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

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Abbreviations: PAR, protease-activated receptor; GPCR, G-protein coupled receptor; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; TXA₂, thromboxane A₂; ADP, adenosine 5'-diphosphate; TP α , thromboxane/prostanoid receptor α ; AC, adenylyl cyclase; cAMP, cyclic adenosine-monophosphate; PAI-1, plasminogen activator inhibitor 1.

SYNOPSIS

Protease-activated receptors 1 and 4 belong to the family of G protein-coupled receptors which induce both $G\alpha_{12/13}$ and $G\alpha_q$ 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 Ca^{2+} mobilization, protein kinase C signalling, α granules secretion, as well as a complete lack of dense granules secretion. Interestingly, subthreshold PAR4 activation rapidly abrogated PAR1 signalling-desensitization by differentially reconstituting these affected signalling events and functional responses, sufficient to re-establish aggregation. The lack of ADP release and P2Y12 receptor-induced $G\alpha_i$ signalling accounted for the loss in the aggregation response, as mimicking $G\alpha_{i/z}$ signalling with 2-MeS-ADP or epinephrine, respectively, 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. Our studies further elucidate differences in human platelet PAR signalling regulation and provide evidence for a cross-talk in which PAR4 signalling counteracts mechanisms involved in PAR1 signalling down-regulation.

INTRODUCTION

Human platelets express the two protease-activated receptor (PAR) 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 4 belong to the family of G-protein coupled receptors (GPCRs) and induce $G\alpha_{12/13}$ and $G\alpha_q$ signalling [2]. The initial re-organization of the platelet cytoskeleton ("shape change") involves $G\alpha_{12/13}$ -mediated calcium/calmodulin and Rho/Rho-kinase signalling pathways [3, 4], whereas $G\alpha_q$ signalling is required for platelet granules secretion [5] which is accomplished via phospholipase C β (PLC β) signalling and subsequent both Ca^{2+} - and diacylglycerol (DAG)-mediated activation of protein kinase C (PKC).

Platelet activation in general involves the generation and release of lipid mediators such as thromboxane A_2 (TXA $_2$) as well as of adenine nucleotides such as adenosine 5'-diphosphate (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 TXA $_2$ the thromboxane/prostanoid receptor α (TP α) is the predominant isoform expressed on platelets and couples, like PAR1 and 4, to $G\alpha_{12/13}$ as well as $G\alpha_q$ [6, 7]. For ADP, the two GPCRs P2Y1 and P2Y12 have been identified on human platelets [8]. The P2Y1 receptor couples to $G\alpha_q$, whereas the P2Y12 receptor couples to $G\alpha_{i2}$ [9], which, by negatively regulating adenylyl cyclase (AC) and therefore cyclic adenosine mono-phosphate (cAMP) levels essentially contributes to full and sustained platelet aggregation and thrombus formation. Along this line,

there are dissenting findings whether PAR1 also contributes to platelet activation via $G\alpha_i$ [10, 11].

However, although PAR1 and 4 couple to the same G-protein subtypes, they apparently differ in terms of affinity for thrombin and the duration of intracellular signalling. For long 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 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 4 form heterodimers, and a model has been proposed in which thrombin binding to PAR1, beside inducing respective receptor activation, facilitate subsequently PAR4 cleavage and signalling [14]. This biphasic model would serve to explain, for instance, the profile of intracellular $[Ca^{2+}]_i$ 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, still 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 to the sustained signalling via PAR4 has been attributed to differential receptor phosphorylation and velocity of receptor internalization [16].

Due 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 TXA_2 -provoked $TP\alpha$ $G\alpha_{12/13/q}$ -coupling aspirinated platelets were used; and due 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 Ca^{2+} mobilization, PKC-signalling, as well as α and dense granules secretion, we refer to our findings as PAR1 signalling-desensitization rather than receptor-desensitization.

Strikingly, we found that subthreshold PAR4 signalling counteract PAR1 signalling-desensitization resulting in a partial reconstitution of Ca^{2+} mobilization and PKC-mediated granules 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). Acetylsalicylic acid (aspirin), apyrase (Grade III, from potato), TXA_2 -mimetic U46619, α -lysophosphatidic acid (oleoyl-sn-glycero-3-phosphate; LPA), human α -1-acid glycoprotein (AGP), serotonin, 2-MeS-ADP, α,β -MeATP, epinephrine, fura-2/AM, Ro31-8220, Y27632, and yohimbine were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). BAPTA/AM and Ro31-8425 were from Calbiochem (La Jolla, CA, USA); collagen (type I, from equine tendons) and the

luciferin/luciferase bioluminescent kit were from Chrono-Log (Haverston, PA, USA). Cangrelor was kindly provided by The Medicines Company (Parsippany, NJ, USA). Human fibrinogen was from IMCO Corporation Ltd. AB (Stockholm, Sweden). All other reagents were of analytical grade.

The Phospho-(Ser) PKC substrate antibody (#2261) was from Cell Signaling Technology Inc. (Danvers, MA, USA), the antibody detecting unmodified pleckstrin (ab17020) from abcam (Cambridge, UK), and secondary HRP-conjugated goat anti-rabbit and rabbit anti-goat antibodies from Jackson ImmunoResearch Europe Ltd. (Suffolk, UK) and Pierce/Thermo Fisher Scientific Inc. (Rockford, IL, USA), 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 acid-citrate-dextrose (ACD: 71 mM citric acid, 85 mM sodium citrate, 111 mM glucose) and centrifuged at 220 g for 20 min. The platelet-rich plasma (PRP) thus obtained was incubated with 100 μ M aspirin for 30 min, supplemented with 0.5 U/ml apyrase, and platelets were collected by centrifugation at 520 g for 25 min. Platelet pellets were carefully washed three times with Krebs-Ringer Glucose (KRG) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, 10 mM glucose; pH 7.3), and finally resuspended in KRG containing 1 U/ml apyrase. The platelet count was determined using an automatic blood cell counter (ABX Micros 60 (ABX Diagnostics, Montpellier, France)) and was adjusted to a final assay concentration of 2.5×10^8 platelets/ml. Suspensions were supplemented with 1 mM CaCl₂ 30 min prior to experimentation. All isolation steps were carried out at room temperature (RT).

Platelet aggregation and ATP release

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

PAR1 surface expression

Measurements were performed as previously described in detail [17] using a Coulter Epics XL.MCL flow cytometer and Expo 32 ADC software (Beckman Coulter, Miami, USA). For the experimental procedure the Platelet Calibrator kit from Biocytex (Marseille, France) and the monoclonal PAR1 antibody WEDE15 (Immunotech, Marseille, France) 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. Due to inter-individual variations in PAR1 expression [17] the number of exposed receptors assessed on resting platelets were set as 100%.

Increase in cytosolic Ca²⁺

Platelets were loaded with fura-2 by incubating PRP with 3 μ M fura-2/AM for 45 min at RT 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 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) at 510 nm with simultaneous excitation at 340 nm and 380 nm. Cytosolic calcium [Ca²⁺]_i is expressed in fluorescence ratio (340/380 nm).

PAI-1 release

Platelets were stimulated as indicated at 37°C in a thermoshaker rotating at 600 rpm in a total volume of 600 μ l. Samples were subsequently centrifuged at 14,000 *g* for 10 min at 4°C. Supernatants were analyzed applying the tintELIZE PAI-1 kit (Biopool AB, Umeå, Sweden) according to the manufacturer's protocol using a Multiskan Spectrum spectrophotometer (Thermo/Electron Corporation, Thermo Fisher Scientific Inc., Göteborg, Sweden). Experiments were carried out in duplicates and mean values were used to calculate the amount of PAI-1 in ng released from 1x10⁶ platelets.

Immuno(Western)blotting

Stimulation of platelets were carried out at 37°C in a total volume of 200 μ l in 2 ml round-bottom tubes in a thermoshaker rotating at 900 rpm; pre-incubations at 500 rpm. Reactions were stopped by the addition of 50 μ l 5x SDS sample buffer, and proteins were denaturated at 95°C for 5 min. Proteins were separated on 4-12% NuPAGE[®] Novex Bis-Tris gels with MOPS running buffer (Invitrogen, Stockholm, Sweden). To determine apparent molecular protein masses MagicMark[™] XP Western Protein Standard (Invitrogen) was used. Proteins were blotted onto Immun-Blot[™] PVDF membranes (0.2 μ m) (BioRad, Hercules, CA, USA). For further steps TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% (w/v) Tween-20) was used. Membranes were incubated with the phospho-(Ser) PKC substrate antibody followed by peroxidase-conjugated goat anti-rabbit IgGs (both diluted 1:3000). Membranes were stripped and re-probed with the pleckstrin antibody followed by peroxidase-coupled rabbit anti-goat IgGs (both diluted 1:4000). Protein bands were visualized by the use of Immobilon[™] Western Chemiluminescent HRP Substrate solution from Millipore (Billerica, MA, USA), and chemiluminescence was recorded by a Fuji LAS 1000 system (Fuji Photo Film, Tokyo, Japan).

Statistical analysis

Data analysis was performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Results are presented as mean \pm standard error of the mean (S.E.M.) and statistical significances were calculated as indicated.

RESULTS

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

Under our conditions, as shown in Figs. 1A and 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 Fig. 1B (upper trace) platelets exposed to SFLLRN cumulatively from 0.1 μM up to 30 μM in 3 min intervals lose their ability to form aggregates. Whereas SFLLRN at 0.1 μM had no effect, the following 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 μM and 30 μM SFLLRN they fail to respond.

To determine the conditions under which PAR1-mediated aggregation becomes abrogated platelets were preincubated with increasing concentrations of SFLLRN for various periods of time prior to the addition of 30 μM SFLLRN (Fig. 1C and D). We observed that platelets incubated with 3 μM SFLLRN for 8 to 10 minutes (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 Fig. 1B (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 (data not shown), as well as in platelet suspension supplemented with exogenous fibrinogen (100 $\mu\text{g}/\text{ml}$) (compare Fig. 7C).

In contrast, no effects on platelet aggregation were observed when platelets were stimulated with AYPGKF (Fig. 1F). In cumulative dose-response studies applying AYPGKF from 1 up to 300 μM (upper trace) we observed a stepwise increase in shape change up to 30 μM , followed by platelet aggregation triggered by 100 μM , 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 (lower trace); which was likewise true when platelets were pre-exposed to 30 μM AYPGKF for up to 60 min (data 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 properly responded to subsequently added 300 μM AYPGKF (data 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.

The loss in responsiveness is not due to PAR1 internalization

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

PAR1 signalling-desensitization affects Ca^{2+} mobilization in a protein kinase C-dependent manner

An immediate platelet response to PAR1 activation is the mobilization of Ca^{2+} from intracellular stores and influx of Ca^{2+} across the plasma membrane. As such, we investigated the impact of the observed desensitizing effect on PAR1 signalling on the elevation of intracellular Ca^{2+} -concentrations; results are given as fluorescence ratios (340/380 nm). As shown in Fig. 3A, pre-exposure of platelets to 3 μM SFLLRN for 10 min decreased the response to 30 μM SFLLRN about 50% (from 3.1 ± 0.2 down to 1.5 ± 0.2), which was not further diminished when platelets were pretreated for up to 30 min (data 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 the exposure to 3 μM SFLLRN. Fig. 3B shows representative traces of Ca^{2+} mobilization induced by 30 μM SFLLRN, the effects of PKC inhibition by 1 μM Ro31-8425 on PAR1 desensitization, as well as the summary of obtained results as a bar graph. In non-pretreated platelets the response to 30 μM SFLLRN was slightly increased when pre-incubated with 0.3 μM Ro31-8220 or 1 μM Ro31-8425, respectively. In this set of experiments the response induced by 30 μM SFLLRN in PAR1 signalling-desensitized platelets was diminished about 60%, and both Ro31-8220 and Ro31-8425 partially but significantly re-established Ca^{2+} mobilization. In parallel we assessed platelet aggregation in this experimental setting (Fig. 3C). As expected, both 0.3 μM Ro31-8220 and 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 marginally or no effects, respectively.

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

PAR1 signalling-desensitization affects PKC signalling

Due to the above reported observation, the impact of PAR1 signalling-desensitization on that of PKC was evaluated by immuno(Western)blotting applying a phospho(Ser) PKC substrate antibody. A detailed time-course of PKC substrate serine phosphorylation up to 20 min is shown

in supplemental data 2A; and as the maximal phosphorylation pattern detected peaked after 30 sec of stimulation this time point was chosen for further experiments.

As illustrated here in Fig. 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 to 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 supplemental data 1, we evaluated the specificity of the phospho(Ser) 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 the 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 μM and 3 μM , respectively, were required, which corresponds to the inhibitory potencies of the applied compounds on platelet aggregation (Fig. 3C).

Desensitized PAR1 signalling is re-sensitized via PAR4 signalling in a Ca^{2+} - and Rho-kinase-dependent manner

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

Regarding $\text{G}\alpha_{12/13}$ -mediated calcium and Rho-kinase signalling pathways, and as shown in Fig. 5B, this re-sensitizing effect was abolished or prominently diminished by pre-incubating platelets with the cytosolic Ca^{2+} -chelator BAPTA/AM (10 μM) or the Rho-kinase inhibitor Y27632 (10 μM), respectively.

To investigate if 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 TXA_2 -mimetic U46619. As mentioned above, platelet TXA_2 signalling is mediated via $\text{TP}\alpha$ receptors, which, like PAR1 and 4, induces $\text{G}_{12/13}$ and Gq signalling. As demonstrated in Fig 5C, U46619 at 0.03 μM , although inducing an about 5 times higher Ca^{2+} mobilization compared to 30 μM AYPGKF (inset), did not re-establish aggregation in PAR1 signalling-desensitized platelets.

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

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

Peptide concentrations which de- and re-sensitize PAR1 signalling, i.e. 3 μ M SFLLRN and 30 μ M 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 μ M SFLLRN still elicits Ca²⁺ mobilization and about half-maximal PAI-1 release, indicating that residual PAR1 signalling still elicits minor cellular responses, but again no ATP release has been observed. Finally, the intermediate addition of 30 μ M AYPGKF resulted in a slight but significant increase in Ca²⁺ mobilization, an almost entire reconstitution of PAI-1 release, as well as an about 15% recovery of ATP release. Along this line, as shown in Fig. 6D as original traces and as summary in the bar graph (Fig. 6E), in PAR1 signalling-desensitized platelets the combination of 0.01 μ M of the stable ADP-analogue 2-MeS-ADP and 30 μ M SFLLRN provoked an aggregation response to an extent similar to that provoked by 30 μ M SFLLRN alone under non-desensitized conditions. In addition, when mimicking G $\alpha_{i/z}$ signalling by replacing 2-MeS-ADP with 10 μ M epinephrine platelet aggregation was likewise fully restored. Taken together, these findings indicate that the loss in responsiveness in PAR1 signalling-desensitized platelets is due do a lack in ADP secretion and P2Y₁₂ receptor G α_i signalling. In addition, they likewise demonstrate that residual PAR1 signalling is still present, as neither 0.01 μ M 2-MeS-ADP nor 10 μ M epinephrine when added alone, or when added 10 min after 3 μ M SFLLRN, evoke platelet aggregation.

Finally, the effect of epinephrine was abolished by the α_{2A} adrenoceptor-antagonist yohimbine (1 μ M); and the reconstituting effect of 30 μ M AYPGKF on aggregation of desensitized platelets induced by 30 μ M SFLLRN was, as to be expected, completely abrogated in the presence of the specific P2Y₁₂ receptor-antagonist cangrelor (10 μ M).

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

Due to the above findings 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 intracellular Ca²⁺ rises and platelet shape change, but no ATP secretion and aggregation. As summarized in Tab. 1, except for 3 μ M SFLLRN, neither the pretreatment with 0.03 μ M TXA₂-mimetic U46619 (compare Fig. 5 C), 10 μ M lysophosphatidic acid (LPA), 20 μ M serotonin, 0.5 mg/ml α -1-acid glycoprotein (AGP) [21], 0.01 μ M of the ADP-analogue 2-MeS-ADP, 10 μ M epinephrine, 1 μ M of the ATP-analogue α,β -MeATP, nor 1 μ g/ml collagen affected platelet aggregation in response to 30 μ M SFLLRN. Furthermore, none of these tested agonists were able to replace AYPGKF in its capability to re-sensitize PAR1 signalling; whereas the reconstituting effects of 2-MeS-ADP and epinephrine (compare Fig. 6D and E) are rather due to substituting or mimicking the lack of ADP release and P2Y₁₂ receptor-mediated G α_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 both dense and α granules secretion

PKC signalling is essentially implicated in platelet activation by signalling mechanisms leading to the secretion of dense and α granules.

As shown in Fig. 7A, and with respect to Fig. 4, we observed that the PAR1 signalling-desensitization-dependent 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 μ M AYPGKF (supplemental data 2). As still little is known about signalling pathways specifically induced by either PAR1 or PAR4, it will remain difficult to differentiate if 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.

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 (Fig. 7B and C). In suspension of isolated platelets the only source for fibrinogen is that which is stored in α granules. As PKC signalling is prominently involved in both dense and α granules secretion we performed our experiments in the absence or presence of the pan-PKC inhibitor Ro31-8425 (10 μ M) without and with exogenously added fibrinogen (100 μ g/ml) (Figs. 7B and C).

Under non-desensitized conditions (left panels) platelet aggregation induced by 30 μ M 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 due to missing both dense and α granules secretion, as mimicking dense granules secretion by the addition of 0.01 μ M 2-MeS-ADP fails to reconstitute aggregation in response to 30 μ M SFLLRN in the absence of fibrinogen, whereas when fibrinogen was supplemented full platelet aggregation occurred.

When PAR1 signalling has been desensitized by pretreated with 3 μ M SFLLRN (right panels) the second treatment with 30 μ M 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 G α_i signalling is missing, which supports our findings shown in Fig. 6C. Intermediate PAR4 signalling induced by 30 μ M AYPGKF re-established SFLLRN-triggered aggregation of PAR1 signalling-desensitized platelets in the absence of supplemented fibrinogen about 70 %, and fully when fibrinogen was surplus added, indicating that secretion of both types of granules preceded (compare Fig. 6 B, C). 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 granules secretion in the absence of supplemented fibrinogen, and completely when α granules secretion was mimicked by fibrinogen supplementation.

Taken together, these findings clearly demonstrate that PAR1 signalling-desensitization results in a reduction in PKC signalling-mediated granules secretion, and that this effect can be re-established by intermediate PAR4 signalling. In addition, as 0.01 μ M 2-MeS-ADP alone (compare Fig. 5D), even in the presence of exogenously added fibrinogen, did not induce platelet

aggregation (data not shown), these findings once more show that residual PAR1 signalling upstream of PKC 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 this 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 general decline in platelet sensitiveness as SFLLRN-pretreated platelets still normally responded 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 G protein-coupled receptor kinases (GRKs), 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 activation. 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 stimulation [23, 24, 17], in our 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 further observed that Ca^{2+} 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 12-*O*-tetradecanoylphorbol-13-acetate (TPA) precludes thrombin-induced Ca^{2+} mobilization [19]; an effect that was also found in platelets pretreated with TPA and challenged with SFLLRN (M. Grenegård, unpublished observation). 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 $\text{G}\alpha_q$ signalling, such as AYPGKF and TXA_2 -mimetic U46619, or via G-protein unrelated signalling, such as collagen-induced GPVI 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 Ca^{2+} 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, Ca^{2+} mobilization was not further re-established, suggesting that a PKC-independent mechanism may account for most of the loss in PAR1-induced Ca^{2+} mobilization capacity.

However, PKC signalling is crucially involved in platelet activation and aggregation by, beside other events, promoting platelet granules 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 granules 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 granules secretion was about 55% reduced, whereas secretion of dense granules was completely abolished. Indeed, we identified that the loss in PAR1-induced platelet aggregation was due to a lack of ADP secretion and subsequent P2Y12 receptor-mediated $G\alpha_i$ -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 α_{2A} adrenoceptors likewise induces signalling via inhibitory $G\alpha_z$ proteins [28].

Neither epinephrine [29] nor 0.01 μ M 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 $G\alpha_q$, which is required for granules secretion [5], that concomitant signalling via $G\alpha_{12/13}$ and P2Y12 receptor $G\alpha_i$ -coupling is sufficient for inducing integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation [30].

In a Rat1 fibroblast model it has been shown that PAR4 does not undergo agonist-promoted phosphorylation and internalize much slower than PAR1 [16]. Accordingly, we did not observe any signs of desensitization in PAR4-mediated aggregation; neither in cumulative dose-response studies nor when platelets were pre-exposed to subthreshold concentrations of AYPGKF; which is, however, opposite to a previous report [31].

Most intriguingly, we found that AYPGKF at a subthreshold concentration of 30 μ M re-established SFLLRN-induced platelet aggregation in PAR1 signalling-desensitized platelets. AYPGKF at this concentration alone induced only marginal Ca^{2+} mobilization and α granules secretion, as well as prominent and sustained platelet shape change, but no dense granules secretion, indicating that the observed responses presumably were evoked by $G\alpha_{12/13}$ signalling. And indeed, the inhibition of either Ca^{2+} -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 granules secretion, only slightly both enhanced residual Ca^{2+} mobilization and re-constituted dense granule secretion in response to 30 μ M SFLLRN. Given a ratio of 1.5 in ADP:ATP content in dense granules [32] it could be roughly estimated that 0.45 μ M 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 due to indirect effects such as ADP release and subsequent P2Y12 signalling [33], or fibrinogen-binding and integrin $\alpha_{IIb}\beta_3$ outside-in signalling [34, 35] was, as beyond the scope of this study, 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 PKC isoform α (PKC α) is a major regulator of dense and α granules secretion [36].

Finally, it is important to note that the re-constitution 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 closely

related TP α signalling no re-sensitization of PAR1 signalling could be observed; although TXA $_2$ -mimetic U46619 at the applied concentration (0.03 μ M) induced an about 5fold higher Ca $^{2+}$ mobilization compared to 30 μ M AYPGKF. In addition, in non-desensitized platelets 30 μ M AYPGKF did not augment Ca $^{2+}$ mobilization evoked by 3 or 30 μ M SFLLRN (supplemental data figure 3). Furthermore, when we replaced AYPGKF with AGP, which has been shown to induce platelet shape change via prominent Rho-kinase signalling independently of Ca $^{2+}$ mobilization [21], we likewise did not observe any reconstituting effect (data 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 Ca $^{2+}$ -mediated signalling, whereas U46619, which likewise induce both signalling cascades had no re-constituting effect, we can not 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 data demonstrate that PAR1 signalling-desensitization results in a marked diminution of PKC signalling-mediated granules 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.

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AUTHOR CONTRIBUTION

Knut Fälker, Linda Haglund, Peter Gunnarsson, Martina Nylander, and Magnus Grenegård designed the experiments, performed research and data analysis. Tomas L. Lindahl provided critical input, 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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FIGURE LEGENDS

Table 1 Impacts of various platelet agonists on PAR1 de- and re-sensitization on platelet aggregation induced by SFLLRN

Aggregation of aspirinated, isolated human platelets was assessed as described in "Experimental Procedures". Platelets were either preincubated with the listed agonists at stated concentrations for 10 min prior to the addition of 30 μ M SFLLRN, and "Desensitizing effects on PAR1-mediated platelet aggregation" are indicated as "+" (desensitizing/inhibitory effect) and "-" (no effect). Or platelets pretreated with 3 μ M SFLLRN for 10 min were stimulated with the listed agonists for 30 sec prior to the addition of 30 μ M SFLLRN, and "Re-sensitizing effects on PAR1-mediated platelet aggregation" are indicated as "+" (re-sensitizing effect) and "-" (no effect). The parenthesis used for the "re-sensitizing effects" 2-MeS-ADP and epinephrine ("(+)") indicate an indirect positive effect for both agonists.

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 "Experimental Procedures". **A**, **B**, **E**, and **F** show original traces of platelet aggregation induced by SFLLRN or AYPGKF, at indicated concentrations, which are representative of ≥ 3 independent experiments. **C** and **D** show bar graphs of maximal platelet aggregation under indicated conditions in mean \pm S.E.M.. **A**: Platelet aggregation in response to 3, 10, and 30 μ M SFLLRN. **B**: Platelet aggregation provoked by cumulatively added concentrations of 0.1 to 30 μ M SFLLRN as indicated in 3 min intervals (upper trace). Aggregation of platelets exposed to 3 μ M SFLLRN for 10 min prior to the addition of 30 μ M SFLLRN, followed by 300 μ M AYPGKF after another 5 min (lower trace). **C**: Platelets were pretreated with increasing, subthreshold SFLLRN-concentrations (0.1 to 3 μ M) for 10 min and subsequently stimulated with 30 μ M SFLLRN (n=3). **D**: Platelets were pretreated with 3 μ M SFLLRN for increasing periods of time (2-90 min) as indicated and subsequently challenged with 30 μ M SFLLRN (n=3). **E**: Platelet aggregation induced by 30, 100, and 300 μ M AYPGKF. **F**: Platelet aggregation evoked by cumulatively added concentrations of 1 to 300 μ M AYPGKF in 3 min intervals as indicated (upper trace), and that induced by 300 μ M AYPGKF after pre-treatment with 30 μ M AYPGKF for 10 min (lower trace).

Figure 2 Platelet PAR1 surface expression upon homologous receptor activation

Diluted whole blood was stimulated with (**A**) 3 μ M or (**B**) 30 μ M SFLLRN for various periods of time as indicated and PAR1 surface expression was assessed as described in "Experimental Procedures". Due to inter-individual variations [17] PAR1 surface expression on untreated platelets were set as 100%. Data are given in mean \pm S.E.M. of n=4 individual experiments.

Figure 3 Effects of PAR1 signalling-desensitization on SFLLRN-induced intracellular Ca^{2+} mobilization: Involvement of PKC

A: Bar graphs of maximal peak rises in intracellular Ca^{2+} under indicated conditions determined as described in "Experimental procedures" in mean±S.E.M.. *Left bar graph:* Aspirinated, isolated platelets were incubated with 3 μ M SFLLRN for increasing periods of time (up to 10 min) followed by stimulation with 30 μ M SFLLRN (n=3). *Right bar graph:* Platelets were pretreated with increasing subthreshold SFLLRN-concentrations (0.1 to 3 μ M) for 10 min and subsequently stimulated with 30 μ M SFLLRN (n=3). In **B** original traces of platelet $[Ca^{2+}]_i$ mobilization are presented. Platelets were stimulated with 30 μ M alone (upper trace); or pretreated with 3 μ M SFLLRN for 10 min prior to the addition of 30 μ M SFLLRN (middle trace), or preincubated with 1 μ M Ro31-8425 for 5 min, followed by incubation with 3 μ M SFLLRN for 10 min and stimulation with 30 μ M SFLLRN (lower trace). The bar graph shows the summarized data (n=4) of intracellular Ca^{2+} mobilization determined in platelets incubated with buffer or 3 μ M SFLLRN for 10 min prior to the addition of 30 μ M SFLLRN (left bars), as well as the effects of pre-incubation for 5 min with either 0.3 μ M Ro31-8220 (middle bars) or 1 μ M Ro31-8425 (right bars) before adding 3 and 30 μ M SFLLRN (p values were determined by two-tailed paired *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 (mean±S.E.M.) of n=5-7 individual experiments. All samples in **B** and **C** contained 0.1% DMSO as Ro-compounds solvent control.

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

Aspirinated, isolated human platelets were preincubated at 37°C with 3 μ M SFLLRN (or buffer) for 10 min prior to stimulation with 3, 10, and 30 μ M SFLLRN for 30 sec with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting as described in "Experimental Procedures" using antibodies recognizing serine-phosphorylated PKC substrates or unmodified pleckstrin. The shown result 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 phospho(Ser) PKC substrate antibody: upper part 3 sec, lower parts 10 sec; anti-pleckstrin antibody: 1 sec).

Figure 5 Effects of intermediated, subthreshold PAR4 activation on SFLLRN-induced aggregation of PAR1 signalling-desensitized platelets: Impacts of the inhibition of $[Ca^{2+}]_i$ mobilization or Rho-kinase signalling. Effects of intermediated, subthreshold TP α activation on SFLLRN-induced aggregation of PAR1 signalling-desensitized platelets

Aggregation of aspirinated, isolated human platelets was assessed as described in "Experimental Procedures". **A:** Platelets were stimulated with 30 μ M SFLLRN alone (left trace), or with 3 μ M SFLLRN for 10 min followed by 30 μ M AYPGKF (right upper trace), or by 30 μ M AYPGKF

for 30 sec and finally with 30 μM SFLLRN (right lower trace). Shown traces are representative of at least 3 independent experiments. Bar graph **B**: platelets were, as indicated, incubated with buffer or 3 μM SFLLRN for 10 min followed by treatment with 10 μM BAPTA/AM or 10 μM Y27632 for 5 min. Platelets were subsequently stimulated with 30 μM SFLLRN alone, or with 30 μM AYPGKF for 30 sec followed by 30 μM SFLLRN, respectively. Maximal aggregation of $n=3-5$ independent experiments are given as mean \pm S.E.M.; all samples contained 0.1% DMSO as solvent control for BAPTA/AM. **C**: Platelets were stimulated with 30 μM SFLLRN (left trace) or 0.03 μM U46619 (right upper trace) alone; or incubated with 3 μM SFLLRN for 10 min followed by 0.03 μM U46619 (right middle trace). Finally, as shown in the right lower trace, platelets were incubated with 3 μM SFLLRN for 10 min followed by 0.03 μM U46619 for 30 sec prior to 30 μM SFLLRN. Shown original traces are representative of $n=5$ independent experiments. The insert shows original traces of platelet $[\text{Ca}^{2+}]_i$ mobilization (in fluorescence ratio (340/380 nm)) induced by either 30 μM AYPGKF or 0.03 μM U46619, respectively, and summarized data are given in mean \pm S.E.M. from $n=3$ independent experiments.

Figure 6 Effects of intermediate, subthreshold PAR4 activation on SFLLRN-provoked $[\text{Ca}^{2+}]_i$ mobilization, PAI-1 release, and ATP release in PAR1 signalling-desensitized platelets. Effects of 2-MeS-ADP and epinephrine on aggregation of PAR1 signalling-desensitized platelets challenged with SFLLRN

Bar graphs **A**, **B**, and **C**: Platelets were stimulated with 3 μM SFLLRN, 30 μM AYPGKF, or 30 μM SFLLRN alone, or with 3 μM SFLLRN for 10 min followed by either 30 μM SFLLRN alone, or with 3 μM SFLLRN for 10 min followed by 30 μM AYPGKF for 30 sec prior to 30 μM SFLLRN. $[\text{Ca}^{2+}]_i$ mobilization (**A**), PAI-1 release (**B**), and ATP release (**C**) was monitored as described in "Experimental procedures" and results are given in mean \pm S.E.M. of $n=3$ (**A**), $n=5$ (**B**), $n=3$ (**C**). P values were determined by two-tailed paired *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 "Experimental Procedures". Platelets were stimulated with 30 μM SFLLRN (left trace), or 0.01 μM 2-MeS-ADP (middle upper trace), or 10 μM epinephrine (right upper trace) alone. Platelets were incubated with 3 μM SFLLRN for 10 min followed by 0.01 μM 2-MeS-ADP (middle middle trace) or 10 μM epinephrine (right middle trace). Finally, platelets were incubated with 3 μM SFLLRN for 10 min followed by 0.01 μM 2-MeS-ADP (middle lower trace) or 10 μM epinephrine (right lower trace) for 30 sec prior to 30 μM SFLLRN. Bar graph **E**: Platelets were either stimulated with 30 μM SFLLRN alone, or preincubated with 3 μM SFLLRN for 10 min followed by 30 μM SFLLRN, or preincubated with 3 μM SFLLRN for 10 min followed by either 30 μM AYPGKF, 0.01 μM 2-MeS-ADP, or 10 μM epinephrine, respectively, before the addition of 30 μM SFLLRN. In the experiments performed with 10 μM cangrelor or 1 μM yohimbine, platelets were treated with 3 μM SFLLRN for 10 min followed by the addition of the receptor-antagonists for 2 min, and subsequently stimulated as indicated. Summarized aggregation results were obtained in $n=3$ individual experiments and data are given as mean \pm S.E.M..

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 pretreated with the PKC-inhibitor Ro31-8425

A: Aspirinated, isolated human platelets were preincubated 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 analyzed by SDS-PAGE followed by Western blotting as described in "Experimental Procedures" 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 lines (applying the phospho(Ser) PKC substrate antibody: upper part 2 sec, lower part 10 sec; (anti-pleckstrin antibody: 1 sec)). **B** and **C:** Aggregation of aspirinated, isolated human platelets was monitored as described in "Experimental Procedures". Pretreatments 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 mean±S.E.M. of 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.

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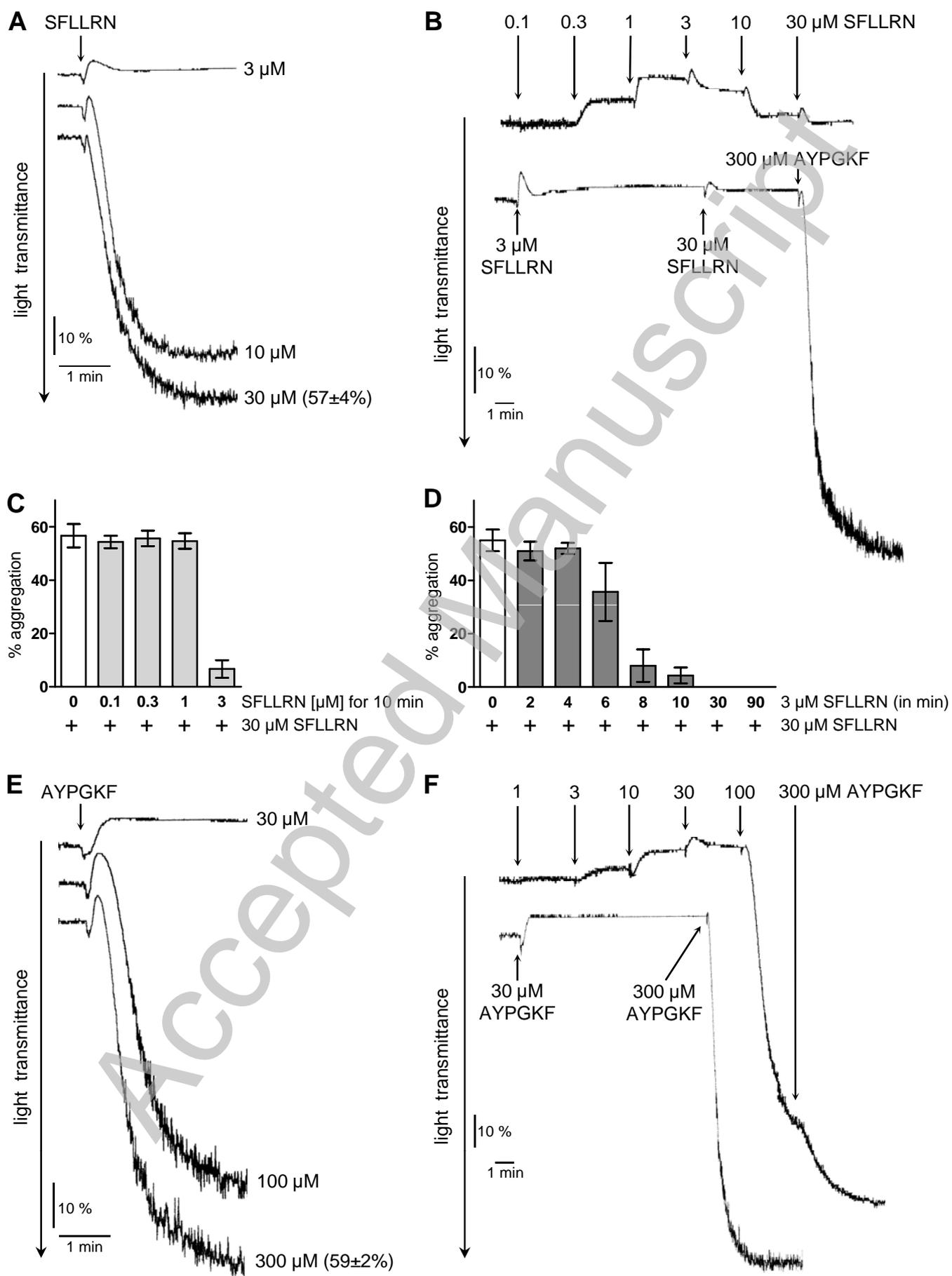
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Agonist	Desensitizing effect on PAR1-mediated platelet aggregation	Re-sensitizing effect on PAR1-mediated platelet aggregation
3 μ M SFLLRN	+	-
30 μ M AYPGKF	-	+
0.03 μ M U46619	-	-
10 μ M LPA	-	-
20 μ M serotonin	-	-
0.5 mg/ml AGP	-	-
0.01 μ M 2-MeS-ADP	-	(+)
10 μ M epinephrine	-	(+)
2 μ M α,β -MeATP	-	-
1 μ g/ml collagen	-	-

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Figure 2, Fälker et al. 2010 R.1

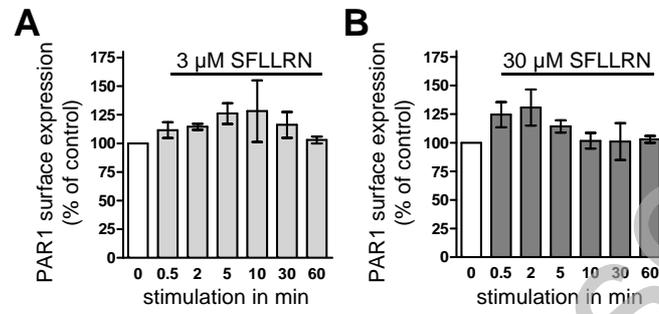


Figure 3, Falker et al. 2010 R.1

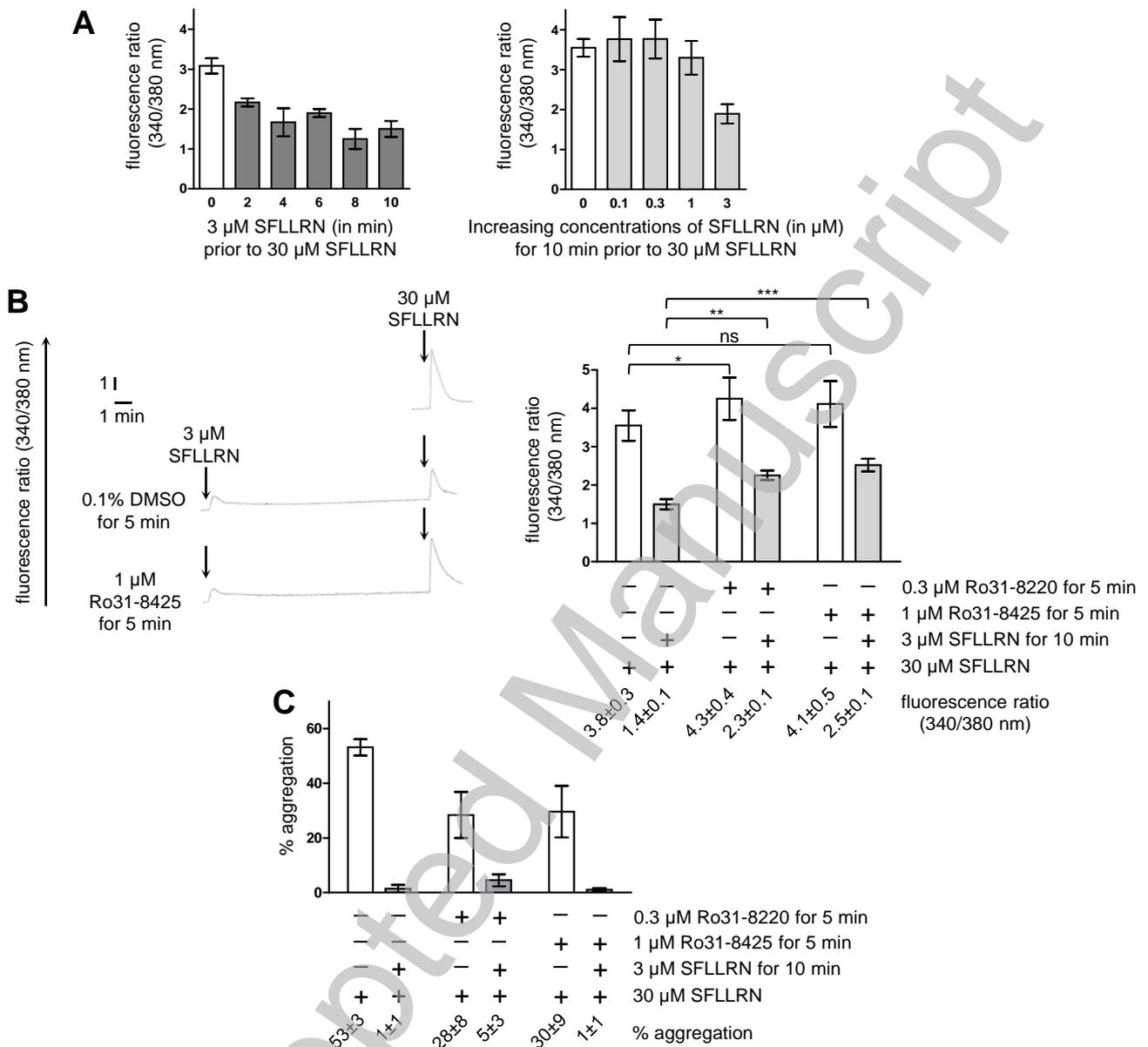
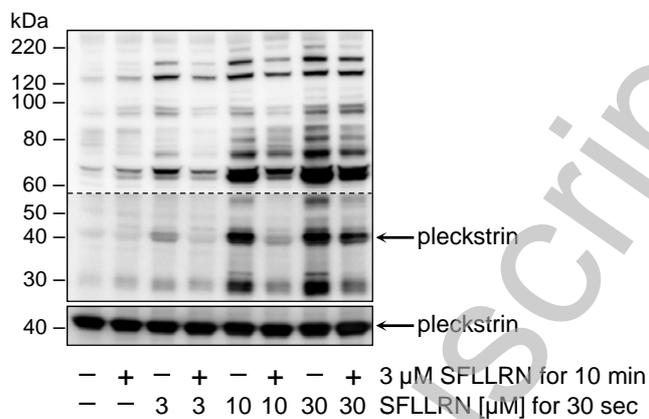


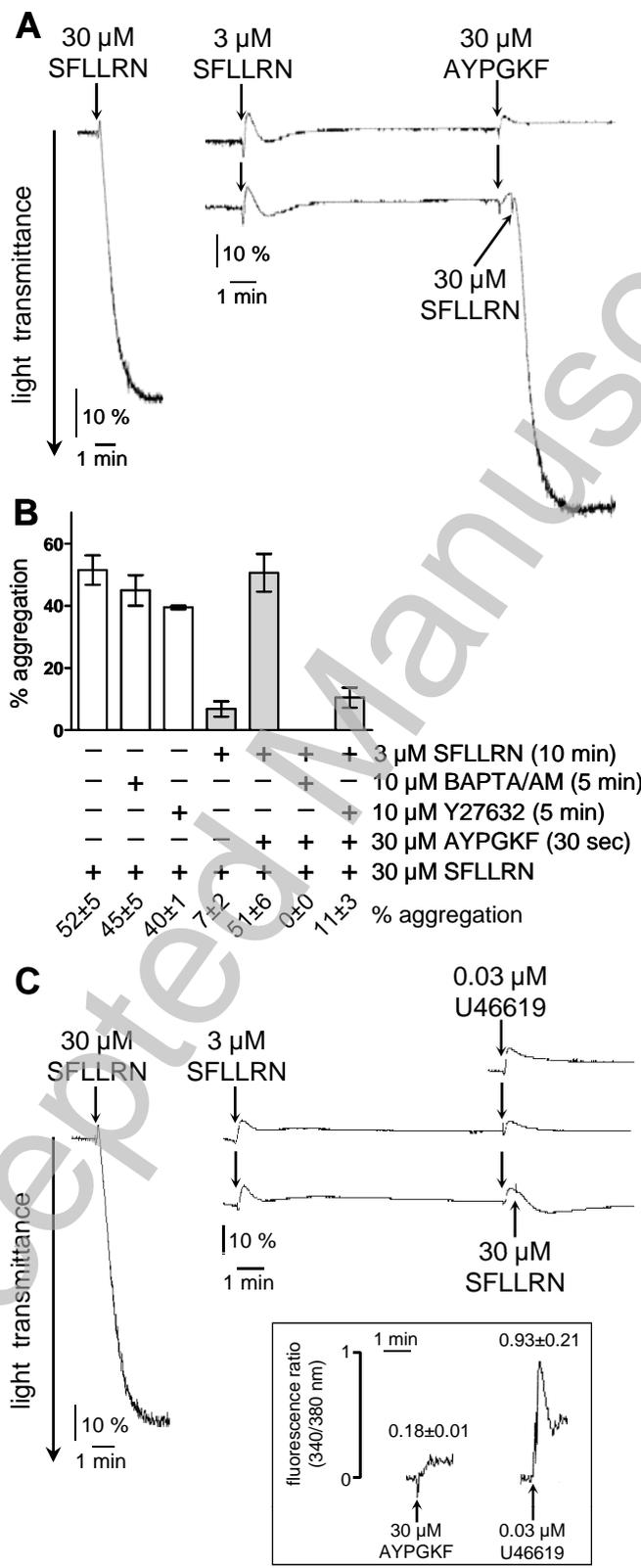
Figure 4, Fälker et al. 2010 R.1



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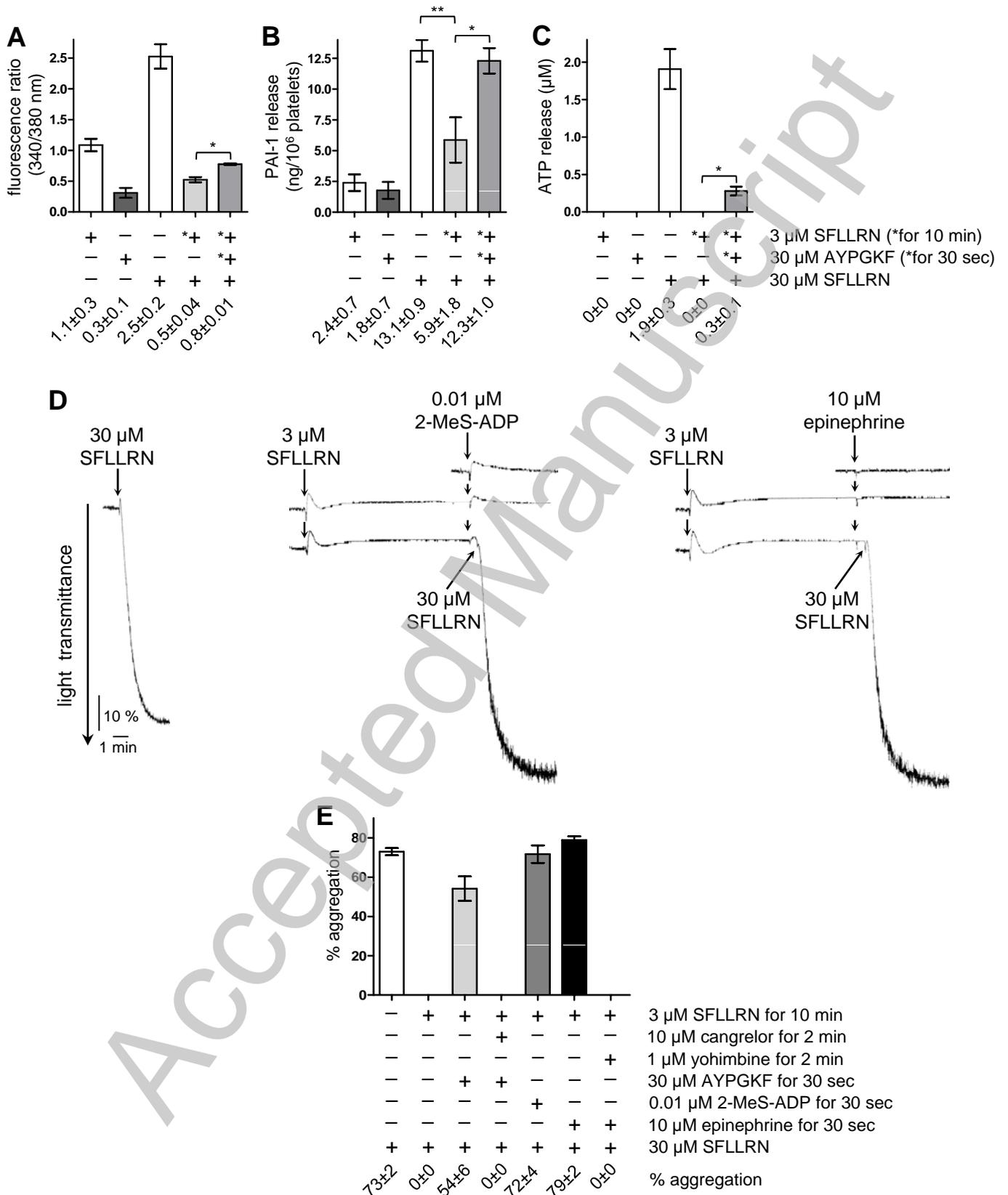
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Figure 5, Fälker et al. 2010 R.1



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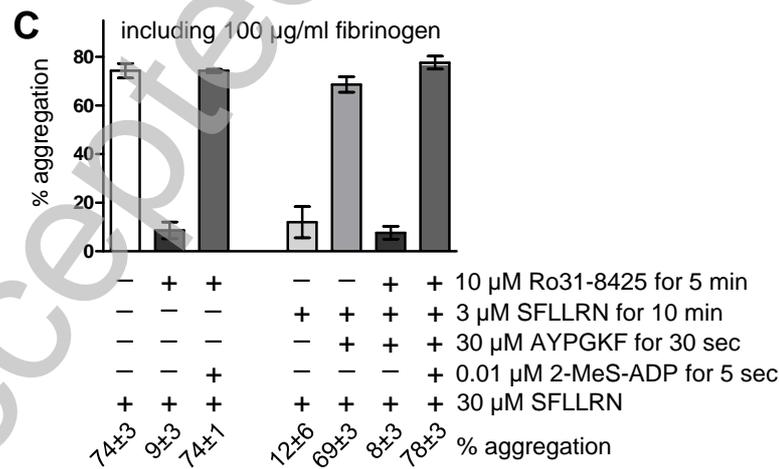
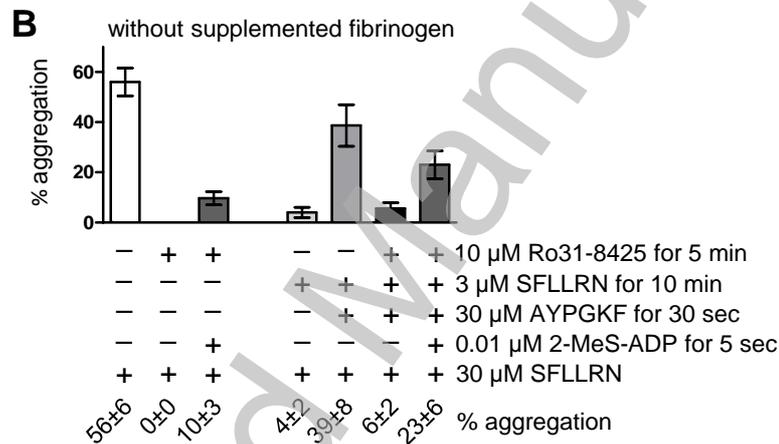
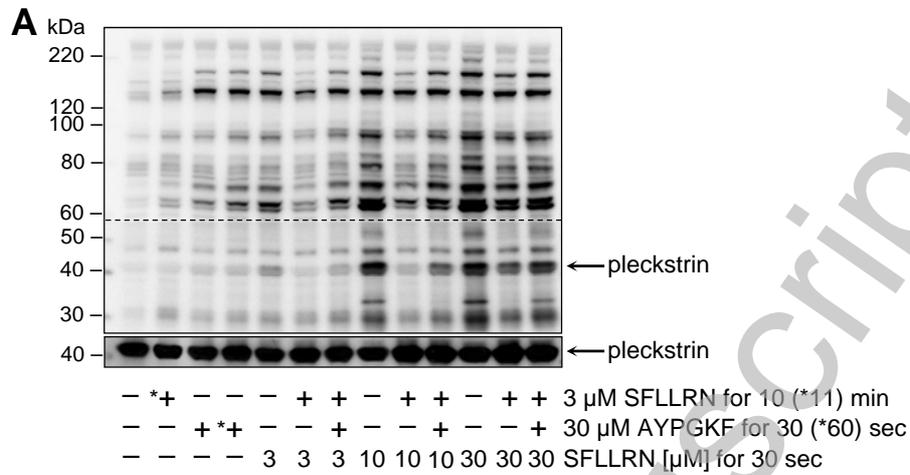
Figure 6, Fälker et al. 2010 R.1



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Figure 7, Fälker et al. 2010 R.1



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