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Thrombin receptor signalling in platelets: PAR1, but not PAR4, is rapidly desensitized

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Sammanfattning
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Platelets play a key role in primary haemostasis but are also related to the pathogenesis of arterial thrombosis. Thrombin is the most effective agonist inducing platelet activation. Human platelets express two G-protein coupled thrombin receptors (GPCRs), called protease activated receptor (PAR)1 and PAR4. The aim of this study was to clarify differences in the activities of PAR1 and PAR4, especially focusing on their resistance towards the platelet inhibitor nitric oxide (NO) and their ability to undergo desensitization. For this, PAR1- and PAR4- activating peptides (APs) (SFLLRN and AYPGKF, respectively) were used. Different aspects of platelet activities were studied: aggregation and the rise in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Aggregation was analyzed with lumiaggregometry, and $[Ca^{2+}]_i$ were studied using the fura-2 method. PKC substrate phosphorylation and the expression of PAR1 surface receptors were also analyzed, using Western blot and flow cytometry, respectively. The results from this study showed that NO exerted similar inhibitory effects on the two thrombin receptors. However, PAR1 and PAR4 differed in their ability to undergo desensitization. In cumulative dose-response studies, a low concentration of PAR1-AP induced desensitization of platelets towards higher PAR1-AP concentrations. This was not the case when studying PAR4-AP. The mechanism behind the desensitization of PAR1 to some part involved PKC, at least when studying the mobilization of intracellular Ca^{2+}. PAR1 desensitization did not seem to involve receptor internalization and neither did it affect the activity of PAR4. This thus suggests that PAR4 might be a more suitable therapeutic target in the future management of thrombosis.

Nyckelord
Keyword:
Nitric oxide, PAR1, PAR4, platelets, receptor desensitization

Content

1 Abstract

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Keywords:
Nitric oxide, PAR1, PAR4, platelets, receptor desensitization

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2 List of abbreviations

$[Ca^{2+}]_i$ - Intracellular Ca^{2+} Concentration
AP - Activating Peptide
ASA - Acetyl Salicylic Acid
DAG - Diacylglycerol
Fura-2 - AM, Fura-2 Acetoxymethylester
GPCR - G-protein Coupled Receptor
GRK - G-protein Receptor Kinase
IP_3 - Inositol Triphosphate
NO - Nitric Oxide
PAR - Protease Activate Receptor
PI3K - Phoshoinositide 3-Kinase
PKC - Protein Kinase C
PLC - Phospholipase C
SNAP - S-Nitroso-Acetylpenicillamine

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3 Introduction

Platelet aggregation plays a key role in primary haemostasis but is also related to the pathogenesis of cardiovascular diseases such as atherothrombosis. Platelets are regulated through various signalling pathways and thrombin is the most effective platelet activator (Chung et al., 2002; Davey and Lüscher, 1967; Leger et al., 2006b). To date, four protease activated receptors (PARs) are known; PAR1-4, of which all except for PAR2 are known to be receptors for thrombin (Coughlin, 1999a; Ishihara et al., 1997; Kahn et al., 1998; Rasmussen et al., 1991; Vu et al., 1991; Xu et al., 1998). The expression of the receptors is species specific, and human platelets are known to express PAR1 and PAR4, while mouse platelets express PAR3 and PAR4 (Huang et al., 2007; Kahn et al., 1998, 1999). The PARs are G-protein coupled receptors (GPCRs) and PAR1 and PAR4 share a common mechanism of activation that is unique for GPCRs. Thrombin cleaves a portion of the N-terminal domain of the receptor, which unmasks a new N-terminal sequence that serves as a tethered ligand for

the receptor. This ligand interacts with the second extracellular loop of the receptor and activates a G-protein (by stimulating the binding of GTP to the α-subunit of the G-protein, subsequently leading to dissociation from the βγ- subunit) (Coughlin, 1999b; Vu et al., 1991; Woulfe, 2005). The synthetic peptides SFLLRN-NH_2 (PAR1-AP) and AYPGKF-NH_2 (PAR4-AP) can activate their receptors without the need for receptor cleavage (Mazharian et al., 2007; Vu et al., 1991).

PAR1 is a high affinity receptor because of its hirudin-like sequence in the N-terminal domain, which allows the receptor to compete with fibrinogen (Day et al., 2006; Kahn et al., 1999; Leger et al., 2006b). PAR1 signalling is associated with activation of $G_{12/13}$ and G_q. These trimeric G-proteins both play an important role in activation of the fibrinogen receptor ($\alpha_{IIb}\beta_3$). Further, activation of $G_{12/13}$ causes the platelets to undergo a conformational change due to activation of Rho/Rho-kinase and actin remodeling. Activation of this G-protein also controls the release of dense granules. PAR1 activation of G_q leads to stimulation of phospholipase C (PLC)-β. This in turn leads to a subsequent generation of diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG serves as a stimulatory cofactor for the activation of protein kinase C (PKC), while IP_3 induces a release of Ca^{2+} from intracellular stores in platelets (Quinton et al., 2002). PKC signalling has been suggested to be involved in platelet activation by regulating several mechanisms, such as granule secretion (Strehl el al., 2007), $\alpha_{IIb}\beta_3$ activation (Yacoub et al., 2006) and Ca^{2+} entry (Harper and Sage, 2006). However, PKC signalling has also been shown to negatively regulate platelet function, e.g. by causing receptor desensitization (Mundell et al., 2006) and Ca^{2+} extrusion from the cell (Harper and Poole, 2007; Pollock et al., 1987). PKC exists in several isoforms which are divided into three groups; the conventional, the novel and the atypical ones (Mellor and Parker, 1998). The conventional isoforms; α, β_I, β_{II} and γ are activated by Ca^{2+} and DAG (Newton, 1995a,b), whereas the novel isoforms; δ, ε, η and θ are sensitive only to DAG. The atypical isoforms; μ, ι, λ and ζ are insensitive to both Ca^{2+} and DAG (Ono et al, 1988). The different isoforms play distinct roles in platelet activation (Murugappan et al., 2004), and human platelets probably express at least six of these isoforms; α, β, δ, η, θ and ζ (Quinton et al., 2002; Strehl et al., 2007).

The aggregation caused by PAR1 activation tends to be transient unless it is strengthened by inputs from either the adenosine diphosphate (ADP) receptor $P2Y_{12}$ or from PAR4 activation (Covic et al., 2002; Trumel et al., 1999; Voss et al., 2007). ADP, which is a weaker platelet agonist than thrombin, is stored in dense granules in platelets and is released upon stimulation with e.g. thrombin. ADP activates two GPCRs on the platelet surface, $P2Y_1$ and $P2Y_{12}$ (Jantzen et al., 1999; Jin and Kunapuli, 1998; Savi et al., 1998). $P2Y_1$ signals via G_q and has been shown to activate PLCβ (causing a subsequent rise in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$)) and to induce a platelet shape change. $P2Y_{12}$ signals via $G_{i/o}$, thereby inhibiting adenylyl cyclase. This leads to a decrease in the levels of cyclic adenosine monophosphate (cAMP), which acts as a negative regulator of platelet activation (Murugappan and Kunapuli, 2006; Nylander et al., 2003). It has also been shown that signalling through PAR1 triggers the release of granules and activates $\alpha_{IIb}\beta_3$ via phosphoinositide-3 kinase (PI3K) (Nylander et al., 2003). Inconsistencies exists about whether PAR1 can couple directly to $G_{i/o}$ in human platelets or not (Kim et al., 2002; Voss et al., 2007). However, it is now generally considered that signalling through this G-protein result from ADP secreted from dense granules.

PAR4 is a low affinity receptor since it lacks the hirudin-like sequence in the N-terminal domain. Instead, PAR4 uses proline residues to provide high-affinity interactions with the active site of thrombin (both PAR1 and PAR4 contain a proline residue at the P_2 position, but PAR4 contains an additional proline at P_4). It also contains a negatively charged amino acid cluster which slows the dissociation of PAR4 from the cationic thrombin (Jacques and

Kuliopulos, 2003). PAR4, like PAR1, is thought to be coupled to $G_{12/13}$ and G_q. However, the signalling is quite different from that through PAR1, since PAR4 is cleaved more slowly than PAR1 and generates most of the rise in $[Ca^{2+}]_i$ (Covic et al., 2000; Leger et al., 2006b; Shapiro et al., 2000; Voss et al., 2007; Woulfe, 2005). The aggregation stimulated by PAR4 does not need additional input from the ADP receptor to be stable (Adam et al., 2003; Covic et al., 2002; Leger et al., 2006b).

Proteases like thrombin activate PARs by an irreversible mechanism. This is due to the cleavage of the N-terminal domain and exposure of the tethered ligand, which is then always available to interact with the receptor. To avoid prolonged signalling there are mechanisms to shut it off. Two of these mechanisms are downregulation and desensitization of the receptors. Receptor desensitization is a common feature of GPCRs (Ossovskaya and Bunnett, 2003). The mechanism of desensitization, however, varies between different PARs and is not yet established. Mundell and coworkers (2006) showed that PKC is involved in the desensitization of platelet ADP receptors. Both novel and classic isoforms of PKC (α, β and δ) can cause desensitization of the ADP receptor $P2Y_1$, while only the novel isoform δ can induce desensitization of $P2Y_{12}$. This finding suggests that PKC may be involved in the desensitization mechanism also of other platelet receptors.

Currently, the use of direct thrombin inhibitors (to block platelet activation in pathological conditions) results in unwanted side effects, such as excessive bleeding. An alternative therapeutic strategy would be to inhibit PAR-mediated intracellular signalling pathways. When receptors are possible new targets in medicine, it is always important to clarify their properties and signaling pathways, in order to elucidate all the possible affects of inhibiting that receptor. In this case, either of the platelet protease activated receptors (PARs) responding to thrombin might be suitable future targets. Therefore, to be able target only one of them without affecting the other (which is often preferable), it is important to find differences between them. Previous works have indicated that PAR1 and PAR4 mediate platelet activation through different signalling pathways (Grenegård et al., 2008; Holinstat et al., 2006; Voss et al., 2007). However, it is not fully known how the pathways differ. In our study, we focused on two aspects that might differ between the receptors. The first subject was the platelet inhibitor nitric oxide (NO). Endothelial derived NO induces vasodilation (relaxation of the vessel) and inhibits platelet activation/aggregation. Atherothrombosis is characterized by NO deficiency (a decreased release of NO), which means that these patients have an increased risk of platelet aggregation and plug formation inside the blood vessels (Laursen et al., 2006). Investigating whether this antiplatelet mediator differently affects PAR1 and PAR4 was therefore of great interest. The second aspect of our study was to clarify whether the receptors have different abilities to undergo desensitization. The hypotheses were; 1) NO differently affects PAR1- and PAR4-mediated platelet activation; 2) PAR1 and PAR4 differ in their abilities to undergo desensitization.

4 Materials and methods

4.1 Isolation of human platelets

Human blood was collected from healthy volunteers and immediately and gently mixed with an acid-citrate-dextrose (ACD) solution (5 volumes blood and 1 volume ACD-solution) composed of 85 mM $C_6H_5Na_3O_7$, 71 mM $H_3C_6H_5O_7$ and 111 mM glucose. The blood was centrifuged for 20 min at 220g to obtain platelet-rich plasma. Acetylsalicylic acid (ASA) (100 μM) and apyrase (0.5 U/mL) were added to the platelet-rich plasma to prevent platelet activation by thromboxane A_2 (TxA_2) and ADP, respectively, during the isolation procedure.

The platelet-rich plasma was then centrifuged for 25 min at 520g to obtain a pellet of platelets. The supernatant was removed and the platelets were washed with Ca^{2+}-free Krebs-Ringer glucose (KRG) solution composed of 120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 1 mM $CaCl_2$ and 10 mM glucose (pH 7.3). They were then gently resuspended in Ca^{2+}-free KRG supplemented with apyrase (1 U/mL). The platelet suspensions were stored in plastic tubes at room temperature and were used within 3 h after preparation. The extracellular Ca^{2+} concentration was adjusted to 1 mM immediately before each measurement.

4.2 Experimental setup

4.2.1 Resistance towards NO

In aggregation- and ATP measurements, platelets ($2.5x10^8$/mL) were preincubated and stirred (800 rpm) at 37 °C for 5 min before exposure to different drugs. When measuring $[Ca^{2+}]_i$ using the fura-2 method, platelets ($1\text{-}2x10^8$/mL) were loaded with fura-2 acetoxymethylester (fura-2 AM) and preincubated for 5 min at 37 °C, before exposure to different drugs. After preincubation, platelets were exposed to various concentrations (0.01 μM, 0.1 μM, 1.0 μM or 10 μM) of either of the two NO-containing drugs S-nitroso-acetylpenicillamine (SNAP) or the mesoionic 3-aryl substituted oxatriazole-5-imine GEA 3175. After additional 1 min, PAR-APs were added. Concentrations of the peptides used were 4 μM, 10 μM, 13.4 μM or 30 μM of PAR1-AP (SFLLRN) and 100 μM or 300 μM of PAR4-AP (AYPGKF). In some aggregation experiments, platelets were incubated with fibrinogen (0.1 mg/mL) before exposure to SNAP (1 μM or 10 μM) and stimulation with either PAR1-AP or PAR4-AP in different concentrations. To confirm the optimal time between exposure of platelets to SNAP and stimulation with PAR4-AP, as well the optimal dose of PAR4-AP, a time-study and a dose-study were designed, measuring aggregation. The time intervals tested between exposure to SNAP (1 μM) and stimulation with PAR4-AP was 5 s, 1 min, 10 min and 30 min, and the doses of PAR4-AP used were 100 μM and 300 μM.

4.2.2 The ability of PAR1 and PAR4 to undergo desensitization

In the cumulative dose-response studies platelets were preincubated for 5 min and then exposed to increasing concentrations of the PAR-APs (0.1 μM: 0.3 μM: 1 μM: 3 μM: 10 μM: 30 μM of PAR1-AP or 1 μM: 3 μM: 10 μM: 30 μM: 100 μM: 300 μM of PAR4-AP). The time interval between stimulation with the different doses was 3 min. In the "PAR1 desensitization experiments" platelets were preincubated with either one of five PKC inhibitors; Ro31-8220 (0.3 μM), Ro31-8425 (1 μM), Gö6976 (20 μM), Rottlerin (10 μM) or the PKCε translocation inhibitor protein (PKCεTIP) (10 μM) before stimulation with PAR1-AP in increasing concentrations. Ro31-8220 and Ro31-8425 are inhibitors of the conventional isoforms (α and β) of PKC, as well as some novel (at least θ). Gö6976 is an inhibitor of PKCα and β, rottlerin is an inhibitor of PKCδ and PKCεTIP is an inhibitor of PKCε.

To find out which dose of the PAR1-AP that is needed to initiate desensitization of the receptors, the platelets were treated with first a low dose of PAR1-AP (0.1 μM, 0.3 μM, 1 μM or 3 μM) for 10 min, followed by a high dose of PAR1-AP (30 μM). After defining the specific dose of PAR1-AP needed for initiation of desensitization, the time gap required between the adding of the two peptide concentrations (for a desensitization of PAR1 to occur) was evaluated. This was performed by incubating the platelets (for 30 s up to 90 min) with 3 μM PAR1-AP before stimulation with the high dose (30 μM). After determining the time and dose, the desensitization experiments proceeded. Platelets were preincubated with either one of the PKC inhibitors. They were then stimulated with a low dose of PAR1-AP (3 μM) for 10 min, followed by a high dose of PAR1-AP (30 μM). Since most of the inhibitors had a

suppressive effect on their own on the aggregation induced by PAR1-AP, fibrinogen (0.1 mg/mL) was added to the incubation step. Inhibitors of MEK, PI3K, Rho-kinase, Syk and Src were also used in this protocol to see whether they could restore the sensitivity of PAR1. Two well known inhibitors of MEK were used; PD98059 (5 μM) and U0126 (5 μM). The PI3K inhibitors LY294002 (5 μM) and wortmannin (100 nM), the Src family kinase inhibitor PP2 (5 μM), the Rho-kinase inhibitor Y27632 (10 μM) and the Syk inhibitor piceatannol (5 μM) were used.

4.2.3 PKC substrate phosphorylation

For Western blot analyses, platelets were isolated according to protocol (see section 4.1 "*Isolation of human platelets"*) and adjusted to $5x10^8$/mL. In some cases the platelet-rich plasma was incubated in absence of ASA. Platelets were stimulated with PAR1-AP (30 μM) or PAR4-AP (300 μM) for different lengths of time, to clarify the time-dependency of PKC substrate phosphorylation. The times used were 30 s, 1 min, 3 min and 10 min. A dose-response study was made for both APs, using an incubation time of 30 s for PAR1-AP and 1 min for PAR4-AP. The peptide concentrations used were 1 μM, 3 μM, 10 μM or 30 μM of PAR1-AP and 10 μM, 30 μM, 100 μM or 300 μM of PAR4-AP.

4.2.4 The expression of PAR1 surface receptors

For flow cytometry analyses, blood was collected into tubes containing trisodium citrate (0.129 M). It was then diluted (1:4) with the diluent (Reagent 1) provided in the kit, according to the instructions from the manufacturer. For each sample, one tube with 20 μL WEDE15 and one with 20 μL negative isotypic control IgG_1 (Reagent 2a) was prepared. Also for each sample, 200 μL diluted blood was pipetted into another tube and incubated with PAR1-AP (3 μM or 30 μM) for a certain duration time (30 s, 2 min, 5 min, 10 min, 30 min or 60 min). As a control, unstimulated blood was used. The treated blood was vortexed and 20 μL was added to each sample (the tubes with WEDE15 and Reagent 2a) and incubated for 10 min at room temperature. The secondary antibody (20 μL of Reagent 4) was added to all samples and again they were vortexed and incubated for 10 min at room temperature. To stop the reactions, 2 mL of diluted Reagent 1 was added. For each sample series a calibration tube was prepared. This contained 40 μL Reagent 3 (vortexed for 5 s), to which 20 μL Reagent 4 was added before stopping the reaction with 2 mL of diluted Reagent 1, as described above. Samples were then analyzed in a Coulter Epics XL.MCL flow cytometer with Expo 32 ADC software from Beckman Coulter, Miami, USA.

4.3 Measurements

4.3.1 Measurements of platelet aggregation

Aliquots (0.5 mL) of platelets ($2.5x10^8$/mL) were preincubated at 37 °C and stirred at 800 rpm for 5 min before exposure to different drugs. Changes in light transmission were recorded using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA, USA).

4.3.2 Measurement of ATP secretion

The concentration of extracellular ATP in platelet suspensions (0.5 mL; $2.5x10^8$ platelets/mL, stirred at 800 rpm at 37 °C) was registered using a luciferin/luciferase bioluminescent assay. The platelets were preincubated for 5 min and then treated with different drugs before stimulation with PAR-APs after additionally 1 min. Changes in bioluminescence were

recorded in a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA, USA).

4.3.3 Measurement of cytosolic Ca^{2+} concentrations

Platelets were loaded with fura-2 by incubating platelet-rich plasma with 3 µM fura-2 AM (from a 4 mM stock solution dissolved in dimethyl sulfoxide (DMSO)) for 45 min at 20 °C. The platelets were pelleted and resuspended as described in section 4.1 *"Isolation of human platelets"*. Platelet suspensions (2 mL; 1-2x10^8 platelets/mL) were incubated for 5 min at 37 °C and then exposed to different drugs (see section 4.2 "*Experimental setup*"). Fluorescence signals from platelet suspensions were recorded using a Hitachi F-7000 fluorescence spectrofluorometer specially designed for measurement of $[Ca^{2+}]_i$. Fluorescence emission was measured at 510 nm with simultaneous excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated according to the general equation as described by Grynkiewicz et al., 1985: $K_d(R-R_{min})/(R_{max}-R) \times (F_0-F_S)$. Maximal and minimal ratios were determined by addition of 10 % Triton X-100 and 250 mM EGTA, respectively. In most cases, the ratio value was used as an index of the rise in $[Ca^{2+}]_i$.

4.3.4 Western Blot

PKC substrate- (Ser) phosphorylation was analyzed with Western blot. Platelets were isolated according to protocol (see section 4.1 "*Isolation of human platelets*"). In some experiments, though, the platelet-rich plasma was incubated in absence of ASA. Platelets were adjusted to 5x10^8/mL, and in each sample 180 µL platelet suspension were used. To this, 20 µL $CaCl_2$ buffer (10 mM) with (or without) agonist was added. The reactions were carried out in an Eppendorf Thermomixer at 37 °C (900 rpm). Reactions were stopped by adding 50 µL of ice-cold 5 x SDS-sample buffer. Denaturing of the proteins was accomplished by heating the samples at 95 °C (900 rpm) for 5 min. Samples were loaded on 10 % NuPAGE Bis-Tris Gels (Invitrogen) together with two markers (one in each outer well). The marker-mix was composed of Magic Mark™ XP Western Protein Standard (Invitrogen) and Precision Plus Protein™ Dual Color Standard (Bio-RAD). Gels were run for 1 ½ - 2 h at 140 V with NuPAGE MOPS SDS Running Buffer (Invitrogen). Blotting to a PVDF-membrane was performed at 125 mA (per gel) for 1 h 45 min. Gels were stained with coomassie to confirm that the blotting procedure went well. Membranes were washed in TBS containing 1 % Tween (TBS-T) and then blocked with TBS-T containing 5 % nonfat dry milk for 1 h in room temperature with agitation. They were then washed and incubated with the primary antibody (Phospho-(Ser) PKC substrate AB (Cell Signalling), 1:2000 in TBS-T containing 5 % BSA) over night at 4 °C with agitation. The membranes were washed and incubated with the second antibody (anti rabbit antibody (Jackson Immuno Res), 1:2000 in TBS-T) for 1 h at room temperature with agitation. Detection was performed using ECL-solution (Millipore) and exposure in a FujiFilm LAS-1000 Intelligent Dark Box (Science Imaging, Scandinavia AB) with a CCD camera for 10-50 s.

4.3.5 Flow cytometry

The expression of PAR1 at the surface of the platelets was analyzed using flow cytometry. This was performed in a Coulter Epics XL.MCL flow cytometer with Expo 32 ADC software from Beckman Coulter, Miami, USA. Fluorosphere control samples (Flow-Check™ and Flow-Set™) from the same supplier were analysed before every sample series to verify that instrument optical alignment, fluidics and fluorescence intensity readings were stable over time. For the experimental procedure, the platelet calibrator kit "Platelet Calibrator" from Biocytex (Marseille, France) was used together with the monoclonal antibody WEDE15 (Immunotech, Marseilles, France). WEDE 15 recognizes residues 51-64 of the N-terminal

peptide of PAR1 (it thus recognizes both cleaved and uncleaved receptors) and was used as the primary antibody. The calibrator kit included a mixture of four calibration beads coated with increasing concentrations of mouse IgGs (300, 13000, 39000 and 91000) for the creation of a calibration curve. It also contained a secondary polyclonal antimouse IgG-fluorescein isothiocyanate (FITC) antibody used as a staining reagent, a negative isotypic control IgG_1 and a buffer (diluent). The experiments were performed in room temperature without stirring and according to the instructions from the manufacturer. For every sample series a calibration curve, based on the mean fluorescence intensity (MFI) and the known number of antigen sites for the calibration beads, was created. The number of surface expressed PAR1 could be estimated by using the calibration curve and subtracting the total number of the anti-PAR1 WEDE15 antibody binding sites with the value from the negative isotypic control. All samples (in the same series) were read with the same calibration curve.

4.4 Drugs

The PAR1 thrombin-receptor-agonist peptide TRAP-6 (SFLLRN) and the PAR4 thrombin-receptor-agonist peptide (AYPGKF) were synthesized by the Biotechnology Centre of Oslo, Oslo University, Norway. Aspirin, apyrase, ATP, fura-2 AM, fibrinogen, the luciferin/luciferase bioluminescent kit, piceatannol, Ro31-8220, Y27632 and thrombin, as well as chemicals for the buffers, were obtained from Sigma Chemicals Co (St. Louis, MO, USA). SNAP was from ALEXIS (San Diego, CA, USA). The following drugs were from TOCRIS; Gö6976 (Batch No. 1A); Rottlerin (Batch No. 2A/84832); LY294002 (Batch No. 3A/87849) and Wortmannin (Batch No. 7A/86375). PD98059 and U0126 were from MEK-Inhibitor Tocriset™ (Batch No.1). PKCεTIP (Lot.No. D00027761), Ro31-8425 (Lot.No. D00039627) and PP2 were from Calbiochem. GEA 3175 was from GEA Pharmaceuticals (Copenhangen, Denmark). Phospho-(Ser) PKC substrate AB (polyclonal rabbit antibody); Cell Signalling #2261, Lot.No. 10. Goat anti rabbit antibody; Jackson Immuno Res #111-035-144, Lot.No. 79749. Monoclonal Antibody Anti-Thrombin receptor, PN IM2085- Purified- Freeze dried- 0.2 mg – Clone WEDE15; Immunotech (Marseille, France). Precision Plus Protein™ Dual Color Standards, Bio-RAD. MagicMark™ XP Western Protein Standard, Invitrogen. Immobilon™ Western Chemiluminescent HRP Substrate; Millipore. Platelet Calibrator, Biocytex (Marseille, France).

4.5 Statistical analysis

Results are expressed as mean values (±SEM). Statistical significance was tested with two-way ANOVA and Bonferronis post test for multiple comparisons, or one-way ANOVA and Dunnett's post test for multiple comparisons (for comparison of one column (control values) with all other columns). Data were analyzed using GrapPad Prism™ v. 4.0. for Windows (GrapPad Software, San Diego, California, USA). *P<0.01-0.05; **P<0.001-0.01; ***P<0.001.

5 Results

5.1 The effect of NO on aggregation

First it was interesting to elucidate the role of NO as an inhibitor of platelet aggregation induced by PAR1-AP and PAR4-AP. For this, PAR-APs were used to stimulate platelets exposed to different concentrations of the NO-containing drug SNAP. The optimal time between exposure to SNAP and the following stimulation with the PAR-APs, as well as the concentrations of the peptides used, was investigated in beforehand (results not shown). In the same experimental setup it was also possible to see whether there was a difference between PAR1-AP and PAR4-AP in their resistance/sensitivity towards NO. Figure 1 shows the result

from the aggregation measurements obtained with SNAP and the two PAR-APs. As can be seen, SNAP strongly inhibited PAR-AP induced aggregation.

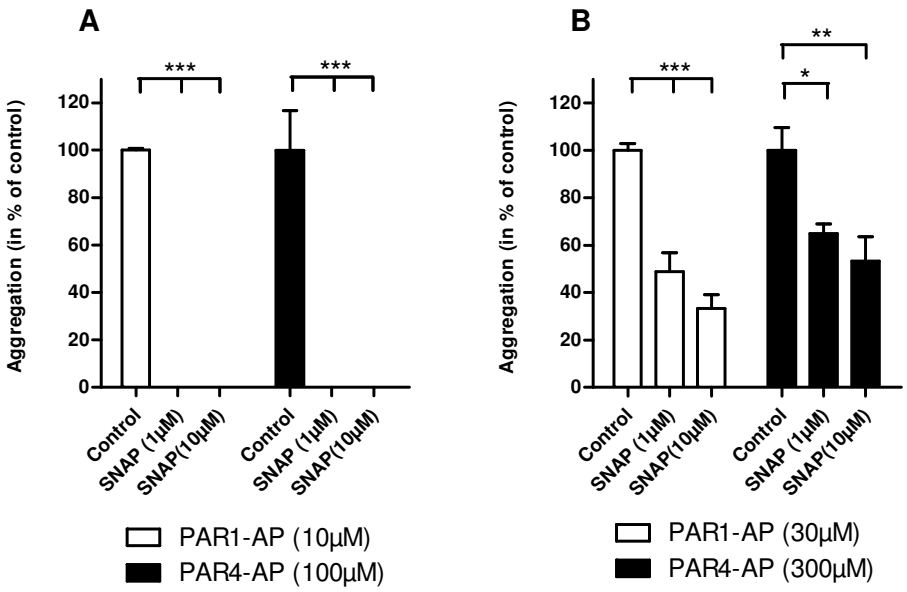

Figure 1. The effects of the NO-containing drug SNAP on aggregation induced by PAR1-AP and PAR4-AP. Isolated human platelets were preincubated for 5 min at 37 °C in the aggregometer and then treated with different concentrations of the NO-containing drug SNAP, followed by stimulation (after 1 min) with PAR-APs (10 µM or 30 µM PAR1-AP; 100 µM or 300 µM PAR4-AP). The controls are platelets preincubated in absence of SNAP. The results are presented as means (±SEM) and as percent aggregation of control. n=3-4. Statistical significance was tested with two-way ANOVA followed by Bonferronis test for multiple comparisons. There was no significant difference between PAR1-AP and PAR4-AP in either (A) or (B).

To further confirm the inhibitory effect of NO on aggregation, the dose-response experiment with 100 µM PAR4-AP was repeated using another well known NO-containing drug, GEA 3175. Figure 2 shows that GEA 3175 dose-dependently suppressed aggregation induced by 100 µM PAR4-AP.

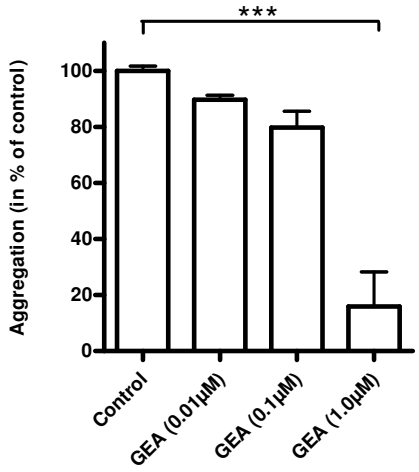

Figure 2. The effects of the NO-containing drug GEA 3175 on aggregation induced by PAR4-AP.Isolated human platelets were preincubated for 5 min at 37 °C in the aggregometer and then treated with different concentrations of the NO-containing drug GEA 3175, followed by stimulation (after 1 min) with PAR4-AP (100 µM). The control represents the response of platelets preincubated in absence of GEA 3175. The results are presented as means (±SEM) and in percent of control. n=4. Statistical significance was tested with one-way ANOVA followed by Dunnett's test for multiple comparisons.

5.2 The effect of NO on intracellular Ca^{2+} responses

The effect of NO on platelet activation induced by PAR1-AP and PAR4-AP was also tested by using the fura-2 method, in which the changes in $[Ca^{2+}]_i$ are measured. PAR-APs (4 µM or 13.4 µM of PAR1-AP and 100 µM or 300 µM of PAR4-AP) were used to stimulate platelets pre-exposed to different concentrations of the NO-containing drug SNAP. The increase in $[Ca^{2+}]_i$ induced by 4 µM PAR1-AP was similar to that induced by 100 µM PAR4-AP. Likewise, the increase in $[Ca^{2+}]_i$ induced by 13.4 µM PAR1-AP was similar to that induced by 300 µM PAR4-AP (results not shown). This is why these concentrations of the APs were chosen to be compared in this experiment. The results (Figure 3) show that SNAP significantly decreased PAR-AP-induced Ca^{2+} mobilization. Further, no significant difference was found between PAR1-AP and PAR4-AP.

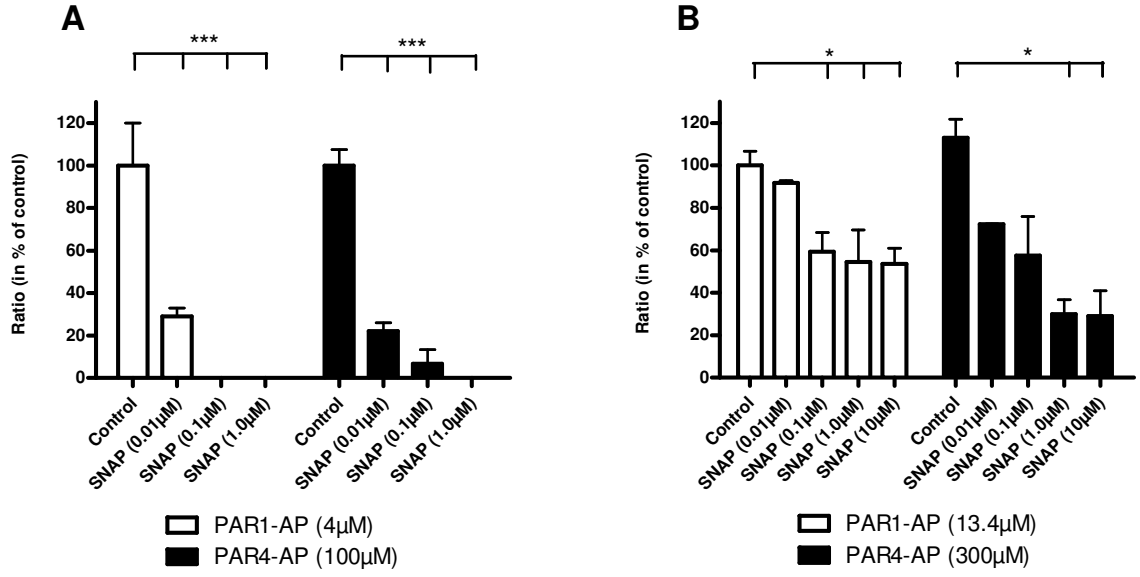

Figure 3. The effects of the NO-containing drug SNAP on the Ca^{2+} response elicited by PAR1-AP and PAR4-AP. Isolated human platelets treated with fura-2 AM were preincubated for 5 min at 37 °C and then exposed to different concentrations of the NO-donor SNAP. Thereafter they were stimulated with (A); a low dose of either PAR1-AP (4 µM) or PAR4-AP (100 µM), or (B); a high dose of PAR1-AP (13.4 µM) or PAR4-AP (300 µM). The controls are platelets preincubated in absence of SNAP. The results are presented as means (±SEM) and in percent ratio (rise in $[Ca^{2+}]_i$) of respective control. n=2 (PAR1-AP (4 µM)), n=3 (PAR1-AP (13.4 µM) and PAR4-AP). Statistical significance was tested with two-way ANOVA followed by Bonferronis test for multiple comparisons. There was no significant difference between PAR1-AP and PAR4-AP in either (A) or (B).

5.3 The effect of NO and the importance of granule secretion

During aggregation studies with PAR4-AP, the ATP released from dense granules were analyzed simultaneously (this was not done with PAR1-AP). It was found that the NO-donor SNAP strongly suppressed PAR4-AP induced ATP secretion (results not shown). This inhibition of secretion (and thereby possibly less secreted fibrinogen) might explain the inhibitory effect of NO on PAR4-AP induced aggregation. An alternative explanation, however, could have been that NO inhibited the conformational change of $\alpha_{IIb}\beta_3$, necessary for fibrinogen binding and aggregation. To elucidate this, the dose-response experiment with various doses of SNAP and PAR-APs (see Figure 1) was repeated in presence of fibrinogen (0.1 mg/mL). The results (Figure 4) show that incubation of the platelets with fibrinogen did not change the response obtained with SNAP and the lower doses of PAR-APs (10 µM PAR1-AP and 100 µM PAR4-AP). However, it did have an affect when using the higher doses (30 µM PAR1-AP and 300 µM PAR4-AP).

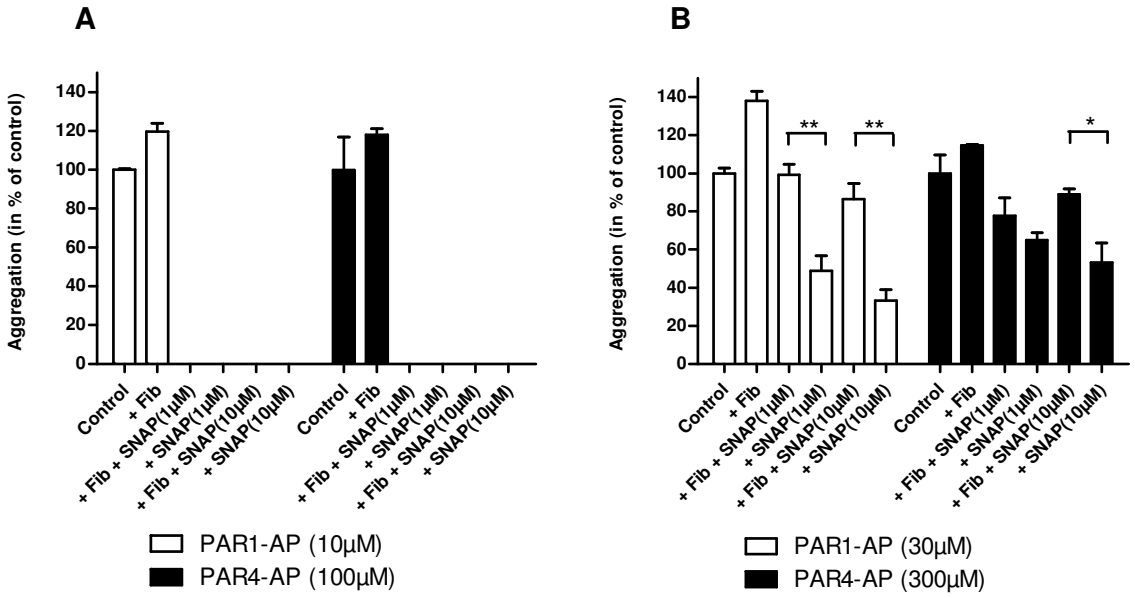

Figure 4. The effects of the NO-containing drug SNAP on aggregation induced by PAR-APs when platelets were preincubated with fibrinogen. Isolated human platelets were preincubated for 5 min at 37 °C in the aggregometer, in presence or absence of fibrinogen. They were then treated with different concentrations of SNAP, followed by stimulation (after 1 min) with PAR1-AP (10 µM or 30 µM) or PAR4-AP (100 µM or 300 µM). Controls are platelets preincubated in absence of SNAP. The result is presented as means (±SEM) and in percent aggregation of respective control. n=3-4. Statistical significance was tested with two-way ANOVA followed by Bonferronis test for multiple comparisons.

5.4 Cumulative dose-response studies

To investigate the second hypothesis, that PAR1 and PAR4 differ in their abilities to undergo desensitization, cumulative dose-response studies were performed. For this, PAR4-AP (1 µM: 3 µM: 10 µM: 30 µM: 100 µM: 300 µM) and PAR1-AP (0.1 µM: 0.3 µM: 1 µM: 3 µM: 10 µM: 30 µM) were used. The results revealed that platelets stimulated with increasing concentrations of PAR4-AP responded in a normal "cumulative dose dependent manner" (i.e. the magnitude of the aggregation response was not underestimated compared to the effect of a single, high dose of PAR4-AP). However, this cumulative dose-response effect was not possible to obtain with PAR1-AP. Specifically it was found that low "non-aggregatory" concentrations of PAR1-AP fully desensitized platelets towards higher concentrations of

PAR1-AP. Another interesting feature found in this experiment was that addition of PAR4-AP (300 µM) after the highest dose of PAR1-AP, mediated a full aggregation response (see Figure 5).

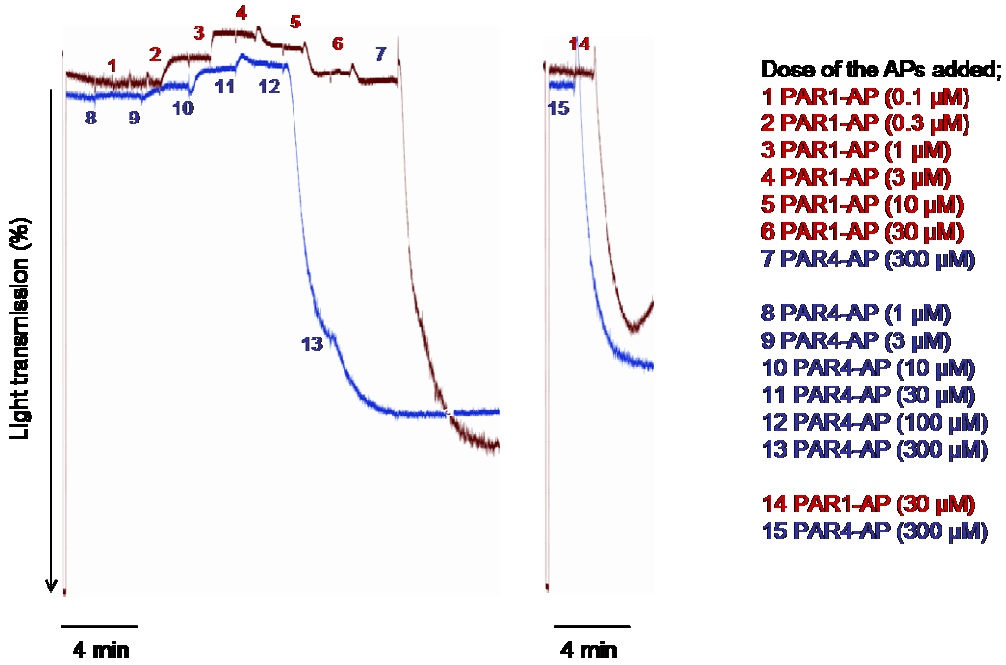

Figure 5. Cumulative dose-response aggregometry study with PAR1-AP and PAR4-AP. Isolated human platelets were preincubated for 5 min at 37 °C in the aggregometer and then exposed to increasing concentrations of either of the PAR-APs (0.1 µM: 0.3 µM: 1 µM: 3 µM: 10 µM: 30 µM of PAR1-AP and 1 µM: 3 µM: 10 µM: 30 µM:100 µM: 300 µM of PAR4-AP). The red curve represents PAR1-AP and the blue curve PAR4-AP. Controls (30 µM PAR-AP and 300 µM PAR4-AP) are shown to the right. The curves are representative of six repeats.

Cumulative dose-response studies were performed also when measuring $[Ca^{2+}]_i$. In accordance with the results from the aggregation study, the rise in $[Ca^{2+}]_i$ were inhibited when adding a high concentration of PAR1-AP after pre-stimulation with lower. However, in this case, the receptor response was only partly desensitized. The response to the highest dose of PAR1-AP (30 µM) in the cumulative study was about 20 % of the control (results not shown). To clarify which dose of the PAR1-AP that was needed to initiate PAR1 desensitization, platelets were treated with first a low dose of PAR1-AP (0.1, 0.3, 1 or 3 µM) for 10 min, followed by a high dose of PAR1-AP (30 µM). Aggregation and $[Ca^{2+}]_i$ were measured and the results are shown in Figure 6. Clearly, the dose needed for initiation of desensitization (3 µM) lies within a narrow range of sub-threshold concentrations.

After defining the specific dose of PAR1-AP needed for inducing desensitization, the time gap required between the addition of the lower and the higher concentration of PAR1-AP was evaluated. This was accomplished by incubating the platelets with 3 µM PAR1-AP for different lengths of time (from 30 s up to 90 min) before stimulation with the high dose (30 µM). Again, both aggregation and $[Ca^{2+}]_i$ were measured and the results are shown in Figure 7. As can be seen, when measuring aggregation, PAR1 desensitization was initiated approximately 5 min after stimulation with 3 µM PAR1-AP, and a full desensitization was

achieved after about 10 min. When measuring Ca^{2+} mobilization, however, the desensitization was only partial, and a full desensitization mechanism was not obtained even after 30 min.

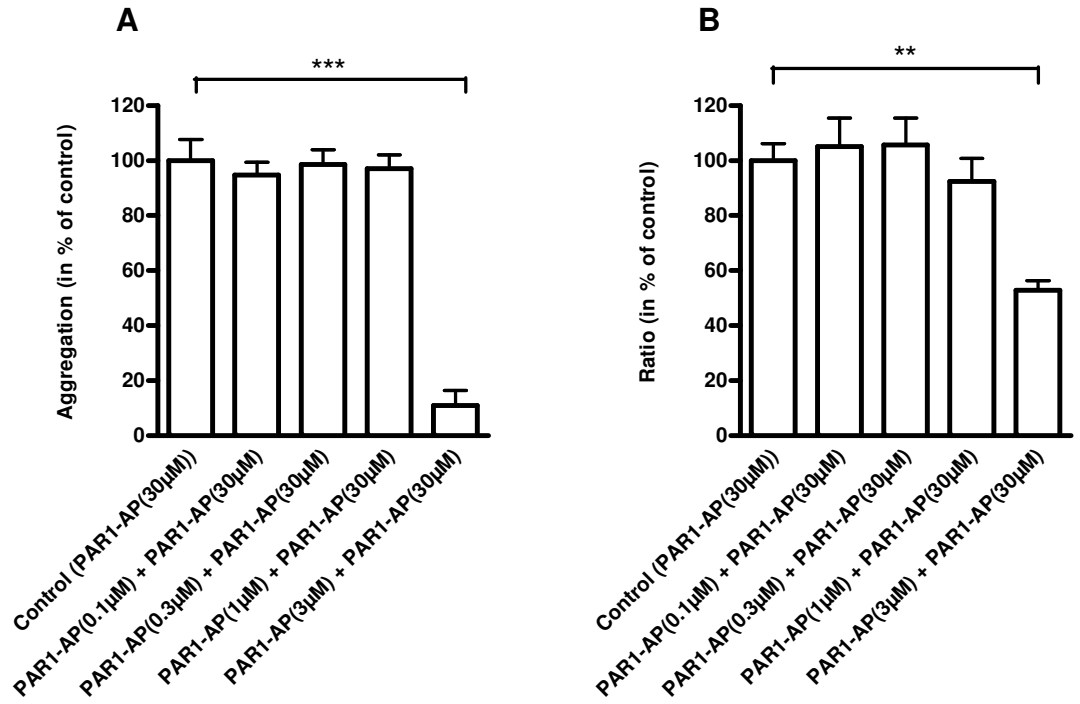

Figure 6. Dose-study on PAR1 desensitization when measuring (A): aggregation and (B): intracellular Ca^{2+} concentrations. Isolated human platelets (treated with fura-2 AM for measurement of $[Ca^{2+}]_i$) were preincubated for 5 min at 37 °C. They were then exposed to a low concentration of PAR1-AP (0.1, 0.3, 1 or 3 µM) 10 min previous to stimulation with 30 µM PAR1-AP. The controls are platelets stimulated with only the high dose of PAR1-AP (30 µM). Aggregation or the ratio (rise in $[Ca^{2+}]_i$) was measured and the results are presented as means (±SEM) and as percent aggregation or percent ratio of control. n=3. Statistical significance was tested with one-way ANOVA followed by Dunnett's test for multiple comparisons

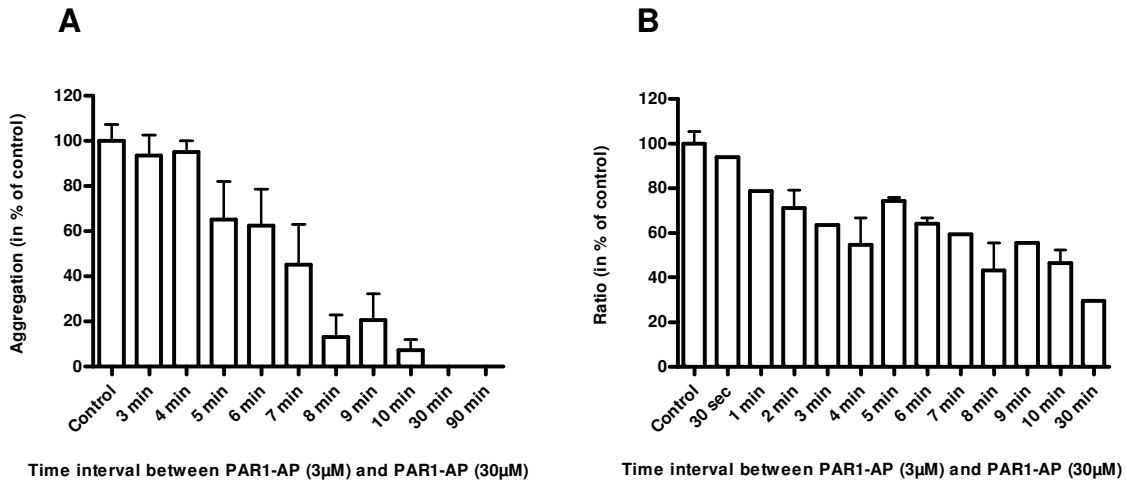

Figure 7. Time-study of the initiation of PAR1 desensitization when measuring (A): aggregation and (B): intracellular Ca^{2+} concentrations. Isolated human platelets (treated with fura-2 AM for measuring $[Ca^{2+}]_i$) were preincubated for 5 min at 37 °C. They were then exposed to 3 µM PAR1-AP, followed by stimulation with 30 µM PAR1-AP at different time points (from 30 s up to 90 min). The controls are platelets stimulated with only the high dose of PAR1-AP (30 µM). Aggregation or the ratio (rise in $[Ca^{2+}]_i$) was measured and the results are presented as means (±SEM) and as percent aggregation or percent ratio of control. n=1-3.

5.5 The involvement of PKC in PAR1 desensitization

One of the mechanisms behind the desensitization of PAR1 may involve PKC, since this kinase has been linked to the desensitization of other platelet receptors (Mundell et al., 2006). This role of PKC was tested by preincubating the platelets with inhibitors against different PKC isoforms prior to stimulation with PAR1-AP in increasing concentrations. The inhibitors used were Ro31-8220 (0.3 µM), Ro31-8425 (1 µM), Gö6976 (20 µM), Rottlerin (10 µM) and PKCεTIP (10 µM). None of the PKC inhibitors had any affect on the cumulative dose-response curve obtained with PAR1-AP when measuring aggregation (results not shown). Again, adding PAR4-AP (300 µM) after the highest dose of PAR1-AP, triggered a full aggregation response. The results from the $[Ca^{2+}]_i$ measurements (Figure 8) show that the desensitization of PAR1 was partly reversed by two Ro-compounds.

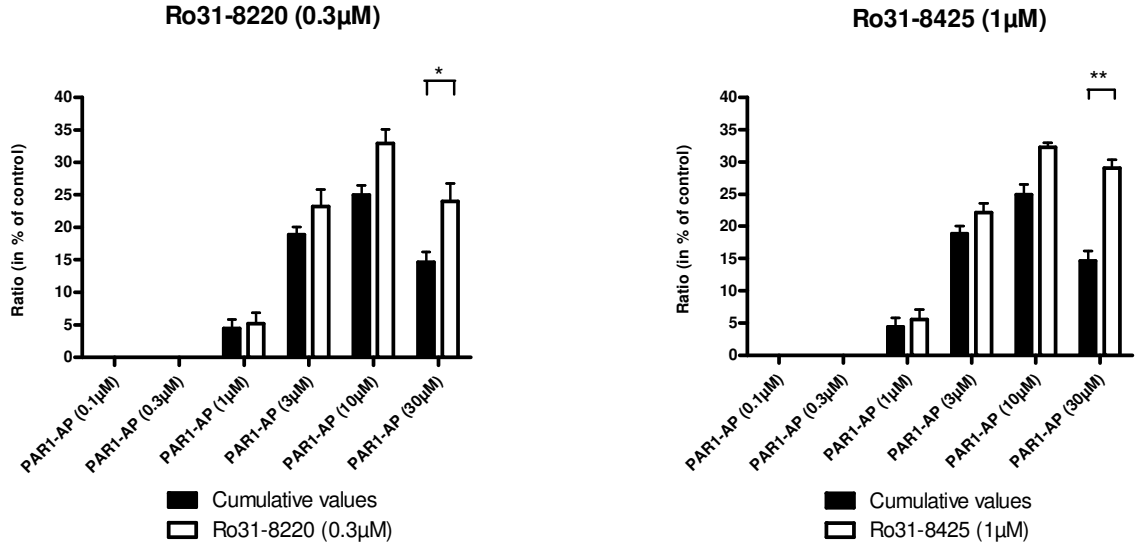

Figure 8. The effect of two different PKC inhibitors on PAR1 desensitization in cumulative dose- response studies when measuring intracellular Ca^{2+} concentrations. Isolated human platelets treated with fura-2 AM were preincubated with either of two different PKC inhibitors (Ro31-8220 or Ro31-8425) for 5 min at 37 °C and then exposed to increasing concentrations of PAR1-AP. The ratio (rise in $[Ca^{2+}]_i$) was measured. The cumulative values (black bars) are platelets preincubated in absence of inhibitor. Results are presented as means (±SEM) and as percent ratio of control. The controls (not shown) representing 100 % ratio were platelets preincubated in absence of inhibitor and stimulated only with 30 µM of PAR1-AP. n=4. Statistical significance was tested with two-way ANOVA followed by Bonferronis test for multiple comparisons.

The experiments clarifying the ability of PAR1 to undergo desensitization proceeded, using both aggregation- and Ca^{2+} studies. When measuring $[Ca^{2+}]_i$, however, rottlerin and Gö6976 had to be excluded since they interfered with the fluorescence properties of fura-2. The platelets were preincubated with PKC inhibitors before exposure to 3 µM PAR1-AP, followed by 10 min incubation and then stimulation with 30 µM PAR1-AP (PAR1-AP concentrations and incubation time evaluated in section 5.4). The PKC inhibitors used were the same as mentioned above. Since most of the drugs had an inhibitory effect on their own on platelet aggregation induced by PAR1-AP, fibrinogen was added to the incubation step. The results are shown in Figure 9A. Interestingly, adding PAR4-AP (300 µM) after the highest dose of PAR1-AP, again caused a full aggregation response (result not shown). The results obtained when measuring $[Ca^{2+}]_i$ are shown in Figure 9B, an original data from one of these experiments, showing the mobilization of intracellular Ca^{2+}, is shown in Figure 10. As can be seen, none of the inhibitors had any effect when measuring aggregation (Figure 9A). However, when measuring $[Ca^{2+}]_i$ (Figure 9B and 10), the Ro-compounds to some part restored the sensitivity of PAR1.

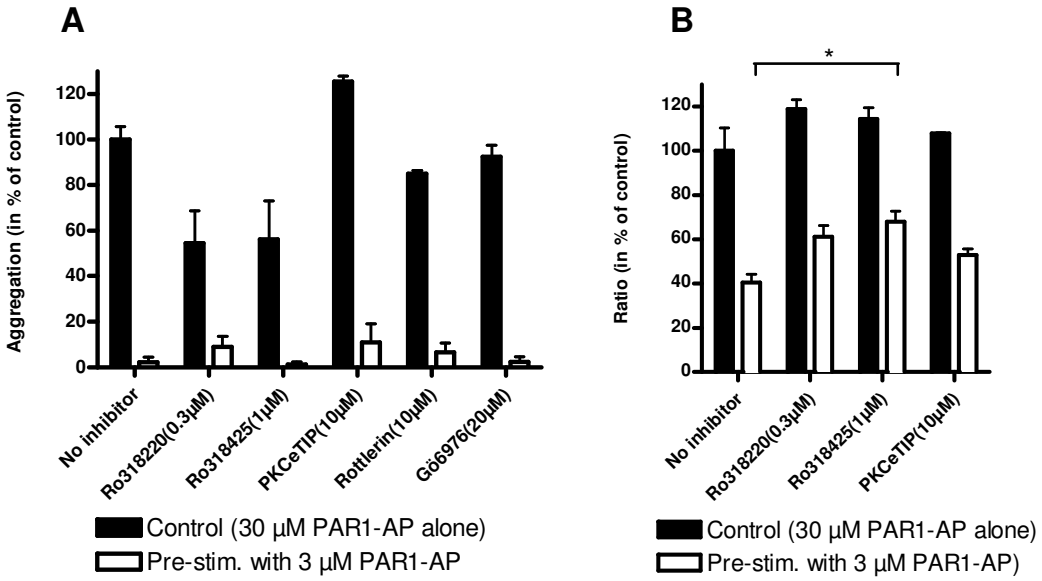

Figure 9. The effect of different PKC inhibitors on PAR1 desensitization when measuring (A): aggregation and (B): intracellular Ca^{2+} concentrations. Isolated human platelets (treated with fura-2 AM for measurement of $[Ca^{2+}]_i$) were preincubated for 5 min at 37 °C in the presence (or absence) of different PKC inhibitors. They were then exposed to 3 µM PAR1-AP, followed by 10 min incubation previous to stimulation with the second dose (30 µM) of PAR1-AP (white bars). Black bars represent platelets stimulated with only the high dose of PAR1-AP (30 µM). Aggregation or the ratio (rise in $[Ca^{2+}]_i$) was measured and the results are presented as means (±SEM) and as percent aggregation or percent ratio of the control value (black bar for "no inhibitor"). n=3-4. Statistical significance was tested with one-way ANOVA followed by Dunnett's test for multiple comparisons. No significant difference was found between platelets incubated in absence or presence of PKC inhibitors when measuring aggregation.

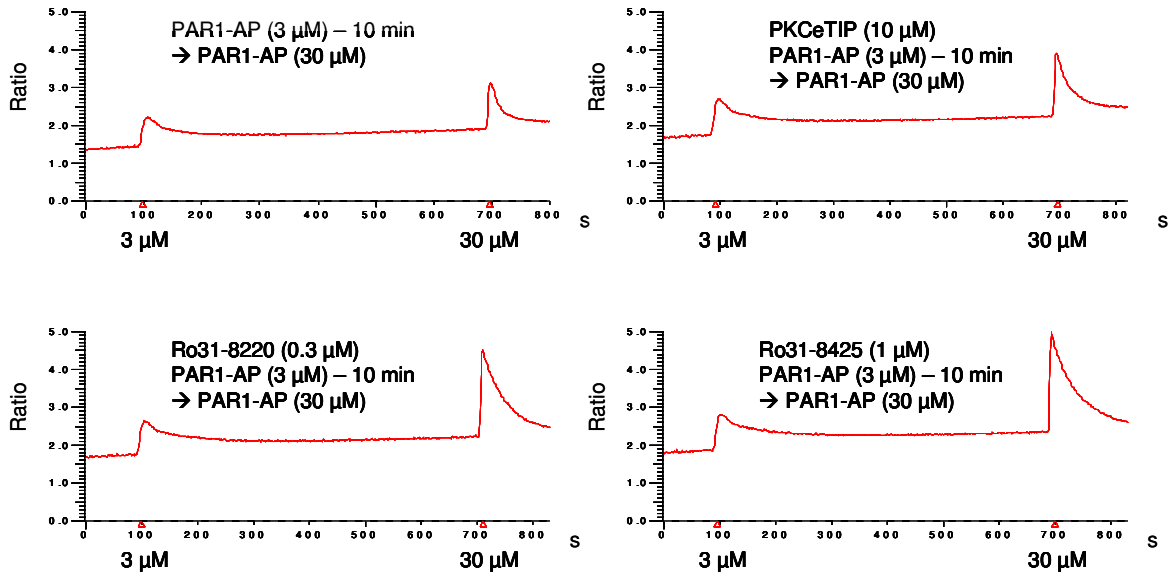

Figure 10. The effect of different PKC inhibitors on PAR1 desensitization when measuring intracellular Ca^{2+} concentrations. Isolated human platelets treated with fura-2 AM were preincubated for 5 min at 37 °C in the presence (or absence) of either one of three PKC inhibitors. They were then exposed to 3 µM PAR1-AP, followed by 10 min incubation previous to stimulation with the second dose (30 µM) of PAR1-AP. The ratio (rise in $[Ca^{2+}]_i$) was analyzed and the traces are representative of four repeats.

Continuing with the desensitization experiments when measuring aggregation, the platelets were preincubated with inhibitors affecting different signalling pathways associated with PAR1 and PAR4 activation. They were then exposed to 3 µM PAR1-AP, followed by 10 min incubation and stimulation with 30 µM PAR1-AP. The inhibitors used were two well characterized inhibitors of MEK; PD98059 (5 µM) and U0126 (5 µM), the PI3K inhibitors LY294002 (5 µM) and wortmannin (100 nM), the Src family kinase inhibitor PP2 (5 µM), the Rho-kinase inhibitor Y27632 (10 µM) and the Syk inhibitor piceatannol (5 µM). Interestingly, adding PAR4-AP (300 µM) after the highest dose of PAR1-AP, again caused a full aggregation response (results not shown). The same protocol was used also for measuring $[Ca^{2+}]_i$, however, only the most promising inhibitors from the aggregation experiments were used (U0126, PD98059 and LY294002). The results (Figure 11) show that none of the inhibitors significantly restored the sensitivity of PAR1.

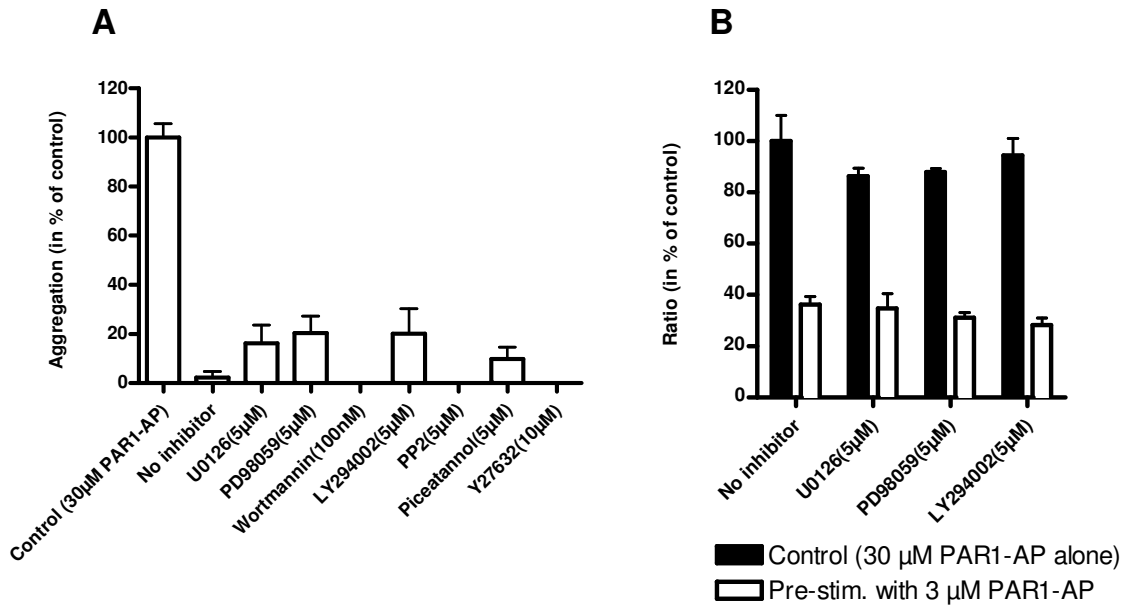

Figure 11. Comparison of the effect of different inhibitors on PAR1 desensitization when measuring (A): aggregation and (B): intracellular Ca^{2+} concentrations. Isolated human platelets (treated with fura-2 AM for measurement of $[Ca^{2+}]_i$) were preincubated for 5 min at 37 °C in the presence (or absence) of different PKC inhibitors. They were then exposed to 3 µM PAR1-AP, followed by 10 min incubation previous to stimulation with the second dose (30 µM) of PAR1-AP. The control in (A) is platelets preincubated in absence of inhibitor and stimulated with only the high dose of PAR1-AP (30 µM). The black bars in (B) represents the ratio obtained with platelets stimulated with only the high dose of PAR1-AP (30 µM). Aggregation or the ratio (rise in $[Ca^{2+}]_i$) was measured and the results are presented as means (±SEM) and as percent aggregation or percent ratio of control. For the aggregation measurements: n=4 (U0126, LY294002 and PD98059), n=3 (PP2, Y27632, wortmannin and piceatannol). For the measurements of $[Ca^{2+}]_i$: n=3. Statistical significance was tested with one-way ANOVA followed by Dunnett's test for multiple comparisons. No significant difference was found between platelets incubated in absence or presence of PKC inhibitors in either aggregation- or $[Ca^{2+}]_i$ measurements.

5.6 PKC substrate phosphorylation

So far, the results indicated that PKC activation to some part contributed to desensitization of PAR1. Therefore, the role of this kinase was further investigated. Western Blot was performed with an antibody directed towards PKC substrate-(Ser) phosphorylation, which was used as an indicator of PKC activity. A dose-response study was made with both PAR1-AP and PAR4-AP to clarify whether the activation of PKC was detectable, if it was dose dependent, and whether it was different for the two PARs. The peptide concentrations used were 1 µM, 3 µM, 10 µM and 30 µM of PAR1-AP and 10 µM, 30 µM, 100 µM and 300 µM of PAR4-AP. The incubation times used was 30 s for PAR1-AP and 1 min for PAR4-AP. Platelets used in this experimental setup were isolated either in presence or absence of ASA, to see whether this aspect affected the outcome. The results are shown in Figure 12 (PAR1-AP) and 13 (PAR4-AP). As can be seen, PKC substrate phosphorylation occurred in a "dose-response manner" when stimulated with the PAR-APs.

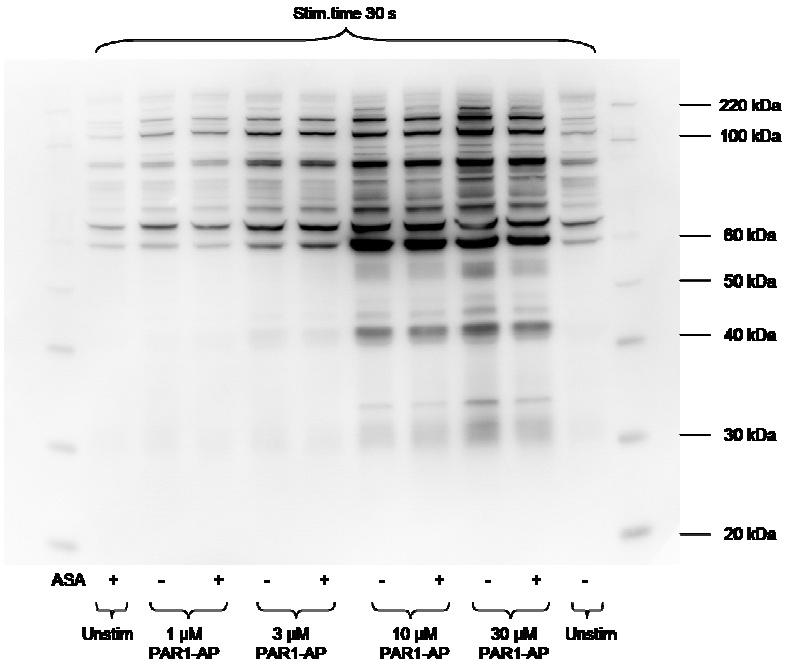

Figure 12. Western Blot on PKC substrate-(Ser) phosphorylation. Isolated platelets (isolated in the presence or absence of ASA) were stimulated for 30 s with different doses of PAR1-AP (1 µM, 3 µM, 10 µM or 30 µM). Samples were denatured and proteins separated with SDS-PAGE. Proteins were blotted onto a PVDF-membrane and incubated with an antibody against PKC substrate-(Ser) phosphorylation. The second antibody was a HRP conjugated goat anti-rabbit antibody. Detection was performed in a FujiFilm LAS-1000 Intelligent Dark Box with an exposure time of 30 s. The blot is representative of two repeats.

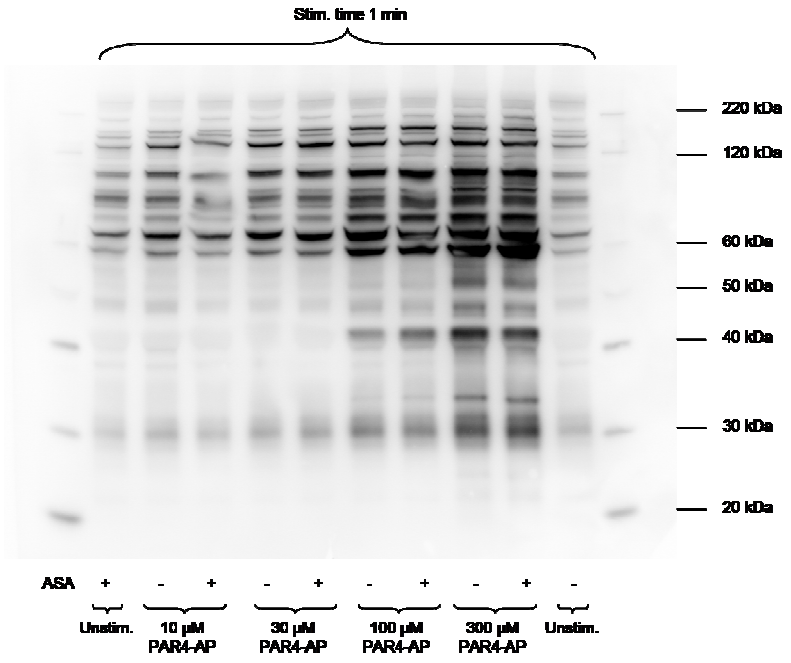

Figure 13. Western Blot on PKC substrate-(Ser) phosphorylation. Isolated platelets (isolated in the presence or absence of ASA) were stimulated for 1 min with different doses of PAR4-AP

(10 µM, 30 µM, 100 µM or 300 µM). Samples were denatured and proteins separated with SDS-PAGE. Proteins were blotted onto a PVDF-membrane and incubated with an antibody against PKC substrate-(Ser) phosphorylation. The second antibody was a HRP conjugated goat anti-rabbit antibody. Detection was performed in a FujiFilm LAS-1000 Intelligent Dark Box with an exposure time of 30 s. The blot is representative of two repeats.

Too see whether the PKC substrate phosphorylation was time-dependent and whether this differed between the PARs, platelets were incubated and stimulated with 30 µM PAR1-AP or 300 µM PAR4-AP for different lengths of time. The times used were 30 s, 1 min, 3 min and 10 min. Again, the platelets were isolated in either the presence or absence of ASA. The results are shown in Figure 14 (PAR1-AP) and 15 (PAR4-AP) and reveal that PAR1-AP induced a transient PKC activation whereas PAR4-AP induced a sustained activation. Further, ASA to some extent prolonged the time needed for activation of PKC when stimulated with PAR-APs, but only for a short time (less than 1 min).

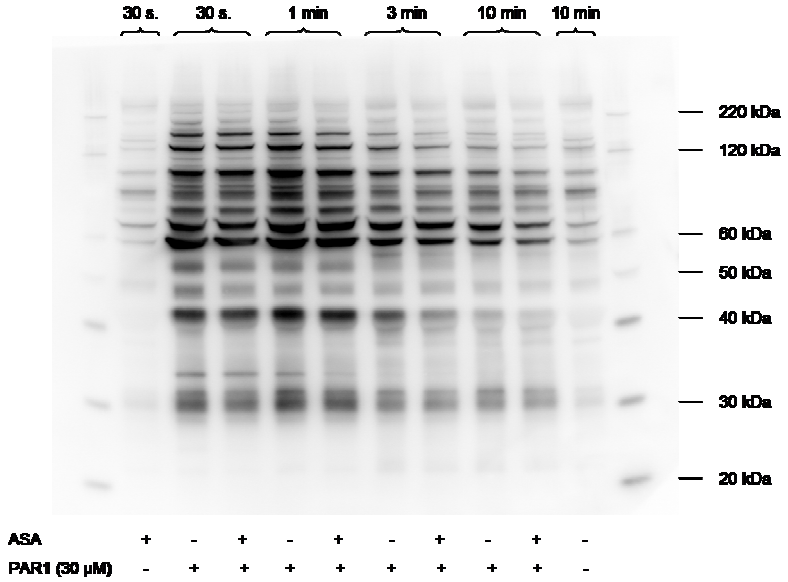

Figure 14. Western Blot on PKC substrate-(Ser) phosphorylation. Isolated platelets (isolated in the presence or absence of ASA) were stimulated (for 30 s, 1 min, 3 min or 10 min) with PAR1-AP (30 µM). Samples were denatured and proteins separated with SDS-PAGE. Proteins were blotted onto a PVDF-membrane and incubated with an antibody against PKC substrate-(Ser) phosphorylation. The second antibody was a HRP conjugated goat anti-rabbit antibody. Detection was performed in a FujiFilm LAS-1000 Intelligent Dark Box with an exposure time of 30 s. The blot is representative of two repeats.

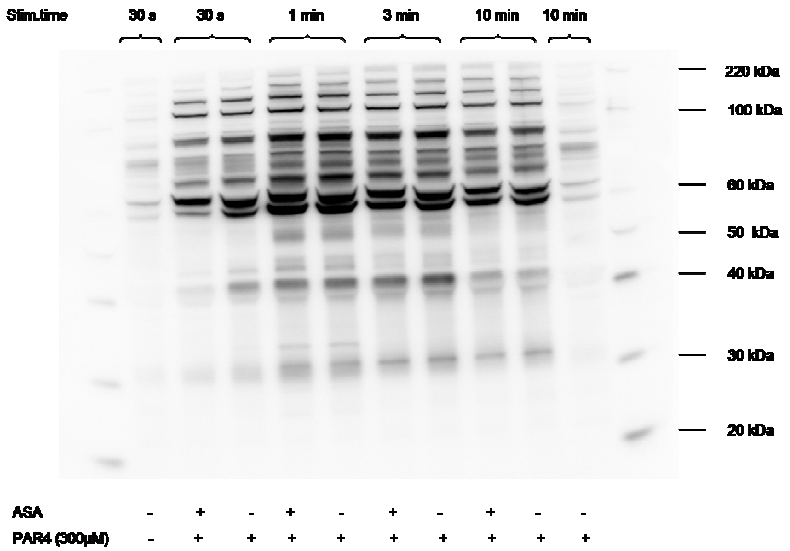

Figure 15. Western Blot on PKC substrate-(Ser) phosphorylation. Isolated platelets (isolated in the presence or absence of ASA) were stimulated (for 30 s, 1 min, 3 min or 10 min) with PAR4-AP (300 µM). Samples were denatured and proteins separated with SDS-PAGE. Proteins were blotted onto a PVDF-membrane and incubated with an antibody against PKC substrate-(Ser) phosphorylation. The second antibody was a HRP conjugated goat anti-rabbit antibody. Detection was performed in a FujiFilm LAS-1000 Intelligent Dark Box with an exposure time of 30 s. The blot is representative of two repeats.

5.7 PAR1 desensitization does not involve receptor internalization

To clarify whether the desensitization of PAR1 involved internalization of the receptors or not, flow cytometry was used. The Platelet Calibrator kit from Biocytex was used to analyze the expression of PAR1 at the platelet surface. Analyzes were made after stimulating the platelets (in diluted whole blood) with PAR1-AP (3 µM or 30 µM) for different duration times (30 s, 2 min, 5 min, 10 min, 30 min or 60 min). The results (Figure 16) show that stimulation of platelets with PAR1-AP did not cause a reduction of surface PAR1.

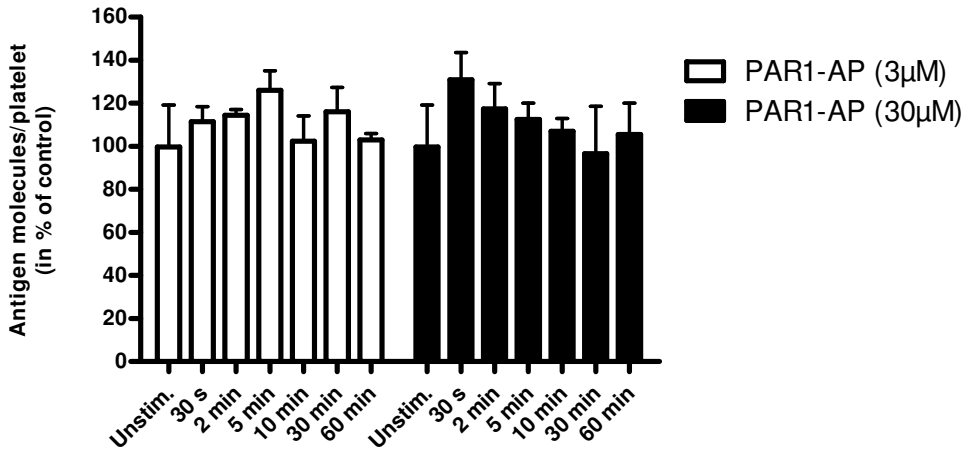

Figure 16. Flow cytometry on the expression of surface PAR1. Whole blood was incubated and stimulated with PAR1-AP (3 µM or 30 µM) for a certain duration time (30 s, 2 min, 5 min, 10 min, 30 min or 60 min). The expression of surface PAR1 was analyzed using the platelet calibrator kit "Platelet Calibrator" from Biocytex (Marseille, France) and the

monoclonal antibody WEDE15 from Immunotech (Marseille, France). Measurements were performed with a Coulter Epics XL.MCL flow cytometer with Expo 32 ADC software from Beckman Coulter, Miami, USA. n=3-4. Statistical significance was tested with two-way ANOVA followed by Bonferronis test for multiple comparisons. No significant difference was found between the numbers of antigen molecules per platelet, depending on the time of stimulation. Neither was there a difference between the doses used of the PAR-APs.

6 Discussion

The thrombin receptors PAR1 and PAR4 might be suitable therapeutic targets for future management of thrombosis. It has previously been shown that PAR1 and PAR4 activate the same variants of G-proteins, but that they differ in affinity to thrombin and in the duration of signalling (Covic et al., 2000; Kahn et al., 1999; Shapiro et al., 2000; Voss et al., 2007; Woulfe, 2005). Hence, this study was designed to further clarify differences in the activities of the two receptors, especially focusing on their resistance/sensitivity towards NO and their ability to undergo desensitization. Firstly, we hypothesized that NO may have variable capacity to suppress PAR1-AP- and PAR4-AP activated platelets. The result in the present study, however, showed that NO exerted a similar inhibitory effect on PAR1-AP- and PAR4-AP mediated aggregation and Ca^{2+} mobilization (Figure 1 and 3). This was thus in contrast to our hypothesis. When measuring aggregation, the presence of fibrinogen did not change the response obtained with SNAP when stimulating the platelets with the lower peptide concentrations (10 µM PAR1-AP and 100 µM PAR4-AP). However, the presence of fibrinogen slightly diminished the effect of NO when using the high peptide concentrations (30 µM PAR1-AP and 300 µM PAR4-AP) (see Figure 4). This led to the suggestion that the inhibitory effect exerted by NO on aggregation is not solely due to inhibition of granule secretion. The effect is more likely due to an inhibition of the conformational change of the fibrinogen receptor ($\alpha_{IIb}\beta_3$), in combination with an attenuated secretion. The results further showed that the inhibitory effect exerted by NO on aggregation and Ca^{2+} release was statistically significant. The role of NO as a negative regulator of platelet aggregation was further confirmed using another NO-containing drug, GEA 3175. This NO-containing oxatriazole derivative was not as significant as SNAP in inhibiting aggregation induced by PAR4-AP, but even though, the inhibitory effect was strong. Taken together, the results show that PAR1 and PAR4 are equally sensitive to the platelet inhibitor NO. Thus, in the current search for new, better, targets for the management of thrombosis, it does not seem to be worth putting emphasis on NO as a way to differently target PAR1 and PAR4.

During the cumulative dose-response studies it was discovered that it was possible to obtain a cumulative dose-response with PAR4-AP but not with PAR1-AP. The latter receptor was apparently desensitized in this experimental setup (see Figure 5). To show that this effect was not dependent on a release of TxA_2, the experiment was repeated with platelets isolated in the absence of ASA. The result obtained was identical to that with platelets isolated in the presence of ASA, demonstrating that the effect was indeed not dependent on TxA_2. Thus, the hypothesis that the receptors have different abilities to undergo desensitization seemed to be correct. PAR1 and PAR4 have recently been suggested to exist as a stable heterodimeric complex (Leger et al., 2006a). They used coimmunoprecipitation and fluorescence resonance energy transfer studies as mechanisms to discover the complex. Leger and coworkers (2006a) suggested that PAR1 serves as a thrombin-enhancing cofactor for PAR4, by assisting in the activation and cleavage of PAR4. However, if PAR1 and PAR4 existed in this heterodimeric complex, then desensitization of PAR1 should somehow affect the activity of PAR4 (e.g. decrease the aggregation induced by PAR4-AP). In the present study we showed that addition of PAR4-AP (300 µM) after the addition of PAR1-AP (30 µM), in cumulative dose-response

studies, caused a full aggregation response even though PAR1 was desensitized. This may indicate that the PARs do not exist as heterodimers, however, further studies are needed to clarify this issue.

The dose needed for initiation of desensitization (3 μM) lies within a narrow range of sub-threshold concentrations. Further, the desensitization of PAR1 was initiated approximately 5 min after stimulation with the low, non-aggregatory, dose of PAR1-AP (3 μM). A full desensitization was achieved about 10 min after stimulation with 3 μM PAR1-AP. When measuring $[Ca^{2+}]_i$, however, the response elicited by 30 μM PAR1-AP was reduced only by 50 % 10 min after the adding of 3 μM PAR1-AP, and a complete desensitization was not obtained even after 30 min (see Figure 7). The present data thus indicate that thrombin can activate platelets in a complex and time-dependent manner. Low concentrations of thrombin in the circulation might cause desensitization of platelet PAR1 towards re-exposure of a low dose of thrombin. However, if the concentration is high enough it might still cause platelet activation, through PAR4 signalling. Therefore, PAR4 may be a more suitable future therapeutic target than PAR1.

When measuring aggregation, the mechanism behind the desensitization of PAR1 did not seem to involve PKC (see Figure 9). However, it is well known that PKC is an important signalling enzyme inducing aggregation, for instance since it stimulates the release of granule secretion and $\alpha IIb\beta_3$ activation (Strehl et al., 2007; Yacoub et al., 2006). This means that by inhibiting PKC, the aggregation response will also be suppressed. Therefore it may be hard to interpret the results from the aggregation studies, even though fibrinogen was added. Further, when measuring aggregation it was observed that the MEK inhibitors (U0126 and PD98059) as well as an inhibitor of PI3K (LY294002) partially restored the sensitivity of PAR1 (see Figure 11). This suggests that these kinases and their signalling pathways play a minor role in the desensitization mechanism. It should be remembered, though, that none of the drugs had a marked effect. The results from the Ca^{2+} measurements, however, indicate that a PKC dependent pathway, at least partly, is involved in PAR1 desensitization. More specifically, this pathway probably involves the conventional isoforms of PKC (α and β), since the restoring effect on PAR1 sensitivity was observed with Ro31-8220 and Ro31-8425 (see Figure 8, 9, 10). The results all together thus indicate that aggregation and Ca^{2+} release are differently regulated by mechanisms causing desensitization. Furthermore, a PKC-dependent, as well as a PKC-independent mechanism contributes to PAR1 desensitization.

The results from the Western blots, further investigating the PKC signalling pathway, showed that PKC substrate phosphorylation occurred in a "dose-response manner" when platelets were stimulated with PAR1-AP or PAR4-AP (see Figure 12 and 13). ASA to some extent prolonged the time needed for activation of platelet PKC when stimulated with PAR-APs, but only for a short time (<1 min), indicating that the activation of PKC was not dependent on TxA_2. The lowest dose of PAR1-AP inducing a detectable PKC substrate phosphorylation was 3 μM (see Figure 12). This is the same concentration that induced desensitization of PAR1 when measuring aggregation and Ca^{2+} mobilization, which further indicates a role of PKC in the desensitization. The time-study performed with Western blot showed that the activity of PKC declined after approximately 10 min when platelets were stimulated with PAR1-AP. This decline was not seen with PAR4-AP, which induced a PKC substrate phosphorylation that was more stable over time (see Figure 14 and 15). This suggests that PAR4-AP provokes a much longer signalling inside the platelets. Together, the data shows that both PAR1-AP and PAR4-AP activates PKC, however, this activation of PKC can not induce desensitization of PAR4, although it seems to play a role in PAR1 desensitization.

Results from the flow cytometry studies suggest that the mechanism behind PAR1 desensitization does not involve internalization of the receptors (see Figure 16). Instead, other

mechanisms besides PKC are involved in the desensitization, possibly involving G-protein receptor kinases (GRKs). Receptor desensitization is a common feature of GPCRs. Generally, ligand occupation of the GPCR induces translocation of GRKs from the cytosol to the activated receptor. GRKs phosphorylate the receptor and triggers membrane translocation and interaction with β-arrestins. The interaction of the receptor with β-arrestins mediates uncoupling from heterotrimeric G-proteins and thereby ends the signal transduction. This mechanism of desensitization, however, varies between different PARs. This is probably due to differences in the structure of their third intracellular loop and carboxy terminus, where the GRKs usually phosporylate the receptor (Ossovskaya and Bunnett, 2003). The role of GRKs in homologous desensitization of PAR1 in platelets remains to be determined.

The data all together thus suggests that NO effectively, and with similar potency, inhibit both PAR1-AP and PAR4-AP induced platelet activation. However, PAR1 and PAR4 differ in their ability to undergo desensitization when exposed to increasing concentrations of their corresponding activating peptides. PAR1 is desensitized in cumulative dose-response studies, but not PAR4. Even more interesting, the desensitization of PAR1 does not seem to affect PAR4. This might thus be an objective to focus on in further studies. Finally, the mechanism behind the desensitization of PAR1 to some extent involves PKC, however, it is obvious that other pathways are involved as well. This mechanism does not seem to involve receptor internalization but rather a more rapid mechanism, such as GRK activation. To further enhance the potential future of PAR1 and PAR4 as therapeutic targets, more studies are needed to clarify differences in their activities and mechanisms of desensitization.

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