μM SFLLRN, they failed to respond.

To determine the conditions under which PAR1-mediated aggregation becomes abrogated, platelets were pre-incubated with increasing concentrations of SFLLRN for various periods of time prior to the addition of 30 μM SFLLRN (Figures 1C and 1D). We observed that platelets incubated with 3 μM SFLLRN for 8–10 min (and up to 90 min) lose their sensitivity towards a subsequent addition of 30 μM SFLLRN. A representative aggregation trace for this phenomenon is shown in Figure 1(B, lower trace). SFLLRN at 3 μM provoked a reversible shape change, and the following addition of 30 μM SFLLRN after 10 min induced a second shape change, but completely failed to provoke aggregation. Nonetheless, platelets still properly responded when finally exposed to 300 μM AYPGKF. It is noteworthy that the above described effects were likewise observed in non-aspirinated platelets (results not shown), as well as in platelet suspension supplemented with exogenous fibrinogen (100 μg/ml) (compare with Figure 7C).

In contrast, no effects on platelet aggregation were observed when platelets were stimulated with AYPGKF (Figure 1F).

In cumulative dose–response studies, applying AYPGKF from 1 μM up to 300 μM (Figure 1F, upper trace), we observed a stepwise increase in shape change up to 30 μM, followed by platelet aggregation triggered by 100 μM AYPGKF, which was even further enhanced by subsequently added 300 μM AYPGKF. Likewise, and opposite to the observation made when applying SFLLRN, pre-incubation with the subthreshold concentration of 30 μM AYPGKF for 10 min did not affect platelet aggregation induced by 300 μM AYPGKF (Figure 1F, lower trace); which was likewise true when platelets were pre-exposed to 30 μM AYPGKF for up to 60 min (results not shown). Furthermore, exposure of platelets to 60 μM AYPGKF, a concentration close to the threshold for initiating aggregation, did not cause PAR4 desensitization, as platelets responded to subsequently added 300 μM AYPGKF (results not shown). Finally, the extent of platelet aggregation induced by 300 μM AYPGKF in cumulative dose–response studies was similar to that provoked by this concentration applied alone.

Figure 2 Platelet PAR1 surface expression upon homologous receptor activation

Diluted whole blood was stimulated with 3 μM (A) or 30 μM (B) SFLLRN for various periods of time as indicated and and PAR1 surface expression was assessed as described in the Experimental section. Owing to inter-individual variations [17], PAR1 surface expression on untreated platelets was set as 100 %. Results are given as means ± S.E.M. for n = 4 individual experiments.

The loss in responsiveness is not due to PAR1 internalization

Owing to the results presented above, we next evaluated whether the loss in responsiveness is due to PAR1 internalization. As shown in Figure 2, we did not observe a decrease, but rather a transient increase of surface PAR1 receptors when platelets were stimulated with either 3 μM (Figure 2A) or 30 μM SFLLRN (Figure 2B) for up to 60 min. When platelets were exposed to 3 μM SFLLRN (Figure 2A), we observed an elevation in PAR1 surface expression within 5–10 min from 100 % (control) to 126 ± 9 % and 128 ± 27 % respectively, followed by a decrease to basal expression of an average of 103 ± 3 % after 60 min. In platelets stimulated with 30 μM SFLLRN (Figure 2B), PAR1 exposure reached a maximum of 131 ± 16 % after 2 min of incubation, and a decrease to basal expression levels of 102 ± 7 % was observed within 10 min.

PAR1 signalling desensitization affects Ca2+ mobilization in a PKC-dependent manner

An immediate platelet response to PAR1 activation is the mobilization of Ca2+ from intracellular stores and influx of Ca2+ across the plasma membrane. As such, we investigated the impact of the observed desensitizing effect on PAR1 signalling on the elevation of intracellular Ca2+-concentrations; results are given as fluorescence ratios (340/380 nm). As shown in Figure 3(A), pre-exposure of platelets to 3 μM SFLLRN for 10 min decreased the response to 30 μM SFLLRN by approximately 50 % (from 3.1 ± 0.2 to 1.5 ± 0.2), which was not further diminished when platelets were pre-treated for up to 30 min (results not shown).

It has been reported that PKC signalling contributes to desensitize PAR1 [18,19]. To preclude this effect, platelets were pre-incubated with the pan-PKC inhibitors Ro31-8220 and Ro31-8425 for 5 min prior to the exposure to 3 μM SFLLRN. Figure 3(B) shows representative traces of Ca2+ mobilization induced by 30 μM SFLLRN, the effects of PKC inhibition by 1 μM Ro31-8425 on PAR1 desensitization, as well as a summary of the results obtained as a histogram. In non-pre-treated platelets, the response to 30 μM SFLLRN was slightly increased when pre-incubated with 0.3 μM Ro31-8220 or 1 μM Ro31-8425. In this set of experiments, the response induced by 30 μM SFLLRN in PAR1 signalling-desensitized platelets was diminished by approximately 60 %, and both Ro31-8220 and Ro31-8425 partially but significantly re-established Ca2+ mobilization. In parallel, we assessed platelet aggregation in this experimental setting (Figure 3C). As expected, both 0.3 μM Ro31-8220 and
Figure 3  Effects of PAR1 signalling desensitization on SFLLRN-induced intracellular Ca$^{2+}$ mobilization: involvement of PKC

(A) Histogram of maximal peak rises in intracellular Ca$^{2+}$ under the indicated conditions, determined as described in the Experimental section, in means ± S.E.M. Left-hand panel: aspirinated isolated platelets were incubated with 3 μM SFLLRN for increasing periods of time (up to 10 min) followed by stimulation with 30 μM SFLLRN (n = 3). Right-hand panel: platelets were pre-treated with increasing subthreshold SFLLRN concentrations (0.1–3 μM) for 10 min and subsequently stimulated with 30 μM SFLLRN (n = 3). (B) Left-hand panel: original traces of platelet intracellular Ca$^{2+}$ mobilization. Platelets were stimulated with 30 μM SFLLRN alone (upper trace), pre-treated with 3 μM SFLLRN for 10 min prior to the addition of 30 μM SFLLRN (central trace), or pre-incubated with 1 μM Ro31–8425 for 5 min, followed by incubation with 3 μM SFLLRN for 10 min and stimulation with 30 μM SFLLRN (lower trace). Right-hand panel: the summarized data (n = 4) of intracellular Ca$^{2+}$ mobilization determined in platelets incubated with buffer or 3 μM SFLLRN for 10 min prior to the addition of 30 μM SFLLRN (left-hand bars), as well as the effects of pre-incubation for 5 min with either 0.3 μM Ro31–8220 (central bars) or 1 μM Ro31–8425 (right-hand bars) before adding 3 and 30 μM SFLLRN (P values were determined by two-tailed paired Student's t tests and significances were defined as *P ≤ 0.05, **P ≤ 0.005 and ***P ≤ 0.0005; ns, not significant). Under these experimental conditions, platelet aggregation was also monitored (C), and results are expressed as maximal induced aggregation (means ± S.E.M.) for n = 5–7 individual experiments. All samples in (B) and (C) contained 0.1 % DMSO as the Ro-compound solvent control.

1 μM Ro31–8425 decreased platelet aggregation induced by 30 μM SFLLRN in non-desensitized platelets. Pre-exposure of platelets to 3 μM SFLLRN abolished aggregation in response to 30 μM SFLLRN, and Ro31–8220 or Ro31–8425 had only a marginal or no effect respectively.

Finally, when both PKC inhibitors were applied at 10 μM, they did not reconstitute Ca$^{2+}$ mobilization further, compared with the extents shown in Figure 3(B) (results not shown); but, as expected, almost completely inhibited platelet aggregation (results not shown; for the effects of Ro31–8425 see Figure 7B).

PAR1 signalling desensitization affects PKC signalling

Owing to the above reported observation, the impact of PAR1 signalling desensitization on that of PKC was evaluated by Western blotting applying an anti-phosphoserine PKC substrate.
Ro31-8425, concentrations of 1 and 3 μM applied compounds on platelet aggregation (Figure 3C).

Ro31-8425 (both 0.3–10 μM) inhibited phosphorylation induced by 30 μM SFLLRN for 10 min prior to stimulation with 3, 10 or 30 μM SFLLRN for 30 s with stirring. Reactions were stopped by the addition of SDS sample buffer. Samples were analysed by SDS/PAGE followed by Western blotting as described in the Experimental section using antibodies recognizing serine-phosphorylated PKC substrates or unmodified pleckstrin. The blot shown is from one experiment that is representative of at least three independent experiments. Chemiluminescence was assessed at various time points and results obtained at differing exposure times are divided by the dotted lines (applying the anti-phosphoserine PKC substrate antibody: upper part 10 min, lower parts 20 s; anti-pleckstrin antibody: 2 s). Molecular mass markers are shown on the left-hand side in kDa.

Figure 4 Effect of PAR1 signalling desensitization on SFLLRN-induced PKC substrate serine phosphorylation

Aspirinated isolated human platelets were pre-incubated at 37°C with 3 μM SFLLRN (or buffer) for 10 min prior to stimulation with 3, 10 or 30 μM SFLLRN for 30 s with stirring. Reactions were stopped by the addition of SDS sample buffer. Samples were analysed by SDS/PAGE followed by Western blotting as described in the Experimental section using antibodies recognizing serine-phosphorylated PKC substrates or unmodified pleckstrin. The blot shown is from one experiment that is representative of at least three independent experiments. Chemiluminescence was assessed at various time points and results obtained at differing exposure times are divided by the dotted lines (applying the anti-phosphoserine PKC substrate antibody: upper part 10 min, lower parts 20 s; anti-pleckstrin antibody: 2 s). Molecular mass markers are shown on the left-hand side in kDa.

Antibody. A detailed time course of PKC substrate serine phosphorylation up to 20 min is shown in Supplementary Figure S1(A; http://www.BiochemJ.org/bj/436/bj4360469add.htm) and as the maximal phosphorylation pattern detected peaked after 30 s of stimulation, this time point was chosen for further experiments.

As illustrated in Figure 4, we observed that the subthreshold concentration of 3 μM SFLLRN provoked a restricted and likewise transient pattern of PKC substrate protein serine phosphorylation (lanes 3 and 2 respectively) compared with that detected with 10 and 30 μM SFLLRN (lanes 5 and 7). Exposure of platelets to 3 μM SFLLRN for 10 min prior to a following addition of 3, 10 or 30 μM SFLLRN distinctly, albeit not completely, reduced the number and extent of phosphorylated proteins, including that of pleckstrin (lanes 4, 6 and 8), indicating that residual PKC activation downstream of PAR1 signalling could still occur.

In addition, as shown in Supplementary Figure S2 (at http://www.BiochemJ.org/bj/436/bj4360469add.htm), we evaluated the specificity of the anti-phosphoserine PKC substrate antibody as well as the PKC inhibitors applied. Ro31-8220 and Ro31-8425 (both 0.3–10 μM) inhibited phosphorylation induced by 30 μM SFLLRN of a whole set of detected proteins in a concentration-dependent fashion, except that of one unidentified protein of approximately 160 kDa. Phosphorylation of pleckstrin was already markedly affected by 0.3 μM and completely abolished by 1 μM Ro31-8220. To obtain similar results with Ro31-8425, concentrations of 1 and 3 μM respectively were required, which corresponds to the inhibitory potencies of the applied compounds on platelet aggregation (Figure 3C).

Desensitized PAR1 signalling is re-sensitized via PAR4 signalling in a Ca²⁺- and Rho-kinase-dependent manner

Most surprisingly, as shown in Figure 5(A), we found that in PAR1 signalling-desensitized platelets, the addition of the subthreshold concentration of 30 μM AYPGKF re-established aggregation in response to 30 μM SFLLRN (Figure 5A, lower trace), whereas when AYPGKF was added alone it had no effect (Figure 5A, upper trace). Notably, this re-sensitizing effect strongly relied on the sequence of addition of AYPGKF and SFLLRN. Intermediate incubation with 30 μM AYPGKF from 5 min up to 5 s prior to 30 μM SFLLRN reconstituted platelet aggregation, which was likewise observed when AYPGKF was added 30 s after SFLLRN. In contrast, when AYPGKF was added 2 min after 30 μM SFLLRN, this reconstituting effect was no longer observed (results not shown).

Regarding Gα12/13-mediated calcium and Rho-kinase signalling pathways, and as shown in Figure 5(B), this re-sensitizing effect was abolished or prominently diminished by pre-incubating platelets with the cytosolic Ca²⁺-chelator BAPTA/AM (10 μM) or the Rho-kinase inhibitor Y27632 (10 μM) respectively.

To investigate whether the observed reconstitution of platelet aggregation by 30 μM AYPGKF was due to a synergistic effect of subthreshold PAR4 signalling and residual PAR1 signalling, we replaced AYPGKF with the TXA₂ mimetic U46619. As mentioned above, platelet TXA₂ signalling is mediated via TPα receptors, which, similar to PAR1 and PAR4, induces Gα12/13 and Gαi signalling. As demonstrated in Figure 5(C), U46619 at 0.03 μM, although inducing an approximately 5-fold higher Ca²⁺ mobilization compared with 30 μM AYPGKF (Figure 5C, inset), did not re-establish aggregation in PAR1 signalling-desensitized platelets.

The lack of ADP release and subsequent P2Y₁₂ receptor signalling accounts for the loss of platelet aggregation

ADP released from dense granules and subsequent P2Y₁₂ receptor-mediated Gαi signalling plays a pivotal role in platelet aggregation in response to all known agonists, including thrombin. Therefore, besides Ca²⁺ mobilization (Figure 6A) and α-granule secretion (by assessing PAI-1 release) (Figure 6B) [20,32], platelet dense granules secretion (in terms of ATP release) under de- and re-sensitizing conditions was assessed (Figure 6C).

Peptide concentrations which de- and re-sensitize PAR1 signalling, i.e. 3 μM SFLLRN and 30 μM AYPGKF, when added alone, induced minor initial mobilization of Ca²⁺ and PAI-1 release, whereas no ATP release was detected. Furthermore, under PAR1 signalling-desensitized conditions, 30 μM SFLLRN still elicits Ca²⁺ mobilization and approximately half-maximal PAI-1 release, indicating that residual PAR1 signalling still elicits minor cellular responses, but again no ATP release was observed. Finally, the intermediate addition of 30 μM AYPGKF resulted in a slight but significant increase in Ca²⁺ mobilization compared with 30 μM AYPGKF (Figure 5C, inset), did not re-establish aggregation in PAR1 signalling-desensitized platelets.

In addition, when mimicking Gαi signalling by replacing 2-MeS-ADP with 10 μM epinephrine, platelet aggregation was likewise fully restored. Taken together, these findings indicate that the loss in responsiveness in PAR1 signalling-desensitized platelets is due to a lack of ADP release and P2Y₁₂ receptor Gαi signalling. In addition, we have demonstrated that residual PAR1 signalling is still present, as neither 0.01 μM 2-MeS-ADP nor...
Figure 5  Effects of intermeditated subthreshold PAR4 activation on SFLRN-induced aggregation of PAR1 signalling-desensitized platelets: impacts of the inhibition of intracellular Ca\textsuperscript{2+} mobilization or Rho kinase signalling, and effects of intermeditated, subthreshold TP\textsubscript{α} activation on SFLRN-induced aggregation of PAR1 signalling-desensitized platelets

Aggregation of aspirated isolated human platelets was assessed as described in the Experimental section. (A) Platelets were stimulated with 30 \( \mu \text{M} \) SFLRN alone (left-hand trace), or with 3 \( \mu \text{M} \) SFLRN for 10 min followed by 30 \( \mu \text{M} \) AYPGKF (right-hand upper trace), or by 30 \( \mu \text{M} \) AYPGKF for 30 s and finally with 30 \( \mu \text{M} \) SFLRN (right-hand lower trace). Traces are representative of at least three independent experiments. (B) Platelets were, as indicated, incubated with buffer or 3 \( \mu \text{M} \) SFLRN for 10 min followed by treatment with 10 \( \mu \text{M} \) BAPTA/AM or 10 \( \mu \text{M} \) Y27632 for 5 min. Platelets were subsequently stimulated with 30 \( \mu \text{M} \) SFLRN alone or with 30 \( \mu \text{M} \) AYPGKF for 30 s followed by 30 \( \mu \text{M} \) SFLRN respectively. Maximal aggregation for \( n = 3 \)–5 independent experiments are given as means ± S.E.M.; all samples contained 0.1 % DMSO as solvent control for BAPTA/AM. (C) Platelets were stimulated with 30 \( \mu \text{M} \) SFLRN (left-hand trace) or 0.03 \( \mu \text{M} \) U46619 (right-hand upper trace) alone or incubated with 3 \( \mu \text{M} \) SFLRN for 10 min followed by 0.03 \( \mu \text{M} \) U46619 (right-hand middle trace). Finally, as shown in the right-hand lower trace, platelets were incubated with 3 \( \mu \text{M} \) SFLRN for 10 min followed by 0.03 \( \mu \text{M} \) U46619 for 30 s prior to 30 \( \mu \text{M} \) SFLRN. The original traces shown are representative of \( n = 5 \) independent experiments. The inset shows original traces of platelet intracellular Ca\textsuperscript{2+} mobilization (in fluorescence ratio 340/380 nm) induced by either 30 \( \mu \text{M} \) AYPGKF or 0.03 \( \mu \text{M} \) U46619, and summarized data are given as means ± S.E.M. for \( n = 3 \) independent experiments.

10 \( \mu \text{M} \) epinephrine when added alone, or when added 10 min after 3 \( \mu \text{M} \) SFLRN, evoke platelet aggregation. Finally, the effect of epinephrine was abolished by the \( \alpha_{2A} \) adrenoceptor antagonist yohimbine (1 \( \mu \text{M} \)); and the reconstituting effect of 30 \( \mu \text{M} \) AYPGKF on aggregation of desensitized platelets induced by 30 \( \mu \text{M} \) SFLRN was, as to be expected, completely abrogated in the presence of the specific P2Y\textsubscript{12} receptor antagonist cangrelor (10 \( \mu \text{M} \)) (Figure 6E).

Desensitization of PAR1 signalling-induced platelet aggregation as well as its re-sensitization via PAR4 strictly relies on homologous receptor activation

Owing to the findings presented above, we evaluated a panel of other platelet agonists regarding their impact on PAR1 signalling de- and re-sensitization. Therefore we applied agonist concentrations which were beforehand defined to evoke minor
Figure 6 Effects of intermediate subthreshold PAR4 activation on SFLLRN-provoked intracellular Ca\(^{2+}\) mobilization, PAI-1 release and ATP release in PAR1 signalling-desensitized platelets, and effects of 2-MeS-ADP and epinephrine on aggregation of PAR1 signalling-desensitized platelets challenged with SFLLRN

(A, B and C) Platelets were stimulated with 3 \(\mu\)M SFLLRN, 30 \(\mu\)M AYPGKF or 30 \(\mu\)M SFLLRN alone, or with 3 \(\mu\)M SFLLRN for 10 min followed by 30 \(\mu\)M AYPGKF for 30 s prior to 30 \(\mu\)M SFLLRN. Intracellular Ca\(^{2+}\) mobilization (A), PAI-1 release (B) and ATP release (C) were monitored as described in the Experimental section and results are given as means ± S.E.M. for \(n = 3\) (A and C) or \(n = 5\) (B) individuals. P values were determined by two-tailed paired Student's t tests and significances were defined as *\(P \leq 0.05\) and **\(P \leq 0.005\).

(D) Aggregation of aspirinated isolated human platelets was assessed as described in the Experimental section. Platelets were stimulated with 30 \(\mu\)M SFLLRN (left-hand trace), 0.01 \(\mu\)M 2-MeS-ADP (centre upper trace) or 10 \(\mu\)M epinephrine (right-hand upper trace) alone. Platelets were incubated with 3 \(\mu\)M SFLLRN for 10 min followed by 0.01 \(\mu\)M 2-MeS-ADP (central middle trace) or 10 \(\mu\)M epinephrine (right-hand middle trace). Finally, platelets were incubated with 3 \(\mu\)M SFLLRN for 10 min followed by 0.01 \(\mu\)M 2-MeS-ADP (central lower trace) or 10 \(\mu\)M epinephrine (right-hand lower trace) for 30 s prior to 30 \(\mu\)M SFLLRN. (E) Platelets were either stimulated with 30 \(\mu\)M SFLLRN alone, pre-incubated with 3 \(\mu\)M SFLLRN for 10 min followed by 30 \(\mu\)M AYPGKF, 0.01 \(\mu\)M 2-MeS-ADP or 10 \(\mu\)M epinephrine respectively, before the addition of 30 \(\mu\)M SFLLRN. In the experiments performed with 10 \(\mu\)M cangrelor or 1 \(\mu\)g/ml collagen did not affect platelet aggregation in response to 30 \(\mu\)M SFLLRN. Furthermore, none of these agonists tested was able to replace AYPGKF in its capability to re-sensitize PAR1 signalling, whereas the reconstituting effects of 2-MeS-ADP and epinephrine (compare Figures 6D and 6E) are rather due to substituting or mimicking the lack of ADP release and P2Y\(_{12}\) receptor-mediated G\(_{\alpha_i}\) signalling than to an intracellular molecular mechanism.

The re-sensitizing effect of PAR4 signalling on that of desensitized PAR1 relies on the reconstitution of PKC-mediated dense and \(\alpha\)-granule secretion

PKC signalling is essentially implicated in platelet activation by signalling mechanisms leading to the secretion of dense and \(\alpha\)-granules. As shown in Figure 7(A), and with respect to Figure 4, we observed that the PAR1 signalling desensitization-dependent
Figure 7 Effects of intermediate PAR4 signalling on SFLLRN-induced PKC substrate serine phosphorylation in PAR1 signalling-desensitized platelets: effects of 2-MeS-ADP and fibrinogen substitution on platelet aggregation provoked by SFLLRN in PAR1 signalling de- and re-sensitized platelets pre-treated with the PKC inhibitor Ro31 – 8425

(A) Aspirinated isolated human platelets were pre-incubated and stimulated with SFLLRN and AYPGKF as indicated at 37 °C with stirring. Reactions were stopped by the addition of SDS sample buffer. Samples from three individual experiments were pooled (1:1:1) and analysed by SDS/PAGE followed by Western blotting as described in the Experimental section using antibodies recognizing serine-phosphorylated PKC substrates or unmodified pleckstrin. Chemiluminescence was assessed at various time points and results obtained at differing exposure times are divided by the broken line (applying the anti-phosphoserine PKC substrate antibody: upper section, 20 s; lower section, 30 s; and anti-pleckstrin antibody, 1 s). (B and C) Aggregation of aspirinated isolated human platelets was monitored as described in the Experimental section. Pre-treatments and stimulations were carried out as indicated in the absence (B) or presence (C) of exogenously added 100 μg/ml fibrinogen. The summarized data show aggregation extents in means ± S.E.M. for n = 3 individual experiments assessed after 3 min of stimulation with 30 μM SFLLRN. All samples contain 0.1 % DMSO as solvent control for Ro31-8425.

prominent reduction of PKC substrate serine phosphorylation provoked by increasing concentrations of SFLLRN were essentially reconstituted by intermediate subthreshold PAR4 activation.

We also observed that PAR1-mediated rapid and transient PKC signalling was substantially prolonged when platelets were co-stimulated with 30 μM AYPGKF (Supplementary Figure S1). As little is known about signalling pathways specifically induced by either PAR1 or PAR4, it will remain difficult to differentiate whether this effect is due to a more sustained signalling via PAR1 by counteracting its signalling desensitization via PAR4, or rather to a synergistic or biphasic effect.
Table 1 Impact of various platelet agonists on PAR1 de- and re-sensitization on platelet aggregation induced by SFLLRN

<table>
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<tr>
<th>Agonist</th>
<th>Desensitizing effect on PAR1-mediated platelet aggregation</th>
<th>Re-sensitizing effect on PAR1-mediated platelet aggregation</th>
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<tr>
<td>3 μM SFLLRN</td>
<td>+</td>
<td>-</td>
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<tr>
<td>30 μM AYPGKF</td>
<td>-</td>
<td>+</td>
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<tr>
<td>0.03 μM U46619</td>
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<td>-</td>
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<tr>
<td>10 μM LPA</td>
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<td>20 μM serotonin</td>
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<td>0.5 mg/ml AGP</td>
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<td>-</td>
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<tr>
<td>0.01 μM 2-MeS-ADP</td>
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<td>(+)</td>
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<tr>
<td>10 μM epinephrine</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>2 μM α,β-MeATP</td>
<td>-</td>
<td>-</td>
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<td>1 μg/ml collagen</td>
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However, we further investigated the role of PKC in the mechanism(s) by which PAR4 signalling reconstitutes aggregation of PAR1 signalling-desensitized platelets in more detail (Figures 7B and 7C). In the isolated platelet suspension, the only source for fibrinogen is that which is stored in the α-granules. As PKC signalling is prominently involved in both dense and α-granule secretion, we performed our experiments in the absence or presence of the pan-PKC inhibitor Ro31–8220 (10 μM) and with exogenously added fibrinogen (100 μg/ml) (Figures 7B and 7C).

Under non-desensitized conditions (left-hand panels), platelet aggregation induced by 30 μM SFLLRN is completely or prominently inhibited by Ro31–8425, in the absence or presence respectively, of exogenously added fibrinogen. This has been found to be owing to the lack of both dense and α-granule secretion, as mimicking dense granule secretion by the addition of 0.01 μM 2-MeS-ADP fails to reconstitute aggregation in response to 30 μM SFLLRN in the absence of fibrinogen, whereas when fibrinogen was supplemented, full platelet aggregation occurred.

When PAR1 signalling was desensitized by pre-treated with 3 μM SFLLRN (right-hand panels), the second treatment with 30 μM SFLLRN fails to induce aggregation even in the presence of exogenously added fibrinogen, suggesting that under these conditions ADP released from dense granules and P2Y₁₂ receptor-mediated Gαq signalling is lacking, which supports our findings shown in Figure 6(C). Intermediate PAR4 signalling induced by 30 μM AYPGKF re-established SFLLRN-triggered aggregation of PAR1 signalling-desensitized platelets in the absence of supplemented fibrinogen by approximately 70 %, and fully when fibrinogen was added in excess, indicating that secretion of both types of granules preceded the aggregation response (compare Figure 6B and 6C). This reconstitution of aggregation was nonetheless almost completely abrogated under conditions of PKC signalling inhibition by Ro31–8425, which, in turn, was partially overcome by mimicking dense granule secretion in the absence of supplemented fibrinogen, and completely when α-granule secretion was mimicked by fibrinogen supplementation.

Taken together, these findings clearly demonstrate that PAR1 signalling desensitization results in a reduction in PKC signalling-mediated granule secretion, and that this effect can be re-established by intermediate PAR4 signalling. In addition, as 0.01 μM 2-MeS-ADP alone (compare Figure 6D), even in the presence of exogenously added fibrinogen, did not induce platelet aggregation (results not shown), these findings once more show that residual PAR1 signalling upstream of PCK is still present.

**DISCUSSION**

Since the discovery that human platelets express PAR subtypes 1 and 4, and that both couple to the same G-protein subtypes, many efforts have been undertaken to elucidate differences in PAR1 and PAR4 signalling to understand the functional relevance for receptor co-expression.

In the present study, we found that human platelets lose their ability to aggregate in response to the PAR1-specific peptide SFLLRN upon homologous subthreshold receptor activation. This effect was not due to a general decline in platelet sensitivity, as SFLLRN-pre-treated platelets still responded normally to the PAR4-agonistic peptide AYPGKF.

In several other cell types, PAR1 activation results in rapid receptor phosphorylation via negative-regulatory feedback loops by activated PKC as well as GPCR kinases, β-arrestin-binding, and clathrin- and dynamin-dependent receptor internalization [22]. In human platelets, PAR1, besides being exposed on the cell surface, is also present in the open canalicular system from which receptors are additionally exposed upon platelet aggregation. As such, the number of detectable PAR1 receptors may reflect the net effect of internalized and newly exposed receptors. Whereas there are diverging findings regarding platelet PAR1 surface density upon various stimulations [17,23,24], in the present study we observed no decrease, which is in accordance with a previous report [25], but a transient increase in PAR1 surface expression in SFLLRN-stimulated platelets.

We observed further that Ca²⁺ mobilization provoked by 30 μM SFLLRN was markedly reduced in PAR1 signalling-desensitized platelets. As mentioned above, one mechanism in PAR1 signalling shut-off involves PKC-mediated receptor phosphorylation. In endothelial cells, it has been shown that the direct activation of PKC with the phorbol ester PMA precludes thrombin-induced Ca²⁺ mobilization [19], an effect that was also found in platelets pre-treated with PMA and challenged with SFLLRN (M. Grenegård, unpublished work). Furthermore, PKC activation can also lead to ‘heterologous’ forms of receptor desensitization [26]. However, when applying a panel of platelet agonists which induce PKC activation either via Gαq signalling, such as AYPGKF and the TXA₂ mimetic U46619, or via G-protein-unrelated signalling, such as collagen-induced GPV1 (glycoprotein VI) signalling, we observed no effects on PAR1-induced platelet aggregation.

Nonetheless, when PKC signalling was inhibited by moderate concentrations of the pan-PKC inhibitors Ro31–8220 and Ro31–8425, the reduction in Ca²⁺ mobilization observed in PAR1 signalling-desensitized platelets was slightly but significantly abrogated. At high inhibitor concentrations, which completely abolish PKC substrate phosphorylation and platelet aggregation, Ca²⁺ mobilization was not further re-established, suggesting that a PKC-independent mechanism may account for most of the loss in PAR1-induced Ca²⁺ mobilization capacity.

However, PKC signalling is crucially involved in platelet activation and aggregation by, besides other events, promoting platelet granule secretion. Especially, the release of ADP from dense granules and subsequent P2Y₁₂ receptor activation play a pivotal role in platelet aggregation in response to all known...
agonists [8]. Activation of pleckstrin, one of the most prominent platelet PKC substrates, is, at least in part, associated with granule secretion [27]. We found that PAR1-mediated PKC substrate serine phosphorylation, including that of pleckstrin, was markedly affected in PAR1 signalling-desensitized platelets, and that α-granule secretion was approximately 55% reduced, whereas secretion of dense granules was completely abolished. Indeed, we identified that the loss of PAR1-induced platelet aggregation was due to a lack of ADP release and subsequent P2Y12 receptor-mediated Gαs-coupling, as supplementing ADP (in form of 2-MeS-ADP) fully re-established aggregation. In addition, 2-MeS-ADP could be replaced by epinephrine, which via α2A adrenoceptors likewise induces signalling via inhibitory Gαi proteins [28].

Neither epinephrine [29] nor 0.01 μM 2-MeS-ADP alone induced aggregation of isolated platelets, indicating that PAR1 signalling unrelated to, or upstream of, mechanisms involved in secretion is still functional. Along this line, it has been demonstrated in mouse platelets lacking Gαs, which is required for granule secretion [5], that concomitant signalling via Gα12/13 and P2Y12 receptor Gαs-coupling is sufficient for inducing integrin αIIbβ3 activation and platelet aggregation [30].

In a Rat1 fibroblast model, it was shown that PAR4 does not undergo agonist-promoted phosphorylation and internalizes much slower than PAR1 [16]. Accordingly, we did not observe any signs of desensitization in PAR4-mediated aggregation either in cumulative dose–response studies or when platelets were pre-exposed to subthreshold concentrations of AYPGKF, which is, however, the opposite of that reported previously [31].

Most intriguingly, we found that AYPGKF at a subthreshold concentration of 30 μM re-established SFLLRN-induced platelet aggregation in PAR1 signalling-desensitized platelets. AYPGKF at this concentration alone induced only marginal Ca2+ mobilization and α-granule secretion, as well as prominent and sustained platelet shape change, but no dense granule secretion, indicating that the responses observed presumably were evoked by Gα12/13 signalling. And indeed, the inhibition of either Ca2+-related or Rho-kinase signalling abolished its re-constitutive effect.

Furthermore, in PAR1 signalling-desensitized platelets, intermediate PAR4 signalling, although almost fully re-establishing α-granule secretion, only slightly enhanced both residual Ca2+ mobilization and reconstituted dense granule secretion in response to 30 μM SFLLRN. Given a ratio of 1:5 in ADP/ATP content in dense granules [32], it could be roughly estimated that 0.45 μM ADP is released, which is well above the applied concentration sufficient for the reconstitution of platelet aggregation.

With respect to PKC signalling, we observed that intermediate PAR4 signalling likewise reconstituted SFLLRN-induced protein serine phosphorylation. If this effect was directly linked to signalling events downstream of PAR1/PAR4, or to indirect effects such as ADP release and subsequent P2Y12 signalling [33], or fibrinogen-binding and integrin αIIbβ3 outside-in signalling [34,35] was beyond the scope of the present study and not further investigated.

PKC was nonetheless identified as the key enzyme affected in PAR1 signalling-desensitization and its re-sensitization via PAR4, and that this latter effect, besides the re-constitution of dense granules secretion, also strongly relied on the release of fibrinogen from α-granules. Regarding the PKC isoform most likely involved, it has been demonstrated in a mouse model that PKCo is a major regulator of dense and α-granule secretion [36].

Finally, it is important to note that the reconstitution of PAR1 signalling, on a molecular level, was exclusively limited to PAR4 signalling (Table 1). One might argue that the reconstituting effect of intermediate PAR4 signalling might merely be due to a synergism of subthreshold PAR4 and residual PAR1 signalling. However, when PAR4 signalling was substituted by the closely related TPα signalling, no re-sensitization of PAR1 signalling could be observed, although the TXA2 mimic U46619 at the applied concentration (0.03 μM) induced an approximately 5-fold higher Ca2+ mobilization compared with 30 μM AYPGKF. In addition, in non-desensitized platelets, 30 μM AYPGKF did not augment Ca2+ mobilization evoked by 3 or 30 μM SFLLRN (Supplementary Figure S3 at http://www.BiochemJ.org/bj/436/bj4360469add.htm). Furthermore, when we replaced AYPGKF with AGP, which has been shown to induces platelet shape change via prominent Rho-kinase signalling independently of Ca2+ mobilization [21], we likewise did not observe any reconstituting effect (results not shown; Table 1). Taken together, these findings strongly imply that the observed effect of PAR4 signalling may not simply be explained by synergistic effects.

In contrast, as we observed that the effect of intermediate PAR4 signalling was abolished by inhibiting Rho-kinase signalling as well as by suppressing Ca2+-mediated signalling, whereas U46619, which likewise induces both signalling cascades had no re-constituting effect, we cannot completely rule out a synergistic effect of residual PAR1 and subthreshold PAR4 signalling. To clarify this option, however, an artificial disturbance of the PAR1–PAR4 heterodimeric receptor complex would be required.

In summary, our results of the present study demonstrate that PAR1 signalling desensitization results in a marked diminution of PKC signalling-mediated granule secretion, and that this effect can be re-established by intermediate PAR4 signalling. Although the specific underlying molecular mechanism(s) remain to be elucidated, our findings provide further information about substantial differences in human platelet PAR1 and PAR4 signalling, and may suggest that the co-expression of PAR4 might be crucial for counteracting mechanisms involved in shutting off PAR1 signalling.

AUTHOR CONTRIBUTION
Knut Fälker, Linda Haglund, Peter Gunnarsson, Martina Nylander, and Magnus Grenegård designed the experiments, and performed research and data analysis. Tomas Lindahl designed the experiments, and performed research and data analysis. Tomas Lindahl provided critical input and helpful discussion, and contributed to the experimental design. Magnus Grenegård and Knut Fälker interpreted results, directed the research and drafted the manuscript; Knut Fälker wrote the manuscript.

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REFERENCES
SUPPLEMENTARY ONLINE DATA

Protease-activated receptor 1 (PAR1) signalling desensitization is counteracted via PAR4 signalling in human platelets

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PKC substrate serine phosphorylation induced by SFLLRN via PAR1 is substantially prolonged in platelets co-stimulated with the PAR4-activating peptide AYPGKF

SFLLRN at 30 μM, as shown in Figure 4(A) of the main text, and also in Figure S1(A), provokes a rapid and transient serine phosphorylation of a broad set of proteins including that of pleckstrin. AYPGKF at the subthreshold concentration of 30 μM induces phosphorylation of a limited set of proteins, in a delayed but sustained manner (Figure S1C, compare also Figure 7A of the main text). When platelets were stimulated with the combination of both 30 μM SFLLRN and 30 μM AYPGKF (Figure S1B), phosphorylation of the set of proteins induced by SFLLRN alone (Figure S1A) was markedly prolonged.

The pan-PKC inhibitors Ro31-8220 and Ro31-8425 concentration-dependently affect PKC substrate serine phosphorylation provoked by SFLLRN

To evaluate the specificity of the anti-phosphoserine PKC substrate antibody as well as the PKC inhibitors applied, platelets were incubated with increasing concentrations of Ro31-8220 and Ro31-8425 (up to 10 μM) prior to the stimulation with 30 μM SFLLRN. Both compounds inhibited phosphorylation of the set of detected proteins in a concentration-dependent fashion, except that of one unidentified protein of approximately 160 kDa (Figure S2). Phosphorylation of pleckstrin was markedly affected by 0.3 μM and completely abolished by 1 μM Ro31-8220, whereas respective concentrations of 1 μM and 3 μM Ro31-8425 were required to obtain similar results.

Subthreshold PAR4 activation provoked by 30 μM AYPGKF does not synergize with SFLLRN-induced Ca²⁺ mobilization under non-desensitized conditions

In PAR1 signalling-desensitized platelets, the subthreshold concentration of 30 μM AYPGKF partially, but significantly, reconstituted Ca²⁺ mobilization provoked by 30 μM SFLLRN (see Figure 6A in the main text). Therefore we investigated whether pre-activation of PAR4 results in an enhancement of PAR1-induced Ca²⁺ mobilization under normal (non-desensitized) conditions. As shown in Figure S3, pre-stimulation of platelets for 30 s with AYPGKF did not affect the peak rise in Ca²⁺ induced by 3 or 30 μM SFLLRN, demonstrating that PAR4 signalling, at least in terms of Ca²⁺ mobilization, does not synergize with that of PAR1.

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K. Fälker and others

Figure S1 PKC substrate serine phosphorylation induced by SFLLRN via PAR1 is substantially prolonged in platelets co-stimulated with the PAR4-activating peptide AYPGKF

Aspirated isolated human platelets were stimulated as indicated at 37 °C with stirring. Reactions were stopped by the addition of SDS sample buffer. Samples were analysed by SDS/PAGE followed by Western blotting as described in the Experimental section of the main text using antibodies recognizing serine-phosphorylated PKC substrates or unmodified pleckstrin. Platelets were stimulated for the indicated periods of time with 30 μM SFLLRN (A), 30 μM SFLLRN and 30 μM AYPGKF (B) or 30 μM AYPGKF (C). The blot shown is from one experiment that is representative of three independent experiments. Chemiluminescence was assessed at various time points and results obtained at differing exposure times are divided by the broken lines (for the anti-phosphoserine PKC substrate antibody: upper section, 5 s; lower section, 20 s; or anti-pleckstrin antibody, 5 s).

Figure S2 The pan-PKC inhibitors Ro31-8220 and Ro31-8425 concentration-dependently affect PKC substrate serine phosphorylation provoked by SFLLRN

Aspirated isolated human platelets were pre-incubated with buffer or the pan-PKC inhibitors Ro31-8220 and Ro31-8425 (both applied from 0.3 to 10 μM) for 5 min and stimulated with 30 μM SFLLRN for 30 s at 37 °C with stirring. Reactions were stopped by the addition of SDS sample buffer and samples were analysed by SDS/PAGE followed by Western blotting as described in the Experimental section of the main text using antibodies recognizing serine-phosphorylated PKC substrates or unmodified pleckstrin. Chemiluminescence was assessed at various time points and results obtained at differing exposure times are divided by the dotted line (for the anti-phosphoserine PKC substrate antibody: upper section, 10 s; lower section, 20 s; or anti-pleckstrin antibody, 5 s). The blot shown is from one experiment that is representative of three independent experiments. All samples contained 0.1 % DMSO as solvent control for the Ro-compounds.

Figure S3 Subthreshold PAR4 activation provoked by 30 μM AYPGKF does not synergize with SFLLRN-induced Ca2+ mobilization under non-desensitized conditions

Maximal peak rises in intracellular Ca2+ were determined as described in the Experimental section of the main text. Aspirated isolated platelets were stimulated with 30 μM AYPGKF or 3 μM and 30 μM SFLLRN alone; or were pre-treated with 30 μM AYPGKF for 30 s followed by 3 or 30 μM SFLLRN. Summarized data are given as means ± S.E.M. for n = 3 independent experiments. Statistical analysis was performed by two-tailed paired Student’s t tests; ns, not significant.

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