Activation and regulation of the extracellular signal-regulated kinase 2 (ERK2) in human platelets

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Meinen Eltern Inge und Winfried,
und meinem Bruder Claas

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1 INTRODUCTION

Blood platelets play a crucial role in primary arterial hemostasis through adhesion to the vessel wall, subsequent aggregation and thrombus formation induced by collagen, von Willebrand factor, thrombin, and other factors exposed at sites of vascular injury. Under pathophysiological conditions, such as atherosclerosis, plug formation can cause inappropriate vascular occlusions resulting in myocardial infarctions or stroke, which represent major health risks today. Therefore, during the last decades increasing efforts have been made to elucidate the signalling mechanisms involved in platelet activation not least with the purpose to provide a basis for developing antiplatelet drugs and strategies.

Platelet activation induced by primary agonists involves subsequent secretion of platelet-derived proaggregatory mediators, including the adenine nucleotides adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP), as well as the generation and release of lipid mediators such as thromboxane A_2 (TXA_2).

Once released, these mediators generate stimulatory loops by activating their respective platelet receptors thereby representing important reinforcement mechanisms for platelet functions.

TXA_2 is the major arachidonic acid metabolite endogenously produced by platelets. Arachidonic acid is converted by cyclo-oxygenase generating the prostaglandin endoperoxide PGH_2 which is sequentially transformed into TXA_2 by thromboxane synthase (Samuelsson *et al.* 1978). For TXA_2, the thromboxane/prostanoid receptor α ($TP\alpha$), a member of the G protein-coupled receptor (GPCR) family, is the predominant isoform expressed on platelets and couples to Gq as well as G12/13 proteins (Habib *et al.* 1999, Offermanns *et al.* 1994).

Platelet adenine nucleotide receptors can be distinguished as three separate subtypes of the purinergic type 2 (P2) receptor family, namely P2X1, P2Y1, and P2Y12. The P2Y12 receptor, that has just recently been cloned (Hollopeter *et al.* 2001, Zhang *et al.* 2001) was formerly variously designated as $P2Y_{ADP}$, $P2Y_{AC}$, $P2Y_{cyc}$, or $P2T_{AC}$.

The P2X1 ATP receptor, that on platelets for long has been mistaken for an ADP receptor, is a ligand-gated ion channel, inducing a rapid calcium influx associated with transient shape change of human platelets (Mahaut-Smith *et al.* 2000, Rolf *et al.* 2001).

For ADP, two metabotropic GPCRs are presently known on platelets: P2Y1 and P2Y12 receptors. The signalling principles of these ADP receptor subtypes as well as their distinct contribution to platelet functions are fairly well understood; the number of affected molecules identified within these pathways is growing (for reviews see Gachet 2001, Kunapuli *et al.* 2003, Hechler *et al.* 2005). The P2Y1 ADP receptor couples to Gq, leading to a transient calcium mobilization from intracellular stores and initiating platelet shape change and aggregation. The P2Y12 ADP receptor couples to an inhibitory G protein, identified as Gi_2 (Ohlmann *et al.* 1995). Gi_2 dissociation leads via β/γ-subunits to the activation of phosphoinositide 3-kinase (PI 3-K) which mediates the potentiation of dense granules secretion, and via α_i-subunits to the inhibition of adenylyl cyclase that is essential for full and sustained platelet aggregation and thrombus formation. In addition, P2Y12 receptor-induced Gi_2 signalling plays an important role in the activation of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ (Kauffenstein *et al.* 2001, Jantzen *et al.* 2001, Nieswandt *et al.* 2002).

The P2Y12 receptor is the target of the active metabolites of the thienopyridine drugs ticlopidine and clopidogrel which selectively and irreversibly inhibit its activation by ADP (for review see Savi *et al.* 2005). Besides competitively acting P2Y12 receptor antagonists such as the ATP-analogue AR-C69931MX (now designated cangrelor) these compounds potently inhibit platelet responses to all platelet agonists depending on their stimulatory intensities, emphasizing the crucial implication of P2Y12 receptor signalling in platelet activation and aggregation. In addition, these antagonists were indispensable to determine the specific roles of P2Y12 receptor signalling in platelet function (for reviews see Dorsam *et al.* 2004, Hechler *et al.* 2005).

Studies with exogenously added ADP alone have pinpointed the mechanisms and roles for both the P2Y1 and P2Y12 receptors and revealed that in ADP-induced platelet activation and aggregation the cooperation of both Gq-coupled P2Y1- and Gi-coupled P2Y12-receptor signalling is required (Hechler *et al.*

1998). In contrast, platelet activation and aggregation caused by strong agonists that directly activate Gq-coupled receptors, such as thrombin and TXA_2, is dependent on subsequent P2Y12 receptor signalling rather than on P2Y1 receptor signalling (Nylander *et al.* 2003, Paul *et al.* 1999). The response to collagen, mediated mainly via glycoprotein VI (GP VI) that induces down-stream protein tyrosine kinase cascades, strongly relies on intermediate TXA_2 signalling, subsequent ADP release, and P2Y12 receptor signalling (Nieswandt *et al.* 2001). Thus, Gi coupling in response to thrombin, collagen, and TXA_2, is a subsequent event following ADP secretion and activation of P2Y12 ADP receptors (Kim *et al.* 2002, Paul *et al.* 1999, Nieswandt *et al.* 2001).

Human platelets contain several members of the mitogen-activated protein kinase (MAPK) family, such as p38 MAP kinase (Kramer *et al.* 1995), c-Jun amino-terminal kinases (JNKs) (Bugaud *et al.* 1999), and the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (Papkoff *et al.* 1994) as well as both ERK upstream kinases MEK1 and MEK2 (MAP/ERK kinases 1 and 2) (McNicol *et al.* 2001).

Following platelet stimulation by primary agonists such as thrombin and collagen, all platelet MAPK family members become phosphorylated and therefore are presumably active.

MAPKs represent a family of evolutionary conserved serine/threonine kinases that have been implicated during the last decades in a wide variety of mammalian cellular functions; ranging from gene expression, cell proliferation and differentiation, cell motility, to cell survival and death. Besides nuclear targets, MAPK activation affects substrates in the cell membrane, the cytosol, the cytoskeleton as well as mitochondria.

The most eminent and best examined members of this family are ERK1 and ERK2, also designated p44 MAPK and p42 MAPK, respectively, as well as c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK) and p38 kinases. The diverse signalling pathways leading to MAPK activation as well as the various affected cellular substrates and functions have been substantially summarized and reviewed in detail (Chen Z. *et al.* 2001, Pearson *et al.* 2001, Roux *et al.* 2004).

ERK1 and ERK2 activation is initiated by extracellular stimuli via ligand-gated ion channels, receptor tyrosine kinases (RTKs), such as growth factor receptors, or by G protein-coupled receptors (GPCRs), all leading to the induction of various protein kinase cascades. These pathways finally funnel into the activation of the highly substrate-specific ERK upstream kinases MEK1 and 2. In general, both MEK1 and 2 activate ERK1 and/or 2 by non-processive phosphorylation of tyrosine and threonine residues of a common Thr-Glu-Tyr (TEY) motif.

ERK2, in particular, becomes first phosphorylated at Tyr-185, and after a threshold amount of this non-active form has accumulated, ERK2 is rapidly converted into its active form by additional phosphorylation at Thr-183. In ERK1, which shares over 80% sequence homology to ERK2, the signature motif is flanked by Thr-202 and Tyr-204.

ERK activation induced by RTKs is mediated via the small GTP-binding protein Ras that activates Raf isoforms, such as Raf-1 and B-Raf. The increase in Raf activity is subsequently transduced through the MEK/ERK module.

The mechanisms employed by GPCRs in ERK1/2 activation are multiple due to the various classes of G proteins as well as to the ability of some receptors to activate more than one species of G proteins (for reviews see Gudermann 2001, Pierce *et al.* 2001, Luttrell *et al.* 2003).

The activation of GPCRs, in general, causes the simultaneous activation and dissociation of G protein α- and β/γ-subunits.

Gαq induces the activation of protein kinase C (PKC) isoforms, which in turn triggers the Ras/Raf kinase cascade resulting in the induction of MEK/ERK.

The signalling cascades induced by Gs-coupled receptors are particularly diverse. The increase in cyclic AMP (cAMP) and activation of protein kinase A (PKA) was found to display cell type-specific inhibitory as well as excitatory features on ERK. From outstanding meaning were the findings by Robert J. Levkowitz and colleagues demonstrating that in HEK293 cells overexpressing β_2-adrenoceptors the stimulation with isoproterenol induces a PKA-dependent switch of receptor coupling from Gαs to Gαi, and that ERK activation is finally mediated by the Gi pathway (Daaka *et al.* 1997)

ERK activation via Gi-coupled receptors is mediated by a β/γ subunit-dependent induction of phosphoinositide 3-kinase γ (PI-3Kγ) (Gutkind 1998). However, there is evidence that, besides PI-3Kγ, the non-receptor tyrosine kinase Src is implicated in the induction of the Ras/ERK signalling pathway downstream of Gi β/γ subunits (for review see Gudermann 2001).

Furthermore, GPCRs provoke ERK activation by recruiting RTKs via ligand-independent tyrosine-phosphorylation, a process that has become known as "transactivation". Several GPCRs, such as the bradykin type 2 receptor and D4-dopamin receptor, have been demonstrated to transactivate RTKs such as the epidermal-growth factor receptor (EGFR) and platelet-derived growth factor receptor β (PDGFRβ), respectively (Liebmann *et al.* 2000, Ferguson 2003). Daub *et al.* (1997) demonstrated in various cell types that both Gq- and Gi-signals induce the transactivation of the EGFR. Following tyrosine phosphorylation of the EGFR, both GPCRs and EGFRs mediated ERK activation are indistinguishable as inhibiting the intrinsic EGFR tyrosine kinase activity blocks both EGFR and GPCR mediated ERK activation.

Hence, G proteins appear able to access multiple protein kinase pathways to induce ERK activation.

Although primary platelet agonists such as thrombin and collagen cause the activation of ERK2 (Papkoff *et al.* 1994, Nakashima *et al.* 1994, Börsch-Haubold *et al.* 1995), the signalling pathways involved are not fully elucidated.

Thrombin-induced ERK2 activation in human platelets is dependent on MEK1/2 activity (Börsch-Haubold *et al.* 1996, McNicol *et al.* 2003) and is mediated by protein kinase C (PKC) and calcium but not, in contrast to nucleated cells, by Raf-1 or B-Raf (Börsch-Haubold *et al.* 1995, Nadal-Wollbold *et al.* 2002). Moreover, whereas integrin signalling induces ERK2 activation in various cell types (for reviews see Giancotti *et al.* 1999, Juliano *et al.* 2004), during platelet aggregation fibrinogen binding-induced $\alpha_{IIb}\beta_3$ outside-in signalling inhibits platelet ERK2 activity (Nadal *et al.* 1997, Pawlowski *et al.* 2002).

Thus, although ERK2 activity appears precisely regulated in platelets during activation and aggregation by consecutive signalling pathways, the regulatory principles apparently deviates from those in nucleated cells.

As mentioned above, platelet activation evokes the release of platelet-derived second mediators such as TXA_2 and ADP. In concert with the primary stimulus these mediators support and enhance platelet activation via Gq-coupled TPα TXA_2 receptors, as well as, and even more important, Gi-coupled P2Y12 ADP receptors, respectively.

In a previous study using HEK293T cells as well as mouse and human fibroblasts it has been demonstrated that ERK2 activation by GPCRs relies on the cooperation of Gq and Gi signals (Blaukat *et al.* 2000).

Therefore, within this work the question has been addressed whether this mechanism also applies for ERK2 activation in human platelets. In addition, the respective platelet-specific pathways involved as well as the functional implication of ERK2 in platelet activation and aggregation have been investigated.

2 AIM OF THE WORK

The present work was set up to investigate the signalling pathways implicated in the activation and regulation of the extracellular signal-regulated kinase 2 (ERK2) in human platelets.

Isolated platelets were used to exclude interactions with other blood cells as well as effects of plasma components.

Platelet activation and aggregation induced by primary platelet agonists such as thrombin or collagen are accompanied by the release and autocrine actions of secondary, platelet-derived mediators such as TXA_2 and ADP.

To answer the question if or how these mediators contribute to ERK2 activation several receptor-specific antagonists as well as enzyme inhibitors affecting important signalling pathways should be applied.

ERK2 activity should be assessed by immuno(Western)blotting using activation-specific antibodies.

Finally, the controversially discussed issue whether ERK2 is involved in primary platelet responses such as granule release, integrin $\alpha_{IIb}\beta_3$ activation, and platelet aggregation should be addressed. As isolated platelets were used, appropriate experimental methods and protocols were about to be modified and/or adapted such as secretion assays and fluorescence-activated cell-sorting (FACS) analysis.

3 ABBREVIATIONS

[^{3}H]5-HT	[^{3}H]5-hydroxytryptamine
2-MeS-ADP	2-(methylthio)adenosine 5'-diphosphate
5-HT	5-hydroxytryptamine
A_3P_5P	adenosine 3'5'-diphosphate
ADP	adenosine 5'-diphosphate
APS	ammonium persulfate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumine
cAMP	cyclic adenosine mono-phosphate
CD61	cluster of differentiation 61 (integrin $\alpha_{IIb}\beta_3$)
CD62	cluster of differentiation 62 (P-selectin)
ddH_2O	double-distilled H_2O
DMSO	dimethylsulfoxid
ECL	enhanced chemoluminescence
EGF(R)	epidermal-growth factor (receptor)
ERK(s)	extracellular signal-regulated kinase(s)
FACS	fluorenscence-activated cell sorting
FITC	fluorescin isothiocyanate
GP VI	glycoprotein VI
GPCR(s)	G protein-coupled receptor(s)
Hepes	N-2-hydroxyethylpiperazin-N'-2-ethyan-sulfonic acid
MAPK(s)	mitogen-activated protein kinase(s)
MEK	MAP/ERK kinase
NC(s)	nitrocellulose membrane(s)
PBS	phosphate-buffered saline
PDGF(R)	platelet-derived growth factor (receptor)
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PI 3-K	phosphoinositide 3-kinase
PKA	protein kinase A

PKB	protein kinase B
PKC	protein kinase C
RGDS	Arg-Gly-Asp-Ser (-peptide)
RTK(s)	receptor tyrosine kinase(s)
SDS	sodium dodecylsulfate
TEMED	N, N, N', N'-tetramethylethylenediamine
TP	thromboxane / prostanoid receptor
Tris	tris-(hydroxymethyl)-aminomethan
TXA_2	thromboxane A_2
U	unit(s)

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

Substance	Source
β-Mercaptoethanol	Sigma
2-Propanol	Roth
Acetic acid	Merck
Acrylamid (rotiphorese Gel 30)	Roth
APS (ammonium persulfate)	Merck
BSA (bovine serum albumine, grade V)	Sigma
$CaCl_2$	Merck
Citric acid	AppliChem
Coomassie-R250 (brilliant blue R250)	Roth
DMSO (dimethylsulfoxid)	Sigma
ECL (Enhanced Chemiluminiscence (ECL) Western Blotting Detection System)	Amersham Biosciences
Ethanol	Merck
Formic acid	Merck
Glucose (α-(+)**D**-glucose)	Merck
Glutaraldehyde	Sigma
Glycerol	Serva
Glycine	Serva
HCl, fuming, 37%	Merck
Hepes (N-2-hydroxyethylpiperazin-N'-2-ethyan-sulfonic acid)	Roth
KCl	Merck
Methanol	Merck
$MgCl_2$	Merck
Na_2HPO_4	Merck
NaCl	Merck
NaH_2PO_4	Merck
NaN_3	Merck

NaOH	Merck
Nonfat dry milk	Roth
PBS, (phosphate-buffered saline) without Ca^{2+}, Mg^{2+}	Biochrom AG
Ponceau-S (3-hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo) phenylazo]-2,7-naphthalenedisulfonic acid)	Sigma
SDS (sodium dodecylsulfate)	Serva
Sodium citrate	Merck
TEMED (N, N, N', N'-tetramethylethylenediamine)	Sigma
Tris (tris-(hydroxymethyl)-aminomethan)	Roth
Tween-20 (polyoxyethylenesorbitan monolaurate)	Sigma
Urea	Roth

4.1.2 Radiochemicals

[5-[α,β-^{3}H(N)]-hydroxtryptamine creatinine sulfat (2 Ci/mmol; 1 Ci/ml)	PerkinElmer Life Sciences

4.1.3 Agonists

2-MeS-ADP (2-(methylthio)adenosine 5'-diphosphate)	Sigma
ADP (adenosine 5'-diphosphate)	Sigma
Collagen	Nycomed
Epinephrine	Sigma
Thrombin, from bovine origin	Sigma
U46619 ([9,11-dideoxy-11α, 9 α-epoxy-methanoprosta-glandine (F2α)])	Sigma

4.1.4 Antagonists and Inhibitors

A_3P_5P (adenosine 3'5'-diphosphate)	Sigma
Apyrase, grade III, from potato	Sigma
AR-C69931MX (N^6-methylthioethyl-, 2-trifluoropropyl-thio-**D**-β-γ-dichloromethylene-ATP)	kindly provided by AstraZeneca, Wilmington, DE, USA
Aspirin (acetylsalicylic acid)	Sigma
Fluoxetine (N-methyl-3-[(4-trifluoromethyl)-phenoxy]-3-phenylpropylamine hydrochloride)	Tocris
Hirudin, recombinant	Pentapharm

LY294002 (2-(4-morhpholinyl)-8-phenyl-4H-1-benzopyran-4-one)	Biomol
MRS2179 (N^6-methyl, 2'-deoxyadenosine 3', 5'-biphoshate)	Tocris
PD98059 (2-(2-amino-3-methoxyphenyl) oxanaphtalen-4-one)	Biomol
RGDS (Arg-Gly-Asp-Ser-peptide)	Sigma
S1197 (3-{2-[4-(S)-(4-aminoimino-methyl-phenyl)-4-methyl-2,5-dioxo-imidazolidin-1-yl]acetyl-amino}-3-(S)-phenylpropionic acid hydro-chloride)	kindly provided by Aventis Pharma, Frankfurt a.M.
SQ22536 (9-(tetrahydro-2'-furyl)adenite)	Calbiochem
SQ29548 [1S-[1α, 2α(Z), 3α, 4α]]-7-[[2- [(phenylamino) carbonyl]hydrazino] methyl]-7-oxabicyclo (2.2.1)hept-2-yl]-5-heptenoic acid	Biomol
U0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio] butadiene)	Cell Signaling Technology, Inc.
Wortmannin	Sigma

For all applications double-distilled water (ddH_2O) was used.

4.1.5 Antibodies

Primary antibodies

Anti-pT^{183} MAPK, rabbit polyclonal antibody; recognizing ERK1 and 2 when mono-phosphorylated at Thr-residues	Promega
ERK 1 (K-23), rabbit polyclonal antibody; recognizing unmodified ERK 1 and 2	Santa Cruz Bio technology, Inc.
ERK 2 (D2), mouse monoclonal antibody; recognizing unmodified ERK 2	Santa Cruz Bio technology, Inc.
p-ERK (E4), mouse monoclonal antibody; recognizing ERK1 and 2 when mono-phosphorylated at Tyr-residues	Santa Cruz Bio-technology, Inc.
Phospho-Akt (Ser473) (587F11), mouse monoclonal antibody	Cell Signaling Technology, Inc.
Phospho-p44/p42 MAPK (E10), mouse monoclonal antibody; recognizing ERK 1 and 2 only when dually-phosphorylated at Tyr- and Thr-residues	Cell Signaling Technology, Inc.

Secondary antibodies

Polyclonal swine anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated	DakoCytomation GmbH
Polyclonal rabbit anti-mouse IgG, horseradish peroxidase (HRP)-conjugated	DakoCytomation GmbH

4.1.6 Antibodies for flow cytometry

Antibody	
CD61 (anti-integrin β_3), peridinin chlorophyll protein (PerCP)-conjugated	BD Biosciences
CD62P, phycoerythrin (PE)-conjugated	BD Biosciences
Mouse IgG_1 Platelet Control; phycoerythrin (PE)-conjugated	BD Biosciences
PAC-1, fluorescin isothiocyanate (FITC)-conjugated	BD Biosciences

4.2 Methods

4.2.1 Preparation of washed human platelets

Suspensions of washed human platelets were prepared as previously described (Martinson *et al.* 1995). After received informed consent according to the permission of the Ethic Board of the Martin-Luther-University Halle-Wittenberg, 60-80 ml blood was drawn from healthy volunteers (who denied to have taken any medication 2 weeks prior to donation) from the antecubital vein using 21 gauge winged infusion sets (Becton Dickinson, Helsingborg, Sweden) and 20 ml single-use syringes (B. Braun, Melsungen, Germany). Blood was transferred in a volumetric proportion of 6:1 to acid-citrate-dextrose formula A (ACD-A: 38 mM citric acid, 88 mM sodium citrate, 124 mM glucose) into 13 ml centrifugation tubes (95x16.8mm in diameter, Sarstedt, Nümbrecht, Germany), and centrifuged at 120 x *g* for 15 min. To the platelet-rich plasma thus obtained were added (per ml) 111 µl ACD-A and 1 U apyrase (1 U/µl solution), and platelets were collected by centrifugation at 450 x *g* for 7 min. The platelet pellet was resuspended in modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 0.36 mM NaH_2PO_4, 1 mg/ml glucose; pH 6.4) supplemented with 1 U/ml apyrase and 1 U/ml hirudin, incubated at 37°C for 15 min, and centrifuged at 450 x *g* for 7 min. The washing step was repeated once using the modified Tyrode's buffer without hirudin, and platelets were finally suspended in the same buffer adjusted to pH 7.4 and supplemented with 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.02 U/ml apyrase, designated as suspension buffer. After determining the platelet count using a Neubauer chamber, suspensions were adjusted to platelet concentrations required for different experimental

procedures. All preparation steps were carried out at room temperature if not otherwise indicated. For centrifugation a Hermle Z364 centrifuge with an out-swing bucket rotor was used (Hermle, Wehingen, Germany).

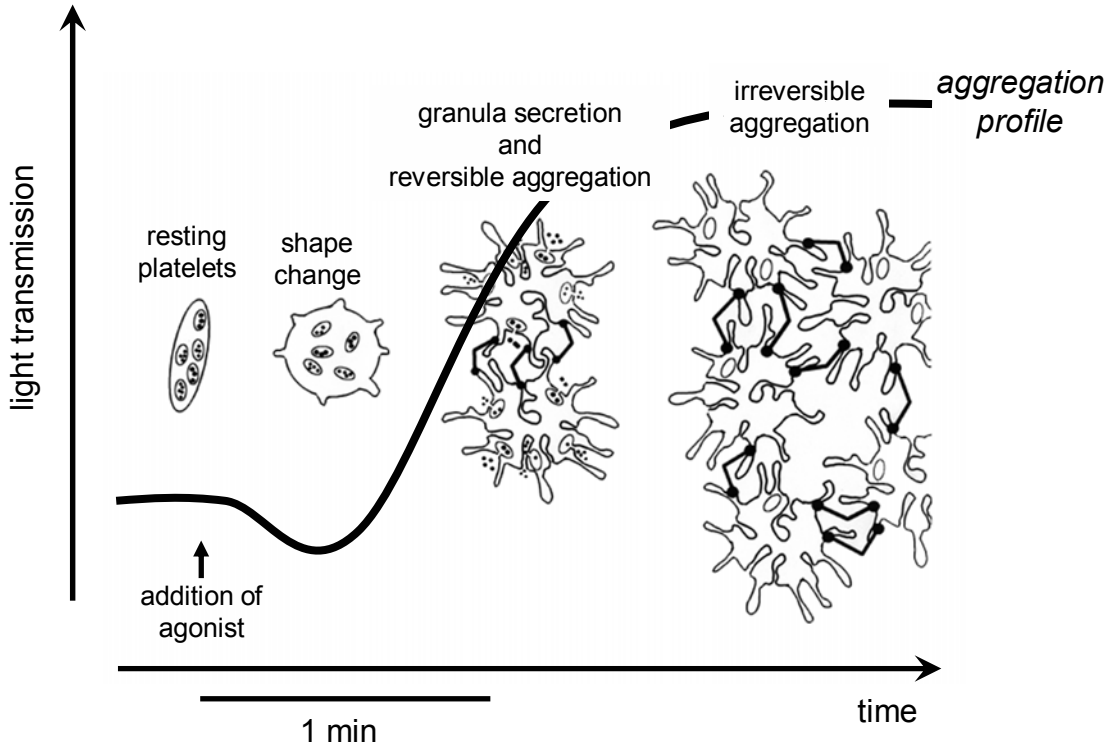
Hirudin was applied in the first washing step to irreversible complex and inactivate residual traces of thrombin. The nucleotidase apyrase (EC 3.6.1.5), scavenging ATP and ADP, was present throughout all preparation steps. Platelet suspensions were finally allowed to rest for 30 min at 37°C prior to experimental procedure for recovering P2Y1 receptors (Baurand *et al.* 2000, Cazenave *et al.* 2004).

All experimentally applied compounds were diluted with suspension buffer. For substances insoluble in water, respective solvent controls were carried out and effects, if any, are mentioned in the Results section.

4.2.2 Platelet aggregation

Prepared platelets were diluted to $3x10^8$ platelets/ml in suspension buffer. Experiments were carried out at 37°C in a platelet aggregation profiler (PAP4 Bio/Data Corp., Hatboro, PA, USA) using siliconized test tubes and magnetic stirrers rotating at 900 r.p.m. according to the method of Born (1962). The principle of this method is monitoring light transmission through a stirred platelet suspension. As shown in the schematic presentation given below, after addition of an agonist such as thrombin the light transmission transiently decreases as platelets undergo a morphological "shape change" from their initial discoid to a spherical form with developing filopodia. During the course of platelet aggregation the light transmission increases due to full platelet aggregation and growing size of aggregates.

Schematic presentation of platelet morphological changes during aggregation and visualization by monitoring light transmission in an aggregometer



Aggregation is expressed as percentage light transmission compared to suspension buffer alone (=100%). Preincubations with antagonists were carried out in the siliconized test tubes as indicated in the respective figure legend in an Eppendorf thermomixer rotating at 700 r.p.m. at 37°C. Platelet aggregation profiles induced by various concentrations of thrombin or respective agonists served as internal controls to asses the quality of each platelet preparation.

4.2.3 Platelet stimulation, detection and quantification of phosphorylated proteins

4.2.3.1 Platelet stimulation

For experiments performed to determine protein phosphorylation washed platelets were adjusted to $5x10^8$ platelets/ml with suspension buffer. Incubations were carried out at 37°C in a total volume of 200 µl in a 2 ml round-bottom tube containing a stainless steel sphere (2 mm diameter; SKF, Schweinfurt,

Germany) in an Eppendorf thermomixer rotating at 700 r.p.m. Except for aspirin, that was present while platelets were allowed to rest for 30 min prior to experimental procedures with occasional gently agitation, preincubations with antagonists or buffer were carried out as indicated in the figure legends at 37°C with stirring at 700 r.p.m. Reactions were stopped by the addition of 50 µl SDS-sample buffer (10% SDS, 1.43 M β-mercaptoethanol, 20% glycerol, 26% urea, 125 mM Tris-HCl, pH 6.8 (all w/v solutions)), and proteins were denaturated at 95°C for 5 min. From these samples 25 µl were used for SDS-PAGE (equal total amount of protein from $1x10^7$ platelets).

4.2.3.2 SDS- Polyacrylamid Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method described by Laemmli (Laemmli 1970). Proteins were separated at room temperature by 10% SDS-PAGE using BioRad Protean II minigel system (BioRad, München, Germany) applying a current of 20 mA per gel. Resolving gels consist of 33.3 % (v/v) acrylamid, 50 % (v/v) Tris-HCl (0.75 M, pH 8.8), 0.5 % (v/v) SDS (20 % solution (w/v)), 0.05 % (v/v) TEMED, 15.45 % (v/v) ddH_2O and 0.7 % (v/v) APS (10 % solution (w/v)) and stacking gels of 16,7 % (v/v) Acrylamid, 12,5 % (v/v) Tris-HCl (0,625 M, pH 6,8), 0,5 % (v/v) SDS (20 % solution (w/v)), 0,1 % (v/v) TEMED, 69,7 % (v/v) ddH_2O und 0,5 % (v/v) APS (10 % solution (w/v)). As running-buffer 25 mM Tris, 200 mM glycine, and 1% (w/v) SDS adjusted to pH 8.3 was used.

To determine apparent molecular protein masses according to Weber and Osborn (1969) the following marker proteins (in SDS-sample buffer) were used: BSA (66 kDa), catalase (60 kDa), ovalbumin (45 kDa), glycerinaldehyd-3-phosphatdehydrogenase (36 kDa), carbonic anhydrase (29 kDa); all purchased from Sigma.

4.2.3.3 Immuno(Western)blotting and detection of phosphorylated proteins

Following SDS-PAGE gels were subjected to immuno(Western)blotting using BioRad Mini Trans-Blot cells (BioRad, München, Germany) and proteins were transferred onto nitrocellulose membranes (NCs) (Protran BA85, Schleicher&Schuell, Dassel, Germany) at 4°C for 2.5 hrs applying a current of

2.5 mA per mm^2 NC. Transfer buffer consisted of 192 mM Glycin, 27 mM Tris, 20 % (v/v) Methanol, 0.015 % (w/v) SDS; pH 8.3-8.4 ,not adjusted.

To ensure proper blotting efficiency gels were dyed for fixing residual proteins with Coomassie brilliant blue (25% (v/v) 2-propanol, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie-R250) for 10 min and gels were rinsed with 10% acetic acid. Proteins transferred to NCs were reversibly dyed with Ponceau-S red, bands of reference proteins were marked with a soft pencil and NCs were subsequently undyed with TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% (w/v) Tween-20). TBS-T was used for all further blocking and washing procedures, as well as antibody dilutions. NCs were gently agitated throughout all steps of analysis. Surplus protein binding sites were saturated to avoid unspecific antibody binding by blocking NCs as indicated with either 5% (w/v) BSA, 5% (w/v) nonfat dry milk, or both, for 2 hours at room temperature.

NCs were incubated with primary antibodies at 4 °C over night. After washing (3 times for 10 min at room temperature), secondary antibodies were applied for 2 hrs at room temperature. Dependent on the applied primary antibody, the different blocking substances, primary, and respective secondary antibody dilutions are listed in the following table:

Primary antibody	**Blocking solution**	**Primary anti-body dilution**	**Secondary antibody / dilution**
Phospho-p44/p42 MAPK (E10)	5% nonfat dry milk	1:500 in 5% nonfat dry milk*	anti-mouse IgG 1:1000
p-ERK (E4)	5% BSA / 5% nonfat dry milk	1 µg/ml in 5% BSA	anti-mouse IgG 1:1000
Anti-pT^{183} MAPK	5% BSA	1:1000 in 5% BSA*	anti-rabbit IgG 1:1000
ERK 1 (K-23)	5% BSA / 5% nonfat dry milk	1:500 in 5% BSA	anti-rabbit IgG 1:2000
ERK 2 (D2)	5% nonfat dry milk	0.4 µg/ml in 5% BSA	anti-mouse IgG 1:1000
Phospho-Akt (Ser473) (587F11)	5% nonfat dry milk	1:1000 in 5% nonfat dry milk*	anti-mouse IgG 1:1000

* concentration cannot be given due to missing details in available product datasheets

After washing of uncoupled secondary antibodies (3 times for 10 min at room temperature), the bands of interest were visualized by the use of the „Enhanced Chemiluminiscence (ECL) Western Blotting Detection System“ (Amersham Biosciences) according to the manufacturer's protocol. The principle of this method is that luminol is formed in the presence of H_2O_2 by peroxidase coupled to the secondary antibody. Resulting chemiluminiscence (light emission) was detected by exposure of NCs to X-ray films (Hyperfilm, Amersham Biosciences). Exposure times varied between 2 and 60 sec.

For re-probing NCs, primary and secondary antibodies were stripped off by incubation in TBS containing 0.05% (w/v) Tween-20, 2% (w/v) SDS, and 1% (v/v) β-mercaptoethanol for 30 min at 70°C followed by re-equilibrating NCs with TBS-T (3 times for 30 min at room temperature).

4.2.3.4 Visualizing and quantification of phosphorylated protein

Emerging X-ray films were scanned with a GS800 densitometer (BioRad, München, Germany). Phosphorylation intensities were evaluated by quantitative densitometry using Quantity One software version 4.2.1 (BioRad, München, Germany) within a determined linear density range from 0 to 160 optical densities (OD) per mm^2 area. Graphics were processed with Adobe Photoshop 5.0 software (Adobe Systems Inc., San Jose, CA, USA).

4.2.4 Determination of platelet dense granule release by [^{3}H]5-hydroxytryptamine release assay

Platelet suspensions were adjusted to $1x10^9$ platelets/ml and labeled with 1.5 µCi/ml [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT; of a 0.1 µCi/µl dilution) in a waterbath at 37°C for 60 min with gentle agitation. Suspensions were centrifuged for 10 min at 700 x *g* (Hermle Z364). Radioactive supernatants were removed and pellets were resuspended in an estimated volume of suspension buffer yielding to approximately $5x10^8$ platelets/ml. Fluoxetine (100 µM) was added 5 min prior to experiments to inhibit [^{3}H]5-HT re-uptake during stimulation. Pretreatments and incubations were carried out as aforementioned (chapter 4.2.3.1). Reactions were stopped by the addition of 200 µl ice-cold 6% (v/v) glutaraldehyd (in NaH_2PO_4/Na_2HPO_4 buffer, pH 7.3). Samples were

centrifuged for 10 min at room temperature in an Eppendorf 5413 centrifuge (at fixed 11.500 r.p.m.). Without disrupting the platelet pellets, supernatants were completely transferred to 6 ml vials (PonyVials, Packard BioScience, (Canberra Packard, Dreieich, Germany)). Pellets were dried for 60 min at room temperature, and digested in 200 µl 80% formic acid at 37°C with occasional vortexing, and debris was also transferred to 6 ml vials. Tubes were rinsed once with 200 µl 80% formic acid (giving total volumes of 400 µl). To all vials 4 ml scintillation-cocktail (Lumasafe Plus, Lumac LSC, Groningen, The Netherlands) were added. After vigorously mixing, samples were equilibrated for 3 hrs before monitoring in a liquid scintillation analyzer (Packard, Canberra Packard, Dreieich, Germany). Radioactive decay was assessed by counting light emission for 10 min and was given as drops per min (dpm). [^{3}H]5-HT secretion was calculated using the following equitation:

$$\frac{100x[\text{dpm supernat. (stim.)} - \text{dpm supernat. (contr.)}]}{\text{dpm pellet (contr.)} - \text{dpm supernat. (contr.)}} = \text{secretion in \% of total incorporated}$$

(supernat. = supernatant; contr. = control; stim. = stimulated)

Radioactive waste was properly discarded.

4.2.5 Determination of platelet α-granule secretion and integrin $\alpha_{IIb}\beta_3$ activation by flow cytometric analysis

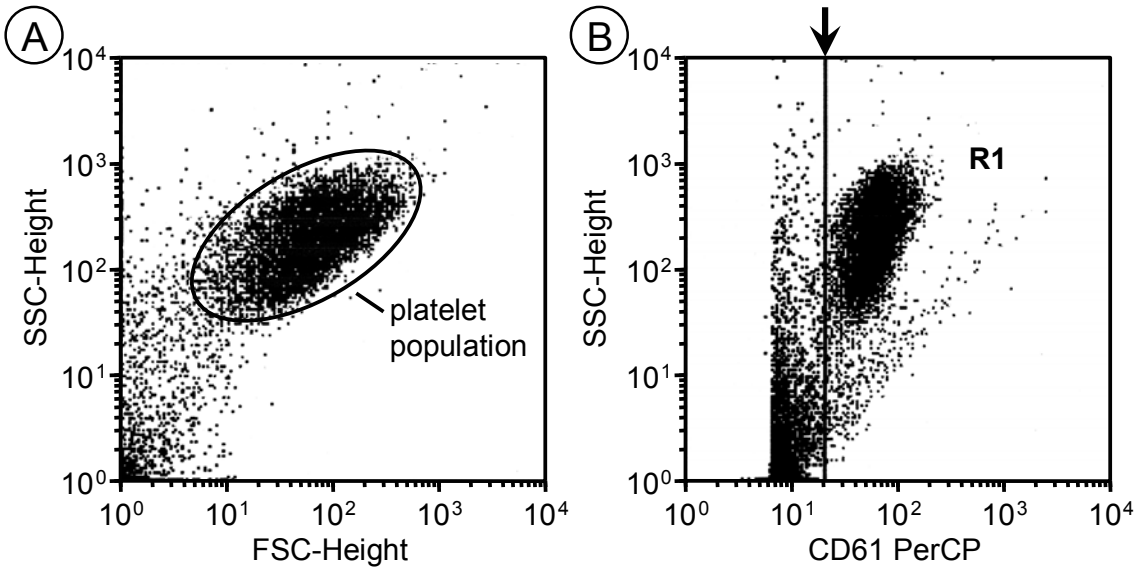
For flow cytometric analysis platelet suspensions were diluted to 1x10^6 platelets/ml. Treatments were carried out at room temperature with gentle agitation in a total volume of 200 µl in a 2 ml round-bottom tube. Pretreatments and incubation times are indicated in the respective figure legends. Reactions were stopped by transferring and therefore diluting 5 µl of the incubated suspensions into 12x75 mm tubes (Falcon, BD Biosciences, Heidelberg, Germany) containing 20 µl PerCP-conjugated CD61 antibody (3 µg/ml solution), 20 µl PE-conjugated CD62P antibody (1.5 µg/ml solution), and 20 µl FITC-conjugated PAC-1 antibody (25 µg/ml) (total volume 65 µl). For control, to detect and exclude nonantigen-specific binding sites, one sample contain 20 µl PE-conjugated Mouse IgG$_1$ (25 µg/ml), 20 µl PerCP-conjugated CD61 antibody

(3 µg/ml solution), 20 µl FITC-conjugated PAC-1 antibody (25 µg/ml), and 10 µl RGDS (10 mg/ml solution) (total volume 75 µl).

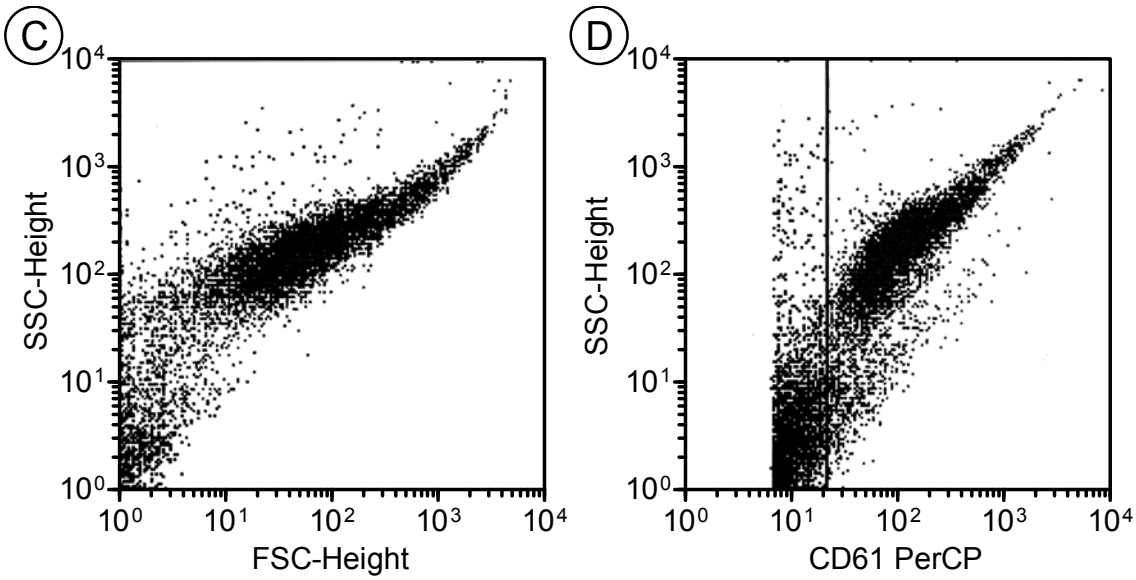
Samples were stained for 20 min at room temperature in the dark. After the addition of 1 ml PBS/0.1% sodium azide (room temperature) samples were stored at 4°C until analyzing within a time period of 5 to 15 min.

Three-colour analysis of platelet activation was monitored by fluorescence-activated cell-sorting (FACS) using a FACScalibur (Becton Dickinson, Heidelberg, Germany) with a laser excitation of 488 nm and specific bandpath filters for 530 nm (FITC), 585 nm (PE) and >650 nm (PerCP).

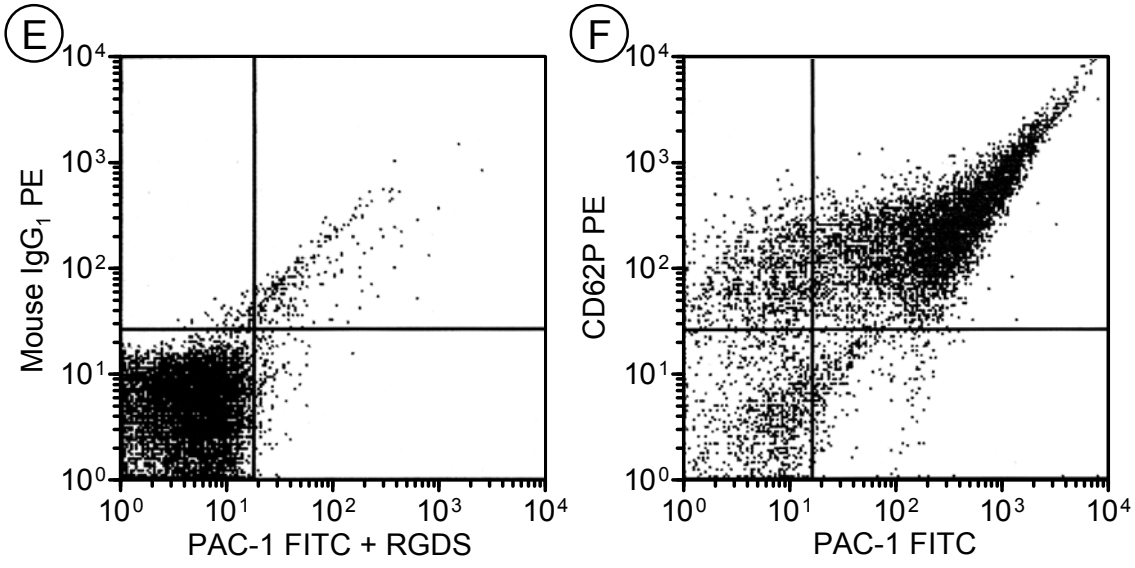
The advantage of a three-colour analysis is the use of one fluorescent color, in this case PerCP conjugated with a CD61 antibody, to delimit data acquisition for analyzing exclusively platelets. The activation-independent but platelet-specific CD61 (integrin β_3) antibody serves by its light-scatter profile to separate the platelet population from other cells, platelet-derived microparticels, or cell debris. The two other fluorescent colors, in this case PE and FITC, conjugated with CD62P and PAC-1 antibodies, respectively, were used to simultaneously asses the binding of platelet activation-dependent antibodies. Before each acquisition the platelet population was defined in a sample of unstimulated platelets according to cellular size and shape by forward scatter (FSC) versus side scatter (SSC) (Fig. A), and validated by fluorescence gating on CD61 positive events (R1), allowing to precisely adjust the >650 nm filter (indicated with the arrow) (Fig. B).



The changes of platelets shape from “discocytes” to “spherocytes” upon stimulation with thrombin (0.1 U/ml) for 2 min within the scatter and fluorescence gating profile (R1) are demonstrated in Figs. C and D.



Within 10.000 events positively gated on CD61 (R1), unstimulated platelets were fluorescent gated on PAC-1 FITC versus CD62P PE positive events to trigger 530 nm (FITC) and 585 nm (PE) filters. Using a sample of unstimulated platelets, the CD61 positive gated platelet population was enclosed to an area >95% negative for binding of Mouse IgG_1 and PAC-1 in the presence of RGDS, respectively (Fig. E).



In this setting, stimulated platelets (2 min with 0.1 U/ml thrombin) were acquired (Fig. F) and the CD61 positive platelet population was sorted on CD62P and/or PAC-1 positive events.

Data were automatically analyzed by Cell Quest software (Becton Dickinson Immunocytometry Systems, San Diego, CA, USA) and CD62P and/or PAC-1 positive assessed platelets were given in % of CD61 positive gated platelets.

4.2.6 Statistical analysis

Emerging data were processed with GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, California, USA. Data is presented as mean±standard error mean (S.E.M.) and statistical analysis (p values) were determined by paired Student's *t* tests.

5 RESULTS

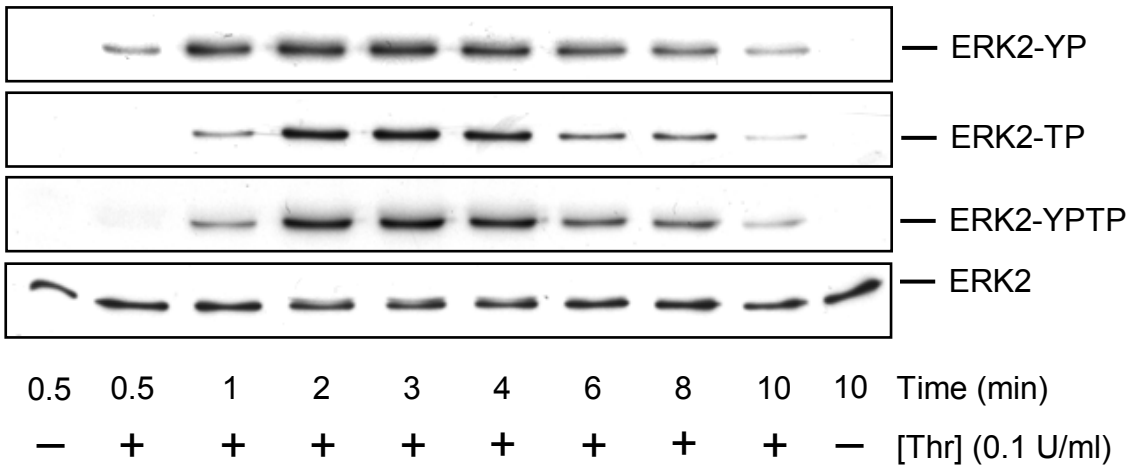
5.1 ERK2 activation induced by primary platelet agonists

5.1.1 ERK2 activation in response to thrombin

5.1.1.1 Time course of ERK2 phosphorylation and activation

As mentioned in the Introduction section, ERK2 becomes phosphorylated by MEK1/2 first on Tyr-185 residues and in a second, non-processive step on Thr-183 residues, thereby converting ERK2 into its dual-phosphorylated active state.

Fig. 1 Time course of platelet ERK2 phosphorylation in response to thrombin



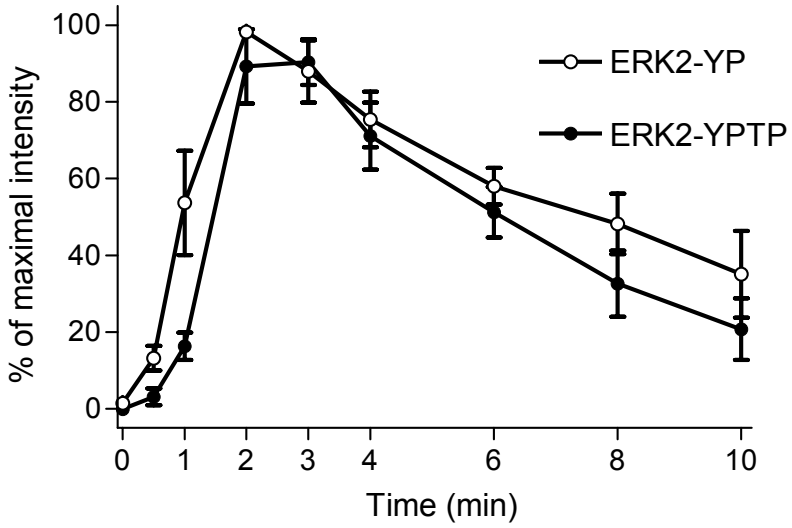
Washed human platelets were challenged with 0.1 U/ml thrombin (Thr) or buffer for control for the indicated times at 37°C with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, phosphorylated at Tyr-185 (-YP), Thr-183 (-TP), Tyr-185/Thr183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 4 similar experiments.

As shown in Fig. 1, ERK2 phosphorylation is not detectable in unstimulated platelets. Upon stimulation with 0.1 U/ml thrombin initial phosphorylation of ERK2 at Tyr-185 residues is observable within 30 sec. The state of maximal Tyr-185 phosphorylation peaks between 2 to 3 min and decreases afterwards

(upper chart). Phosphorylation of ERK2 at Thr-183 is detectable about 1 min after the onset of stimulation, peaks likewise within 2 to 3 min and diminishes subsequently (upper middle chart). The use of an antibody recognizing ERK2 dual-phosphorylated at Tyr-185/Thr-183 reveals an identical time course (lower middle chart). Equal protein loading amounts were ensured by re-probing respective immuno(Western)blots using an antibody recognizing unmodified ERK2 (lower chart).
As the dual-phosphorylated state of ERK2 resembles its active state it is therefore further referred to as activated ERK2.

The time courses of ERK2 mono-phosphorylation at Tyr-185 and dual-phosphorylated at Tyr-185/Thr183 were confirmed by quantitative densitometric analysis (Fig. 2).

Fig. 2 Quantification of the time-dependent changes in the pattern of ERK2 phosphorylation upon thrombin-stimulation

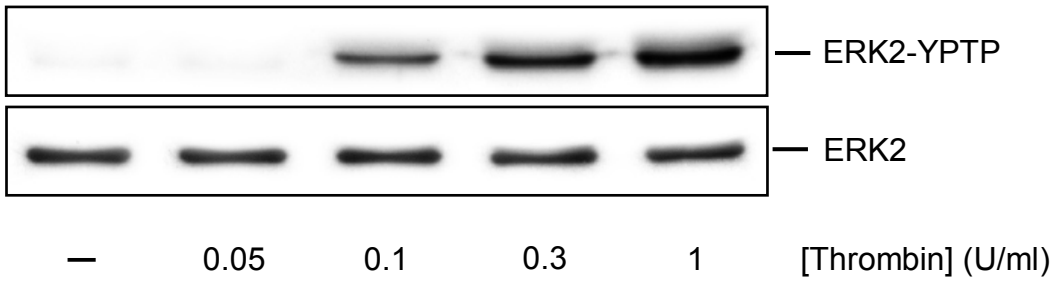


X-ray films from the immuno(Western)blots, of which one is presented in Fig. 1, were densitometrically analyzed as described in “Materials and methods”. Data were processed by setting the maximal optical intensity of the respective band as 100% and are means±S.E.M. Error bars are not shown for data points having error that is smaller than the symbol. Shown graphs of ERK2 phosphorylated at Tyr-185 (-YP) and Tyr-185/Thr-183 (-YPTP) are from 4 experiments.

5.1.1.2 ERK2 activation evoked by increasing concentrations of thrombin

As shown in Fig. 3, incubating platelets with thrombin at a concentration of 0.05 U/ml does not cause detectable ERK2 activation. Thrombin at 0.1 U/ml, more potently at 0.3 U/ml and most effectively at 1 U/ml induces the activation of ERK2. Higher concentrations of thrombin (up to 10 U/ml) do no further enhance ERK2 activation (data not shown).

Fig. 3 ERK2 activation induced by increasing concentrations of thrombin

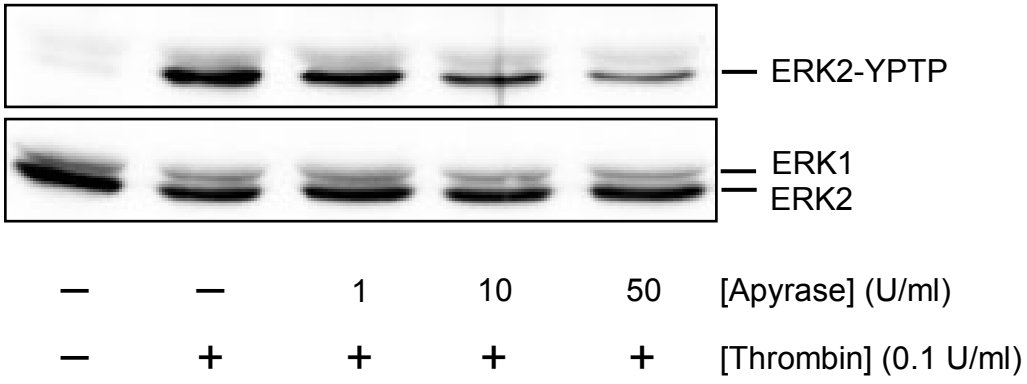


Washed human platelets were stimulated with the indicated concentrations of thrombin for 2 min at 37°C with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 3 similar experiments.

5.1.1.3 Effect of the ATP/ADP scavenger apyrase on ERK2 activation

Apyrase is commonly used during platelet preparations to protect platelets from activation and aggregation induced by released ADP (compare "Materials and methods" chapter 4.2.1). Likewise, apyrase is a useful tool to investigate an overall involvement of released adenine nucleotides in agonist-induced platelet activation. Therefore, platelets were stimulated with thrombin in the presence of increasing concentrations of apyrase. As demonstrated in Fig. 4, ERK2 activation induced by 0.1 U/ml thrombin is diminished by 1 and 10 U/ml and almost completely abolished by 50 U/ml apyrase.

Fig. 4 Effect of apyrase on thrombin-induced ERK2 activation

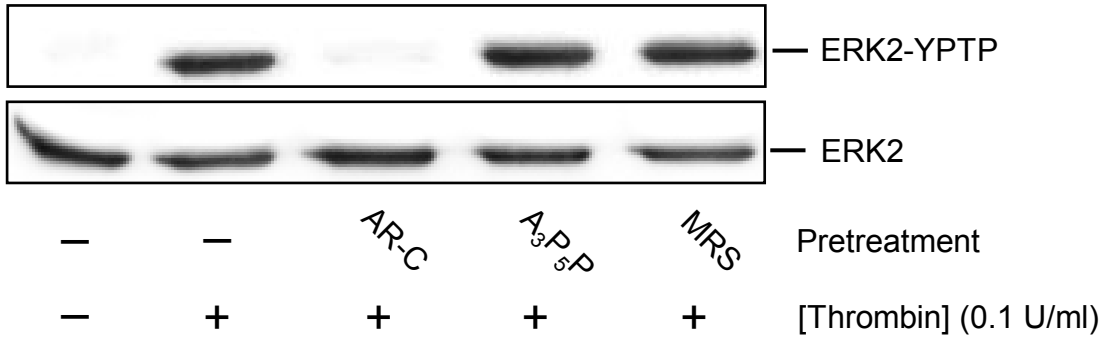


Washed human platelets were preincubated for 5 min at 37°C with buffer or apyrase at the indicated concentrations and stimulated with thrombin for 2 min at 37°C with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Material and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK1 and 2. Shown result is from one experiment that is representative of 3 similar experiments.

5.1.1.4 Effects of specific P2Y receptor antagonists on ERK2 activation

To discriminate between the ADP-receptor subtypes involved in thrombin-induced ERK2 activation, platelets were stimulated in the presence of specific P2Y receptor antagonists (Fig 5).

Fig. 5 Effects of specific P2Y receptor antagonists on ERK2 activation caused by thrombin



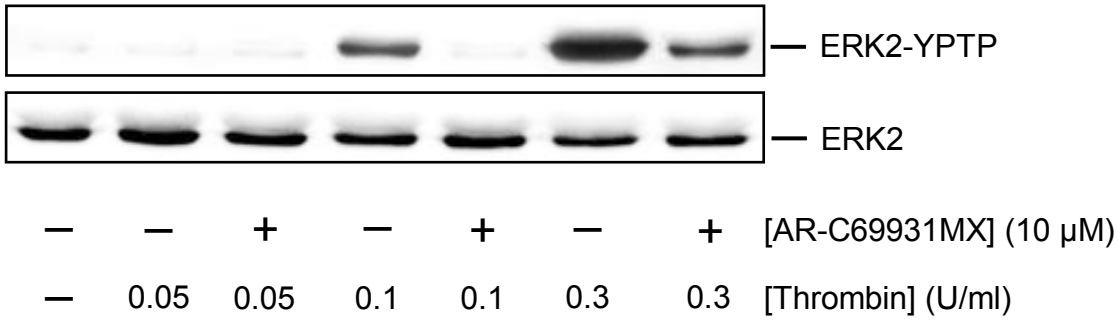
Washed human platelets were preincubated with buffer, 10 µM AR-C69931MX (AR-C), 500 µM A_3P_5P, or 100 µM MRS2179 (MRS) for 5 min at 37°C with stirring and challenged with 0.1 U/ml thrombin for 2 min. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 5 similar experiments.

As demonstrated in Fig. 5, pretreatment with the highly specific, competitively acting P2Y12 receptor antagonist AR-C69931MX (10 µM) completely abrogates ERK2 activation caused by 0.1 U/ml thrombin, whereas the P2Y1 receptor antagonists A_3P_5P (500 µM) and MRS2179 (100 µM) have no effect.

5.1.1.5 Effect of the P2Y12 receptor antagonist AR-C69931MX on ERK2 activation in response to increasing concentrations of thrombin

As demonstrated before, a concentration of 0.05 U/ml thrombin is not sufficient to evoke ERK2 activation (Fig. 3). ERK2 activation induced by 0.1 U/ml thrombin is completely abolished by pretreatment with 10 µM AR-C69931MX (Fig. 5) and, as shown in Fig. 6, the response to 0.3 U/ml thrombin is still markedly attenuated by 10 µM AR-C69931MX. ERK2 activation provoked by 0.3 U/ml thrombin, however, cannot completely be prevented even when AR-C69931MX is applied up to 100 µM (data not shown).

Fig. 6 Effect of AR-C69931MX on ERK2 activation induced by increasing concentrations of thrombin



Washed human platelets were preincubated for 5 min at 37°C with buffer or 10 µM AR-C69931MX and challenged with the indicated concentrations of thrombin for 2 min at 37°C with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 3 similar experiments.

5.1.2 ERK2 activation in response to collagen

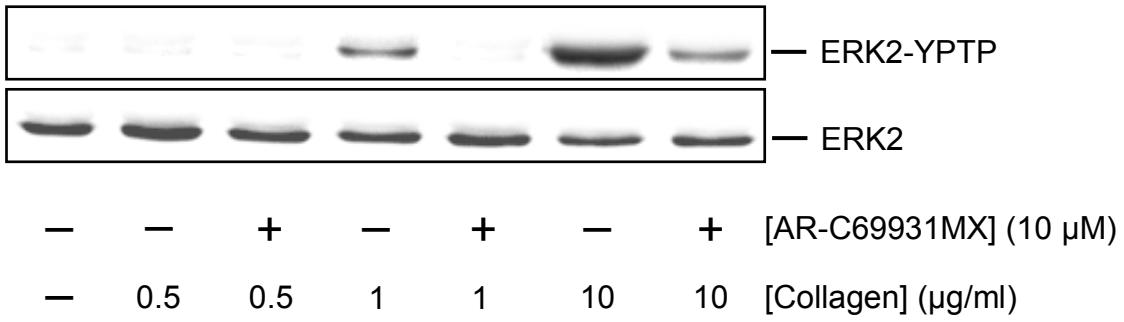
Collagen is indispensable for platelet adhesion to the exposed endothelial matrix at sites of vascular injuries and is, besides thrombin, a strong agonist causing platelet activation and aggregation. Stimulating washed human platelets with collagen (1 and 10 µg/ml) provokes ERK2 activation following a time course similar to that observed with thrombin (Fig. 1; data not shown).

5.1.2.1 Effect of precluding P2Y12 receptor signalling with AR-C69931MX on ERK2 activation

To examine whether collagen-induced ERK2 activation is mediated by released ADP and subsequent P2Y12 receptor signalling platelets were preincubated with AR-C69931MX (10 µM) prior to stimulation with increasing concentrations of collagen (0.5, 1 and 10 µg/ml). Results are shown in Fig. 7.

A concentration of 0.5 µg/ml collagen does not provoke detectable ERK2 activation. Collagen at 1 µg/ml distinctly induces ERK2 activation that is completely abolished by AR-C69931MX. ERK2 activation in response to 10 µg/ml collagen is still remarkably, but not entirely attenuated AR-C69931MX.

Fig. 7 Effect of AR-C69931MX on ERK2 activation in response to increasing concentrations of collagen



Washed human platelets were preincubated for 5 min at 37°C with buffer or 10 µM AR-C 69931MX and challenged with the indicated concentrations of collagen for 2 min at 37°C with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 3 similar experiments.

As with thrombin (Fig. 5), ERK2 activation induced by collagen (1 and 10 µg/ml) is unaffected by pretreatment with the P2Y1 receptor antagonists A_3P_5P (500 µM) or MRS2179 (100 µM) (data not shown).

5.1.2.2 Effect of precluding TXA_2 signalling on ERK2 activation in response to collagen or thrombin

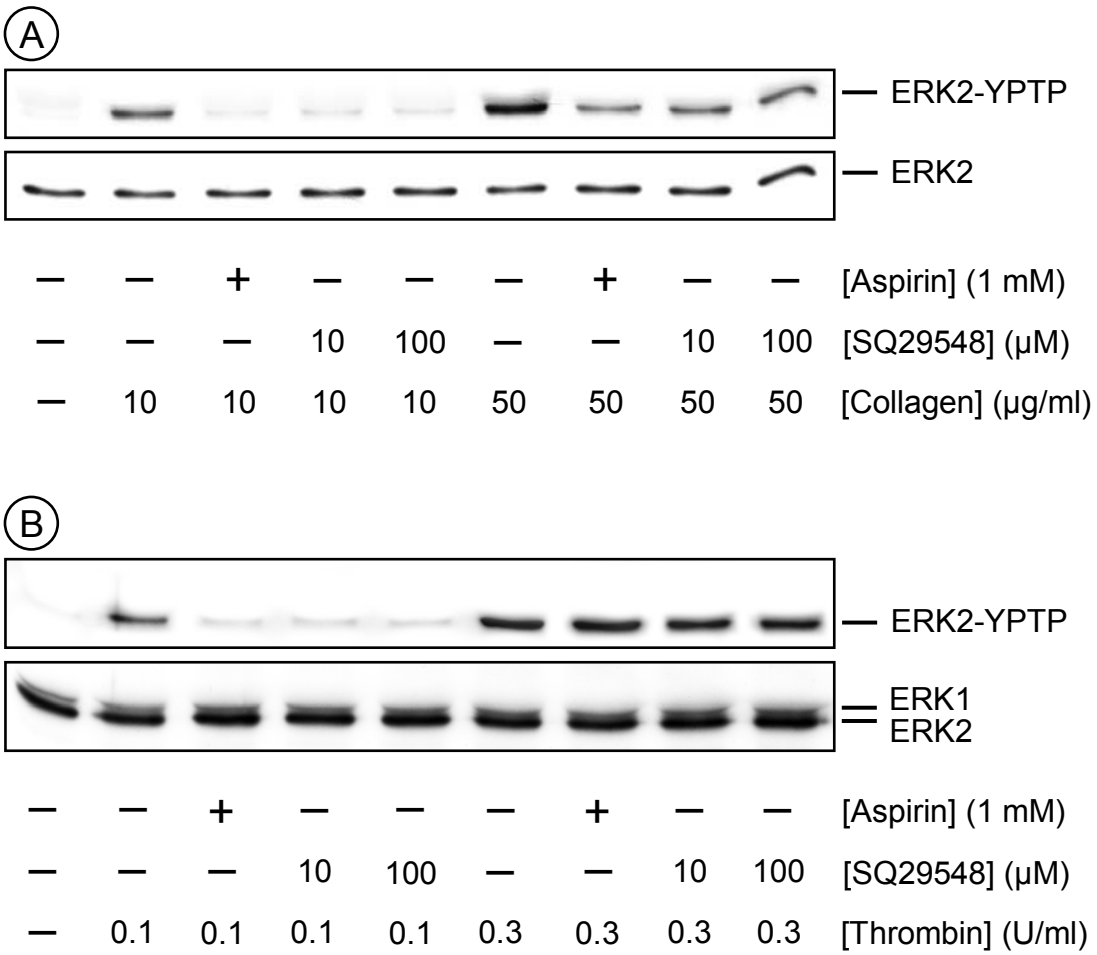
Both collagen and thrombin, although initiating platelet activation via different signalling pathways, evoke the release of TXA_2 that supports the effect of the initial stimulus leading to an enhanced secretion of ADP.

To preclude TXA_2-mediated signalling platelets were pretreated either with aspirin or with the TXA_2-receptor antagonist SQ29548 prior to the addition of collagen or thrombin.

As shown in Fig. 8A, preincubation with aspirin as well as with SQ29548 abrogates the response to 10 µg/ml collagen and remarkably decrease the response to 50 µg/ml collagen. On the other hand (Fig. 8B), preventing TXA_2 signalling, whereas abrogating ERK2 activation provoked by 0.1 U/ml thrombin, has no effect when platelets are challenged with 0.3 U/ml thrombin.

Thus far, the data indicate that ERK2 activation in response to both primary agonists thrombin and collagen strongly, albeit not exclusively, relies on released TXA_2. Furthermore, ERK2 activation caused by thrombin as well as by collagen in the last instance relies on released ADP and P2Y12 receptor-mediated Gi-coupling.

Fig. 8 Effects of aspirin and SQ29548 on ERK2 activation in response to collagen and thrombin



Washed human platelets were pretreated with buffer, 1 mM aspirin for 30 min, or 10 and 100 µM SQ29548 for 5 min at 37°C with stirring and stimulated as indicated with collagen (A) or thrombin (B) for 2 min. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP) (A and B), and unmodified ERK2 (A), or ERK1 and 2, respectively (B). Shown results are each from one experiment that is representative of 2 (A) and 3 (B) experiments.

5.2 Effect of platelet-derived, secondary mediators on ERK2 activation

TXA_2 and ADP, although provoking platelet activation and aggregation on their own, *in vivo* rather represent subsequent mediators as they are released from platelets usually as a consequence of primary stimulation by collagen or thrombin.

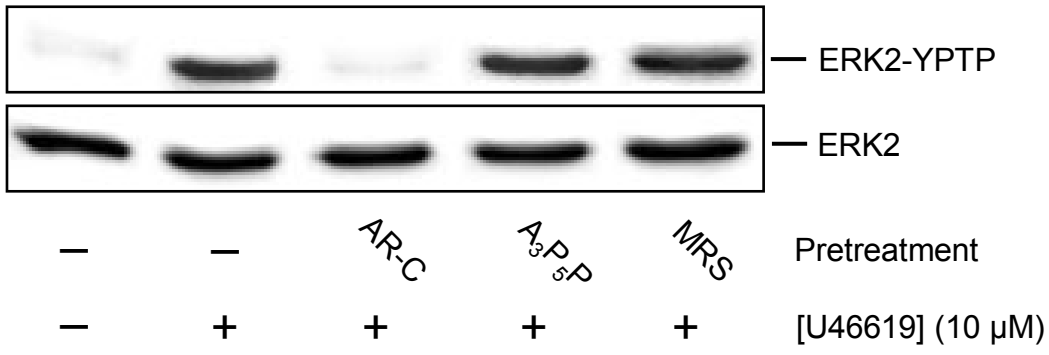
5.2.1 ERK2 activation in response to TXA_2-mimetic U46616

The stable TXA_2-mimetic U46619 applied at 10 µM provokes ERK2 activation peaking between 2 and 3 min of stimulation (data not shown), comparable to the time courses obtained for thrombin and collagen.

5.2.1.1 Effects of P2Y receptor antagonists on U46619-induced ERK2 activation

As shown in Fig. 9, and similar to the effects observed in thrombin-stimulated platelets (Fig. 5), pretreatment with 10 µM AR-C69931MX entirely inhibits ERK2 activation induced by 10 µM U46619, whereas 500 µM A_3P_5P and 100 µM MRS2179 have no effect.

Fig. 9 Effects of P2Y receptor antagonists on U46619-evoked ERK2 activation



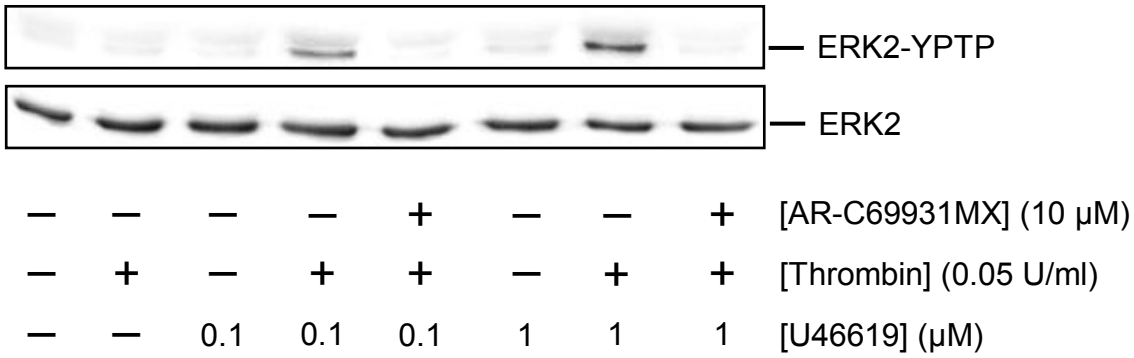
Washed human platelets were preincubated with buffer, 10 µM AR-C69931MX, 500 µM A_3P_5P, or 100 µM MRS2179 for 5 min at 37°C with stirring and stimulated with U46619 for 2 min. All samples contain 0.1 % ethanol as U46619-solvent control. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 4 similar experiments.

5.2.1.2 Effect of a co-stimulation with U46619 and thrombin on ERK2 activation

ERK2 activation induced by 0.1 U/ml thrombin is dependent on intermediate TXA_2 release and signalling (Fig. 8B) and both thrombin- and TXA_2-provoked ERK2 activation depend on ADP release and subsequent P2Y12 receptor signalling (Figs. 5 and 9). To investigate this synergism in more detail platelets were incubated with subthreshold concentrations of thrombin and U46619 alone or in combination in the absence or presence of 10 µM AR-C69931MX.

As shown in Fig. 10, U46619 at 0.1 µM and 1 µM alone does not provoke ERK2 activation. On the other hand, in combination with 0.05 U/ml thrombin that alone likewise has no effect (Fig. 3), U46619 at 0.1 µM and more effectively at 1 µM provokes ERK2 activation that is sensitive to AR-C69931MX.

Fig. 10 ERK2 activation provoked by combinations of subthreshold concentrations of thrombin and U46619



Washed human platelets were pretreated with buffer or 10 µM AR-C69931MX for 5 min at 37°C with stirring and stimulated with both thrombin or U46619 as indicated singly and in combination for 2 min. All samples contain 0.01 % ethanol as U46619-solvent control. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 3 similar experiments.

5.2.2 Effect of ADP on ERK2 activation

The data thus far suggest that TXA_2 released in response to both collagen and thrombin synergizes with the signalling events of the initial stimulus by supporting ADP release and in turn consecutive P2Y12 receptor-mediated ERK2 activation. Interestingly, neither exogenously added ADP alone nor its more potent analogue 2-MeS-ADP, each applied up to 100 µM, are sufficient to provoke any detectable ERK2 activation (data not shown).

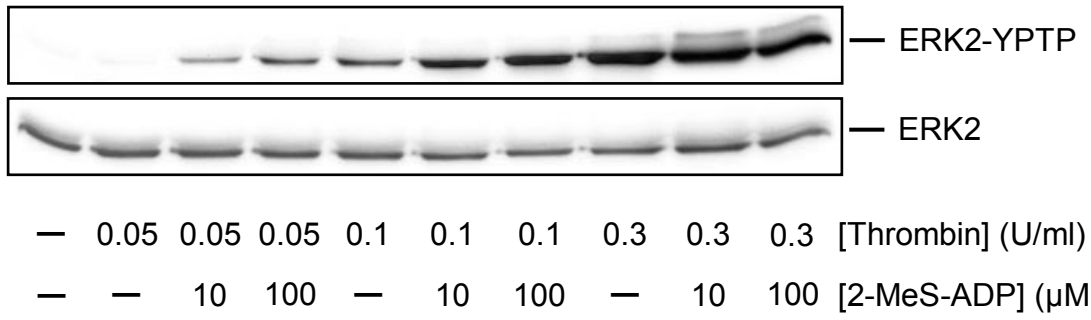
5.2.2.1 Effect of 2-MeS-ADP on thrombin-evoked ERK2 activation

The concentration of a primary agonist such as thrombin or collagen determines the amount of initially released ADP and, as such, the intensity of the subsequent amplifying effects of P2Y12 receptor signalling.

If these limiting mechanisms also apply for ERK2 activation was tested by co-stimulating platelets with increasing concentrations of both thrombin and 2-MeS-ADP.

As demonstrated in Fig. 11, in the presence of 0.05 U/ml thrombin that alone has no effect, the exogenously addition of 2-MeS-ADP at 10 µM and, more potently at 100 µM, provokes in ERK2 activation. This positive synergistic effect is also valid for ERK2 activation in response to 0.1 U/ml thrombin, but little or no amplification is detectable with 0.3 U/ml thrombin.

Fig. 11 Effect of combinations of thrombin and 2-MeS-ADP on ERK2 activation



Washed human platelets were stimulated with thrombin singly or in combination with 2-MeS-ADP (as indicated) at 37°C for 2 min with stirring. Reactions were stopped by the addtion of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and Methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 3 similar experiments.

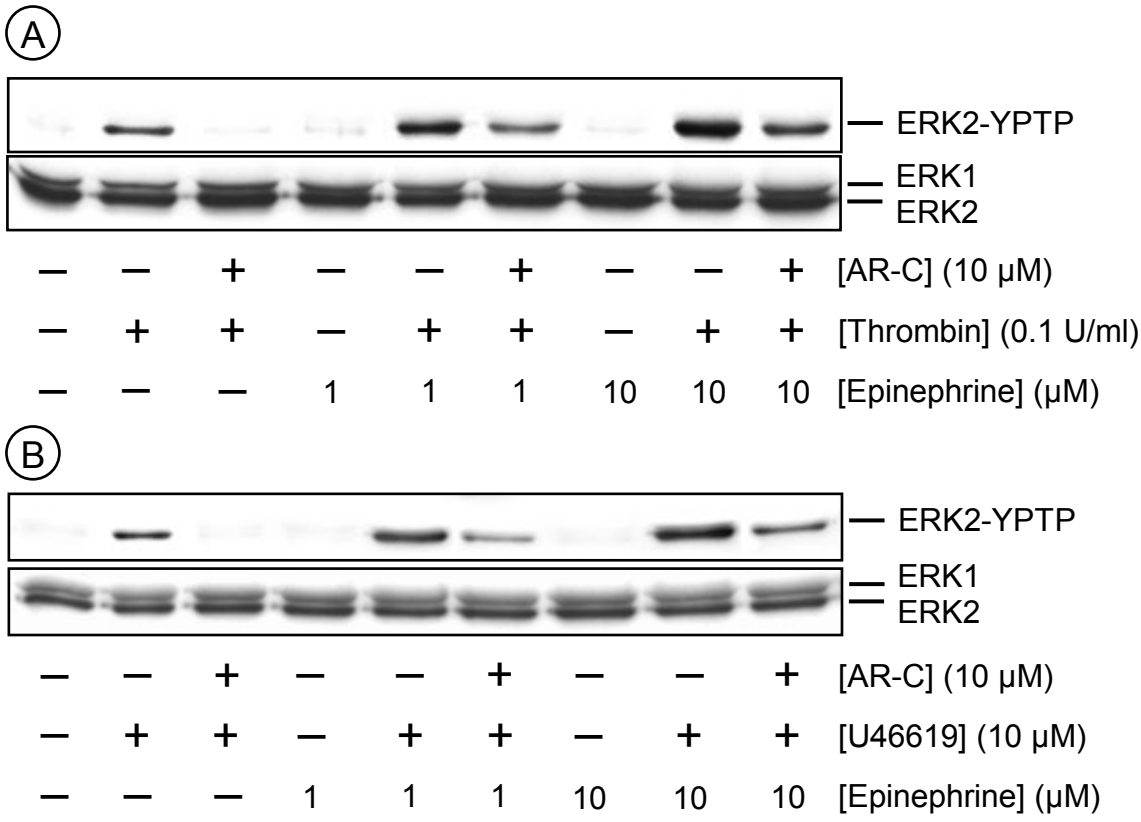
5.3 Mimicking P2Y12 ADP-receptor Gi-coupling by stimulating Gz-coupled platelet α_{2A}-adrenoceptors with epinephrine

Epinephrine, acting on platelets via inhibitory G protein Gz-coupled α_{2A}-adrenoceptors does not initiate activation of isolated platelets on its own, but potentiates platelet responses induced by other agonists.

5.3.1 Effect of epinephrine on ERK2 activation induced by thrombin or U46619 and precluded P2Y12 ADP receptor signalling

In accordance with previous studies, epinephrine-induced α_{2A}-adrenoceptor signalling can mimic P2Y12 receptor-mediated platelet responses (Fig. 12).

Fig. 12 Effect of epinephrine on ERK2 activation in thrombin- or U46619-stimulated and AR-C69931MX-pretreated platelets

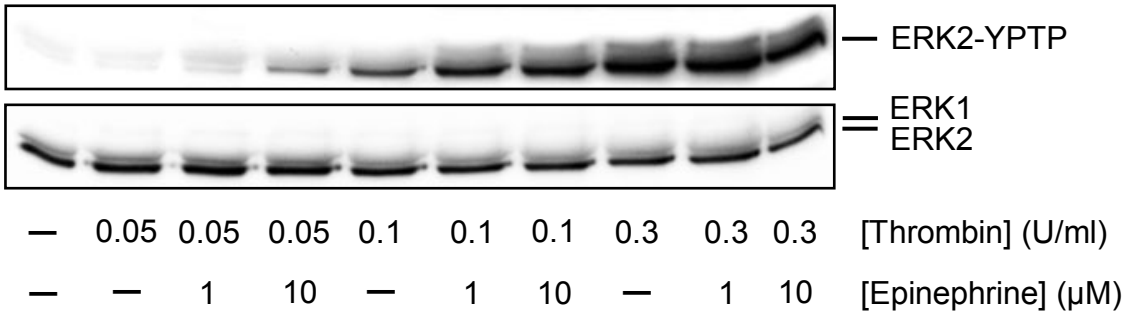


Washed human platelets were pretreated with buffer or 10 µM AR-C69931MX (AR-C) for 5 min at 37°C with stirring and stimulated as indicated with both epinephrine and thrombin (A) or U46619 (B) singly and simultaneously in combination for 2 min. All samples in B contain 0.1 % ethanol as U46619-solvent control. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immono(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr183 (-YPTP), or unmodified ERK1 and 2. Shown results are each from one experiment that is representative of 3 similar experiments.

As shown in Fig. 12 A, epinephrine alone (1 and 10 µM) has no effect on ERK2 activation. In contrast, epinephrine at 1 µM and more effectively at 10 µM enhances ERK2 activation in response to 0.1 U/ml thrombin.
In addition, the inhibitory effect of 10 µM AR-C69931MX on ERK2 activation induced by 0.1 U/ml thrombin can be bypassed by 1 µM and 10 µM epinephrine in a concentration-dependent fashion. Similar results are obtained in platelets stimulated with 10 µM U46619 (Fig. 12 B)

As demonstrated above, epinephrine, although alone is not able to evoke ERK2 activation enhances the effect initially provoked by thrombin as well as by U46619.
A more detailed analysis of this amplifying effect for thrombin-induced ERK2 activation is shown in Fig. 13.

Fig. 13 Effect of combinations of thrombin and epinephrine on ERK2 activation



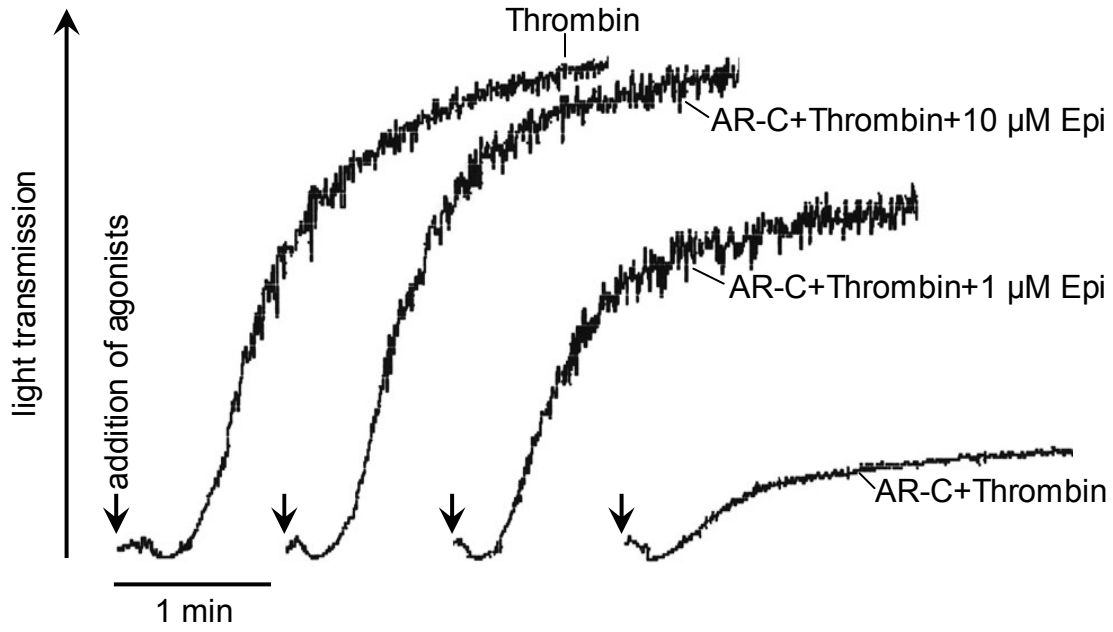
Washed human platelets were stimulated with as indicated thrombin alone or simultaneously in combination with epinephrine at 37°C for 2 min with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK1 and 2. Shown result is from one experiment that is representative of 3 similar experiments.

5.3.2 Effect of epinephrine on platelet aggregation induced by thrombin or U46619 under conditions of precluding P2Y12 ADP receptor signalling

The ATP-analogues of the AR-C series such as AR-C69931MX and AR-C660096MX potently inhibit aggregation of washed human platelets in response to most, if not all, commonly known agonists, including thrombin (Fälker *et al.* 2005) , collagen (Roger *et al.* 2004), and U46619 (Paul *et al.* 1999).

The effect of epinephrine (1 and 10 µM) on aggregation of washed platelets pretreated with AR-C69931MX (10 µM) in response to thrombin (0.1 U/ml) is shown in Fig. 14.

Fig. 14 Effect of epinephrine on platelet aggregation in thrombin-stimulated and AR-C69931MX-pretreated platelets



Washed human platelets were preincubated for 5 min with stirring at 37°C with buffer or 10 µM AR-C69931MX (AR-C) and challenged with 0.1 U/ml thrombin alone or simultaneously in combination with 1 and 10 µM epinephrine (Epi), respectively. Platelet aggregation was monitored as described in "Materials and methods". Shown curves are from one experiment that is representative of 5 similar experiments.

As expected, epinephrine alone (up to 100 µM) does not induce aggregation of washed human platelets (data not shown).

A statistical analysis of the 5 experiments of which one is presented in Fig. 14 revealed that 0.1 U/ml thrombin induces maximal platelet aggregation of 84±2%. Pretreatment with 10 µM AR-C69931MX decreases aggregation in response to thrombin to 11±4%. Epinephrine, when co-applied at 1 µM is able to recover the thrombin-response up to 66±8%, whereas 10 µM epinephrine entirely restores aggregation (82±2%).

Regarding U46619-provoked aggregation of washed platelets comparable results were obtained. U46619 at 10 µM evokes maximal platelet aggregation of 73±5% (n=6); pretreatment with 10 µM AR-C69931MX nearly entirely abrogates aggregation to 2±2% (n=6). Epinephrine is able to fully recover the U46619-response when co-applied at 1 µM (75±6%; n=4). In combination with 10 µM epinephrine aggregation is regained to a minimum of 71% and maximum of 75% (n=2).

Ethanol at 0.1 % (v/v), as solvent control for U46619 were present in all samples and has no effect on platelet aggregation when added alone (data not shown).

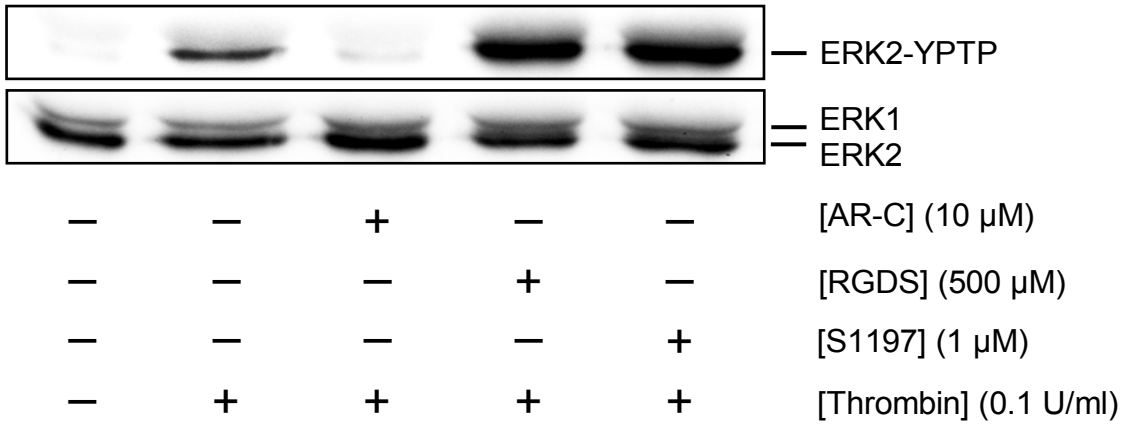
5.4 Effect of integrin $\alpha_{IIb}\beta_3$ outside-in signalling on ERK2 activity

Agonist-induced platelet activation leads to the conformational change and, as such, activation of integrin $\alpha_{IIb}\beta_3$. Fibrinogen binding, besides providing the molecular basis for platelet aggregation induces integrin $\alpha_{IIb}\beta_3$ outside-in signalling that has been demonstrated to regulate a variety of platelet responses and enhance platelet activation and aggregation (Parise 1999, Shattil *et al.* 2004).

5.4.1 Effect of precluding fibrinogen-binding to integrin $\alpha_{IIb}\beta_3$ on ERK2 activation provoked by thrombin

Besides the RGDS peptide the non-peptide RGD mimetic S1197 was used for studying the effect of precluding fibrinogen binding to activated integrin $\alpha_{IIb}\beta_3$ on ERK2 activity. As shown in Fig. 15, both compounds markedly enhance ERK2 activation induced by 0.1 U/ml thrombin.

Fig. 15 Effect of RGDS and S1197 on ERK2 activation in response to thrombin



Washed human platelets were pretreated as indicated with buffer, AR-C69931MX (AR-C), RGDS, or S1197 for 5 min at 37°C with stirring and challenged with thrombin for 2 min. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western) blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK1 and 2. Shown result is from one experiment that is representative of 3 similar experiments.

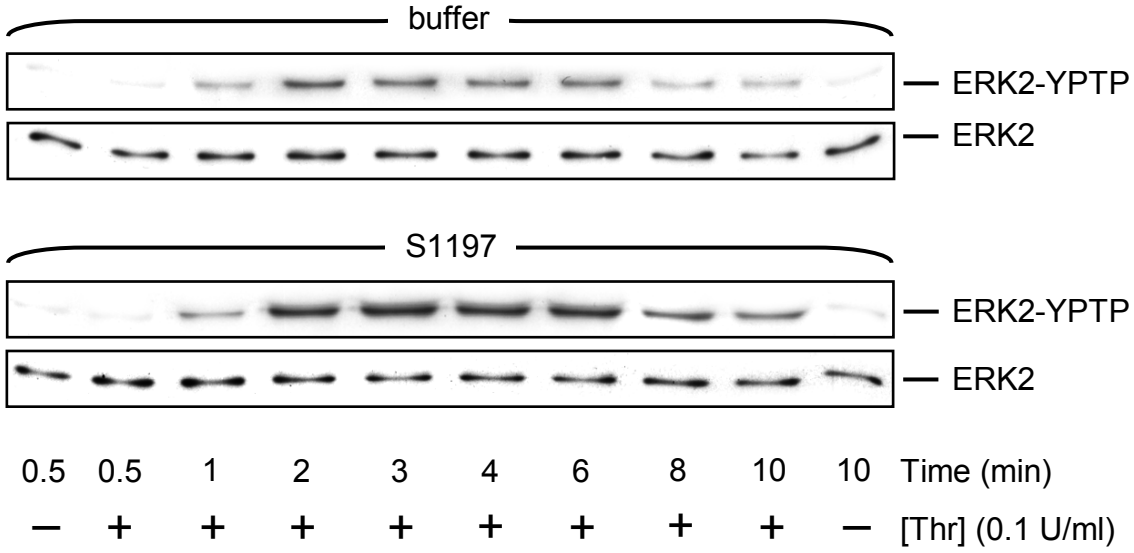
As expected, platelet aggregation in response to 0.1 U/ml thrombin is completely abolished by RGDS (500 µM) as well as S1197 (1 µM) (data not shown).

5.4.2 Effect of S1197 on the time course of thrombin-induced ERK2 activation

To further investigate the effect of integrin $\alpha_{IIb}\beta_3$ outside-in signalling on ERK2 activity a time course was performed stimulating platelets with 0.1 U/ml thrombin in the presence of 1 µM S1197.

As shown in Fig. 16, in the presence of S1197 the activation of ERK2 in response to thrombin is not accelerated but markedly enhanced.

Fig. 16 Effect of S1197 on the time course of ERK2 activation in response to thrombin



Washed human platelets were pretreated with buffer or 1 µM S1197 for 5 min at 37°C with stirring and challenged with 0.1 U/ml thrombin (Thr) or buffer for control for the indicated times. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting with sequential re-probing as described in "Materials and methods" using identical dilutions of antibodies for both NCs, respectively. Applied antibodies recognize ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. NCs were simultaneously exposed to X-ray films. Shown result is from one experiment that is representative of 2 similar experiments.

5.5 Signalling pathways downstream the P2Y12 ADP receptor involved in ERK2 activation

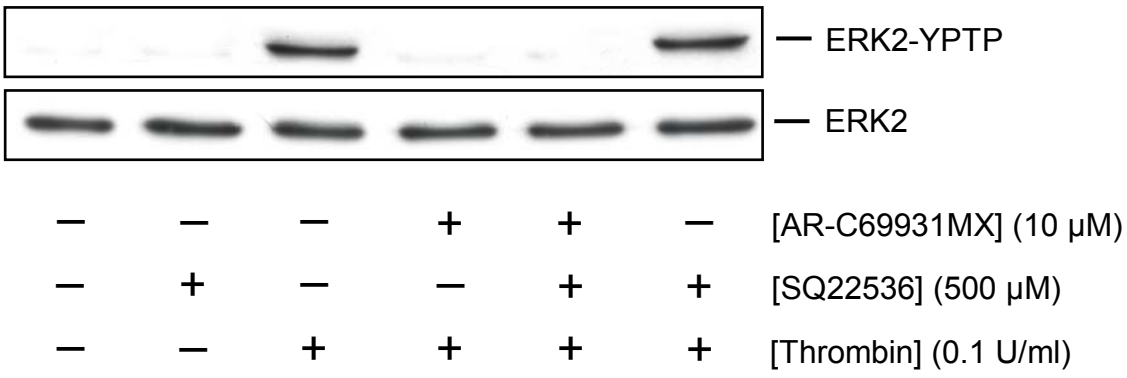
Stimulation of the Gi_2-coupled P2Y12 receptor by ADP induces the activation and therefore dissociation of Gi α- and β/γ-subunits. Whereas α_i-subunits cause the inhibition of adenylyl cyclase, the β/γ-subunits have been demonstrated to activate phosphoinositide 3-kinase (PI 3-K).

5.5.1 Effect of the direct inhibition of adenylyl cyclase on ERK2 activation

Whether the direct inhibition of adenylyl cyclase is sufficient to restore the effects of AR-C69931MX on P2Y12 receptor-mediated G_{i2} α-subunit signalling was investigated. Platelets were incubated with either 10 µM AR-C69931MX or 500 µM SQ22536, an adenosine analogue that potently blocks adenylyl cyclase activity, alone or in combination prior to the stimulation with 0.1 U/ml thrombin.

As demonstrated in Fig. 17, SQ22536 neither recovers the inhibitory effects of AR-C69931MX on thrombin-induced ERK2 activation nor affects the single thrombin response.

Fig. 17 Effect of SQ22536 on thrombin-induced ERK2 activation in AR-C69931MX-pretreated platelets



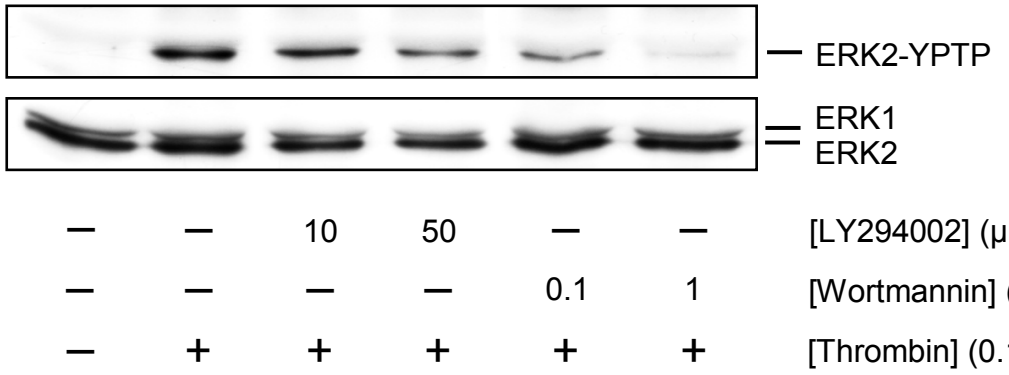
Washed human platelets were pretreated with buffer, 500 µM SQ22536, 10 µM AR-C69931MX or both for 5 min at 37°C with stirring and challenged with thrombin or buffer for 2 min, respectively. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 2 similar experiments.

5.5.2 Effect of the inhibition of phosphoinositide 3-kinase (PI 3-K) on thrombin-induced ERK2 activation

To determine whether PI 3-K activity contributes to P2Y12 receptor-dependent ERK2 activation platelets were incubated with the two structurally unrelated PI 3-K inhibitors wortmannin and LY294002 prior to thrombin-stimulation.

The effects of LY294002 and wortmannin on ERK2 activation provoked by thrombin are shown in Fig. 18.

Fig. 18 *Effects of LY294002 and wortmannin on ERK2 activation induced by thrombin*



Washed human platelets were pretreated with buffer, LY294002 or wortmannin as indicated for 15 min at 37°C with stirring and stimulated with thrombin for 2 min. All samples contain 0.25 % DMSO as solvent control. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK1 and 2. Shown result is from one experiment that is representative of 3 similar experiments.

Both PI 3-K inhibitors attenuate ERK2 activation caused by thrombin in a concentration-dependent fashion. LY294002 at 10 and more potently at 50 µM as well as wortmannin at 0.1 and, most efficiently at 1 µM prevent ERK2 activation in response to 0.1 U/ml thrombin.

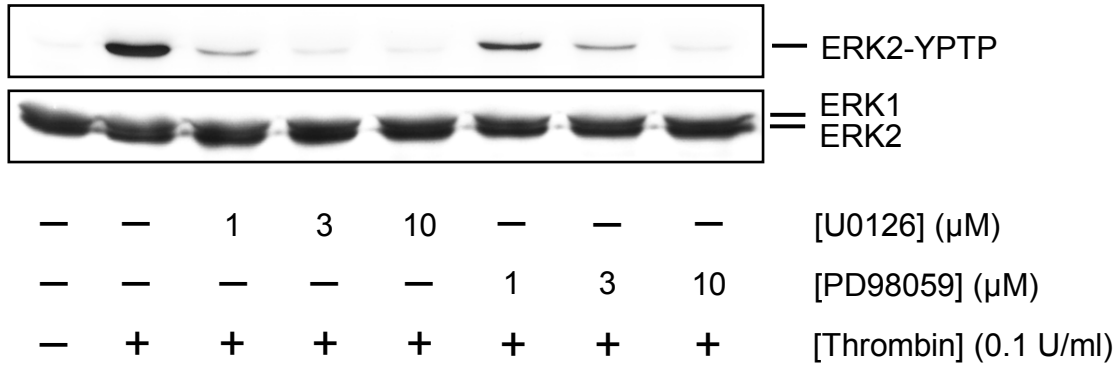
5.5.3 Effects of the inhibition of the MAP/ERK kinases 1 and 2 (MEK1/2) on ERK2 activation evoked by thrombin

ERK1 and 2 both become activated by the dual-specific MAPK/ERK kinases 1 and 2 (MEK1/2).

Two commonly used pharmacologically unrelated MEK1/2 inhibitors were applied within this work, the organic compound U0126 and the synthetic flavone compound PD98059.

ERK2 activation provoked by 0.1 U/ml thrombin is effectively attenuated by 1 µM and almost completely inhibited by 3 µM U0126, whereas PD98059, to achieve comparable effects, is required at higher concentrations of 3 µM and 10 µM, respectively (Fig. 19).

Fig. 19 Effects of U0126 and PD98059 on thrombin-induced ERK2 activation



Washed human platelets were pretreated with buffer or U0126 and PD98059 as indicated for 30 min at 37°C with stirring and stimulated with thrombin for 2 min. All samples contain 0.05% DMSO as solvent control. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK1 and 2. Shown result is from one experiment that is representative of 3 similar experiments.

5.5.4 Akt (protein kinase B) phosphorylation at Ser-473 in response to thrombin

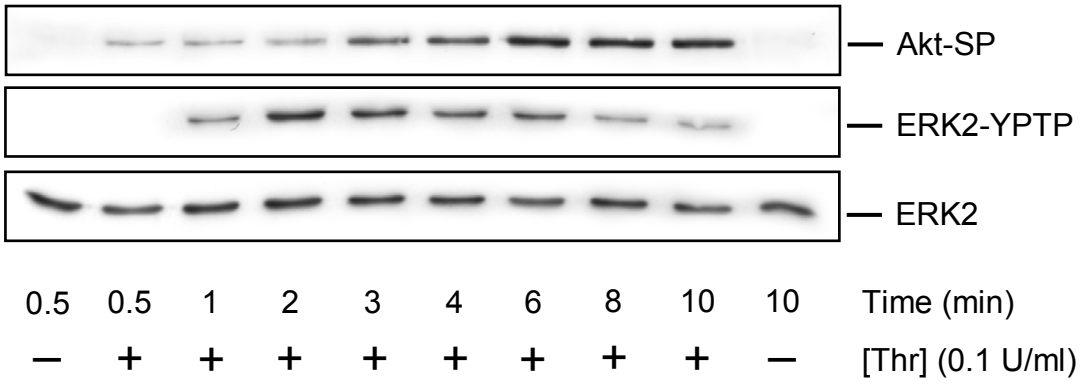
In thrombin-stimulated washed human platelets the serine/threonine kinase Akt, also designated protein kinase B (PKB), gets activated in a P2Y12 receptor/ PI 3-K-dependent manner (Kim *et al.* 2004). Besides its translocation to the cell membrane, independent phosphorylation of Akt at both Thr-308 and Ser-473 residues are required for full enzyme activity (Alessi *et al.* 1996). Akt Thr-308 residues are phosphorylated by the phosphatidylinositol-dependent kinase 1 (PDK1), whereas the kinase which phosphorylates Ser-473 is yet unidentified.

5.5.4.1 Time course of thrombin-induced Akt Ser-473 phosphorylation

The time course of Akt phosphorylation at Ser-473 compared to ERK2 activation in platelets stimulated with 0.1 U/ml thrombin is shown in Fig. 20.

In unstimulated platelets, Akt is not, or in some experiments only marginally, phosphorylated. Upon thrombin stimulation, Akt phosphorylation is detectable within 30 sec, increases within 6 min and remains phosphorylated up to 10 min, demonstrating the Akt activation lags behind that of ERK2.

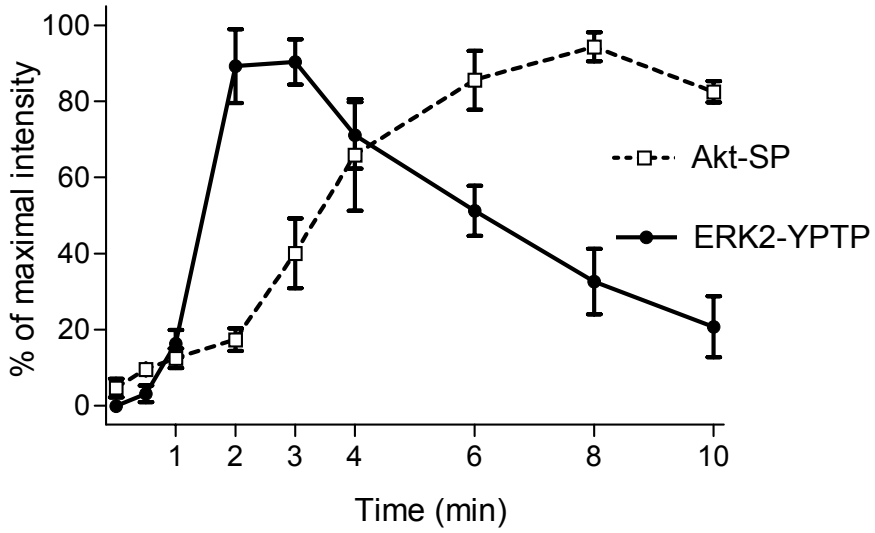
Fig. 20 Time course of Akt serine phosphorylation and ERK2 activation provoked by thrombin



Washed human platelets were incubated with 0.1 U/ml thrombin (Thr) for the indicated times at 37°C with stirring. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in “Materials and methods” using antibodies recognizing Akt, phosphorylated at Ser-473 residues (-SP), ERK1 and 2, dually phosphorylated at Tyr-185/Thr-183 (-YPTP), or unmodified ERK2. Shown result is from one experiment that is representative of 4 similar experiments.

The time-dependent changes of Akt phosphorylation at Ser-473 compared to that of ERK2 at Tyr-185/Thr-183 were quantified by densitometric analysis (Fig. 21).

Fig. 21 Quantification of the time-dependent changes in phosphorylation of Akt at Ser-473 and ERK2 at Tyr-185/Thr183 upon thrombin-stimulation



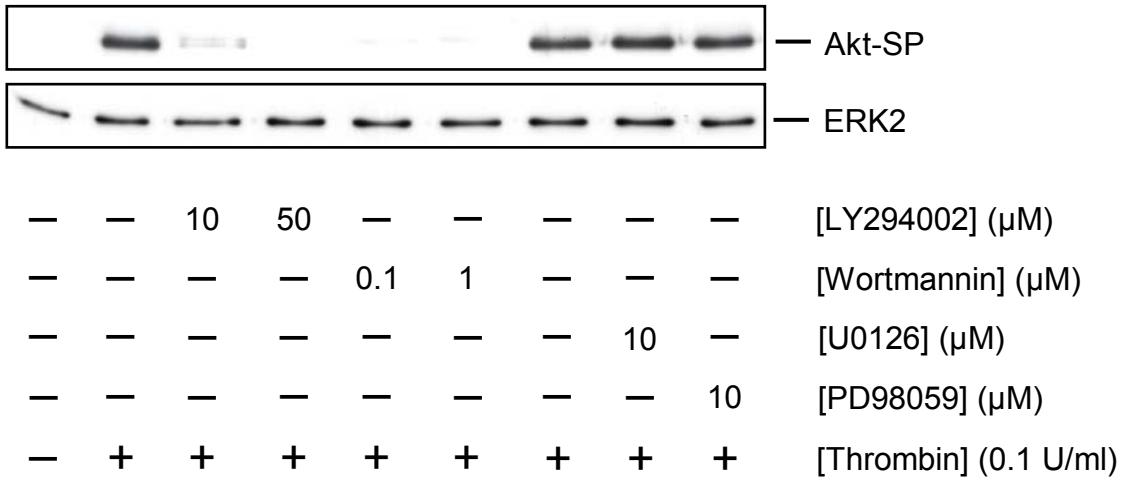
X-ray films from the 4 immuno(Western)blots, of which one is shown in Fig. 20, were densitometrically analyzed as described in "Materials and methods". Data were processed by setting the maximal optical intensity of the respective band as 100% and are means±S.E.M. Error bars are not shown for data points having error that is smaller than the symbol. The graph shows Akt Ser-473 phosphorylation (Akt-SP) as well as ERK2 dually phosphorylated at Tyr-185/Thr-183 (ERK2-YPTP).

5.5.4.2 Effects of inhibitors of PI 3-Kinase and of MEK1/2 on Akt Ser-473 phosphorylation provoked by thrombin

The effects of the PI 3-K inhibitors LY294002 and wortmannin, as well as of the MEK1/2 inhibitors U0126 and PD98059 on thrombin-evoked Akt Ser-473 phosphorylation are shown in Fig. 22.

Akt Ser-473 phosphorylation induced by 0.1 U/ml thrombin is effectively inhibited by the two PI 3-K inhibitors. LY294002 potentially prevents Akt phosphorylation at 10 µM and completely abrogates phosphorylation at 50 µM whereas wortmannin totally blocks Akt phosphorylation already at 0.1 µM.

Fig. 22 Effects of LY294002 and wortmannin as well as U0126 and PD98059 on Akt Ser-473 phosphorylation induced by thrombin



Washed human platelets were pretreated with buffer, or increasing concentrations of LY294002 and wortmannin as indicated for 30 min, or buffer, and U0126 or PD98059 for 15 min, respectively, at 37°C with stirring and challenged with thrombin for 6 min. All samples contain 0.25 % DMSO as solvent control. Reactions were stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by immuno(Western)blotting with sequential re-probing as described in "Materials and methods" using antibodies recognizing Akt phosphorylated at Ser-473 (-SP) or unmodified ERK2. Shown result is from one experiment that is representative of 3 similar experiments.

The MEK1/2 inhibitors U0126 and PD98059 (both at 10 μM), in contrast to their ability to prevent ERK2 activation (Fig. 19), do not have any effect on thrombin-provoked Akt phosphorylation at Ser-473.

5.6 Involvement of ERK2 in primary functional responses associated with platelet activation

Agonist-induced platelet activation, as aforementioned, strongly relies on the release of positive feedback mediators, such as TXA_2 and ADP. These platelet-derived mediators support and enhance the primary signal leading, besides other platelet responses, to the secretion of α- and dense granules and to the activation of integrin $\alpha_{IIa}\beta_3$ as the prerequisite for final platelet aggregation. Therefore, an involvement of ERK2 in these events accompanying primary platelet activation was addressed.

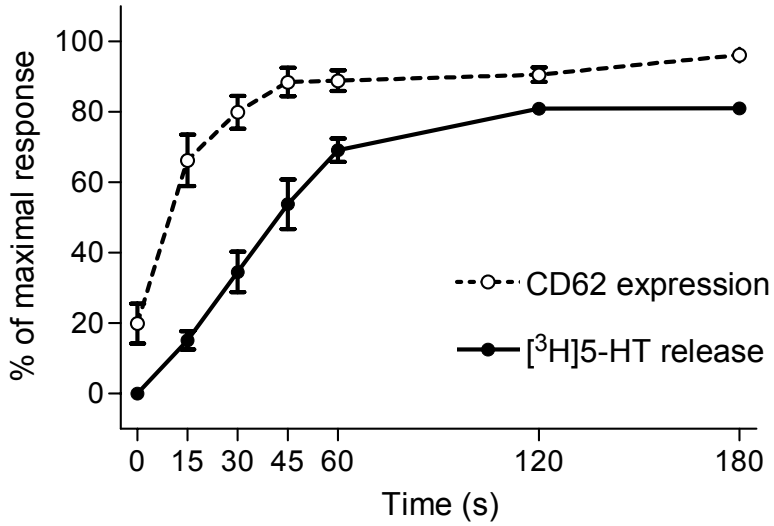
5.6.1 Effects of MEK1/2 inhibitors on α- and dense granule release in response to thrombin

P-selectin, also termed CD62, is localized in the membrane of α-granules of resting platelets and serves as a marker for α-granule release from activated platelets when exposed on the plasma membrane.

Platelet dense granules contain, besides ADP and ATP, serotonin (5-hydroxytryptamine; 5-HT).

The time-courses of thrombin-induced platelet α-granule secretion, determined by P-selectin expression, and the release of dense granule contents, determined by 5-HT-release, are presented in Fig. 23.

Fig. 23 Quantification of the time course of platelet CD62 exposure and [^{3}H]5-HT release provoked by thrombin



Washed human platelets were challenged with 0.1 U/ml thrombin for the indicated times. Platelet [^{3}H]5-HT release and CD62 expression were monitored as described in "Materials and methods". Data are means±S.E.M. and error bars are not shown for data points having error that is smaller than the symbol. Shown curves are each from 4 experiments.

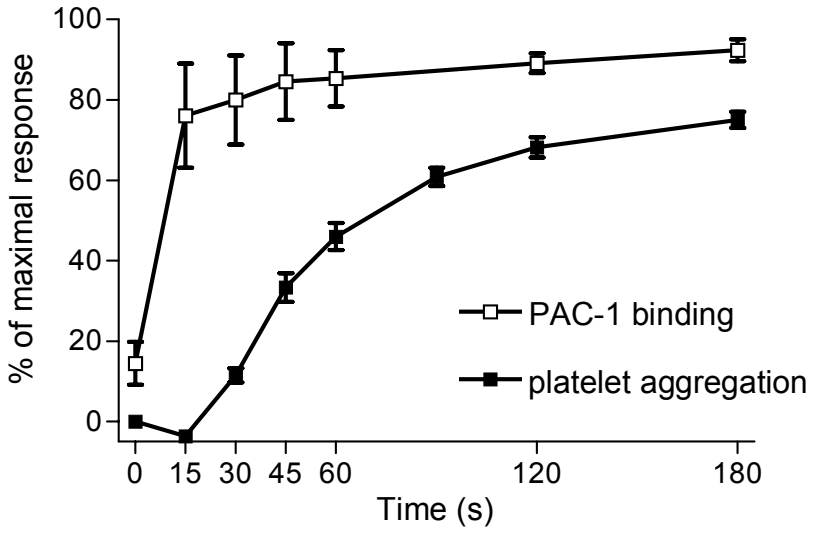
Upon stimulation with 0.1 U/ml thrombin α-granule secretion is completed up to 89±3 % within 1 min after the onset of stimulation. 5-HT from dense granules is released within 1 min up to 70±3 %, and the maximal determined release of 81±1 %is assessed after 2 min.

Pretreatment with U0126 or PD98059, both at 10 µM, does not have any effect on α-granule secretion (determined by CD62 expression; n=3; data not shown). On the other hand, the release of [^{3}H]5-HT from dense granules evoked by 0.1 U/ml thrombin determined after 2 min of stimulation is attenuated about 9±5 % by 10 µM U0126 (p=0,18; n=3) and 16±9 % by 10 µM PD98059 (p=0,03; n=3). DMSO, present in all samples at 0.05 % (v/v) as solvent control for U0126 and PD98059, has no effect.

5.6.2 Effects of MEK1/2 inhibitors on thrombin-provoked integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation

The monoclonal antibody PAC-1 binds exclusively to activated integrin $\alpha_{IIb}\beta_3$ and is consentiently accepted as a quantitative marker for integrin $\alpha_{IIb}\beta_3$ activation by flow cytometric analysis (Schmitz *et al.* 1998). The time courses of PAC-1 binding and platelet aggregation in response to thrombin are shown in Fig. 24.

Fig. 24 Quantification of the time courses of thrombin-induced PAC-1 binding and platelet aggregation



Washed human platelets were stimulated with 0.1 U/ml thrombin for the indicated times. PAC-1 binding and platelet aggregation were monitored as described in "Materials and methods". Data are means±S.E.M. and error bars are not shown for data points having error that is smaller than the symbol. Shown curve for PAC-1 binding is from 4 and that for platelet aggregation from 6 experiments, respectively.

The data reveal that after 30 sec of stimulation with 0.1 U/ml thrombin the activation of integrin $\alpha_{IIb}\beta_3$ (PAC-1 binding) is already completed up to 80±11 %; the maximal response of 92±3 % was determined as after 3 min.
Platelet aggregation induced by thrombin reaches about 46±3 % after 1 min, and 68±3 % after 2 min of stimulation. Maximal platelet aggregation of 82±2 % is reached after 4 to 5 min.

Whether ERK2 contributes to integrin $\alpha_{IIb}\beta_3$ activation or/and platelet aggregation was investigated by pretreating platelets with 10 µM PD98059 or U0126, respectively, prior to the stimulation with 0.1 U/ml thrombin. DMSO, as solvent control for U0126 and PD98059 was present in all samples at 0.05 % (v/v), and had no effect.
Both inhibitors do not have any effect on neither integrin $\alpha_{IIb}\beta_3$ activation (PAC-1 binding) nor the velocity or amplitude of platelet aggregation in response to 0.1 U/ml thrombin (data not shown).

6 DISCUSSION

6.1 ERK2 activation in response to the primary platelet agonists thrombin and collagen

6.1.1 Gq-mediated signalling induced by thrombin initiates ADP secretion and ERK2 activation which is amplified by Gi-coupled P2Y12 ADP receptor signalling

Thrombin, on of the most potent primary platelet agonist induces the activation and aggregation of human platelets via the two Gq-coupled protease-activated receptors (PARs) 1 and 4 (Kahn *et al.* 1999).

In isolated human platelets thrombin has been shown to induce ERK2 activation in a time- and concentration-dependent manner (Papkoff *et al.* 1994, Nakashima *et al.* 1994, Nadal *et al.* 1997). The precise signalling mechanisms involved, however, have not yet been elucidated.

Within this thesis it could be demonstrated that the release of ADP and subsequent P2Y12 ADP receptor signalling are crucial events amplifying thrombin- as well as collagen-induced ERK2 activation.

A detailed analysis of the two phosphorylation steps of ERK2 in thrombin-stimulated washed human platelets (Figs. 1, 2) revealed that initial Thr-185 phosphorylation occurs within 30 sec, whereas the dual-phosphorylated active state is not detectable until 1 min after the onset of stimulation. ERK2 activity peaks between 2 to 3 min and decreases subsequently. Comparing the course of ERK2 deactivation, which will be discussed below (chapter 6.3), reveals that dephosphorylation of threonine-residues precedes that of tyrosine-residues.

The importance of released ADP for thrombin-induced ERK2 activation was implied by the use of apyrase, which scavenges released adenine nucleotides (Fig. 4).

The use of specific P2Y ADP receptor antagonists allows to differentiate between the receptor subtypes involved and revealed that P2Y12 receptors but not P2Y1 receptors mediate the effect of released ADP (Fig. 5). AR-C69931MX, a highly specific, competitively acting P2Y12 receptor antagonist, completely abolishes ERK2 activation in response 0.1 U/ml thrombin.

ERK2 activation induced by higher thrombin concentrations (0.3 U/ml) is still effectively but not completely inhibited by AR-C69931MX (Fig. 6), strongly indicating that an ADP-independent mechanism comes into play under these conditions. This mechanism might involve protein kinase C (PKC)- and calcium-dependent pathways, which have previously been demonstrated to mediate ERK2 activation in thrombin-stimulated platelets (Börsch-Haubold *et al.* 1995, Nadal-Wollbold *et al.* 2002), suggesting that under these conditions Gq-mediated pathways predominate over P2Y12 ADP receptor-induced Gi-signalling.

6.1.1.1 Thrombin and released TXA_2 synergizes in Gq-signalling evoked ADP release

Besides ADP, TXA_2 is released from activated platelet and both mediators support and enhance the platelet response to the initial stimulus.

Human platelets contain mRNA encoding both the thromboxane / prostanoid receptor alpha and beta subtypes (TPα and TPβ), which couple to Gq- and Gi-proteins, respectively (Hirata *et al.* 1996). In 1999, Habib *et al.* demonstrated that the TPα receptor is the predominant isoform expressed on human platelets. Indeed, TXA_2-mimetic U46619-induced inhibition of adenylyl cyclase and platelet aggregation depends upon secretion of ADP and subsequent P2Y12 ADP receptor signalling (Paul *et al.* 1999). U46619 has also been demonstrated to induce ERK2 activation in rabbit and human platelets (Ohkubo *et al.* 1996, McNicol *et al.* 2001).

Like thrombin, U46619 provokes ERK2 activation in a P2Y12 receptor- but not P2Y1 receptor-dependent manner (Fig. 9; Fälker *et al.* 2004, Roger *et al.* 2004). Thrombin and released TXA_2 act through G protein-coupled receptors that in turn activate Gq as well as G12/13 (Offermanns *et al.* 1994). In platelets from Gq-deficient mice, coordinated signalling through G12/13 and Gi proteins has been demonstrated to be sufficient for integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation (Nieswandt *et al.* 2002, Dorsam *et al.* 2002). In contrast, for granules secretion, Gq-induced signalling pathways are required (Offermanns *et al.* 1997). Low concentrations of U46619 induce calcium mobilization and platelet shape change via G12/13, whereas at higher U46619 concentrations

Gq-coupled signalling causes ADP release and, in turn, P2Y12 receptor-dependent platelet aggregation (Paul *et al.* 1999). The observation that U46619-induced ERK2 activation is mediated by P2Y12 receptor signalling allows the conclusion that Gq-mediated ADP secretion precedes ERK2 activation.

The data suggest that Gq signalling, either induced by thrombin or by U46619, is the initial and determining step for ERK2 activation, which in turn is amplified by concomitant ADP release and P2Y12 receptor-mediated Gi-signalling.

This raised the question to what extent released TXA_2 is involved in or contributes to ERK2 activation induced by thrombin.

Subthreshold concentrations of thrombin and U46619 alone have no effect, whereas a co-stimulation provokes ERK2 activation sensitive to AR-C69931MX (Fig. 10). In addition, precluding TXA_2 signalling either by preventing TXA_2 synthesis with aspirin or by pretreatment with the TXA_2 receptor antagonist SQ29548 has no effect on ERK2 activation evoked by high concentrations of thrombin (0.3 U/ml), whereas the response to 0.1 U/ml thrombin is almost completely inhibited (Fig. 8B).

These results provide evidence that released TXA_2 synergizes thrombin-induced ERK2 activation by amplifying initial Gq signalling resulting in enhanced ADP release and subsequent P2Y12 receptor signalling. This synergism, on the other hand, is obviously not required at high concentrations of thrombin.

6.1.2 Cooperative Gq- and Gi-signalling is required for collagen-induced ERK2 activation

Collagen is the most thrombogenic component of the subendothelial matrix. Following vascular damage, collagen is exposed to and induces the activation of circulating platelets. Mainly two receptors are involved in the platelet response to collagen; integrin $\alpha_2\beta_1$ acts to adhere platelets to collagen, allowing platelets to interact with the lower affinity receptor glycoprotein VI (GP VI), which is for the most part responsible for platelet activation. GP VI, a member of the immunoglobulin (Ig) receptor superfamily, builds a complex with the Fc receptor (FcR) γ-chain. The signal induced by collagen via GP VI is transduced through a FcR γ-chain-mediated tyrosine phosphorylation cascade

involving the Src kinase family members Lyn and Fyn, leading to the activation of phospholipase C isoform γ2 (PLCγ2). Although collagen initiates the activation of platelets, the release of TXA_2 and ADP as well as thrombin-generation are required for full platelet aggregation and thrombus formation (for review see Nieswandt *et al.* 2003).

Besides thrombin, collagen has been reported to provoke ERK2 activation in human platelets (Börsch-Haubold *et al.* 1995).

Previous studies by Oury *et al.* (2002) suggest that ERK2 activation induced by low concentrations of collagen (1 µg/ml) is mediated by released ATP and ionophoric P2X1 receptors in an calcium- and PKC-dependent manner. The authors further reported that this effect can be abolished by antagonizing the P2X1 receptors with ADP.

This report contradicts the presented finding that ERK2 activation induced by 1 µg/ml collagen is completely inhibited by 10 µM AR-C69931MX (Fig. 7), indicating that released ADP acts rather supportive than inhibitory. Although it cannot be excluded that the observed inhibitory effect might be due to antagonistic actions of released ADP on P2X1 receptors, the amount of apyrase, which scavenges ADP, applied during platelet preparation within this work is not sufficient to protect platelet P2X1 receptors from desensitization (see the “Materials and methods” section 4.2, and Cazenave *et al.* 2004).

In addition, ERK2 activation provoked by moderate concentrations of collagen (10 µg/ml) is also markedly decreased by AR-C69931MX (Fig. 7).

The data correspond with the observations by Roger *et al.* (2004), who additionally demonstrated that desensitizing P2X1 receptors with α,β-MeATP has no effect on ERK2 activation in response to 10 µg/ml collagen. The authors further showed, in absolute agreement and support with the data presented in Fig. 8A, that intermediate TXA_2 signalling is required for ERK2 activation provoked by 10 mg/ml collagen.

However, at high concentrations of collagen (50 µg/ml) ERK2 activation is still remarkably but not entirely dependent on TXA_2 signalling (Fig. 8A).

Collagen and convulxin, a component of the venom of the tropical rattlesnake (*Crotalus durissus terrificus*), induce via GP VI the activation of integrin $\alpha_{IIb}\beta_3$ by unrelated PKC- and calcium-regulated pathways. These pathways are

supported by, but do not strictly dependent on secreted TXA_2 and/or ADP (Quinton *et al.* 2002, Atkinson *et al.* 2001).

Thus, like in thombin-stimulated platelets (Börsch-Haubold *et al.* 1995, Nadal-Wollbold *et al.* 2002), the observed effects might by explained by a PKC- and/or calcium-mediated activation of ERK2.

6.2 Gi-mediated signalling alone is not sufficient to induce ERK2 activation

6.2.1 ADP does not evoke ERK2 activation but amplifies the Gq-mediated response to thrombin

The presented data thus far clearly demonstrate that released ADP and subsequent P2Y12 receptor-mediated Gi signalling is necessarily required for ERK2 activation in response to low concentrations of thrombin and collagen, whereas this contributing effect becomes less important at higher concentrations of both primary agonists.

This regulatory principle had on a molecular level first been reported for thrombin-induced platelet phospholipase D (Martinson *et al.* 1998).

Neither exogenously added ADP nor its more potent analogue 2-MeS-ADP, however, have any detectable effect on ERK2 activation (data not shown), which agrees with previous observations by Oury et al. (2002) and in part with those by Roger et al. (2004) reporting no or little ERK2 phosphorylation induced by 2-MeS-ADP.

These findings indicate that the cooperation of P2Y1 receptor-mediated Gq coupling and P2Y12 receptor-mediated Gi signalling is not sufficient to evoke ERK2 activation. P2Y1 receptors are considered to induce only weak Gq signalling due to the relatively small amount of approximately 150 P2Y1 receptors per platelet, which is very low as compared with the TPα TXA_2 receptors or the PAR1 thrombin receptors (both 1000-2000 receptors/platelet) (Hechler *et al.* 2005).

In combination with 0.05 U/ml thrombin, that alone has no effect, 2-MeS-ADP evokes ERK2 activation in a concentration-dependent fashion (Fig. 11). This amplifying effect is also valid for ERK2 activation induced by 0.1 U/ml

thrombin, but becomes less prominent at a concentration of 0.3 U/ml. This finding suggests that the amount of endogenous ADP released at higher concentrations of thrombin is sufficient to support maximal ERK2 activation.
Thus far, the data provide evidence that ERK2 activation appears not to be simply the consequence of cooperative Gq- and Gi-signalling, but that the quality and/or intensity of the initial Gq-signal critically determines the amplifying capacity of the subsequent Gi-signalling.

6.2.2 P2Y12 ADP receptor coupling to Gi can be mimicked by stimulating α_{2A}-adrenoceptors with epinephrine

To further focus on the significance of Gi signalling, platelets were stimulated with epinephrine (adrenaline). On platelets epinephrine acts mainly via α_{2A}-adrenergic receptors coupling to an inhibitory G protein that have been identified as Gz (Yang *et al.* 2000). In isolated human platelets epinephrine potentiates platelet activation but is not an agonist by itself (Lanza *et al.* 1988, Steen *et al.* 1993). Along this line it has been demonstrated that epinephrine is able to mimic and amplify ADP-induced activation of washed human platelets from volunteers after ticlopidine administration (Gachet *et al.* 1990). In addition, platelets isolated from epinephrine "nonresponders" also showed an impaired response to ADP, strongly suggesting that both α_{2A}-adrenoceptors and P2Y12 purinoceptors share intracellular pathways (Nakahashi *et al.* 2001).
As expected, epinephrine alone has neither any effect on ERK2 activation (Fig. 12 A and B) nor on platelet aggregation (data not shown).
By contrast, in combination with low concentrations of thrombin (0.05 U/ml), that alone has no effect, epinephrine is able to evoke ERK2 activation. The enhancing effect of epinephrine is attenuated at higher concentrations of thrombin (Fig. 13). Furthermore, epinephrine is able to abrogate the inhibitory effect of AR-C69931MX on ERK2 activation in response to thrombin and U46619 (Figs. 12 A and B) as well as on platelet aggregation (Fig. 14).
Regarding thrombin-induced ERK2 activation, as well as platelet aggregation, the data demonstrate that P2Y12 ADP receptor coupling to Gi can be mimicked by stimulating α_{2A}-adrenoceptors with epinephrine.

Likewise, it has been previously reported that epinephrine is able replace ADP in terms of platelet PI 3-K activation (Selheim *et al.* 2000) and PI 3-K-dependent integrin $\alpha_{IIb}\beta_3$ activation (Kauffenstein *et al.* 2001).
That the effect of epinephrine on ERK2 activation is mediated by inhibitory Gz-coupled α-adrenergic receptors has been positively tested by challenging AR-C69931MX-pretreated and thrombin-stimulated platelets with isoproterenol (10 µM). Isoproterenol selectively stimulates platelet β_2-adrenergic Gs-coupled receptors. In contrast to epinephrine, isoproterenol is not able to recover ERK2 activation or platelet aggregation (data not shown). In addition, and as described previously, isoproterenol attenuates thrombin-induced platelet aggregation due to an increase of cAMP (Mills *et al.* 1971, Kerry *et al.* 1983).

As AR-C69931MX potently inhibits thrombin-induced platelet aggregation (Fig. 14, Fälker *et al.* 2005) one might argue that the attenuation of ERK2 activation is simply due to the inhibition of platelet aggregation. Under conditions of thrombin-induced platelet activation with precluding aggregation by preventing fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ with the tetrapeptide RGDS or the non-petidergic S1197, the activity of ERK2 is markedly enhanced (Figs. 15 and 16, discussed below in 6.3). Therefore, the inhibitory effect of AR-C69931MX on thrombin-stimulated ERK2 activation cannot be due to indirect actions of the antagonist on integrin $\alpha_{IIb}\beta_3$ outside-in signalling, which itself is inhibitory to ERK2 activation.

Taken together, the observation that neither ADP nor epinephrine alone activates ERK2 but both are able to amplify the response to thrombin and the TXA_2-mimetic U46619 indicate that Gi-coupling alone is not sufficient to provoke ERK2 activation; a quantitatively-regulated cooperation of initial Gq as well as subsequent Gi signalling is required.
Furthermore, the findings support the suggestion that the amplifying effects of Gi signalling are in the first instance determined by the intensity of the initial Gq signal.

6.3 ERK2 activity is regulated by integrin $\alpha_{IIb}\beta_3$ outside-in signalling

In several cell types such as fibroblasts, endothelial cells, and osteoblasts integrin-mediated signalling causes the activation of the ERK cascade (Chen Q. *et al.* 1994, Ishida *et al.* 1996, Schmidt *et al.* 1998).

In contrast, and most noteworthy, by preventing fibrinogen binding to platelet integrin $\alpha_{IIb}\beta_3$ with the tetrapeptide RGDS or by the use of platelets from patients with Glanzmann's thrombasthenia (which lack functional integrin $\alpha_{IIb}\beta_3$ complexes) it has been demonstrated that integrin $\alpha_{IIb}\beta_3$ outside-in signalling down-regulates ERK2 activity (Nadal *et al.* 1997, Bugaud *et al.* 1999). This negative regulation has further been reported to be mediated via an unknown cytosolic serin/threonine phosphatase by selective dephosphorylation of Thr-183, whereas the regulation of Tyr-185 phosphorylation does not engage integrin $\alpha_{IIb}\beta_3$ signalling (Pawlowski *et al.* 2002).

Several antagonists of the integrin $\alpha_{IIb}\beta_3$, such as the RGDS peptide, couple to the fibrinogen-binding motif (RGD) within the integrin α-subunit and, although precluding platelet aggregation, exert intrinsic properties and are therefore able to induce outside-in signalling (Du *et al.* 1991).

Besides RGDS, the non-peptide RGD mimetic S1197 has been used (Stilz *et al.* 2001). Due to its chemical composition, this compound is considered not to bear intrinsic features. S1197, similar to RGDS, markedly enhances ERK2 activation in response to thrombin (Fig. 15), supporting and broaden the above mentioned reports (Nadal *et al.* 1997, Bugaud *et al.* 1999).

The time-course of thrombin-induced ERK2 activity in S1197-pretreated platelets (Fig. 16) reveals that ERK2 deactivation is inhibited and delayed but not entirely abrogated, suggesting that besides integrin $\alpha_{IIb}\beta_3$ outside-in signalling other pathways may mediate ERK2 dephosphorylation.

6.4 Gi β/γ-subunit-induced phosphoinositide 3-K activity mediates ERK2 activation downstream of the P2Y12 ADP receptor

It is well established that P2Y12 receptor Gi_2-coupling affects adenylyl cyclase via Gi α-subunits and induces activation of PI 3-K isoforms via Gi β/γ-subunits (Dorsam *et al.* 2004, Hechler *et al.* 2005). The precise mechanisms for the Gi-mediated potentiation of platelet responses, however, are yet not fully understood.

Neither ADP nor epinephrine, in contrast to their ability to inhibit PGI_2-induced adenylyl cyclase activity, have been found to cause a decrease in basal cAMP levels (Savi *et al.* 1996, Yang *et al.* 2002).

The adenosine analogue SQ22536 potently blocks adenylyl cyclase activity in intact human platelets (Haslam *et al.* 1978a). The direct inhibition of adenylyl cyclase by SQ22536 or 2', 5'-dideoxyadenosine exerts pro-aggregatory effects only when cAMP levels were pre-elevated by PGI_2, but does neither induce platelet aggregation on its own nor potentiates agonist-induced platelet responses (Haslam *et al.* 1978b). In addition, the negative regulation of adenylyl cyclase alone has been reported not to be able to replace neither P2Y12 receptor nor α_{2A}-adrenoceptor signalling (Savi *et al.* 1996, Yang *et al.* 2002, Daniel *et al.* 1999). The inhibition of adenylyl cyclase, as such, is not considered to be directly involved in platelet activation but to be required to counteract anti-aggregatory effects of mediators causing elevation of cAMP.

P2Y12 receptor-mediated activation of PI 3-K, on the other hand, induces and/or potentiates the activation of Rap1B (Lova *et al.* 2003), Akt (Kim *et al.* 2004), as well as secretion of dense granule contents (Dangelmaier *et al.* 2001), activation of integrin $\alpha_{IIa}\beta_3$ (Kauffenstein *et al.* 2001) and finally platelet aggregation (Trumel *et al.* 1999).

Regarding the negative regulation of adenylyl cyclase we have just recently been able to demonstrate that SQ22536, at a concentration that maximally blocks forskolin-induced adenyly cyclase (Daniel *et al.* 1999), is able to abrogate inhibitory effects of AR-C69931MX on thrombin-induced tyrosine phosphorylation of unidentified proteins with apparent molecular masses of 27 and 31 kDa (Fälker *et al.* 2005).

However, SQ22536 has no effect on ERK2 activation on its own nor circumvents the inhibitory effect of AR-C69931MX on thrombin-provoked ERK2 activation (Fig. 18).

On the other hand, the two structurally unrelated PI 3-K inhibitors LY294002 and wortmannin prevent thrombin-induced ERK2 activation in a concentration-dependent fashion (Fig. 19). These data contradict previous findings by Nadal-Wollbold *et al.* (2002) negotiating an involvement of PI 3-K in ERK2 activation in response to thrombin. Within their studies the authors used lower concentrations of LY294002 (25 µM) and wortmannin (50 nM) and stimulated with 1 U/ml thrombin. Especially the use of the high concentration of thrombin might lead to the misinterpretation of their findings, as under these conditions the enhancing effect of released ADP and subsequent P2Y12 receptor signalling becomes insignificant (compare chapter 6.1.1 and Fig. 6).

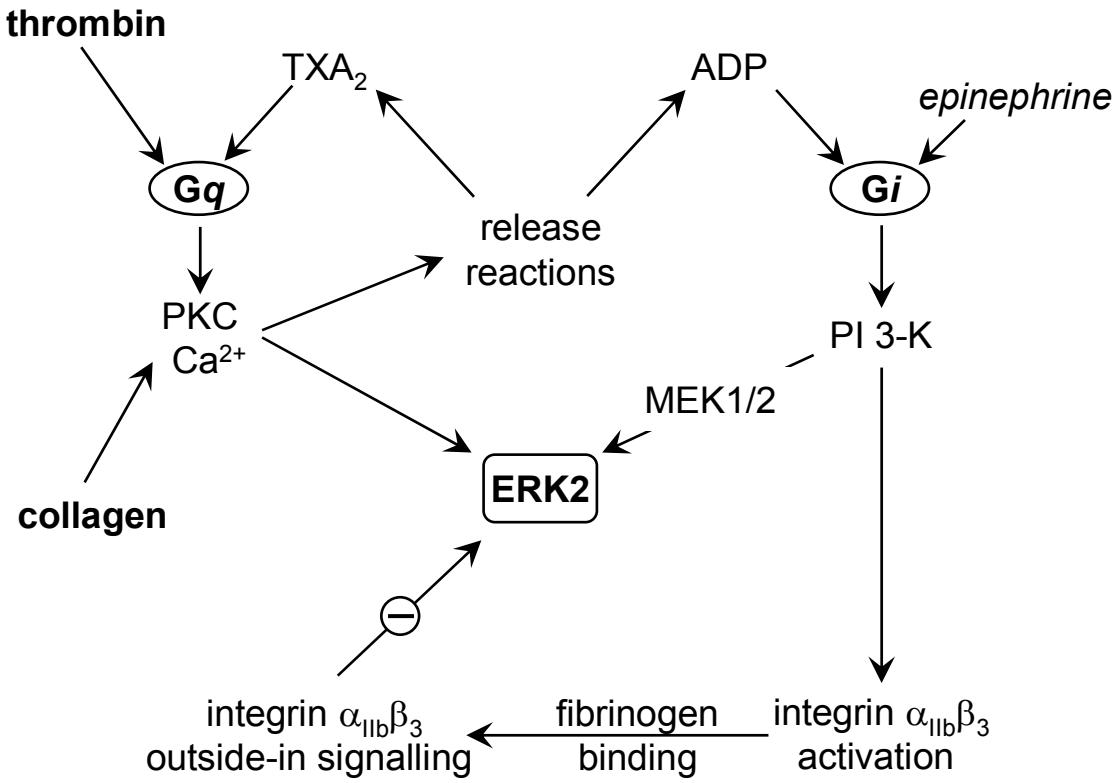
In support of our observations, Soulet *et al.* (2004) demonstrated in chinese hamster ovary cells stably expressing the P2Y12 receptor, although an artificially created system, that ERK2 is activated by 2-MeS-ADP via P2Y12 receptor signalling in a PI 3-K-dependent manner.

As integrin $\alpha_{IIb}\beta_3$, that upon fibrinogen binding negatively affects ERK2 activation, is likewise activated by P2Y12 receptor-mediated PI 3-K activation, a key role for PI 3-K for the regulation of ERK2 activity can be proposed.

As mentioned in the Introduction section, ERK2 becomes activated by the highly substrate- and dual-specific ERK upstream kinases MEK1 and 2.

In this study, the two pharmacologically unrelated MEK1/2 inhibitors U0126 and PD98059 were applied. As expected and previously shown, both inhibitors prevent thrombin-induced ERK2 activation in concentration-dependent fashions (Fig. 20, Börsch-Haubold *et al.* 1996, Rosado *et al.* 2001). Whereas U0126 at 3 µM almost completely prevents ERK2 activation in response to 0.1 U/ml thrombin, a concentration of 10 µM of PD98059 is required to receive a comparable effect.

On the level of the cooperation of G protein-mediated signalling the following simplified scheme summarizes the basic mechanisms involved in platelet ERK2 activation and regulation in response to the primary platelet agonists thrombin and collagen:



In brief, thrombin initiates ERK2 activation as well as the release of TXA_2 and ADP via Gq signalling, which in turn is synergized by TXA_2. Released ADP accomplishes ERK2 activation by PY212 receptor mediated Gi signalling. Collagen-provoked ERK2 activation also requires Gq- and Gi-coupling which are achieved by released TXA_2 and ADP, respectively. PKC and/or calcium trigger ERK2 activation as well as the release of the platelet-derived secondary mediators TXA_2 and ADP in response to both thrombin and collagen. Gi-coupling, that alone is insufficient to evoke ERK2 activation, can be mimicked by non-platelet-derived epinephrine. Gi-amplified ERK2 activation is mediated by PI 3-K and MEK1/2. PI 3-K activation by Gi-signalling is also a prerequisite for integrin $\alpha_{IIb}\beta_3$ outside-in signalling that down-regulates ERK2 activity, implying a key role for PI 3-K in the regulation of ERK2 activity.

6.5 Protein kinase B (Akt) is not a downstream target of ERK2

The serin/threonine kinase Akt, also designated protein kinase B (PKB), becomes activated in platelets stimulated with thrombin, the TXA_2-mimetic U46619, and collagen (Kroner *et al.* 2000, Cho *et al.* 2002, Barry *et al.* 2002).

Human platelets contain two isoforms of Akt, which are Akt1 (PKBα) and Akt2 (PKBβ) (Kroner *et al.* 2000). For full Akt activity, besides its translocation to the cell membrane, the independent phosphorylation of both Thr-308 and Ser-473 is required. Akt activation is prevented by PI 3-K inhibitors (Burgering *et al.* 1995) and in thrombin-stimulated platelets dual-phosphorylation of Akt strongly relies on P2Y12 receptor signalling (Kim *et al.* 2004). Whereas the phosphatidylinositol-dependent kinase 1 (PDK1) has been demonstrated to phosphorylate Thr-308 (Stokoe *et al.* 1997), the kinase that phosphorylates Ser-473 is not yet identified. Nevertheless, due to its function it has been designated PDK2.

As thrombin-induced Akt Ser-473 phosphorylation lags behind ERK2 activation (Figs. 21 and 22, Kim *et al.* 2004) an involvement of ERK2 in Akt Ser-473 has been investigated. As mentioned above and shown in Fig. 19, the PI 3-K inhibitors LY294002 and wortmannin both effectively decreases and abrogates ERK2 activation as well as, and even more potently, Akt Ser-473 phosphorylation (Fig. 25).

On the other hand, the MEK1/2 inhibitors U0126 and PD98059, both at a concentration of 10 µM that entirely inhibit thrombin-induced ERK2 activation (Fig. 20), have no effect on Akt Ser-473 phosphorylation (Fig. 23).

These findings indicate that ERK2 and Akt are activated by different signalling pathways diverging downstream of P2Y12 receptor-mediated PI 3-K activation.

Interestingly, whereas thrombin-induced Akt phosphorylation at Ser-473 is already inhibited by LY294002 and wortmannin when applied at 10 and 0.1 µM, respectively, higher inhibitor-concentrations are obviously required for ERK2 inhibition (Figs. 22 and 19). As such, different PI 3-K isoforms might be involved in P2Y12 receptor signalling. Platelets express three of the four cell surface receptor-activated “class I” isoforms, namely PI 3-Kα, PI 3-Kβ, and PI 3-Kγ (Jackson *et al.* 2004). Akt phosphorylation in response to thrombin, U46619, and ADP has been reported to be impaired in PI 3-Kγ-deficient mouse platelets

(Hirsch *et al.* 2001, Li *et al.* 2003). On the other hand, the P2Y12 receptor-dependent and PI 3K-mediated activation of the small guanosine tri-phosphatase Rap1B is not affected in mouse platelets lacking PI 3-Kγ, indicating that a different isoforms of PI 3-K is involved (Woulfe *et al.* 2002, Lova *et al.* 2002, Lova *et al.* 2003).

6.6 ERK2 is neither involved in α- and dense granule secretion nor in integrin $\alpha_{IIb}\beta_3$ activation or platelet aggregation

The relevance of ERK2 activation in non-nucleated platelets is not yet clear. Whether ERK2 participates in platelet activation and aggregation is controversially discussed.

Therefore, the consequence of the preclusion of ERK2 activation with MEK1/2 inhibitors on primary platelet responses such as secretion of α- and dense granules as well as integrin $\alpha_{IIb}\beta_3$ and final platelet aggregation has been investigated. Pretreatment with U0126 or PD98059, both at 10 µM which completely blocks ERK2 activation (Fig. 20), slightly but significantly attenuates dense granule secretion but does neither affect α-granule secretion, nor integrin $\alpha_{IIb}\beta_3$ activation, or platelet aggregation (compare “Results” section, chapters 5.6.1 and 5.6.2). In addition, all the examined events associated with platelet activation and aggregation are in most part completed prior to the onset of ERK2 activation. Finally, low concentrations of the examined agonists such as 0.05 U/ml thrombin, 1 µM U46619, or 1 µg/ml collagen, which do not or only marginally induce ERK2 activation, were sufficient to cause aggregation in most platelet preparations. These findings support previous reports suggesting that ERK2 activity is dissociated from primary platelet activation and aggregation (Börsch-Haubold *et al.* 1996, McNicol *et al.* 2003).

In contrast, it has been reported by Börsch-Haubold *et al.* (1998) that inhibitors of the MAP/ERK-activating kinases MEK1/2 affect platelet aggregation induced by low concentrations of collagen. Similar observation were made by Li *et al.* (2001) in platelets stimulated with low concentrations of thrombin and the authors further even proposed a role for ERK2 in integrin $\alpha_{IIb}\beta_3$ activation.

However, extensive studies, in particular from Phillip Cohen's group, determining the specificity of various kinase inhibitors suggest that most, if not all inhibitors available affect one or more enzymes besides their specific or actual targets (Davies *et al.* 2000, Bain *et al.* 2003). Hence, data obtained applying such inhibitors should be interpreted with caution and the use of various, structurally and mechanistically unrelated inhibitors is strongly recommended.

Along this line, U0126, besides its ability to inhibit ERK2 activation, seems to affect a protein kinase C-dependent but ERK2-unrelated pathway (McNicol *et al.* 2003), and therefore may interfere with ADP secretion induced via Gq-coupled receptors. This might explain the observed attenuation of thrombin-induced dense granule secretion by U0126 as well as the by Li *et al.* (2001) reported inhibition of thrombin-induced platelet aggregation and proposed engagement of ERK2 in integrin $\alpha_{IIb}\beta_3$ activation.

PD98059 has been demonstrated to directly inhibit cyclooxygenase-1 and -2 (Börsch-Haubold *et al.* 1998) and, as such, interferes with TXA_2 synthesis that is essentially required for platelet activation and aggregation in response to moderate concentrations of collagen (for review see Nieswandt *et al.* 2003) and synergizes with thrombin in ERK2 activation (Fig. 10).

Although P2Y12 receptor signalling is involved in platelet activation and aggregation in response to thrombin, collagen, and TXA_2, the presented data further indicate that ERK2 activation is dissociated from these events.

Whereas p38 MAPK isoforms have been implicated in platelet activation and aggregation in response to thrombin, collagen, TXA_2 mimetics, and calcium ionophores (Kramer *et al.* 1995, Saklatvala *et al.* 1996), still less in known about the role(s) and substrate(s) of ERK1 and ERK2.

The ERK pathway has recently been demonstrated the be involved in the activation of store-mediated calcium entry and, therefore, platelet calcium homeostasis (Rosado *et al.* 2001, 2002), representing the first functional implication for ERK in human platelets.

It has further been reported that ERK2 translocates into the cytoskeleton of activated platelets and been suggested that ERK2 might play a role in cytoskeletal rearrangement or that the cytoskeleton serves as a frame to align ERK2 with its substrates (McNicol *et al.* 2001). Recently, more detailed studies

by Pawlowski *et al.* (2002) demonstrated that only small amounts of ERK2 (about 14%), and mostly in a mono-phosphorylated and therefore inactive form, translocates to the cytoskeleton whereas the dual-phosphorylated active form was found in the cytosolic fraction.

Taken together, the present work clearly demonstrates that ERK2 is a target molecule markedly affected by the P2Y12 ADP receptor-induced signalling pathway.
In addition, it has been elucidated that the quantitatively fine-tuned cooperation of primary signalling through Gq, which evokes ADP secretion, as well as subsequent coupling via Gi by the P2Y12 ADP receptor are required for ERK2 activation in human platelets.

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8 SUMMARY

The study elucidates the signalling pathways involved in the activation and regulation of the extracellular signal-regulated kinase 2 (ERK2) in human platelets.

ERK2 activation in response to primary platelet agonists such as thrombin and collagen strongly, albeit not exclusively, relies on the release and subsequent action of the platelet-derived secondary mediators thromboxane A_2 (TXA_2) and adenosine 5'-diphosphate (ADP). The cooperation of Gq-signalling, induced by thrombin or TXA_2, and Gi-signalling by the P2Y12 ADP receptor is required.

Both Gq and Gi are indispensable for ERK2 activation. The intensity of one pathway limits the effect of the other. The quality and strength of the Gq signal determines the release of ADP and, in turn, the amplifying potency of P2Y12 receptor-mediated Gi signalling. Gi signalling alone is not sufficient to evoke ERK2 activation. On the other hand, subthreshold Gq signalling input is amplified by Gi signalling when induced by exogenously added 2-MeS-ADP or epinephrine.

As such, platelet ERK2 activation is achieved by a fine-tuned and well-balanced synergism of Gq and Gi.

Gq- and Gi-induced pathways are likewise required for integrin $\alpha_{IIb}\beta_3$ activation.

In platelets, fibrinogen binding and subsequent integrin $\alpha_{IIb}\beta_3$ outside-in signalling down-regulates ERK2 activity. Phosphoinositide 3-kinase (PI 3-K) mediates Gi-induced activation of ERK2 as well as of integrin $\alpha_{IIb}\beta_3$, implying a regulatory key role of PI 3-K in ERK2 activity.

ERK2 net activity is determined by Gq/Gi-induced ERK2 phosphorylation and integrin $\alpha_{IIb}\beta_3$-dependent dephosphorylation.

Platelet responses such as the release of α- and dense granules, activation of integrin $\alpha_{IIb}\beta_3$, as well as platelet aggregation are dissociated from ERK2 activation.

In summary, the present work demonstrates that platelet ERK2 activation is precisely regulated by cooperation of Gq- and Gi-mediated signalling pathways but is not implicated in granules secretion and platelet aggregation.

Hiermit versichere ich, daß ich die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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10 PUBLICATIONS

Original articles

Fälker K, Lange D, Presek P. P2Y12 ADP receptor-dependent tyrosine phosphorylation of proteins of 27 and 31 kDa in thrombin-stimulated human platelets. Thromb Haemost 2005; 93:880-8

Fälker K, Lange D, Presek P. ADP secretion and subsequent P2Y12 receptor signalling play a crucial role in thrombin-induced ERK2 activation in human platelets. Thromb Haemost 2004; 92:114-23

Hanke S, Valkova C, Stirnweiß J, Drube S, **Fälker K**, Presek P, Liebmann C. Epidermal growth factor-induced tyrosine phosphorylation of bradykinin B2 receptor regulates its coupling to Gi proteins. under revision

Aktas B, **Fälker K**, Sapper M, Presek P, Geiger J, Walter U. P2Y12 Receptor Stimulates Phospholipase D during Secretion in Human Platelets. under revision

Oral presentations

Fälker K, Lange D, Presek P. P2Y12 ADP receptor-mediated negative regulation of adenyly cyclase affects tyrosine phosphorylation of 27 and 31 kDa proteins in thrombin-stimulated human platelets. presented at the XXth European Platelet Meeting 2005, Ede, The Netherlands, abstract to be published in Platelets 2006

Fälker K, Lange D, Presek P. Signalling pathways and functional implications mediated by the P2Y12 ADP receptor upon thrombin-stimulation in human platelets. presented at the XXI. Bilateralen Symposium Poznan-Halle „COR ET VASA II“ 2005; Poznan, Poland

Fälker K, Lange D, Presek P. ERK2 activation through cooperation of Gq and Gi signalling in human platelets. presented at the 10th Erfurt Conference on Platelets 2004, Erfurt; Germany, abstract in Platelets 2004; 15(8)

Fälker K, Lange D, Presek P. P2Y12-signalling affects ERK2 activation in thrombin-stimulated human platelets. presented at the XVIIIth European Platelet Meeting 2003, Romrodt, Germany, abstract in Platelets 2004; 15(3)

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Poster presentations

Fälker K, Lange D, Frühauf A, Wolf HH, Presek P. What is the role of ERK2 MAP kinase in human platelets? presented at the XIXth European Platelet Meeting 2004, Bad Brückenau, Germany, abstract in Platelets 2005; 16(5)

Fälker K, Lange D, Marx-Grunwitz A, Martinson EA, Presek P. ADP receptor signalling, phospholipase D regulation, and protein tyrosine phosphorylation in human platelets. presented at the 7th International Dahlem Symposium "Cellular Signal Recognition and Transduction" 2001, Berlin-Dahlem, Germany

Presek P, **Fälker K**, Haas MA, Marx-Grunwitz A, Martinson EA. Regulation of phospholipase D in human platelets. presented at the XVth European Platelet Meeting 2000, Le Bischenberg (Alsace), France; abstract in Platelets 2001; 1(1)

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Haas MA, **Fälker K**, Martinson EA, Presek P. Regulation of phospholipase D in human megakaryoblastic cell lines. presented at the XIIIth European Platelet Meeting 1998, Seggau Castle, Austria; abstract in Platelets 1999; 10(4)

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