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NOVEL AGONISTS AND ANTAGONISTS OF
PLATELET RECEPTORS



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2011

Cover illustration: Docking of a farnesyl pyrophosphate (FPP) molecule (yellow and red) in the P2Y₁₂-receptors ligand binding site.

Lund University, Faculty of Medicine

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*Yesterday is history. Tomorrow is a mystery. Today is a gift.
That's why it is called the present.*

-Master Oogway

*This thesis is dedicated to Karin, my
loving wife.*

Abstract

Platelets are central for the primary haemostasis. The platelet has surface bound receptors that are vital for the platelet activation. One of these receptors, the purinergic ADP-activated G-protein coupled receptor (GPCR) P2Y₁₂ is a key receptor for platelet activation, and thus for thrombus formation, which makes P2Y₁₂ a target in pharmaceutical antiplatelet therapies. As a complement during revascularization surgery, mild hypothermia has been accepted, but its effect on platelet activation is unclear. We performed a comparative *ex vivo* study on platelets, treated with mild hypothermia and normothermia in the presence of P2Y₁₂ inhibitors. We found that mild hypothermia increased platelet P2Y₁₂ activation and that the P2Y₁₂ inhibitor, clopidogrel, lost in efficiency. The P2Y₁₂ receptor is also present on vascular smooth muscle cells (VSMC), promoting contractions. We initiated a comparative study on human and murine artery tissue to evaluate if any of the antiplatelet substances, clopidogrel and ticagrelor (AZD6140) a reversibly non-prodrug P2Y₁₂ inhibitor, could inhibit P2Y₁₂ signaling also on VSMC. The results showed that ticagrelor was able to inhibit P2Y₁₂ stimulated vasoconstriction, while clopidogrel had no effect. The succinate GPCR (SUCNR1) mRNA has been found at high levels in platelets. We performed a study on SUCNR1 to investigate its function in platelets. The study showed that the succinate receptor was expressed as a membrane bound G α i-protein. Succinate *per se* could mediate platelet activation. It was dependent on P2Y₁₂ activation, thromboxane A₂ release and PI3K β signaling. By screening intermediates in the mevalonate pathway, i.e. the cholesterol synthesis, we found that one of them, farnesyl pyrophosphate (FPP), had an antagonistic effect on ADP induced platelet activation. Further investigation on FPP with [³⁵S]GTP γ S binding and docking studies confirmed that FPP is an endogenous antagonist on the platelet P2Y₁₂ receptor.

Keywords: Platelets, P2Y₁₂, SUCNR1, hypothermia, FPP

List of publications

This thesis is based on four studies, referred to in the text by their Roman numerals. Papers are appended at the end of the thesis.

- I.** Carl Högberg, David Erlinge and Oscar Ö Braun
Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment
Thrombosis Journal 2009; 7:2

- II.** Carl Högberg, Helen Svensson, Ronny Gustafsson; Atli Eyjolfsson, and David Erlinge
The reversible oral P2Y₁₂ antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature
International Journal of Cardiology 2010, vol 142 pp 187-192

- III.** Carl Högberg, Olof Gidlöf, Chanyuan Tan, Siv Svensson, Jenny Nilsson-Öhman, David Erlinge and Björn Olde
Succinate independently stimulates full platelet activation via cAMP and PI3β kinase signaling
Journal of Thrombosis and Haemostasis (in press)

- IV.** Carl Högberg, Olof Gidlöf, Francesca Deflorian, Kenneth A. Jacobson, Björn Olde and David Erlinge
Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y₁₂ receptor mediated platelet aggregation
(*Manuscript*)

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Abbreviations

ADP	adenosine 5'-diphosphate
2-MeSADP	2'-methylsulfa adenosine 5'-diphosphate
ASA	ascorbic acid
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-monophosphate
CHO	chinese hamster ovary cells
COX-1	cyclooxygenase-1
DAG	diacylglycerol
FPP	farnesyl pyrophosphate
GDP	guanine 5'-diphosphate
GPIIb/IIIa	glycoprotein IIb/IIIa
GPCR	G-protein coupled receptor
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
IP₃	inositol 1', 4', 5'-triphosphate
LTA	light transmission aggregometry
MI	myocardial infarction
NE	norepinephrine
NO	nitric oxide
OCS	open canalicular system
PAC-1	procaspase activating compound-1
PCI	percutaneous coronary intervention
PDGF	platelet derived growth factor
PGI₂	prostacyclin I₂
PIP₂	phosphatidylinositol 4', 5'-phosphate
PLC	phospholipase C
PPP	platelet poor plasma
PRP	platelet rich plasma
P-selectin	platelet selectin
5-TH	serotonin
TXA₂	thromboxane A₂
VASP	vasodilator-stimulated phosphoprotein
VSMC	vascular smooth muscle cells
vWf	von Willebrand factor

Introduction

Cardiovascular disease is the main cause of death in the developed world [1]. Myocardial infarction and stroke are caused by haemostatic complications, in which the blood platelet has an essential role [2, 3]. Atherosclerosis is a chronic long-term disease which originates during late childhood and progresses all throughout life [4]. It is caused by a number of synergistically acting variables that effectively promotes the accumulation of inflammatory atherosclerotic plaque (atheromas). The two most important factors are lifestyle (physiological and psychosocial) and genetic factors. Variables related to lifestyle are smoking, physical inactivity and high intake of calorie rich food (fat and carbohydrates) leading to diabetes mellitus and the metabolic syndrome [5]. Factors related to the psychosocial negative lifestyle are stress and severe forms of psychosocial illness, i.e. depression [6]. Other inherited important risk factors are: gender, genetic polymorphisms, type 1-diabetes, hypertension and age [7].

Atherosclerosis, initiation and pathophysiology

Atheromas are initiated by migration of low density lipids (LDL), through the endothelial cell (EC) barrier into the extracellular matrix (ECM) of the intima [8]. The cholesterol rich LDL-particles collected in the intima, becomes oxidized and initiate inflammation by promoting local release of chemotactic cytokines, particularly macrophage chemoattractant protein -1 (MCP-1). Macrophages, recruited by MCP-1, will accumulate in the intima matrix where they will phagocyte the oxidized LDL-particles [9]. Doing so, the macrophages change their chemical constitution and morphology ending up as so called foam cells. The foam cells are not able to process the oxidized-LDL, and eventually, they will enter apoptosis depositing a greater amount of oxidized cholesterol into the artery wall. The macrophage migration and turnover into foam cells is the origin of the lipid rich core of the atheroma. Platelet recruitment occurs in the parts of the artery where atheromas rupture and the EC layer is damaged [10]. Platelets release mediators, e.g. platelet derived growth factor (PDGF), which promotes migration

of vascular smooth muscle cells (VSMC) into the intima, and also inflammatory ligands, for example CD154, Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) which regulates activation of CD40 which, in turn, promotes expression of pro-atherogenic mediators [11]. Platelet also release Transforming Growth Factor beta (TGF- β) which promotes collagen I production in the ECM [12]. Collagen production in the ECM is stimulated by recruitment of VSMC into the intima [13]. Artherosclerotic plaque can, depending on their ability to promote thrombus formation, be categorized as “stable” or “vulnerable”. If the plaque contains a fibrous cap of VSMC that safely withhold the lipid core from the lumen, the plaque is characterized as a “stable” one. If, on the other hand the plaque has a large lipid core with a poor developed fibrous cap, the plaque is considered to be “vulnerable” [14] (Fig 1).

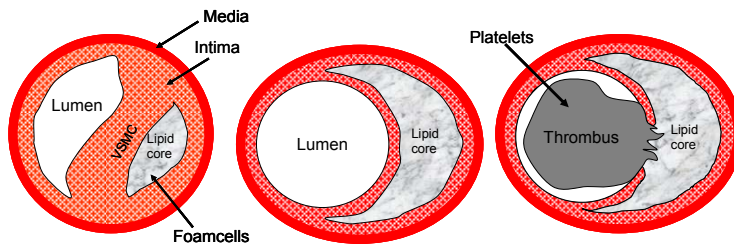


Fig 1. Left: Illustrating a “stable” plaque. Centre: Illustrating a “vulnerable” plaque. Right: Illustrating a vulnerable eruptive plaque which initiate a thrombus formation and thus, vascular occlusion which in turn can onset angina pectoris, stroke or a MI.

If a plaque ruptures, collagen and vWf are exposed and will rapidly adhere and recruit platelets, creating large thrombi. The thrombi can initiate a massive coronary occlusion subsequently leading to a stroke or an acute MI, causing a massive necrosis of the myocardium with a possible mortal outcome [15]. To save a patient with MI the coronary vessels must be revascularized, either pharmacologically through fibrinolysis [16] or mechanical through percutaneous coronary intervention (PCI) [17]. During PCI, the occlusive lesion area in the coronary artery is expanded by balloon angioplasty and stenting. However, the metal stent may stimulate platelet aggregation and cause in-stent thrombosis [18]. Patients receiving a metal stent must take a dual antiplatelet therapy ascorbic acid

(ASA) and an ADP-receptor antagonist [17]. If the patient suffers from multivessel disease, Coronary Artery Bypass Graft (CABG) surgery can be performed where an artery or a vein is used to bypass the stenotic area [17].

Pharmacological approaches to inhibit primary and secondary haemostasis

Patient suffering from coronary heart disease are treated with several medications to prevent progression of the disease and the development of myocardial infarction. The therapy is usually aimed at inhibition of both the primary (platelets) and secondary phase (coagulation) in haemostasis. Platelet inhibition is achieved through direct inhibition of enzymes or receptors, such as the thromboxane A_2 (TXA₂) generating enzyme, (COX-1) using ASA, or the ADP receptor P2Y₁₂, present on the platelet surface. Another target is the glycoprotein (GP) IIb/IIIa integrin also situated on the platelet surface. GPIIb/IIIa is a membrane-protein with a central role in the platelet-platelet aggregation but also in the initiating adhesion stage to vWf in ECM [19]. Common inhibitors of the fibrin generation phase are warfarin, heparin, fractionated heparin (LMWH), thrombin-inhibitors (bivalirudin) or Xa-inhibitors (fondaparinux) [16, 19]. Other useful substances in the treatment of coronary heart disease are Nitroglycerine which promotes dilatation of blood vessels and Beta blocking agents (metoprolol, propranolol) that inhibits β -receptor activity, thus reducing heart rate and blood pressure [20]. Therapies aimed at lowering the cholesterol levels are often used as a way of controlling the development of atherosclerosis. Cholesterol reducing compounds such as simvastatin, atorvastatin and rosuvastatin belong to a group called statins which function by inhibiting the rate limiting enzyme in the mevalonate pathway, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) [21].

Platelet physiology

The platelets or thrombocytes (after the Greek word thrombos - clot and cytos - cell) have a central role in the haemostatic machinery. Platelets main function is to stop vascular hemorrhage, but platelets also contribute by increasing the inflammatory and atherosclerotic process. Once activated, from a resting state, the platelets work in concert with the cross linked fibrin network to repair liaisons in the EC barrier of the vascular wall lumen.

The platelets circulate in the blood plasma, where they are present in high concentrations (150 - 400,000 cells/ μ L in whole blood) [22]. Platelets are created through endocytosis from larger polynuclear cells called megakaryocytes [23] and have a life span of approximately 7-10 days. The megakaryocytes are present in the bone marrow matrix and produce 5,000 to 10,000 platelets per day [23]. Old platelets are removed either by macrophages, through phagocytosis, or by the liver and spleen.

The resting platelet is discoid with the dimension of 3 times 0.5 μ m in width, respectively [22]. The platelet is an anuclear cell, but have retained many cell structures, e.g. plasma membrane, mitochondria, dense tubular system (store of $[Ca^{2+}]_i$), and occasional Golgi elements and ribosomes (Fig 2). Platelets also contain membrane-bound granules that, depending on type, have different composition of active substances. The alpha-granules contain several proteins i.e. growth factors like platelet derived growth factor (PDGF) important for endothelial activation, coagulation proteins like thromboglobulin, fibronectin and fibrinogen. The alpha granules contain adhesion factors, as vWF and GPIIb/IIIa, and they hold platelet specific proteins, e.g. coagulation factor V (which activates thrombin) and CD62P (P-selectin) [24]. P-selectin is an important protein that facilitates platelet-platelet and platelet-leukocyte binding. Platelets also contain delta-granules, sometimes referred to as dense opaque core because of their elevated electron density when observed through an electron microscope. The delta granules holds high concentration of small molecules, for instance ATP, ADP pyrophosphate (P_i), 5-HT and ions like Ca^{2+} and Mg^{2+} [24]. These small molecules are vital for the platelets further activation and recruitment of other platelets.

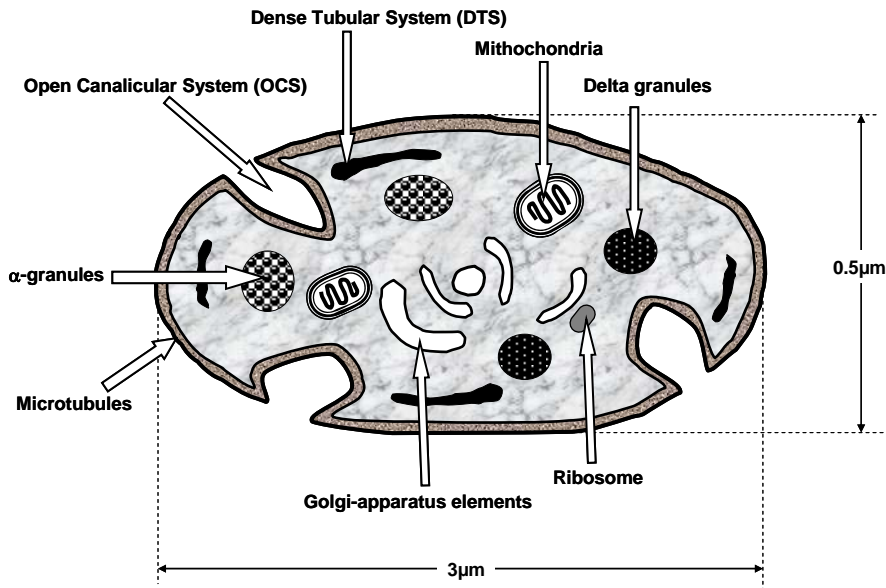


Fig 2. Illustrative figure describing the physiology of a resting platelet.

Initiated platelet activation and subsequent deactivation

Platelet activation is initiated when the disrupted endothelial cell layer exposes collagen and vWf, that is present in the subendothelial intima [25]. Collagen is an important part of the connective tissue and also one of the most expressed proteins in the intima [25]. The platelet activation starts initially by rolling and tethering platelets on the surface of the exposed subendothelial area. The platelet GPIb-IX-V integrin complex connects to exposed collagen-bound vFf. This initial adhesion stage becomes stabilized through $\alpha_2\beta_1$ integrin-collagen interaction and further GPIIb/IIIa-vWf attachment [25, 26]. The stable adhesion enables a conformational shape change of the platelet which irreversibly alters the cell platelet morphology. The components that reorganizes the cytoskeleton, the sub-membrane microtubulin and the cytoplasmic actin filaments, alters the cell structure of the

platelet, and the once discoid shape becomes spherical. The spherical shape then flattens and lamellae begin to stretch out from the inner core of the platelet and the organelles and granules get squeezed into the center of the activated platelet [27]. The alpha and delta granules are fused by exocytosis through an open canalicular system (OCS). Molecules released from the platelet vesicles, ADP, P-selectin and fibrinogen, now interacts with membrane bound receptors on other cell types present in the vascular lumen, e.g. leukocytes, endothelial cells and other platelets. The meshwork of platelets and fibrin grows in the ulcerated plaque, which might end up in forming a thrombus-generated occlusion. These processes are inhibited by the vessel wall endothelium which releases nitric oxide (NO) and prostacyclin I_2 (PGI_2) into the lumen and thereby stop and de-activate the thrombus formation [28] (Fig 3). This suppression of the platelet is simultaneously followed by release of endogenous plasmin. Plasmin is generated from thrombin-induced cleavage of plasminogen into plasmin, which lyses the fibrin cross linked meshwork and disperse the thrombus [29].

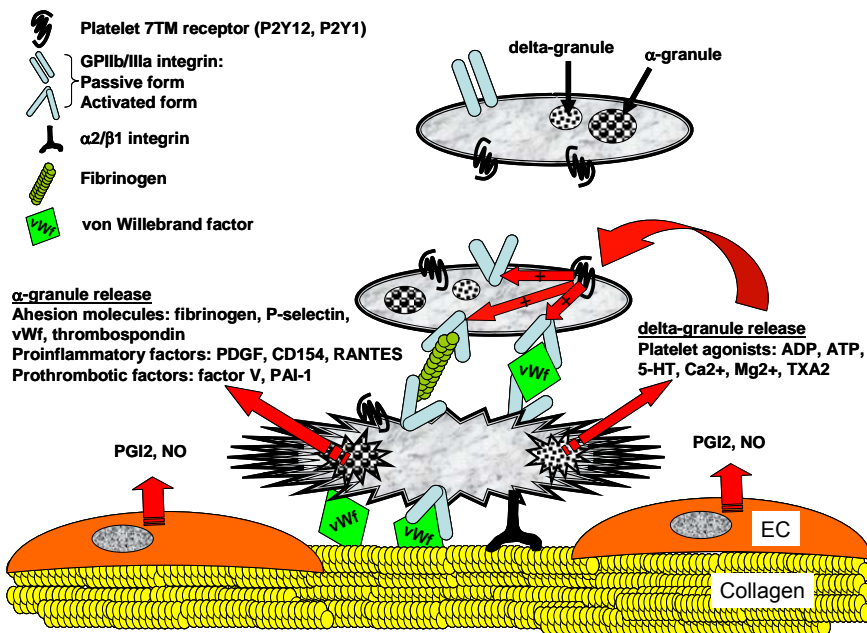


Fig 3. Illustrating platelet activation in artery lumen where a vascular lesion exposes the collagen rich subendothelial matrix. The circulating resting platelets activates by collagen and surface expressed vWf. During total activation the platelets release their granular content which recruits and activates new platelets to the damaged area. The surrounding endothelium releases anti-thrombotic mediators as nitric oxide (NO) and prostacyclin (PGI_2) to inhibit further thrombus formation.

General classification, structure and regulation of purinergic receptors: Ionic channel receptors and G-protein coupled receptors (GPCR) on platelets

The purinergic receptor present on the platelet is either an ionic channel receptor or a G-protein coupled receptor (GPCR). The ionic channel receptor P2X₁ is a transmembrane protein that becomes activated upon ATP binding to its extracellular binding site [30]. The activated receptor protein opens an inward rectifying ionic gate whereby ionic-currents of Ca²⁺ leaks in and promotes an initiation of platelet shape change. The P2X₁ is an oligomer that is built up by three subunits where every subunit contains two transmembrane (TM) α -helices (Fig 4). The purinergic GPCR is also activated by mediators from the extracellular side, initiating an intracellular G-protein coupled signaling cascade. In the platelet, these GPCR activated transduction cascades often lead to massive platelet activation resulting in shape change, aggregation and vesicular release.

A GPCR is also known as a seven transmembrane receptor (7TM). The general structure of a GPCR is characterized by a single protein chain with an extracellular N-terminal followed by 7TM α -helices connected by three extracellular and three intracellular loops and an intracellular C-terminal tail. The tertiary structure of a GPCR resembles a barrel with the 7TM helices surrounding a central cavity forming the ligand binding domain (Fig 4). The intracellular side of the GPCR is normally bound to a heterotrimeric G-protein composed of a G α -GTP subunit and a G $\beta\gamma$ -subunit. Once the GPCR is activated, by binding of an agonist, the receptor shifts conformation thus activating the G-protein causing it to detach from the receptor. The activated G α -subunit releases guanine 5'-diphosphate nucleotide (GDP) from the binding pocket and exchanges it into a guanine 5'-triphosphate nucleotide (GTP). This exchange promotes separation of the G $\beta\gamma$ -subunit from the G α -subunit. Both the G α - and the G $\beta\gamma$ -subunit promote or inhibit enzymatic activity by interacting with key enzymes. Depending on the type of G α -subunit different second messenger systems will be activated. For example, activation of G α_s -subunits will stimulate adenylate cyclase (AC) and cAMP production while G α_i -subunit will inhibit AC, causing a decrease in the cytoplasmic levels of cAMP.

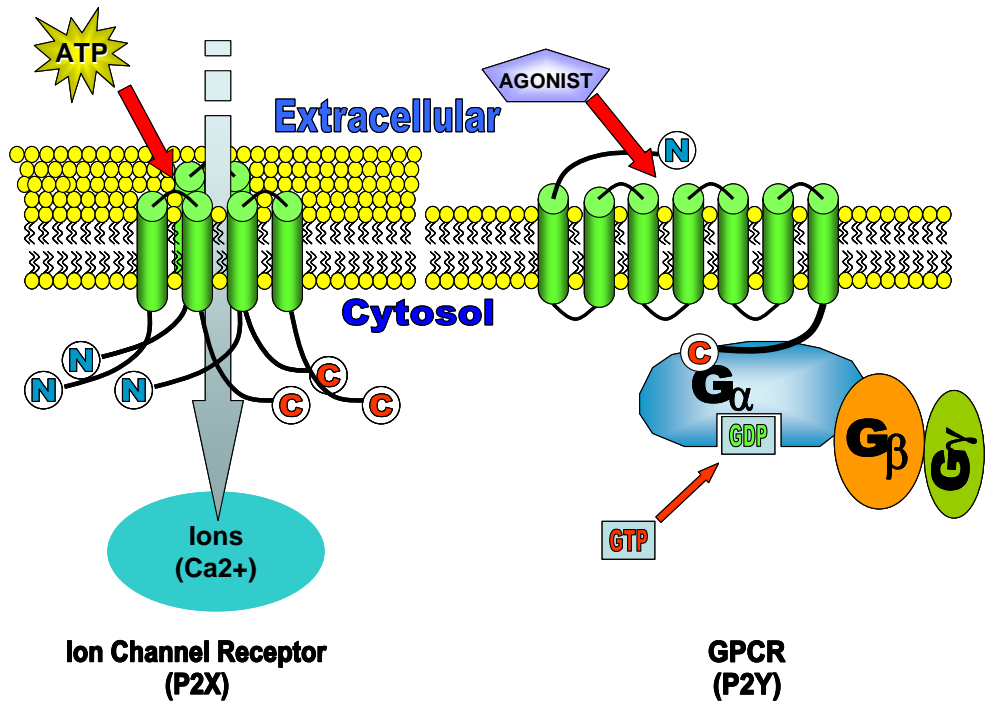


Fig 4. Illustration of structures of a typical ATP activated ion channel receptor like the P2X₁ and a classic GPCR like the P2Y₁ or P2Y₁₂ receptor.

Purinergic platelet GPCR and their agonists

The main purinoreceptors responsible for platelet activation are the P2Y₁ and P2Y₁₂ receptors. The primary agonist for both these receptors is the adenine nucleotide adenosine 5'-diphosphate (ADP) (Fig 7). The P2Y₁ receptor is a Gαq-coupled receptor. Gαq-proteins regulate the activation of the intracellular Phospholipase C (PLC) which hydrolyzes the membranebound phosphatidylinositol 4', 5'-phosphate (PIP₂) into diacylglycerol (DAG) and the second messenger molecule inositol 1', 4', 5'-triphosphate (IP₃) [30]. Released IP₃ activate IP₃ receptors present on the membrane of the calcium stores in the platelets dense tubular system [31, 32] and trigger calcium release. This calcium flux initiate the reorganization of the cytoskeleton and thus the platelet shape change [32]. P2Y₁ is a low affinity receptor and is not so highly expressed on platelets as P2Y₁₂ [33]. The high affinity P2Y₁₂ receptor is a Gαi-coupled receptor which inhibits adenylate cyclase resulting in a reduction of the intracellular signaling messenger cAMP (Fig 7), while the Gβγ-subunit activates an enzyme known as phosphatidylinositol-3-kinase (PI3K). PI3K promotes the phosphorylation and activation of the downstream effector enzyme Protein Kinase B (PKB) also known as Akt1, a protein that has been reported to play a role in optimal activation of GPIIb/IIIa [34].

Synthetic antagonists for the purinergic receptors P2Y₁ and P2Y₁₂

Two of the most frequently used synthetic P2Y₁ antagonists are MRS2179 and MRS2500. These substances are both competitive antagonists that bind reversibly to the active site of the receptor and thus inhibit receptor activation [35, 36] Half maximal inhibiting concentration values (IC₅₀) are 0.23μM [35] and 1nM [36] for MRS2179 and MRS2500, respectively.

A clinically used antagonist for the P2Y₁₂ receptor is clopidogrel (Fig 5). Clopidogrel, distributed as Plavix[®] is together with ASA (ascorbic acid) used as standard antithrombotic treatment in coronary heart disease and as follow up treatment after PCI to reduce post-stent thrombotic complications [37, 38]. Clopidogrel is a prodrug, and needs to be converted by the liver enzyme cytochrome P450 (CYP450) into its functionally active metabolic form [38]. The

active metabolite of clopidogrel is an irreversible ligand that binds covalently to the binding site, blocking the receptor for interaction with native agonists [39]. Clopidogrel resistance, caused by individual differences in the enzymatic conversion capacity of CYP450, has become a serious problem in anti-thrombotic treatment [40, 41]. As much as 50 percent of the patients are non- or poor responders to clopidogrel [41]. Another problem is that the irreversible binding of this compound can complicate acute surgical operations. This has called for the development of new precise, stable antithrombotic drugs with less variability in effect. Amongst them is the substance cyclopentyl-triazolopyrimidine, ticagrelor (AZD6140) (Fig 5). Ticagrelor is not a prodrug and it binds reversibly to the P2Y₁₂ receptor and can also reach high plasma concentrations, which makes it a possible antagonist for P2Y₁₂ receptors present on VSMC, preventing ADP mediated artery contractions [39, 42]. Ticagrelor has also the benefit in fast plasma clearance and thus enables acute interventions in patients. Clinical studies as the PLATelet inhibition and patient Outcome (PLATO) 2010 [43] confirmed the results from the earlier Dose confirmation Study assessing anti Platelet Effects of AZD6140 vs. clopidogrel in non-ST segment Elevation myocardial infarction – 2 (DISPERSE-2) study[44] where the advantages of ticagrelor vs. clopidogrel, as antiplatelet therapy in patients suffering from acute coronary syndromes, were evaluated and confirmed.

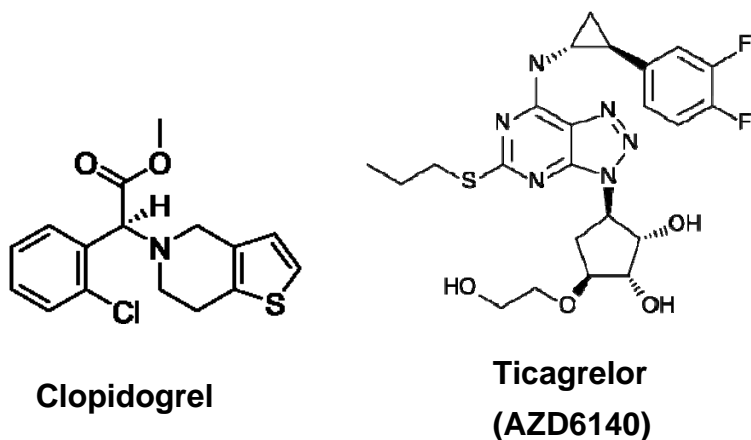


Fig 5. Chemical structures of synthetic P2Y₁₂ receptor antagonists.

Succinate and its receptor SUCNR1

The succinate receptor number 1 (SUCNR1), is a GPCR. SUCNR1 is expressed in most body organs with high expression in liver and kidneys [45]. SUCNR1 is a protein composed of a 330 amino acids. The SUCNR1 is a low affinity receptor needing high agonist concentrations to become activated. The key ligand for the SUCNR1 is succinate [46] (Fig 7). Succinate is a physiological important compound generally known as an intracellular intermediate in the citric acid cycle [47]. Levels of succinate present in the plasma are approximately 20 μ M [48, 49]. During pathophysiological conditions like ischemia and metabolic stress, succinate plasma levels have been reported to increase and reach millimolar concentrations [49-52]. Succinate has also in a recent rapport been identified to be a paracrine signal molecule released upon high levels of glucose, induces SUCNR1-stimulating renin release from kidneys and activates the renin-angiotensin system, thus leading to an increase in blood pressure.[49]. However, succinate has previously been reported to have an potentiating effect in ADP and epinephrine induced platelet activation [53], but the succinate receptor and mechanism of action was unknown until SUCNR1 were characterized on the platelet surface [46].

Farnesyl pyrophosphate (FPP)

The isoprenoid farnesyl pyrophosphate (FPP) (Fig 7) is an important intermediate in the mevalonate pathway (Fig 6). The mevalonate pathway is central for the cholesterol synthesis mainly taking place in the liver and intestines [54]. FPP is identified as a precursor molecule for the synthesis of haem A, ubiquinone (important mediators in the electron transport chain) and dolichol, which functions as a membrane anchor for the formation of oligosaccharides [54]. FPP is further involved in farnesylation of several proteins (e.g., Ras, Rho, Rac) that modulate a variety of cellular processes including signaling, differentiation, and proliferation [55]. In the farnesylation process, Farnesyl Transferase (FTas) catalyzes the transfer of the non-polar 15-carbon backbone of FPP to the protein thus enabling the protein to attach to the hydrophobic membrane structure where it later becomes activated [56]. The small intracellular protein GTPase Ras functions as downstream effectors for gene transcription and rapid cell proliferation. Ras have been characterized as a protein involved in the development of several malign

forms of cancer [55, 56], identify FTAs as target protein in drug development [57]. FPP has lately been assigned a new role, namely as a potential agonist. Free FPP has been reported to function as an agonist for the Lysophosphatic acid GPCR LPA₅ (GPR92) having potentiating effects on LPA-mediated shape and change. This we could confirm in our studies [58]. Recent work has also identified FPP as a potent agonist on the ion channel receptor, the Temperature-sensitive transient receptor (TRPV1) [59].

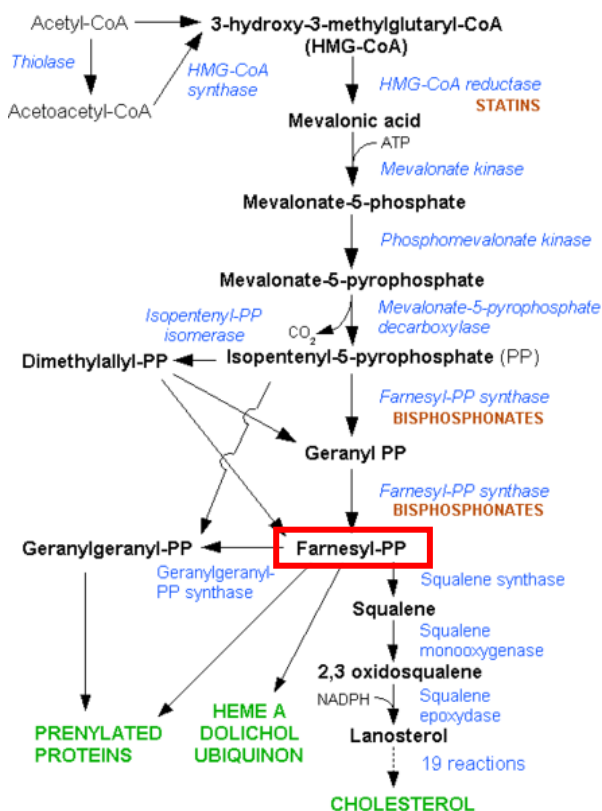


Fig 6. Schematic view over the mevalonate pathway. FPP (in red square) is a central mediator in the mevalonate pathway.

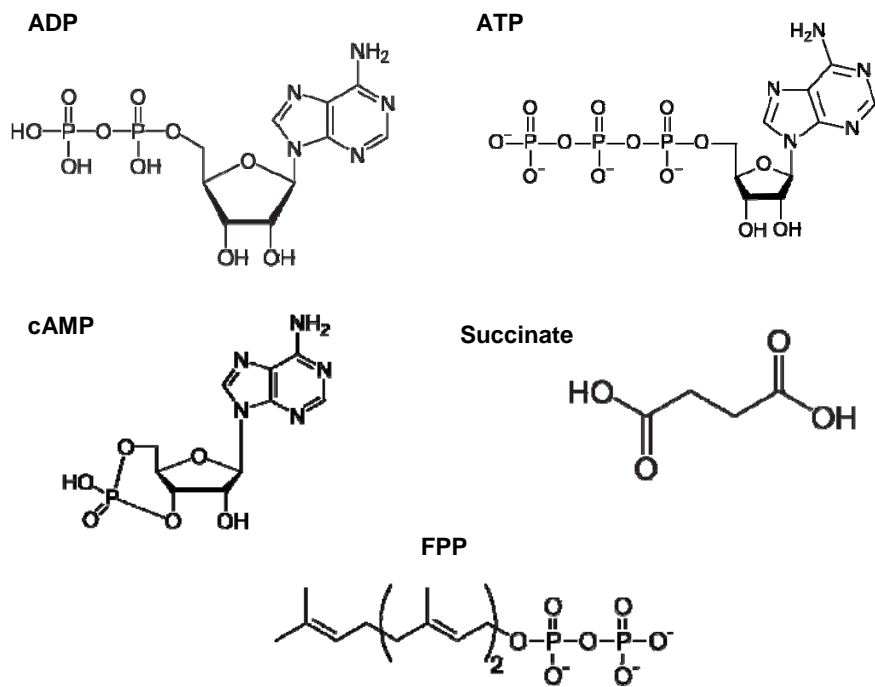


Fig 7. Chemical structures for agonists and secondary messengers.

Aims

The aim of this thesis was to examine different aspects of the platelet activation, except in paper II where focus was on how an important platelet receptor (P2Y₁₂) activates smooth muscle cells in the vascular wall. Furthermore, the thesis concerns different aspects of platelet GPCR activation; with a central aspect on the purinergic ADP activated P2Y₁₂ receptor.

Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment (Paper I)

Mild hypothermia is used as a recommended standard treatment for patients treated for a cardiac arrest [60]. Although several mechanisms probably are involved, the general hypothesis is that by cooling the body core temperature the metabolism becomes depressed whereby damaged tissue recovers easier. Clinical studies have reported beneficial effects of hypothermia with reduced mortality and improved neurological outcome in unconscious patients affected by cardiac arrest [61-64]. Hypothermia could also have effects on the haemostatic system. Coagulation is impaired since the activity of involved coagulation enzymes is reduced. For platelets it has been reported both reduced [65, 66] and increased activity [67-70]. Most patients with cardiac arrest are in need of anti-thrombotic treatment, because myocardial infarction is often the primary cause of the disease. It is therefore important to know how hypothermia affects platelet function and platelet inhibiting drugs.

From this background we initiated a study named “Cool Platelets” This study had its origin from a clinical perspective and aimed to investigate the effect of mild hypothermia on platelet activation *ex vivo*. Moreover, a study was also performed with the P2Y₁₂ blocking agents’ clopidogrel and AZD6140 (ticagrelor) to

investigate if there were any differences in their ability to oppose platelet activation during hypothermic comparing to normothermic conditions.

The reversible oral P2Y₁₂ antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature (Paper II)

The purinergic ADP activated P2Y₁₂ receptor is well studied on platelets where it plays a central part in the platelet activation machinery [71]. The P2Y₁₂ receptor is also identified on VSMC where it mediates contraction upon activation. Paper II aimed to investigate if antiplatelet substances with the P2Y₁₂ receptor as target could inhibit P2Y₁₂ receptor signalling in platelets as well as inhibiting VSMC contractions, having a dual pharmacological therapeutic effect. The study also aimed to compare the two antiplatelet drugs, clopidogrel and ticagrelor, in order to evaluate the therapeutic effect on ADP mediated vascular contractions.

Succinate independently stimulates full platelet activation via cAMP and PI3 β kinase signaling (Paper III)

The succinate receptor SUCNR1 (GPR91), belongs to the gene superfamily of GPCR, and He *et al.* [46] has in an earlier report recognized SUCNR1 as a major target for physiological succinate. Succinate was studied by Huang *et al.* 1984 [53] and was identified as a substance with a potentiating effect for known platelet agonists e.g. ADP. The SUCNR1 is expressed in many organs and tissues, e.g. in the liver, spleen, blood vessels and is most abundant in the kidneys [45]. In an earlier report, aiming at identifying all GPCR (including orphan receptors) on the platelet membrane [33], the succinate receptor was found to be the third highest expressed GPCRs of all on the platelet. With this knowledge about high transcript expression of the succinate receptor in platelets we intended to examine the function of SUCNR1s in platelets. We also aimed to evaluate to which G-protein

SUCNR1 is linked and thus, to study the intracellular pathways that the SUCNR1 stimulates.

Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y₁₂ receptor mediated platelet aggregation (Paper IV)

Paper IV is based on an earlier report aimed to identify all GPCRs on the platelet membrane [33]. The study recognized high expression of one of the GPCR suggested being a receptor for farnesyl pyrophosphate (FPP). FPP is an intermediate substance in the mevalonate pathway. Intermediates in the mevalonate pathway have, in earlier studies, been suggested to be involved also in functions other than the synthesis of cholesterol. These facts captured our interest. In Paper IV we performed a study aimed to evaluate if FPP had any function in platelet activation.

Methods

This chapter is presenting a general description of methods applied throughout the thesis.

Myograph experiments

A myograph is a technical device that measures and record tension from the VSMC in blood vessels. Blood vessels are cut into small segments with a few mm in width. The registered contractile force is measured in Volt, which further can be transformed into Newton - defined as the amount of net force required to accelerate a weight of one kilo (kg) at a rate of one meter per square second (s^2). The vessels are dissected from connective tissue and further cut into vascular segments. Segments are later mounted between two holders with help of a metal thread; where one of the holders are connected to a force – displacement transducer for continuous recording of the isomeric tension. The other segment-holder allows fine adjustments of the vascular resting tension. The segments are held in temperature-controlled (37°C) tissue baths with a physiological Krebs buffer solution, pH at 7.4. The resting force (the baseline) of the vessel segments is fixed after 30 – 45 minutes. After the baseline has been set the vessel segments are stimulated with a potassium rich organic buffer which allows the VSMC to contract and reach its tension peak value. After the segment peak value is registered in the myograph the resting tension is restored through repeated washing with potassium free physiological Krebs buffer. The dilated vessel segments are allowed to rest for 25-30 minutes before an additional washing with buffer. The vessel segments are now ready to be stimulated with agonists in the presence or absence of selective antagonists.

Light transmission aggregometry

Light Transmission Aggregometry (LTA) is a method for *ex vivo* measurement of platelet activation in platelet rich plasma (PRP) or washed platelets, when stimulated with natural or synthetic agonists. LTA is based on turbidometry, and was first used and invented by Gustav Born (1962) [72]. In the turbidometric LTA technique the PRP is under constant stirring in a quartz cuvette through which a beam of visible light pass and the ability of the PRP block light is compared with an unstimulated control PRP. The turbidity of the PRP is proportional to the degree of aggregation and PRP becomes more transparent in the presence of activating compounds.

Real Time-PCR (RT-PCR)

Quantitative RT-PCR (qRT-PCR), is a method to measure the level of mRNA in a sample. The RT-PCR methodology is based on the PCR technique where complimentary DNA (cDNA) is synthesized using isolated cellular mRNA as template. The enzyme reverse transcriptase reads the mRNA transcript and builds the complementary fragment of single stranded cDNA. The cDNA is then amplified using primers constructed to give a product of approximately 200 base pairs (bp). The gene amplification occurs in cycles where each cycle consists of three steps; Step 1 -denaturation of the dsDNA into ssDNA (95C, 1 sec); Step 2 - annealing of primers to the ssDNA; Step 3 - elongation where the annealed primers are extended to a product. A fluorescent dye usually SYBR Green, with the ability to emit light when bound dsDNA is included in the reaction mixture. The principle of the quantification is simple. As the PCR product accumulates it will bind more dye and the fluorescence will increase. The result is normalized to a housekeeping gene, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or Actin β , which is present in all types of cells at relatively constant levels. A convenient way to evaluate the ratio of gene expression is to use the comparative $2^{-\Delta\Delta CT}$ method, further described by Livak and Schmittgen [73].

Flow cytometry

Flow cytometry is a technique where single types of cells are sorted by their physical characteristics, e.g. size or density. One of the most used methods in flow cytometry is a technique known as Fluorescence-activated cell sorting, (FACS). The FACS technique is based on specific antibodies that covalently bind to the proteins of interest and to which later secondary antibodies marked by different fluorescence dyes attach. Cells that have become marked by the fluorophore labeled proteins can be detected, sorted and isolated. The cellsorting technique is divided into several steps: 1. The cells flow in a narrow rapid stream of liquid and becomes separated relative to the diameter; 2. Cells are separated and becomes further separated into droplets with one cell per droplet; 3. Cells contained in droplets will pass through a fluorescence-monitor where a laser will excite the fluorophore-labeled cells and then isolate them through an electrostatic mechanism. The FACS technique can be used to study if a specific protein in the platelet activation machinery becomes activated in the presence of different agonists or antagonists.

The Procaspase Activating Compound 1 (PAC-1) antibody is used for measuring the activation of membrane-bound GPIIb/IIIa [74]. The activation of GPIIb/IIIa is regulated through phosphorylation by an upstream affecting secondary messenger protein, the VAsodilating-Stimulated Phosphoprotein (VASP) [75]. VASP is also involved in the regulation of the cytoskeleton reorganization of the activated platelet, which initiates the platelet shape change [76]. The rate of phosphorylated (activated) and de-phosphorylated (inactivated) form of VASP can be measured using certain antibodies. The α -granule exocytose, following platelet activation, releases the unique P-selectin (CD62P) protein that becomes expressed on the platelet surface and hence promote platelet-leukocyte adhesion [77]. The rate of platelet activation can be measured through the presence of P-selectin by using directed specific antibodies.

Calcium flux analysis

Intracellular calcium $[Ca^{2+}]_i$ release is vital in the primary stage of platelet activation. The release of $[Ca^{2+}]_i$ is initiated by extracellular stimulation and hence activation of a receptor linked Gαq-protein [78]. The Gαq-protein regulates activation of the enzyme Phospho Lipase C (PLC) which promotes hydrolysis of PIP_2 into the membrane-bound DAG and the secondary messenger IP_3 . The IP_3 interacts with a specific IP_3 receptor (IP_3R) situated on the membrane of the dense tubular system (DTS), which regulates the $[Ca^{2+}]_i$ release from the calcium stores into the cytosol. The increasing concentration of calcium in the platelet causes reorganization of the cytoskeleton, i.e. platelet shape change. The intracellular calcium signal from an activated platelet in a calcium free buffer is measured through fluorescence. A calcium ion indicator, e.g. the non-fluorescent Fluo-4 acetoxymethyl ester (AM), passes the plasma membrane into the cytosol where an esterase hydrolyzes the ester yielding the fluorescent Fluo-4. As the product Fluo-4 is unable to traverse the plasma membrane, it becomes intracellular trapped. Upon combination with Ca^{2+} the Fluo-4 becomes highly fluorescent at certain wavelengths (Fig 8).

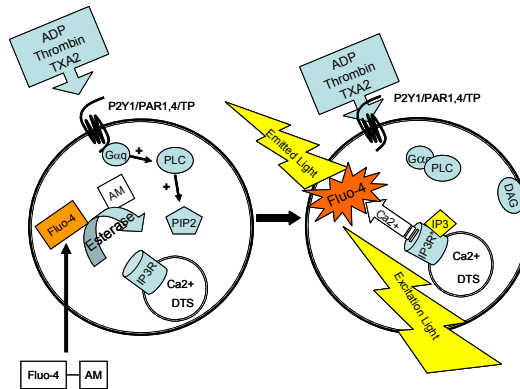


Fig 8. Extracellular stimuli on the platelet Gαq-receptor initiate a mobilization of cytosolic calcium through IP_3 turnover. The calcium ions activate the incorporated Fluo-4 molecule into a more excitable formation whereby excitation light becomes emitted and read spectrophotometric at 488 nm.

[³⁵S]GTPγS binding

Several of the membrane bound receptors present on the platelets are members of the super family of receptor proteins, known as the G-protein coupled receptors (GPCR). The GDP is a nucleotide that is bound to the inactive form of the Gα subunit in a GPCR. When the agonist activates the GPCR the receptor goes through a conformational change, which ends up in the Gα -subunit exchanging the GDP for GTP [79]. The GTP enhances dissociation of the Gα -subunit from the receptor, activating the Gα-GTP and Gβγ subunits (Fig 9). The activated subunits will in turn regulate intracellular downstream effectors. The cycle is completed by deactivation of the Gα –GTP, through the action of a GTPase, followed by re-association of the receptor/heterotrimeric G-protein complex [79]. Exchanging the native, active GTP nucleotide for a stable isotopic labeled form, i.e. [³⁵S]GTPγS, makes it possible to detect and measure the amount of activated Gi-protein. As the [³⁵S]GTPγS-molecule is resistant to hydrolysis by the GTPase, the accumulation of G-protein occupied by [³⁵S]GTPγS in the plasma membrane upon receptor stimulation, provides a simple quantification of receptor linked Gi-protein activation [80].

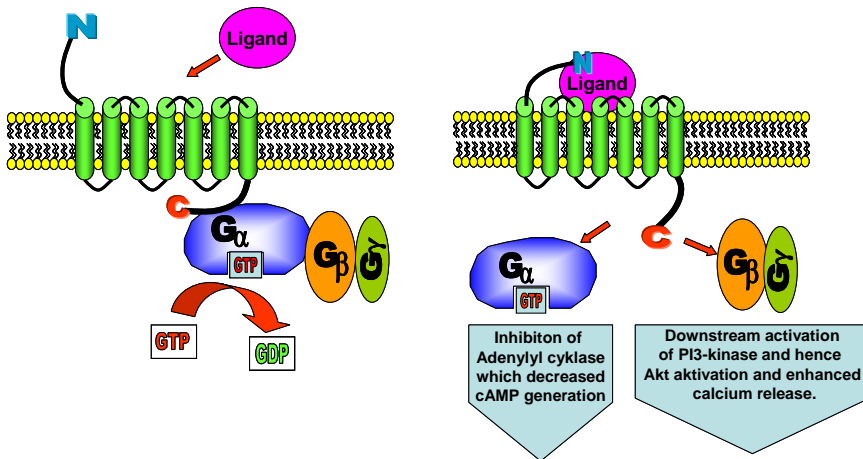


Fig 9. Illustration of the intracellular transduction pathway in a GPCR. When the agonist docks into the GPCR binding site, it initiates a conformational change in the inward rectifying cytosolic part of the receptor, which in turn enhances a nucleotide exchange from GDP into GTP in a receptor subunit protein, defined as Gα. This nucleotide exchange triggers an uncoupling mechanism which accomplishes dissociation of the receptor/heterotrimeric (Gα and Gβγ) G-protein complex. The subunit dissociation becomes the start signal for several intracellular transduction cascades that finally will modulate the platelet morphology.

Results and discussion

Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment (Paper I)

The strategy employed in this paper was to investigate platelets from freely healthy, non medicated free volunteers (n=8) that received a maximal dose, 600mg *per os* (p.o.) of the thiopyridine clopidogrel, trademarked as Plavix®. The volunteers donated blood samples the day before as a control. The collected PRP samples – both control and sample, were incubated at two different temperatures, 33C and 37C before LTA testing with several agonists as collagen, epinephrine, 5-TH, ADP and antagonists, MRS2500 (inhibit P2Y₁ signaling) and ticagrelor (inhibit P2Y₁₂ signaling).

P2Y₁₂ receptor activation inhibits the synthesis of cyclic adenosine 3', 5'-monophosphate (cAMP) by blocking the ATP turnover into cAMP by adenylyl cyclase. cAMP is a regulating secondary messenger for the enzyme phosphokinase A (PKA). PKA is a regulator for the phosphorylation of a downstream effector protein known as VASP. A FACS study was performed with a specific VASP assay to investigate how mild hypothermia affects platelet activation. No significant effects could be seen between mild hypothermia compared to normothermia in the presence or absence of clopidogrel in ADP mediated inhibition of VASP phosphorylation.

Although ADP activation in platelet was not attenuated by mild hypothermia the study resulted in interesting data (Fig 10). We observed a slightly increased activity in platelets exposed to mild hypothermia compared to normothermic circumstances (Fig 11). This observation has been reported by Xavier *et al.* [69] and Hartwig *et al* [22] whereas the latter described that cold induced platelet activation by shape change mimics the agonistic response. Secondly, platelet inhibition by clopidogrel lost some of its blocking potency effect in the presence of mild hypothermia. This pattern of decreased effect was also seen in the presence of ticagrelor and by the potent P2Y₁-inhibitor MRS2500. The problem with clopidogrel resistance was first reported by Järemo *et al.* [40] suggesting that

it could be beneficial to use a more potent ADP inhibitor to patients with clopidogrel resistance. In conclusion, mild hypothermia can not independently be relied on as an anti-platelet therapy and as the potency of clopidogrel is decreased by mild hypothermia. The antiplatelet therapy should not be discontinued; instead hypothermic patients may even need more potent antagonists.

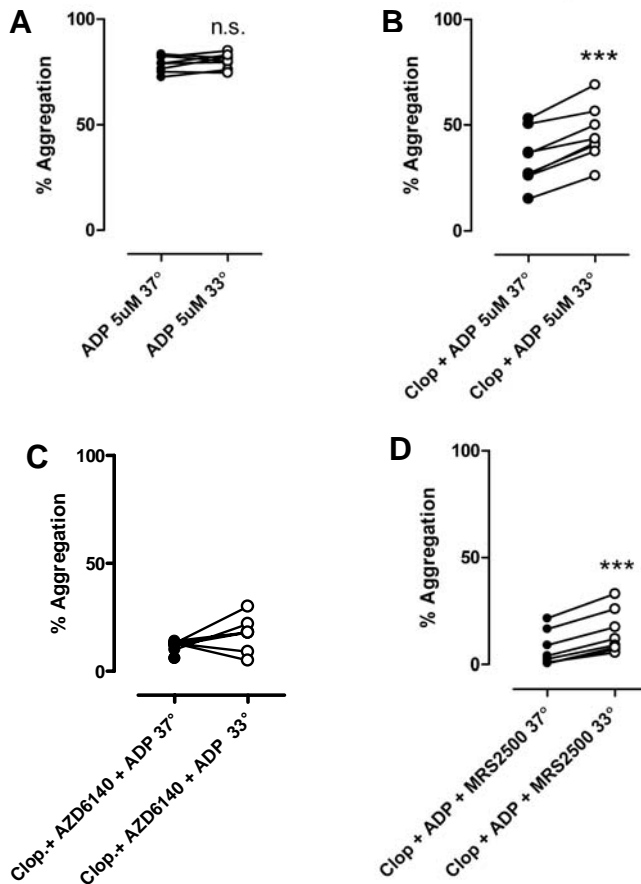


Fig 10. LTA study on platelets, ex vivo, normothermia (37°) (●) vs. mild hypothermia (33°) (○). (A) No difference in ADP induced platelet activation in control between 37C vs. 33C. (B): Significant difference in platelet activation was seen between 37C vs. 33C in platelets treated with clopidogrel, p.o. (C) Ticagrelor 10μM, ex vivo, lowered the platelet activation further in both 37C and 33C. No significance was seen between 37C vs. 33C. (D): When platelets treated with clopidogrel, p.o, were treated with 1μM of the P2Y₁ blocker MRS2500 the significance withholds and the platelet activation decreased in both 37C and 33C.

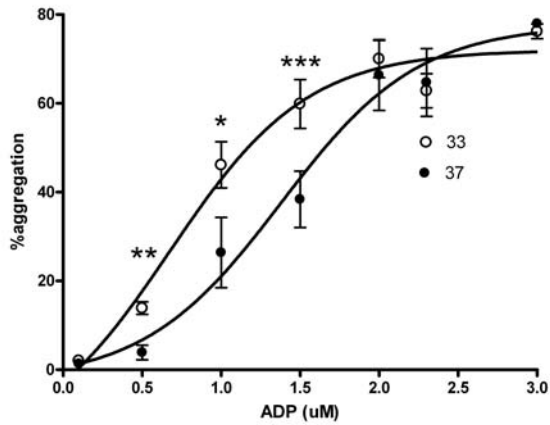


Fig 11. Dose-response in ADP activation on platelets in normothermia compared to mild hypothermia. A shift to the left in the diagram on platelets incubated in 33°C (○) compared to 37°C (●), indicates an increased activation in platelets during conditions of mild hypothermia.

The reversible oral P2Y₁₂ antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature (Paper II)

Both a murine *in vivo* and a human *ex vivo* model were used for the study. In the murine animal model, aorta was used to measure inhibition of 2-MeSADP induced contractility in the presence of clopidogrel and ticagrelor (AZD6140). In the human model small omental arteries and Left Interior Mammary Arteries (LIMA) were obtained during bypass surgery from 9 patients, mean age = 72 years of which 7 were men. The blood vessels were prepared and used in the same manner as vessels in the murine model. Female mice in the groups of two (5 test animals and 4 vehicles) were used in the experiment. The test animals were treated per os. twice with clopidogrel (50mg/kg). The administration of clopidogrel took place the day before and 2 hours before sacrifice of both groups of animals. Thoracic aortas were harvested through dissection. The connective tissue was removed from the vessels and they were cut into smaller segments. The aorta segments were then denuded, and the endothelial cells removed by gentle rubbing with a wooden stick.

The segments from mice aorta and human small arteries were mounted in a myograph in a 37°C controlled organ bath. The myograph settings have been described by Malmsjö *et al.* [81] and Wihlborg *et al.* [82]. After a period of rest the mice segments and small human arteries were pre-contracted to 1 mN while the LIMAs, with wider total diameter, were pre-contracted to 2 mN. Maximum contraction of segments were obtained in the presence of 60 mM K⁺ after pre-treatment with ticagrelor and vehicle segments with 1:1000 dimethyl sulfoxide (DMSO). To study antagonistic effects of clopidogrel and ticagrelor, the blood vessels were first slightly pre-contracted with 10nM norepinephrine (NE), and then further contracted by using the stable synthetic ADP analog, 2-MeSADP. To exclude contractile response from the P2X₁ receptor, the P2X₁ receptors present in the VSMCs were all desensitized with 10μM α,β-methylene ATP (αβ-MeATP) for 10 min as described by Kasakov and Burnstock [83] (Fig 12). The results, generated by the myograph studies, are presented as percent of the K⁺ mediated contractions.

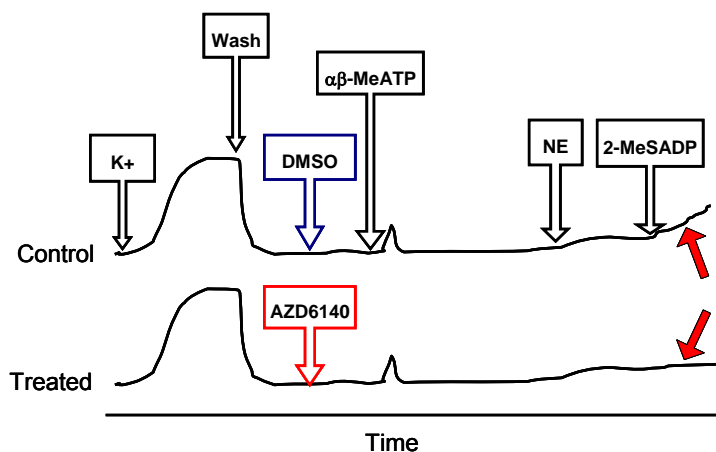


Fig 12. Illustrating a representative myograph experiment, where AZD6140 (ticagrelor) where added in the treated artery segments and DMSO in equal volumes were used in the control segments. Red arrows indicate the myograph-registered blocking effect of ticagrelor on P2Y₁₂- induced vascular contractions.

The study showed a dual therapeutic action by ticagrelor with effects also on ADP-mediated contraction apart from the previously shown platelet inhibitory effects. In contrast, clopidogrel did not have any effect on vascular contractions. This was seen both in the murine and the human arteries. Murine aortas from clopidogrel *in vivo* treated trial animals displayed no difference in 10μM 2-MeSADP mediated contractile responses compared to vehicle animals. However 1μM ticagrelor inhibited vascular contractions in the absence and presence of clopidogrel when added *ex vivo* (Fig 13). Ticagrelor added *ex vivo* to segments from human LIMA significantly differed from control in reducing 2-MeSADP initiated contractions. This decrease in vascular contractility was also seen in the pericardial fat (small) arteries (Fig 14). The specificity of ticagrelor on the P2Y₁₂ receptor were evaluated as there were no difference seen between NE induced α_{2a} mediated contractions in either human LIMAs or human small artery vessels. This study is based on the observation that the P2Y₁₂-receptor is expressed on the VSMC where it promotes artery-contractions [84]. The clinical advantage with a drug that simultaneously acts on two target areas is obvious. The interpretations of the outcome of this study are that the orally administrated prodrug clopidogrel is not able to block P2Y₁₂ mediated vessel-contractions in murine vessels, even if present in high concentrations. However, ticagrelor added *ex vivo* displayed distinct effects on all types of vessels tested in both murine and human tissue. Ticagrelor has been shown to have effect on various cell types. The antiplatelet potential is clearly recognized through several clinical studies. This study confirms

that ticagrelor also prevents from dangerous vasospasm which is especially harmful in stenotic arteries. Also, ticagrelor could be a successful tool in areas where the endothelial cell-layer is affected by intervention from revascularization through angioplasty or stenting.

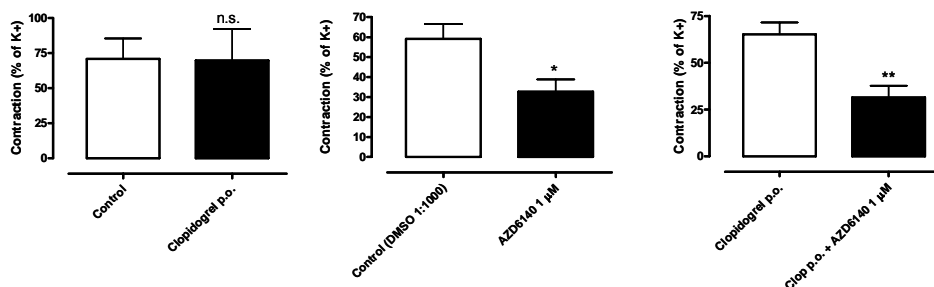


Fig 13. Bar graphs presenting results on contractility response from 2-MeSADP in murine aorta segments. Left bar graph shows no significant difference between control artery segments from vehicles vs. artery segments from mice pre-treated with clopidogrel per os. ($P=n.s.$, $n=5$). Centre bar graph shows that ticagrelor (AZD6140) significantly blocking 2-MeSADP induced $P2Y_{12}$ contraction on VSMC (59 ± 7 vs. 33 ± 6 $*P=0.015$). Right bar graph demonstrate results that marks ticagrelors ability to block $P2Y_{12}$ contractions in murine arteries comparing to clopidogrel (64 ± 8 vs. 32 ± 6 $*P=0.002$).

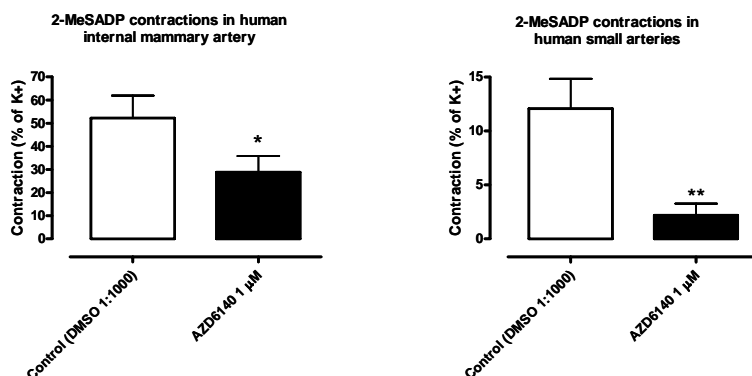


Fig 14. Left Bar graph demonstrate results on human LIMA (29 ± 7 vs. 52 ± 1 , $*P<0.05$, $n=15$) and small arteries (2 ± 1 vs. 12 ± 3 $**P=0.01$, $n=10$) control vs. treated with ticagrelor (AZD6140), ex vivo. 2-MeSADP induced contractions are both reduced in LIMA and small human artery in the presence of ticagrelor.

Succinate independently stimulates full platelet activation via cAMP and PI3 β kinase signalling: (Paper III)

The results from a previous study, showing expression of SUCNR1 receptor in platelets, was confirmed using qPCR and the SUCNR1 gene was found to be transcribed at the same level as the one for P2Y₁. The SUCNR1 expression on platelets was further confirmed on protein level by western blot and confocal immunofluorescence (Fig 15). Investigation with LTA confirmed that the effect of succinate mediated platelet activation was dependent on both TXA₂ generation and P2Y₁₂ activation as it was abolished in the presence of both ASA and ticagrelor. To evaluate if succinate activates any G $\alpha_{12/13}$ -protein we used a specific inhibitor to a downstream effector for the G $\alpha_{12/13}$ -protein, p160 Rho-associated coiled-coil containing kinase (Rho-kinase) p160ROCK [85]. Activation of the enzyme p160ROCK is important for the platelet shape change [85]. Pre-incubating platelets with the p160ROCK inhibitor Y27632 before succinate stimulation proved to have no effect on the platelet activation thus proving that succinate does not signal through G $\alpha_{12/13}$ -proteins.

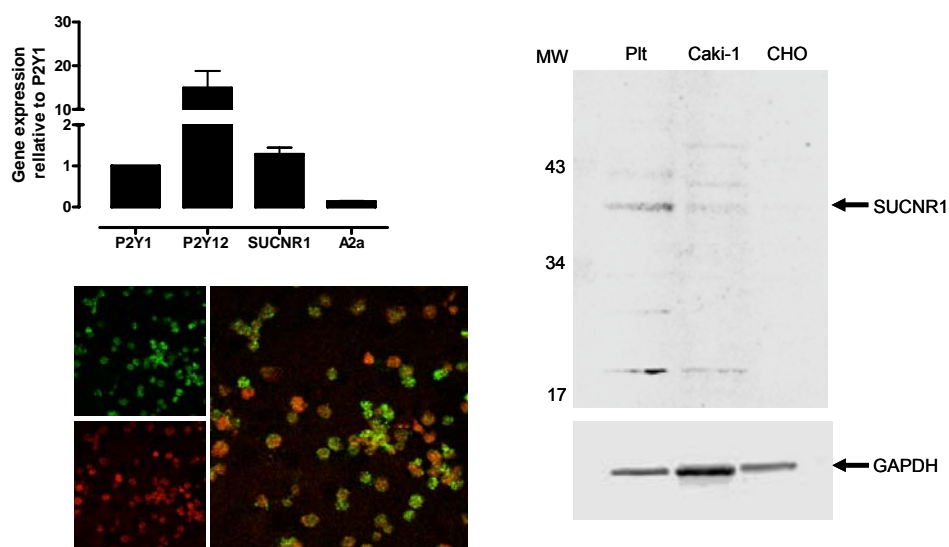


Fig 15. Upper left bar graph: Expression of SUCNR1 on mRNA level studied through quantitative RT-PCR. The expression of SUCNR1, P2Y₁₂ and A2a were normalized against P2Y₁. Lower left panel: Confocal immunofluorescence image platelets specific stained with rhodamine-6G (red) and SUCNR1 stained with primary goat polyclonal anti-SUCNR1 antibody (green). Right panel: Presence of

SUCNR1 on protein level in platelets (Plt) confirmed through western blot. Protein from Caki-1 cells (positive control) and Chinese Hamster Ovary (CHO) cells (negative control. GAPDH used as loading control.

We next evaluated if the platelet SUCNR1 is linked to the Gq-protein, which is a G-protein involved in the regulation of intracellular calcium. Our results showed that the intracellular calcium signaling from platelets was absent in the presence of succinate, and thus exclude the SUCNR1 as a G α q-protein linked receptor. We then investigated if succinate is involved in the regulation of AC and hence the secondary messenger cAMP and its downstream effector VASP. We found that succinate suppresses generation of phosphorylated VASP as well as cAMP in platelets either stimulated by forskoline or prostaglandin E₁ (PGE₁). Furthermore, succinate stimulation increased both the levels of GPIIb/IIIa activation and the release of P-selectin (CD62P) in platelets (Fig 16).

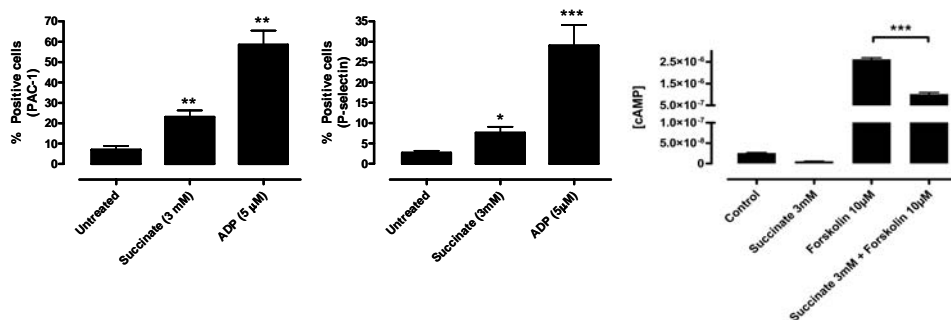


Fig 16. Flow cytometry study on platelets. Left bar graph: GPIIb/IIIa activation in platelets. Marked differences between un-stimulated platelets and platelets stimulated with 3mM succinate or 5μM ADP (ANOVA: ** $P < 0.01$, $n = 16$). Centre bar graph: P-Selectin release from platelets. Vesicle secretion of α -granules mediated by succinate could be statistically confirmed between untreated platelets vs. platelets treated with 3mM succinate or 5μM ADP (ANOVA: * $P < 0.05$, *** $P < 0.001$, $n = 16$). (C) Effects of succinate on forskoline induced cAMP-production in platelets. Significant differences in cAMP production was observed between platelets pretreated with 3mM succinate and 10μM forskolin vs. 10μM forskolin (ANOVA: *** $P < 0.001$, $n = 4$).

Besides inhibition of cAMP generation, Gi activation generates G $\beta\gamma$ subunits that can regulate certain isoforms of PI3K. Blocking PI3K using a general PI3K inhibitor, wortmannin, resulted in a total block of succinate induced platelet stimulation, showing that SUCNR1 platelet activation is dependent on PI3-kinase

activation. Several isoforms of PI3K (PI3K α , PI3K β and PI3K γ) are present in the human platelets [86]. We next evaluated which isoform of PI3K that was regulated by SUCNR1 activation. The results showed that platelets treated with a specific inhibitor for PI3K β (TGX221) displayed significantly reduced aggregation upon stimulation with succinate. The PI3K initiates downstream phosphorylation of the enzyme Akt1, also known as Protein Kinase B (PKB α). Using western blot analysis we confirmed that succinate stimulated phosphorylation of PKB/Akt and that this effect could be primarily assigned to PI3K β (Fig 17).

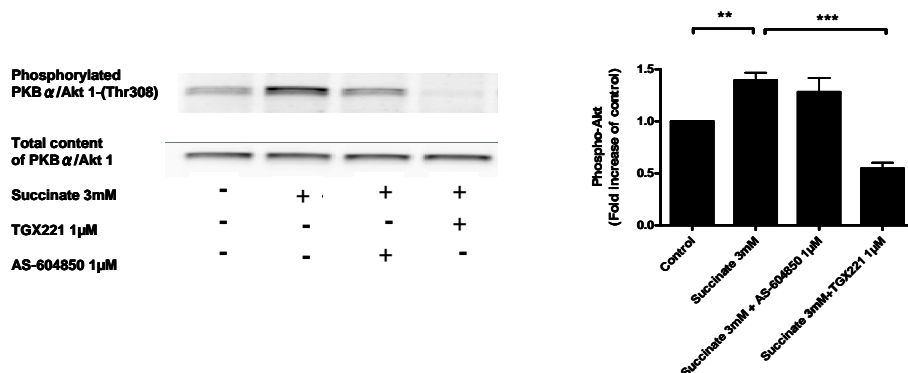


Fig 17. Western blot on washed platelets. Left fig: Presenting a representative result from a western blot on phosphorylated PKB α /Akt1 where the total content of PKB α /Akt1-protein serves as a loading control. Platelets treated with 1 μ M of the PI3K γ inhibitor AS-604850, or 1 μ M of the PI3K β inhibitor TGX221. All platelet samples, except the control, were stimulated with 3mM succinate. Right bar graph: Complete results from western blot where ratio is normalized towards control, (ANOVA: *** P <0.001, ** P <0.01, n =3).

Our results also showed that the succinate stimulated SUCNR1 activated receptor responded in a biphasic manner and that the response was relative to the rising concentration of succinate with an EC₅₀ value equal to approximately 0.5 mM. This latter finding was confirmed by [³⁵S]GTP γ S-binding performed on isolated platelet membranes. [³⁵S]GTP γ S binding is, as previously described, a tool for functional receptor studies. The [³⁵S]GTP γ S-binding confirmed that SUCNR1 receptor activation in platelets is linked to the Gai protein. Furthermore, results from the [³⁵S]GTP γ S binding proved that the SUCNR1 receptor can be desensitized not only homologously by succinate, but also heterologously by ADP (Fig 18).

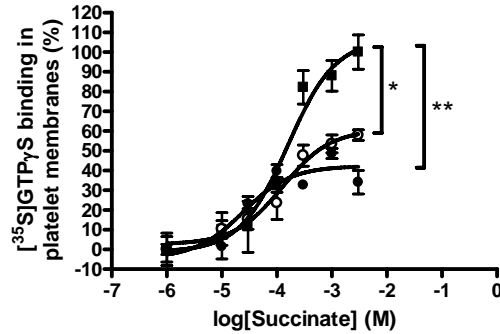


Fig 18. Dose-response of succinate-stimulated [^{35}S]GTP γ S-binding in platelet membranes. EC_{50} -value equals $110\mu\text{M}$. untreated platelet membranes (■), platelet membranes pre-treated with $10\mu\text{M}$ 2MeSADP (○), platelet membranes pre-treated with 3mM succinate (●). The maximal response, obtained for membranes pretreated with either $10\mu\text{M}$ 2MeSADP or 3mM succinate, was significantly reduced compared to untreated membranes (ANOVA: $**P<0.001$, $*P<0.01$, $n=3$).

More precise future functional studies on SUCNR1 activation demand better tools in the form of effective antagonists. SUCNR1 takes part in the platelet activation and might be an interesting target in the development of new antiplatelet therapies.

Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y₁₂ receptor mediated platelet aggregation (Paper IV)

In this study we screened several of the intermediates of the mevalonate pathway for effects on platelet aggregation. One of them, farnesyl pyrophosphate (FPP) was found to have a blocking effect on ADP mediated platelet aggregation. Other platelet agonists like Thrombin Activating Peptide (TRAP) and epinephrine were also tested on FPP pre-incubated platelets, but without any difference in the inhibiting effect on platelet activation. However, FPPs inhibiting effect on ADP mediated platelet activation was further strengthened by the result from FACS on GPIIb/IIIa and P-selectin. Both GPIIb/IIIa and P-selectin activation was down-regulated compared to platelets stimulated only by ADP (Fig 17). Studies on cAMP generation also proved that FPP had a suppressing effect on ADP repressed forskoline stimulated cAMP production in platelets (Fig 19).

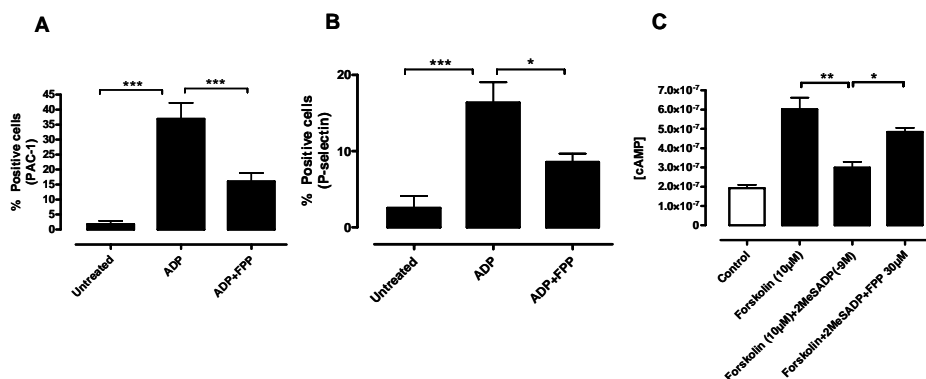


Fig 19. Bar graphs demonstrating ADP mediated platelet activation in presence of FPP. (A) GPIIb/IIIa activation, (B) P-selectin activation, (C) cAMP assay with FPP present.

To identify the mechanism of FPP effect on platelets, we studied [³⁵S]GTPγS binding both on preparations of membranes from platelets and from P2Y₁₂ transfected Chinese Hamster Ovary (CHO) -1 cells. The platelet membranes and the CHO-1-cells were preincubated with different concentrations of FPP before

stimulation with increasing concentrations of 2-MeSADP. The presence of FPP resulted in a decline in 2-MeSADP potency and also in a significant decrease in efficacy (Fig 20). These results prove that FPP interferes with the signaling of the P2Y₁₂ receptor.

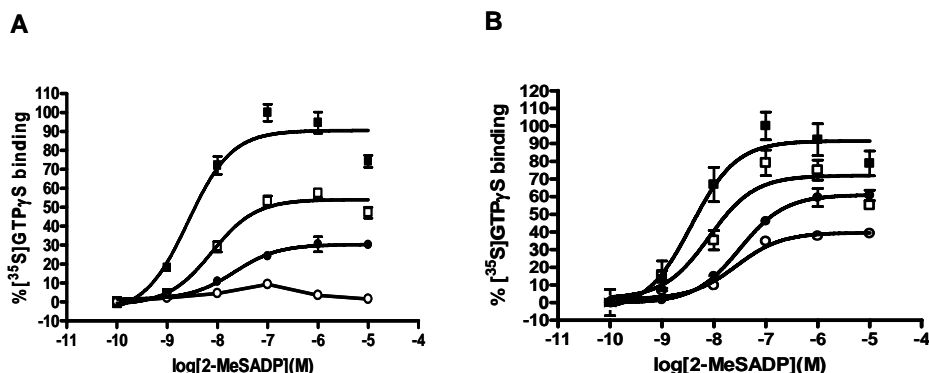


Fig 20. [³⁵S]GTP γ S binding assay on (A) platelet membranes and (B) P2Y₁₂ transfected CHOK1-cells with different concentrations of FPP. (■) Control (2-MeSADP), (□) [FPP]=10 μ M, (●) [FPP]=50 μ M and (○) [FPP]=100 μ M

The most obvious similarity between FPP and ADP is the organization of both molecules into a pyrophosphate group and a hydrophobic backbone. However, the hydrophobic part of FPP consists of a 15-carbon chain that is quite different from the rings of ADP. In order to understand the way FPP and 2-MeSADP bind to the same receptor and to find the common rational basis for the binding modes, a molecular modeling study was conducted. (Fig 21). The results from the docking supported the hypothesis by showing that FPP is able to bind to the same binding cavity as ADP, sharing several aminoacid residues in the process.

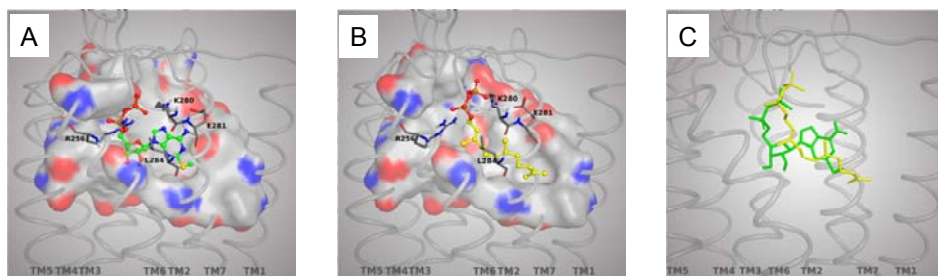


Fig 21. Docking model of FPP and the P2Y₁₂ receptor. (A) The 2-MeSADP molecule (green and with red phosphate group) docks with the receptor in the active site. (B) The FPP molecule (yellow and with red phosphate group) docks with the receptor in the active site. (C) Merge of 2-MeSADP and FPP.

The idea that FPP, besides its role in the mevalonate pathway, also have other functions is not new. FPP has also in an earlier report by Williams *et al.* [87] been identified as a potential ligand for the LPA₅ receptor, previously known as GPR92. It has also been shown, by Amisten *et al.* [33], that LPA₅/GPR92 is present on mRNA level in platelets. It is likely that this receptor is responsible for the promoting effects of FPP on LPA mediated platelet shape-change. In this work we have identified FPP as an endogenous non-competitive antagonist on the P2Y₁₂ receptor. However, we have also noticed that low concentrations of FPP seem to have a potentiating effect on ADP mediated platelet activation. This suggests that FPP is a partial agonist which in the presence of ADP acts as an antagonist. The concentration of free FPP in plasma under normal and pathogenic conditions is still not known and could constitute the key to the role of FPP in the haemostatic/thrombotic pathway. At low concentrations FPP may act as a platelet activator, while at high concentrations FPP could act as an inhibitor of platelet activation.

General Discussion

Mild hypothermia is used as complement method in the acute standard care treatment of patients affected by ACS or MI. In paper I we evaluated how mild hypothermia also influences the platelet activation. The study was performed in an *in vivo* milieu where maximal doses of the antiplatelet drug Plavix® (clopidogrel) were administrated, 600 mg *per os* in healthy volunteers. We found in this experiment that mild hypothermia does not attenuate platelet ADP-mediated response; on the contrary, we saw that mild hypothermia resulted in increased platelet activation.

The interaction between the vascular wall and platelets are central in the thrombotic process. The P2Y₁₂ receptor which is essential for platelet activation is also present on the vascular smooth muscle cell (VSMC) where it - if activated – can respond by initiating contractions. From this knowledge we initiated a comparative study (paper II) where we examined the possibility with a dual therapy. We compared the ability of two antiplatelet P2Y₁₂ receptor blocking substances (clopidogrel and ticagrelor) which both inhibit ADP induced platelet aggregation to inhibit ADP induced VSMC contractility in murine and human arteries. The results showed that the prodrug clopidogrel, the dominating P2Y₁₂-blocking pharmaceutical treatment on the market, had no affect on P2Y₁₂ mediated contractility on VSMC *in vivo*. However ticagrelor, administrated *ex vivo*, proved to have the capability to decrease ADP mediated VSMC contractions. The platelets ability to activate is dependent on extracellular stimuli via membrane bound receptors. The extracellular signals are further amplified through several intracellular mechanisms.

In an earlier report by Amisten *et al.* [33] a number of membrane-bound receptors expressed at the platelet surface were identified, of which several were characterized as *orphan* GPCRs. In paper III we evaluated the intracellular response from one orphan GPCRs, the low affinity succinate receptor SUCNR1. SUCNR1 has in a previous report been identified as linked to both G α i and G α q [46]. Succinate has in moderate concentrations proved to independently activate the platelet through SUCNR1-activation. However, the succinate activation requires TXA₂ generation and P2Y₁₂ activation for initiation of full scale platelet activation. Moreover, in a further examination of the intracellular signaling pathways of SUCNR1 it was able to couple to the G α i-protein and hence inhibit the membrane bound adenylate cyclase. The study also identified SUCNR1 as an

activator for PI3 β -kinase, an isoform of PI3-kinase. Earlier reports have confirmed that the isoform PI3K β can both be regulated by G $\beta\gamma$ -subunits and by tyrosine kinases [88]. We believe that receptor mediated selection of PI3K isoforms is platelet specific and depend on the constellation of G β and G γ -subunits [89, 90].

FPP has earlier been reported by Williams *et al.* [87] to be an agonist on LPA₅ (GPR92) and thus have additive effects on LPA-mediated shape and change, a quality of FPP which agreed well with our findings. In an attempt to investigate if FPP or any other of the mevalonate intermediates had any effect on ADP mediated platelet aggregation, we screened the majority of them with LTA. Only FPP had any effect, and was inhibitory. Also, LTA studies with epinephrine and TRAP confirmed that the inhibitory effect was true only for ADP induced platelet activation. Calcium-influx on washed platelets confirmed that ADP mediated P2Y₁ signaling was not attenuated in platelets pre-treated by 30 μ M FPP. However, the attenuated activation of P-selectin and PAC-1 in the presence of FPP together with the fact that FPP affected cAMP levels indicated that the purinergic receptor P2Y₁₂ is the target for FPP. [³⁵S]GTP γ S binding assay performed on membranes isolated from human platelets as well as from P2Y₁₂-transfected CHO-K1 cells showed that 2-MeSADP mediated P2Y₁₂ activation loses both potency and efficacy in presence of FPP. The molecular resemblance between the P2Y₁₂ receptors endogenous agonist ADP and the FPP molecule is not obvious, but they both share a pyrophosphate group. A docking model between the P2Y₁₂ receptor and the synthetic stable ADP-analog 2-MeSADP and FPP concluded that FPP was able to fit into the active site of P2Y₁₂ and thus attenuate the functionality of the receptor.

The studies throughout the thesis have aimed to investigate different aspects of platelet activation in haemostasis. The mechanisms that trigger the important and sensitive platelet activation are complex and sometimes they appear elusive. Studies on platelets are a vital tool for the understanding of how platelets fit into the haemostatic puzzle and hence, guide us to develop better antiplatelet therapies.

Main Conclusions

- Mild hypothermia does not attenuate platelet activation. Hypothermia has an augmenting effect on ADP induced platelet activation.
- Hypothermia induced clopidogrel resistance is reduced in the presence of the P2Y₁₂ receptor blocking substance ticagrelor (AZD6140).
- Mild hypothermia can not independently be used clinically as an anti-platelet therapy. Instead patients treated with mild hypothermia might even need more potent antagonists.
- Clopidogrel administrated *in vivo* have no inhibiting effect on ADP induced VSMC contractions in murine aorta and human LIMA or small arteries. However, ticagrelor, *ex vivo* has the ability to block ADP mediated VSMC contractions.
- Succinate has *per se* the capacity to induce full platelet activation. Succinate activation is dependent of TXA₂ generation, ADP mediated activation of the P2Y₁₂ receptor, and PI3K β activation.
- The intracellular signaling pathways of the succinate receptor, SUCNR1, inhibit adenylate cyclase cAMP production and regulate Akt1 activation through phosphorylation by the PI3K isoform PI3K β .
- FPP is an endogenous antagonist of the P2Y₁₂ receptor in high concentrations (>30 μ M), but seems to have a potentiating effect in ADP mediated platelet activation if present at low concentrations (partial agonist and antagonist).

Populärvetenskaplig sammanfattning

Hjärt- och kärlsjukdomar är den främsta orsaken till en för tidig död för både män och kvinnor i dagens industrialiserade samhällen. I det akuta förloppet av dessa sjukdomar spelar blodplättan (trombocyten) en viktig roll då den är inblandad i bildandet av blodproppar. Den kärnlösa och kortlivade trombocyten roll är vanligen en annan och central i läkningsprocessen av skadade blodkärl. Vid en kärlskada organiserar sig trombocyterna med trådverk av samverkande proteiner för att levra (koagulera) blodet och därmed blodförlust. Trombocyterna använder sig av ytbundna receptorer och frisättning av signalmolekyler för att kommunicera med sin omgivning. En av dessa receptorer, $P2Y_{12}$ är viktig för att aktivera trombocyten. $P2Y_{12}$ finns även på ytan av glatt muskulatur i blodkärlsväggen, där den svarar med att kontrahera muskelcellen. Flera läkemedel, däribland clopidogrel och ticagrelor har utvecklats för att förhindra att $P2Y_{12}$ aktiveras. Clopidogrel måste omvandlas via levern för att aktiveras, vilket innebär att clopidogrel föreligger i låga koncentrationer i blodplasman. Clopidogrel har också påvisats ha en låg effektivitet vid behandling. Ticagrelor är en substans som tillhör den nya generationen $P2Y_{12}$ blockerare som visat sig ha en bättre blockerande effekt på trombocyter än sina föregångare.

Det finns ett behov av att ta fram nya terapier för att sänka trombocytaktiveringen. Genom att sänka kroppstemperaturen från 37°C till 33°C (mild hypotermi) sänks kroppsmetabolismen och behovet av syre till organ och vävnader. Detta har gett positiva resultat vid hjärt- och kärlkirurgi, och visat sig vara en metod för att skona vävnad strax innan samt under de kirurgiska ingreppen. Dock har studier på trombocyten svar vid mild hypotermi visat på både en ökad respektive minskad aktivering. Vid en undersökning kunde vi konstatera en ökad trombocyt aktivering vid 33°C jämfört med 37°C. Vid samma undersökning kunde vi bekräfta att även effektiviteten hos clopidogrel sänktes vid 33°C jämfört med 37°C.

Eftersom $P2Y_{12}$ receptorn även uttrycks i glatt muskulatur så skulle i teorin en blockerande substans för $P2Y_{12}$ receptorn, antingen clopidogrel eller ticagrelor kunna blockera dess aktivering på muskelcellerna i kärlen och förhindra skadliga kärlsammandragningar. Våra undersökningar visade att kontraktionerna i den glatta muskulaturen hämmades utav ticagrelor, medan clopidogrel var verkningslöst. Vi har vid tidigare undersökningar på trombocyter identifierat höga transkriptnivåer för succinatreceptorn (SUCNR1). Vid vidare undersökningar

upptäckte vi att denna receptor fanns uttryckt på trombocyts yta och har en egen såväl som förstärkande effekt på trombocyt-aktivering. Aktivering av SUCNR1 visade sig vara beroende av P2Y₁₂ aktivering, frisättning av tromboxan A₂, såväl som det SUCNR1 aktiverade PI3Kβ enzymet.

Ett något oväntat fynd gjordes vid en undersökning av signalmolekyler, inblandade i kolesterol-syntesen. Det visade sig att en av signalmolekylerna, farnesyl pyrofosfat (FPP), uppvisade en hämmande effekt på ADP medierad trombocyt aktivering. Preliminära försök visade att det endast var den ADP aktiverade P2Y₁₂-receptorn som blockerades genom närvaro av FPP. Vid senare underökningar kunde vi bekräfta att FPP, vid moderata koncentrationer, verkade hämmande på P2Y₁₂-receptorn, men också att FPP vid låga koncentrationer kan fungera additivt på ADP/P2Y₁₂-medierad trombocyt-aktivering.

Sammanfattningsvis har dessa arbeten, var och en på sitt vis, verkat för en kliniskt och prekliniskt ökad kunskap om trombocytaktivering. Genom studierna angående trombocytaktivering under påverkan av mild hypotermi, har vi visat att kyla ökar trombocytaktivering samt att clopidogrel förlorar en del av sin P2Y₁₂-blockerande effekt. Den kliniska relevansen består i att behålla doserna av anti-trombotiska läkemedel även i de fall där mild kylning används som ett komplement vid operativa ingrepp. Vidare har våra försök visat att P2Y₁₂-hämmaren ticagrelor även har en hämmande effekt på ADP stimulerad P2Y₁₂-receptor inducerad kärlkontraktion, vilket kan vara en viktig terapeutisk egenskap i de fall där kärlväggen är skadad med frilagd underliggande glatt muskulatur. Vi har också identifierat succinat receptorns bakomliggande funktioner för medverkan i trombocytaktivering. Till sist har våra studier visat en ny roll hos en viktig molekyl i kolesterol syntesen, farnesyl pyrofosfat, nämligen den att farnesyl pyrofosfat även visat på antagonistiska- såväl som agonistiska egenskaper på den för trombocyt-aktivering centrala P2Y₁₂ receptorn.

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Appendix: Original Papers I-IV

- I. Carl Högberg, David Erlinge and Oscar Ö Braun
Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment
Thrombosis Journal 2009; 7:2
- II. Carl Högberg, Helen Svensson, Ronny Gustafsson; Atli Eyjolfsson, and David Erlinge
The reversible oral P2Y₁₂ antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature
International Journal of Cardiology 2010, vol 142 pp 187-192
- III. Carl Högberg, Olof Gidlöf, Chanyuan Tan, Siv Svensson, Jenny Nilsson-Öhman, David Erlinge and Björn Olde
Succinate independently stimulates full platelet activation via cAMP and PI3 β kinase signaling
Journal of Thrombosis and Haemostasis (in press)
- IV. Carl Högberg, Olof Gidlöf, Francesca Deflorian, Kenneth A. Jacobson, Björn Olde and David Erlinge
Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y₁₂ receptor mediated platelet aggregation
(*Manuscript*)

Paper I

Original basic research

Open Access

Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment

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Abstract

Background: Mild hypothermia is currently standard of care for cardiac arrest patients in many hospitals and a common belief is that hypothermia attenuates platelet aggregation. We wanted to examine the effects of clopidogrel on platelet aggregation during hypothermia.

Methods: Platelet reactivity at 37°C and 33°C was evaluated by light transmission aggregometry and vasodilator-stimulated phosphoprotein (VASP) in blood from healthy volunteers before, and 24 hours after, a 600 mg loading dose of clopidogrel.

Results: Collagen, 5-HT, epinephrine, U46619 and ADP-induced platelet aggregation was unaltered or even increased by hypothermia. After clopidogrel, there was a significant increase in platelet aggregation for 5 and 20 µM ADP at 33°C compared to 37°C (46 ± 5 vs. $34 \pm 5\%$ and 58 ± 4 vs. $47 \pm 4\%$, $p < 0.001$, $n = 8$). Hypothermia also increased ADP-induced aggregation after pretreatment with the P2Y₁ antagonist MRS2500. The decreased responsiveness to clopidogrel during hypothermia could be overcome by addition of the reversible P2Y₁₂ antagonist AZD6140. ADP-induced inhibition of VASP-phosphorylation was unaffected by hypothermia both in the presence and absence of clopidogrel. A dose-response curve for ADP-induced platelet aggregation revealed increased potency for ADP during hypothermia with no difference in efficacy.

Conclusion: Mild hypothermia did not attenuate platelet aggregation, instead it even increased ADP-stimulated platelet aggregation after clopidogrel treatment. Dual platelet inhibition with aspirin and a P2Y₁₂ receptor antagonist is probably needed for patients with acute coronary syndromes treated with mild hypothermia, and it is possible that future ADP blockers could be of benefit.

Introduction

Hypothermia is a condition in which many biological reactions are altered. Even a minor change of temperature in a cell can alter the response to stimuli. Mild hypothermia (33–35°C) has been shown to reduce mortality and improve neurological outcome in unconscious patients

with cardiac arrest [1,2], and is recommended by treatment guidelines [3]. Mild hypothermia is already standard of care in many hospitals for cardiac arrest patients. Furthermore, mild hypothermia has been shown to reduce myocardial infarct size in animal models, and clinical studies are ongoing to determine whether this strategy

can preserve not only the brain but also the heart [4]. For many patients with cardiac arrest, the aetiology is an acute coronary syndrome, which needs to be treated with platelet inhibitors. The cornerstone of this treatment is dual antiplatelet therapy with aspirin and clopidogrel [3]. However, to our knowledge, the pharmacodynamic effects of clopidogrel during hypothermia have never been studied.

Conventional wisdom holds that hypothermia reduces platelet activation, and it has been suggested that antiplatelet treatment should be reduced during hypothermia. There are some studies supporting this notion [5-7]. However, there are more recent reports suggesting increased platelet reactivity during mild hypothermia [8-11]. It is of great importance to select the correct level of platelet inhibition for this patient category. Insufficient platelet inhibition may result in stent thrombosis, acute myocardial infarction, and cardiac death. It is therefore important to understand the effect of hypothermia on clopidogrel treatment.

To examine the effect of clopidogrel during mild hypothermia, we analyzed platelet reactivity as measured by light transmission aggregometry (LTA), vasodilator-stimulated phosphoprotein (VASP)-phosphorylation, and a P2Y₁₂ specific flow cytometry assay, in healthy volunteers at 37° and 33°C before and 24 hours after a 600 mg dose of clopidogrel.

Materials and methods

Platelet preparation for LTA analysis

Whole blood samples (40 mL) were collected from healthy voluntary blood donors (n = 8). The blood samples were collected from an antecubital vein into Becton Dickinson (BD) Vacutainer™ tubes containing 0.129 M sodium citrate. A second blood sample was collected 24 hours after a 600 mg oral loading dose of clopidogrel. To obtain platelet rich plasma (PRP), whole blood was centrifuged (10 min, 140 × g, room temperature (RT)). After centrifugation the supernatant containing the PRP was collected and transferred to a 15 mL polypropylene tube. To obtain reference platelet poor plasma (PPP), whole blood was centrifuged (10 min, 2260 × g, RT). The supernatant containing PPP was collected into a 15 mL polypropylene tube.

LTA analyses

Two serial connected aggregometers (Chrono-Log 490, Chrono-Log Corporation, Havertown, PA, USA) were pre-heated to 37°C and 33°C respectively. To allow for temperature equilibration, the PRP was incubated for 5 minutes in the aggregometers prior to addition of an agonist. In experiments with ex vivo antagonists, addition of these compounds was followed by incubation for 5 min

prior to ADP stimulation. Analyses were performed using AGGRO/LINK® software (Chrono-Log Corporation, Havertown, PA, USA.). Stirring was set to 1200 rpm and the optical band was in the range of 600 OD. Each of the experiments continued for 6 minutes (to observe if a secondary aggregation took place). The test volumes containing PRP were set to 250 µL, while the PPP volume in the reference wells of the two aggregometers was set to 500 µL.

Flow cytometric analysis of VASP-phosphorylation

Whole blood was collected from the antecubital vein into BD Vacutainer™ tubes containing 0.129 M sodium citrate. The blood was incubated for 10 min at 33°C and 37°C. The VASP assay was performed using the platelet VASP/P2Y₁₂ kit (Biocytex Platelet VASP kit, Marseille, FR). The experimental procedure was carried out according to the manufacturer's instructions except that the samples were prepared at 33°C and 37°C instead of room temperature until the fixation step. Mean Fluorescence Intensity (MFI) was measured with a flow cytometer (FACScalibur®, BD, USA). Platelet reactivity index (PRI) was calculated from the corrected MFI (cMFI) of prostaglandin E₁ (PGE₁) and ADP- and PGE₁- treated samples according to the following equation:

$$PRI = [(cMFI_{(PGE_1)} - cMFI_{(PGE_1+ADP)})/cMFI_{(PGE_1)}] \times 100$$

Drugs

ADP, epinephrine, collagen, and thrombin were from Chrono-Log Corporation, USA. AZD6140 was a gift from Astra-Zeneca, Sweden. MRS2500 was from Tocris Bioscience, UK. U-46619 was from Sigma-Aldrich, USA. Clopidogrel was from Sanofi Pharma Bristol-Myers Squibb SNC, France. All drugs (clopidogrel excluded) were dissolved in 0.9% saline; AZD6140 was dissolved in DMSO at 10⁻² M and then further diluted in 0.9% saline.

Ethics

The Ethics Committee of Lund University approved the project. All blood donors provided signed informed consent to participate in the study.

Calculation and statistics

Statistical analyses were performed using the GraphPad Prism 4.0 software (Graph Pad Software, USA). LTA and VASP data were analyzed using paired Student's t-test. P-values less than 0.05 were regarded as statistically significant. Values are presented as mean ± s.e.m.

Results

ADP-stimulated platelet aggregation evaluated by LTA

There was no significant difference in maximum platelet aggregation (MPA) levels when PRP was stimulated with 5 µM ADP at 33°C compared to 37°C (79 ± 1 vs. 80 ± 1%,

P = NS, n = 8) or with 20 μ M ADP at 33°C compared to 37°C (80 ± 2 vs. $80 \pm 1\%$, P = NS, n = 8) [Figure 1a, b].

ADP-stimulated platelet aggregation before and after clopidogrel treatment

24 hours after a loading dose of 600 mg clopidogrel, there was a significant increase in MPA after both 5 and 20 μ M ADP at 33°C compared to 37°C (46 ± 5 vs. $34 \pm 5\%$, P < 0.001, n = 8, and 58 ± 4 vs. $47 \pm 4\%$, P < 0.001, n = 8) [Figure 1c, d]. There was a significant increase in MPA when PRP treated with the P2Y₁ receptor antagonist MRS2500 (1 μ M) was stimulated with 5 μ M ADP at 33°C compared to 37°C (57 ± 7 vs. $41 \pm 9\%$, P < 0.05, n = 8) [Figure 2a] and with combined treatment with oral clopidogrel and MRS2500 *ex vivo* (15 ± 4 vs. $7 \pm 3\%$, P < 0.001, n = 8) [Figure 2b]. 10 μ M AZD6140 *ex vivo* in addition to per oral clopidogrel markedly reduced MPA after 5 μ M ADP with no significant difference in MPA at 33°C compared to 37°C [Figure 2c].

Collagen-, 5-HT-, epinephrine-, and U46619-stimulated platelet aggregation before and after clopidogrel treatment

There was no significant difference in MPA when PRP was stimulated with 2 μ g/ml collagen without clopidogrel at 33°C compared to 37°C (79 ± 1 vs. $83 \pm 2\%$, P = NS, n = 8) or after oral clopidogrel (71 ± 5 vs. $71 \pm 4\%$, P = NS, n = 8) [Figure 3a, b]. There was a significant increase in MPA levels with 10 μ M 5-HT at 33°C compared to 37°C both without clopidogrel (9.5 ± 1.5 vs. $6.3 \pm 1.8\%$, P < 0.001, n = 8) and after oral clopidogrel (9.6 ± 2.4 vs. $6.0 \pm 2.2\%$, P < 0.001, n = 8) [Figure 3c, d]. No differences in MPA between temperatures were observed with epinephrine 10 μ M without clopidogrel (73 ± 6 vs. $69 \pm 7\%$, P = NS, n = 8) or after clopidogrel (61 ± 8 vs. $62 \pm 7\%$, P = NS, n = 8) [Figure 4a, b] or with U46619 10 μ M without clopidogrel (27 ± 14 vs. $38 \pm 14\%$, P = NS, n = 8) or after clopidogrel (15 ± 5 vs. $11 \pm 4\%$, P = NS, n = 8) [Figure 4c, d].

Inhibition of P2Y₁₂ receptors evaluated by VASP-phosphorylation

PRI (%) as assessed by the VASP kit was similar at 33°C compared to 37°C at baseline (71 ± 3 vs. $65 \pm 7\%$, P = NS, n = 8) and after a 600 mg loading dose of clopidogrel (30 ± 7 vs. $27 \pm 5\%$, P = NS, n = 8) [Figure 5a, b]. PRI (%) VASP was also similar at 33°C compared to 37°C with ADP and 10 μ M AZD6140 added *ex vivo* [Figure 5c].

Dose response for ADP-stimulated platelet aggregation evaluated by LTA

To further evaluate the mechanism of increased sensitivity to ADP during hypothermia, we performed a narrow dose response curve for ADP-induced platelet aggregation in a new subset of healthy individuals. There was no significant increase in the plateau phase of MPA at 33°C com-

pared to 37°C, 72.0% (95% confidence interval (CI) 66.9–77.2) vs 77.8%, CI 66.9–88.6), P = NS, n = 7) [Figure 6]. However, there was leftward shift of the dose response curve indicating increased potency for ADP during hypothermia ($EC_{50} = 0.68$ μ M (confidence interval 0.41–0.93)) compared to normothermia ($EC_{50} = 1.38$ μ M (confidence interval 1.06–1.70)). MPA was significantly higher during hypothermia for 0.5, 1.0, and 1.5 μ M of ADP.

Discussion

We examined platelet reactivity before and after clopidogrel treatment at 37°C and at 33°C, a temperature used for the treatment of patients resuscitated after cardiac arrest. Mild hypothermia did not attenuate platelet aggregation. Instead, collagen, 5-HT, epinephrine, the thromboxane analogue U46619 and ADP-induced platelet aggregation was unaltered or even increased by hypothermia. Dose-response curves revealed increased potency of ADP during hypothermia with no change in efficacy (maximum effect) compared with normothermia. Furthermore, the inhibitory effect of clopidogrel was attenuated. Similar attenuation of effect on ADP-induced aggregation was also seen after inhibition with the P2Y₁ antagonist MRS2500. The decreased responsiveness to clopidogrel during hypothermia could be overcome by addition of the P2Y₁₂ antagonist AZD6140.

Dual antiplatelet therapy with aspirin and clopidogrel is recommended treatment for patients with acute coronary syndromes and after stent implantation [3]. A more rapid onset and higher level of platelet inhibition can be obtained with a 600 mg loading dose of clopidogrel, a dose recently endorsed for acute coronary syndromes to obtain better protection during percutaneous coronary interventions. A large number of patients with cardiac arrest have an acute coronary syndrome; some of them are treated with direct-PCI and stenting because of ST-elevation myocardial infarction, and therefore receive clopidogrel treatment. Despite this, the effect of clopidogrel has to our knowledge never been examined during mild hypothermia.

There is a general belief that mild hypothermia attenuates platelet aggregation [6,12], and it was somewhat surprising to find that collagen, 5-HT, epinephrine, the thromboxane analogue U46619 and ADP-stimulated platelet aggregation was unaltered or even increased by mild hypothermia. Most of the previous studies have been performed at lower temperatures, eg, 28°, 22°, or even 2°C [6,7,13]. Our interest was in the growing use of mild hypothermia (33–35°C) for the treatment of cardiac arrest, and perhaps in the future to reduce infarct size in patients with acute myocardial infarction. One study demonstrated a reduction in ADP-stimulated platelet

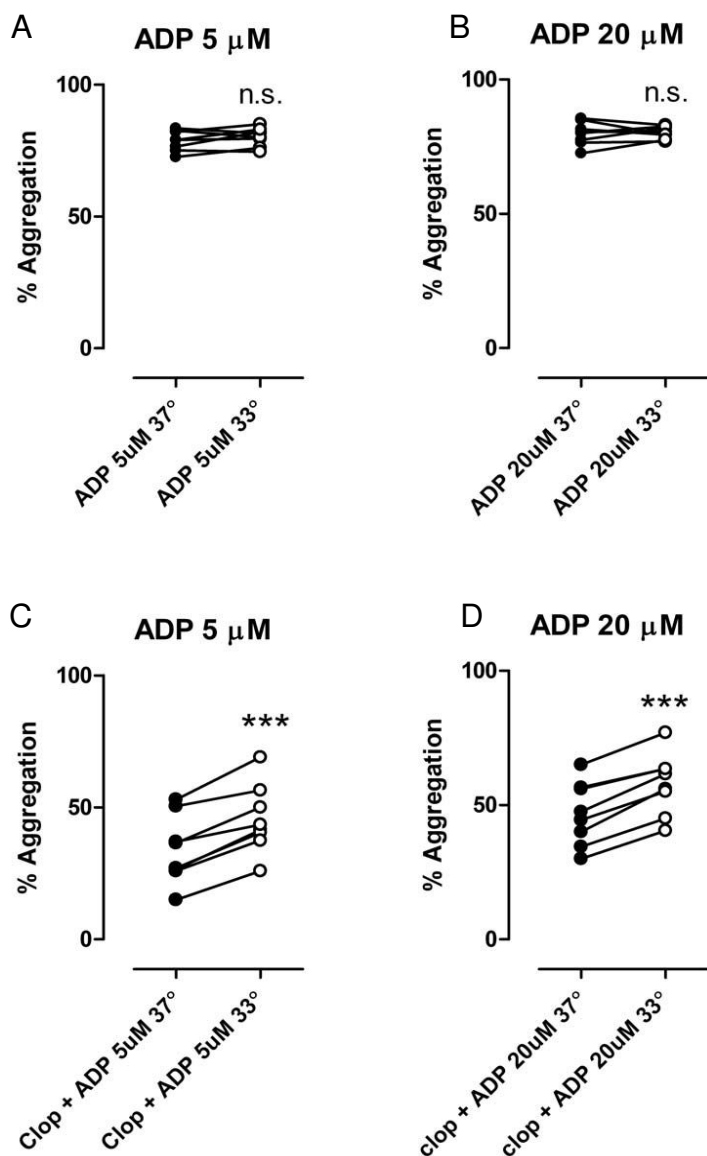
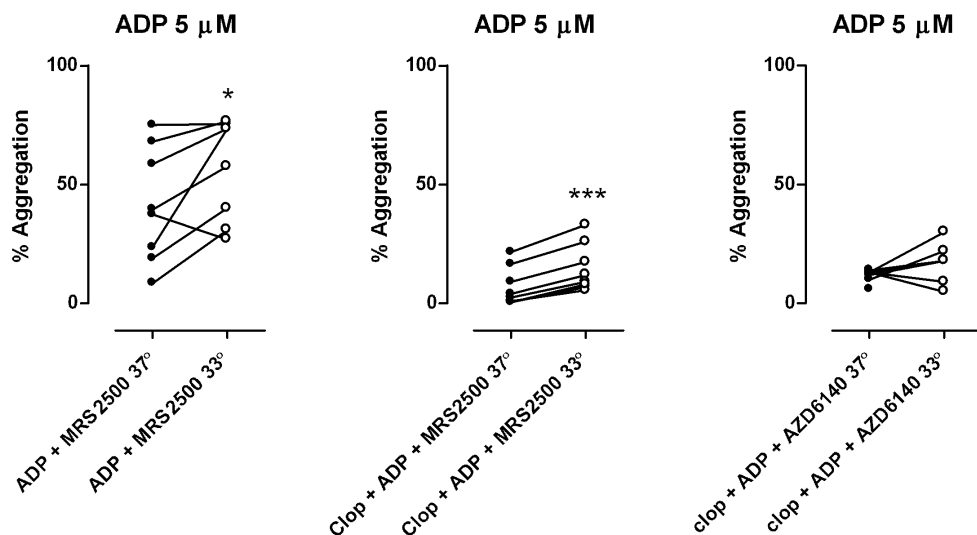


Figure 1
ADP-stimulated platelet aggregation at 5 and 20 μM [a, b] and after treatment with clopidogrel (600 mg po [c, d]. n = 8. filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant, *** = P < 0.001.

**Figure 2**

ADP (5 μ M)-stimulated platelet aggregation after pretreatment with MRS2500 (1 μ M) *ex vivo* [a], after pretreatment with clopidogrel (600 mg) *po* and MRS2500 *ex vivo* [b], and after pretreatment with clopidogrel (600 mg) *po* and AZD6140 (10 μ M) *ex vivo* [c]. n = 8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant, * = P < 0.05, *** = P < 0.001.

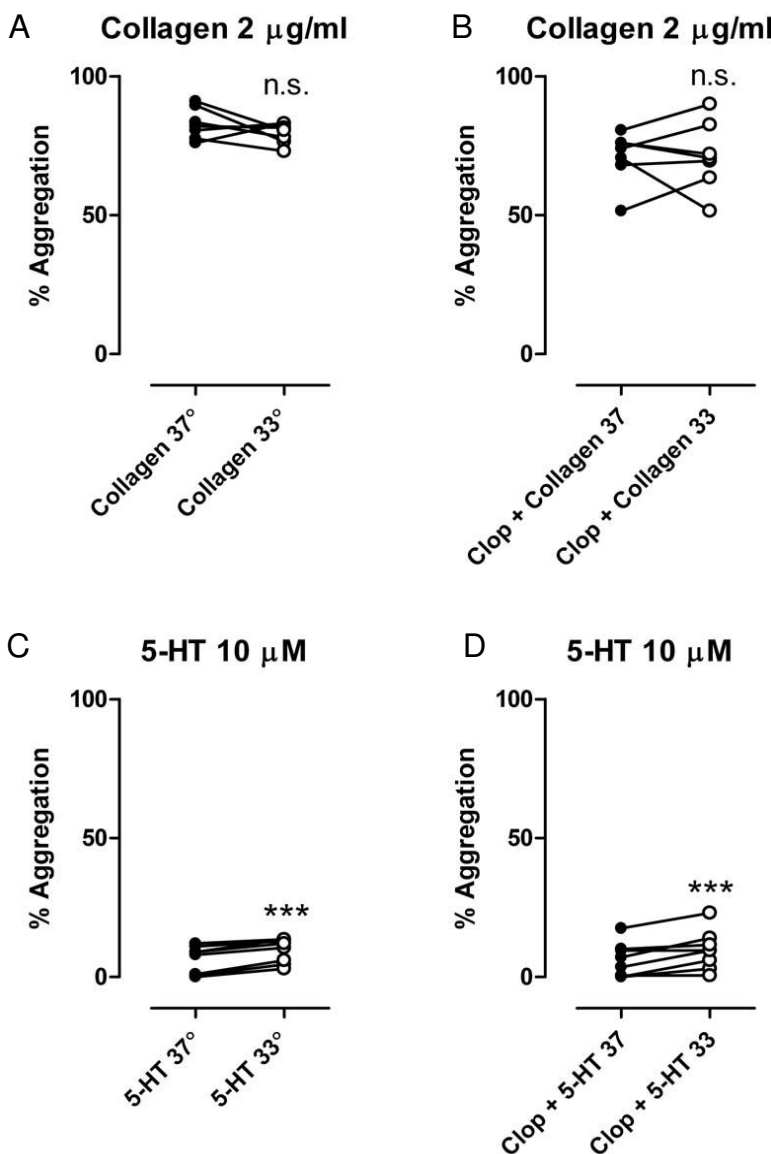
aggregation by 16% at 32°C compared to 37°C [5]. In contrast, a recent study by Heptinstall et al. demonstrated increased ADP-stimulated platelet aggregation at 28°C [9]. Lindenblatt et al [8] found increased *ex vivo* stimulated GpIIb/IIIa expression after TRAP stimulation at 34°C compared to normothermia, as well as accelerated thrombus formation *in vivo* in mice at 34°C. Our findings are also in agreement with Scharbert and co-workers [10], who found that platelet aggregation was increased with ADP, but unaltered with collagen during hypothermia. Further, shear stress-induced aggregation has been shown to increase at 32°C and 35°C [11].

In the present study, we found an attenuated effect of clopidogrel on ADP-stimulated platelet aggregation during mild hypothermia. The effect was highly significant and consistent at both doses of ADP used. This is in agreement with previous studies that have shown increased ADP responses during hypothermia in the presence of aspirin [9,10]. However, mild hypothermia has also been shown to augment the inhibitory effect of the reversible GpIIb/IIIa-blockers eptifibatide and tirofiban (but not that of the irreversible blocker abciximab) [5]. It could be hypothesized that hypothermia affects different classes of

platelet inhibitors differently. Such information is of course important in the selection of adequate platelet inhibition for patients treated with mild hypothermia.

Since the first report of a variable response to clopidogrel [14], a large number of studies have found a high prevalence of patients with pharmacodynamic poor responsiveness to clopidogrel [15]. Our data indicate that hypothermia induces a situation in which the effect of clopidogrel is reduced. The decreased responsiveness to clopidogrel during hypothermia could be overcome by addition of the reversible P2Y₁₂ antagonist AZD6140.

ADP activates two receptors on the platelet: the Gi-coupled P2Y₁₂ receptor coupled to inhibition of cAMP; and the Gq-coupled P2Y₁ receptor, which stimulates IP₃ and increases intracellular Ca²⁺. We tried to elucidate the mechanisms of the platelet effects induced by hypothermia by testing whether they were P2Y₁₂-specific. We added the selective P2Y₁-receptor antagonist MRS2500. Even in this situation, increased ADP sensitivity was seen during mild hypothermia. The effect was even more pronounced when MRS2500 was combined with clopidogrel.

**Figure 3**

Platelet aggregation stimulated by collagen (2 $\mu\text{g/ml}$) [a, b] and by 5-HT (10 μM) [c, d] before and after pre-treatment with clopidogrel (600 mg) *po*. n = 8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant, *** = $p < 0.001$.

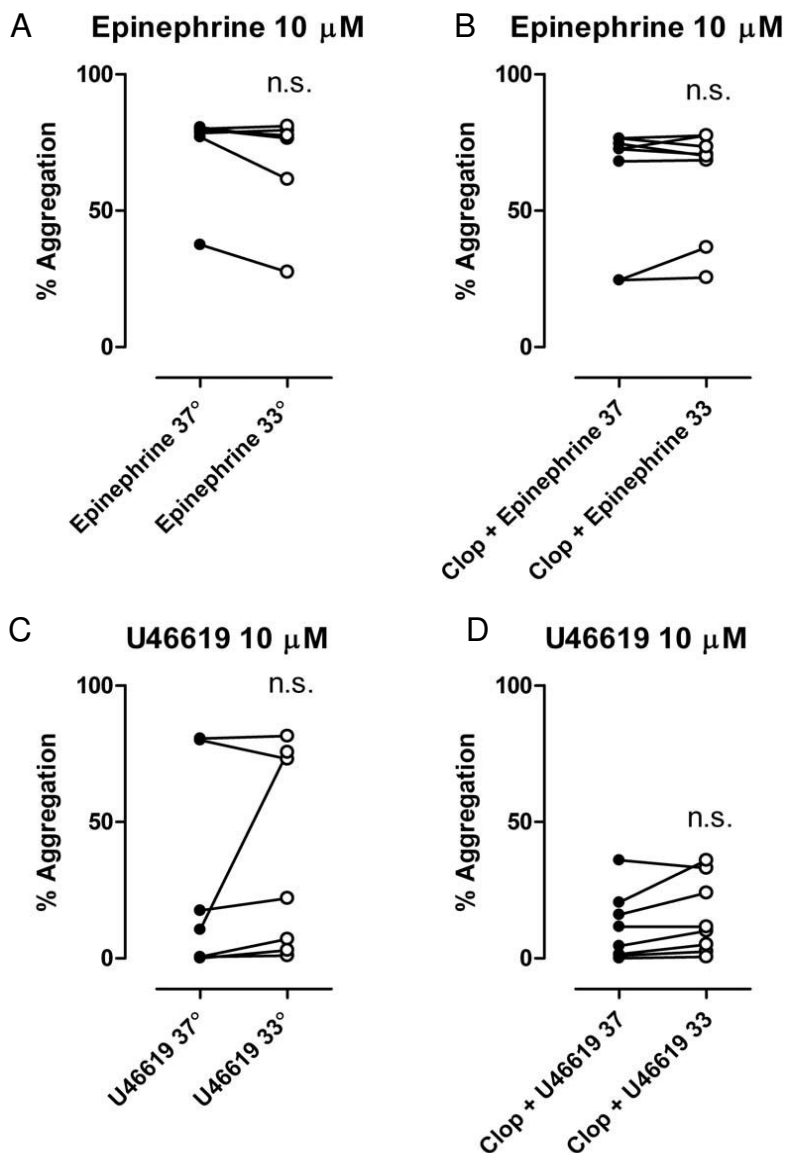
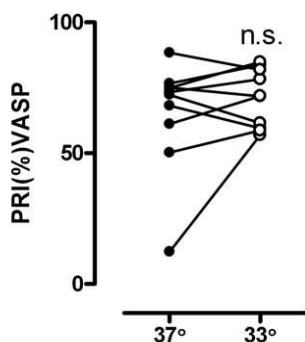
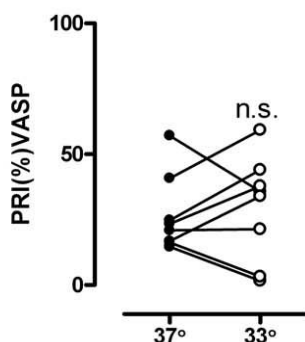
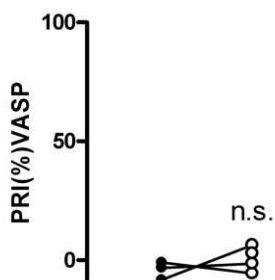


Figure 4
Platelet aggregation stimulated by epinephrine (10 μ M) [a, b] and U46619 (10 μ M) [c, d] before and after pre-treatment with clopidogrel (600 mg po). N = 6–8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant.

A Baseline ADP stimulation**B ADP after clopidogrel p.o.****C ADP and AZD6140 ex vivo****Figure 5****Figure 5**

Flow cytometry study in which phosphorylation of VASP (and hence inhibition of platelet activation) is stimulated by PGE₁ and dephosphorylation (and hence augmentation of platelet activation) is mediated by ADP. Platelet activation is represented as platelet reactivity index (%PRI). ADP-stimulated platelet aggregation at baseline [a], after pretreatment with clopidogrel *po* [b], and after pretreatment with AZD6140 (10 μ M) *ex vivo* [c]. n = 8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant.

Thus, the increased platelet stimulatory effect of ADP can be seen regardless of which ADP receptor is blocked.

ADP activation of the P2Y₁₂ receptor results in reduction of cAMP. VASP is an intracellular actin regulatory protein [16,17] that is phosphorylated into its P-VASP form by cAMP dependent protein kinases. ADP, the natural agonist to the P2Y₁₂ receptor, inhibits VASP phosphorylation through inhibition of adenylate cyclase and downregulation of cAMP production. We examined if the mechanism of the platelet effects of hypothermia are mediated at the level of VASP-phosphorylation. However, we saw no effect of hypothermia on VASP-phosphorylation when whole blood was stimulated by ADP alone or in combination with clopidogrel or AZD6140.

The mechanism of the increased ADP-induced platelet aggregation during hypothermia observed in our study remains elusive. It does not seem to depend on antagonist-receptor interaction or intracellular second messenger systems. Another possible explanation would be altered degradation rates of ADP. However, we repeated the experiments with the stable ADP analogue 2-MeSADP and obtained similar results (data not shown). To understand the receptor pharmacology during hypothermia, we assessed dose-response curves for ADP. Platelets at 33°C displayed an increased sensitivity to ADP and epinephrine compared to 37°C, with a leftward shift of the dose-response curve and a significantly lower EC₅₀ value for platelets at 33°C. Thus, it was only possible to see a difference in effect when doses in the sigmoidal part of the curve were tested, with the maximum effect being unaltered compared with normothermia. This observation may explain some of the conflicting data in the literature. It also indicates that it is important to take the concentration of the agonist into consideration when studying hypothermia and platelets.

Haemostasis during hypothermia is complex, and it is possible that platelet responses differ at different temperatures. We found unaltered or even increased platelet

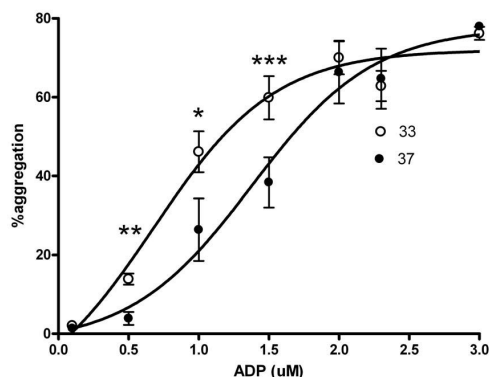


Figure 6
ADP dose response curve for platelet aggregation at normothermia and hypothermia. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$.

responses during mild hypothermia. The literature contains conflicting data, but in support of our findings, increased platelet activation has been seen during profound hypothermia [13] and increased aggregation has been reported at a more intermediate temperature of 28°C [9]. However, prolongation of bleeding time has been reported in clinical situations with hypothermia [18-20]. It is possible that this primarily reflects effects of hypothermia on the coagulation system, especially a decrease in the fibrinolysis-inhibiting α_2 -macroglobulin levels [21]. On the other hand, unintentional perioperative hypothermia is associated with postoperative myocardial ischemia, indicating a prothrombotic effect of hypothermia, and platelet count and platelet activity have been shown to be increased in this setting [12,21].

Conclusion

In conclusion, our study indicates that platelet reactivity is unaltered and in some situations increased during mild hypothermia. The inhibitory effect of clopidogrel was attenuated in our study. The clinical conclusion is that we cannot rely on hypothermia per se as a platelet inhibitor. Based on current *ex vivo* evidence, dual platelet inhibition with aspirin and clopidogrel is probably needed for patients with acute coronary syndromes treated with mild hypothermia. Since hypothermia induces a state of reduced clopidogrel responsiveness, it is possible that new reversible ADP blockers such as AZD6140 could be beneficial. Clinical studies are needed to determine the best use of platelet and coagulation inhibitors for the growing number of patients treated with mild hypothermia.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CH carried out the platelet function studies, analyzed and participated in writing the manuscript. DE and OB designed the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Paper II

The reversible oral P2Y₁₂ antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature

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Abstract

Objectives: The platelet ADP P2Y₁₂ receptor which is a target for the antithrombotic drug clopidogrel is also distributed on vascular smooth muscle cells and stimulate contraction. This study investigates whether AZD6140, in contrast to clopidogrel, can inhibit ADP-mediated arterial contractions.

Methods: Mice were treated with clopidogrel, 50 mg/kg, 24 and 2 h before experiment. Thoracic aorta ring segments from both clopidogrel-treated ($n=5$) and untreated ($n=4$) mice were mounted in myograph baths. Contractions of human left internal mammary arteries (IMA) and small arteries were studied in an identical manner.

Results: Clopidogrel treatment per os did not inhibit contractions by the stable ADP analogue 2-MeSADP (10 μ M). However, addition of 1 μ M AZD6140 in vitro inhibited ADP contraction (% of maximal contraction by 60 mM K⁺) both in the clopidogrel-treated, from 64% to 32% ($P=0.002$) and in the untreated group, from 59% to 33% ($P=0.015$). 2-MeSADP contractions in human IMA and small arteries were inhibited by AZD6140.

Conclusions: The antiplatelet drug AZD6140 blocks the contractile effects of ADP in both murine and human vasculature. These effects of AZD6140 could be beneficial in the management of conditions in which vasospasm may play a role.

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Keywords: Vasoconstriction; Receptors; Platelets

1. Introduction

The platelet P2Y₁₂ receptor is an established target for inhibiting ADP-mediated platelet aggregation, thus preventing thrombosis. The thienopyridine ticlopidine has been shown to prevent stent thrombosis, and the second-generation thienopyridine clopidogrel to reduce the incidence of myocardial infarction in patients with acute coronary syndromes (ACS) (CURE) and mortality in patients with ST-elevation myocardial infarction (COMMIT) [1,2]. The third-generation thienopyridine prasugrel is associated with significantly reduced rates of ischaemic events, including stent thrombosis in ACS patients with scheduled percuta-

neous coronary intervention, compared to clopidogrel (TRITON) [3].

The thienopyridines ticlopidine, clopidogrel, and prasugrel are prodrugs with bioactive metabolites that reduce platelet aggregation by irreversibly binding to the P2Y₁₂ receptor [4]. Irreversible binding to the P2Y₁₂ receptor is a problem in patients in need of acute coronary bypass surgery, other invasive procedures, or when bleeding complications occur. To regain P2Y₁₂ receptor function, platelets must be regenerated either by transfusion or through the natural platelet life cycle of approximately 10 days.

AZD6140 is a new, orally active cyclopentyl-triazolopyrimidine (CPTP) that does not require metabolic activation and inhibits platelet aggregation via reversible binding to the P2Y₁₂ receptor. It is currently being studied for its potential to prevent thrombotic events in patients with ACS [5]. In clinical studies to date, AZD6140 showed greater and

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more consistent inhibition of platelet aggregation than clopidogrel, with a similar incidence of total bleeding events [6]. Due to its reversible antagonistic effect, discontinuation of AZD6140 results in rapid return of ADP P2Y₁₂-receptor function as drug plasma levels fall [7].

P2Y₁₂ receptors are also found on vascular smooth muscle cells (VSMC), where they mediate arterial contraction after stimulation by ADP [8]. However, the contractions are not inhibited in blood vessels obtained from patients treated with clopidogrel [8], perhaps because the agent's active metabolite reaches low systemic concentrations and has a short half-life [9]. Furthermore, even the clopidogrel effects on platelets are variable between patients and clopidogrel "resistance" is common [9]. We therefore wanted to repeat the experiments in an animal model where we could maximize the clopidogrel dose to see if inhibition of ADP contractions could be achieved.

A short peak of high systemic levels of the active metabolite could result in an irreversible blockade of the P2Y₁₂ receptors on the VSMC, but the effect is not expected to be as permanent as in platelets, since VSMC constantly synthesize new P2Y₁₂ receptors. In contrast to clopidogrel, AZD6140 reaches continuous high systemic concentrations and has the potential to block vascular P2Y₁₂ receptors. It could therefore have beneficial effects not only by inhibiting platelet aggregation, but also by preventing vasospasm.

The aim of this study was: (1) To evaluate if clopidogrel administered in high doses (1000-fold higher per kg compared to regular clinical doses) could inhibit P2Y₁₂-receptor mediated contractions in a mouse model. (2) To evaluate the effect of AZD6140 on P2Y₁₂-mediated contraction in mice in the presence and absence of orally administered clopidogrel. (3) To examine if AZD6140 could inhibit ADP-mediated contraction in human internal mammary arteries and small human arteries.

2. Methods

2.1. Mouse experiments

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Five of 9 female mice were pretreated with clopidogrel 50 mg/kg the day before and 2 h before sacrifice and tissue harvest; 4 mice received no clopidogrel treatment. Thoracic aorta sections taken from all mice were dissected from connective tissue and denuded by gently rubbing the intimal surface with a wooden stick. Ring segments (7 or 8 per mouse) were mounted into temperature-controlled tissue baths with Krebs buffer (mM): NaCl 119, NaHCO₃ 15, KCl 4.6, MgCl₂ 1.2, NaH₂PO₄ 1.2, CaCl₂ 1.5, and glucose 5.5.

2.2. Human blood vessels

The investigation conforms to the principles outlined in the Declaration of Helsinki [10]. Internal mammary artery

segments and pericardial fat arteries were obtained from 9 patients undergoing coronary bypass surgery (mean age=72 years), 7 were men and 3 had non-insulin-dependent diabetes mellitus. The blood vessels were put in cold buffer solution (see below) and transported to the laboratory where they were dissected free of adhering tissue under a microscope. The vessels were immediately used for experiments. The internal mammary arteries had a relaxed inner diameter of approximately 1 mm while the pericardial fat arteries had a relaxed inner diameter of approximately 0.3–0.5 mm. The endothelial layer of the internal mammary arteries was removed by gently rubbing the intimal surface with a wooden stick before mounting it in the myograph. The endothelium of the pericardial fat arteries was removed by perfusion with TritonX (0.1%, 10 s), followed by perfusion with buffer solution. This was confirmed by demonstrating a lack of dilatory effects of 1 μ M acetylcholine.

2.3. Myograph experiments

The pharmacological responses were analyzed using tissue baths for measurement of isometric tension. The experimental setting has previously been described [11,12]. Mouse aorta and the small human arteries were precontracted to 1 mN and human internal mammary arteries to 2 mN. The isometric tension in each segment was continuously recorded, and the maximum contractile capacity of the segments was determined by exposure to 60 mM K⁺. AZD6140 1 μ M or dimethyl sulfoxide (DMSO) 1:1000 as control was added. After 20 min, segments were slightly precontracted with 10 nM norepinephrine (NE) and then further contracted with 10 μ M of the stable ADP molecule 2-methylthio-ADP (2-MeSADP). Results are presented as percent of K⁺ induced contraction. To study the response to 2-MeSADP without interference of simultaneous P2X₁-receptor-induced responses, all vessels were desensitized with 10 μ M α , β -methylene ATP (α , β -MeATP) for 10 min as described earlier [13].

The experiments were conducted as follows: first, the potassium-induced contraction was tested (and found to be similar between all compared groups). After 1.5 h of resting, the antagonist (AZD6140) or control saline solution with DMSO 1:1000 was added. AZD6140 did not have any effect on resting tone in any of the examined blood vessels. The arteries were then stimulated with 1 μ M of α , β -MeATP, which stimulates the ATP receptor P2X₁, an ion-channel receptor that desensitizes very quickly. Within 5 min the contraction was back at baseline. Norepinephrine was then added to produce a small precontraction. After 10 min, when the NE contraction had stabilised, 2-MeSADP was added. The 2-MeSADP-induced contractions were calculated without including any remaining NE contraction.

2.4. Drugs

α , β -MeATP, 2-MeSADP, and NE were purchased from Sigma Co., USA. Clopidogrel was purchased from Sequoia

Research Products, Ltd., Oxford, UK. AZD6140 was provided by AstraZeneca, Mölndal, Sweden. All drugs were dissolved in 0.9% saline, except AZD6140 and its control saline solution, which were dissolved in DMSO 1:1000. PCR consumables were purchased from Life

Technologies, Inc., USA, or Perkin–Elmer Applied Biosystems Inc, Foster City, CA, USA.

2.5. Ethics

The Ethics Committee of Lund University approved the project. All patients submitted written consent to participate in the study. The study conforms with US National Institutes of Health guidelines, and the Declaration of Helsinki.

2.6. Calculation and statistics

Calculations and statistics were performed using Graph-Pad Prism 4.0 software. Contraction was calculated as the percentage of the contraction with 60 mM K^+ . Statistical significance was accepted when $P < 0.05$, using Student's *t*-test analysing the 2-MeSADP data. Values are presented as means \pm SEM. *n* = number of vessel segments.

3. Results

3.1. The effect of AZD6140 on mouse aorta

10 μ M 2-MeSADP induced prominent contractions in mouse aorta of $59 \pm 7\%$ (measured as % of maximal contraction induced by 60 mM K^+) from otherwise untreated mice. The contractions were significantly reduced by 1 μ M of the P2Y₁₂ antagonist AZD6140 to $33 \pm 6\%$ ($P = 0.015$ vs. clopidogrel, $n = 9$ vessel segments per group from 4 resp 5 mice, Fig. 1A). From mice treated with oral clopidogrel, the 2-MeSADP-induced contractions in mouse aorta were similar to those from untreated mice ($P = n.s.$, $n = 5$ vessel segments per group from 4 resp 5 mice, Fig. 1B). Even in aorta from mice pretreated with oral clopidogrel, did 1 μ M of the P2Y₁₂ antagonist AZD6140 significantly inhibit the contractions induced by 2-MeSADP: $64 \pm 8\%$ vs. $32 \pm 6\%$ ($P = 0.002$, $n = 17$ vessel segments per group from 4 resp 5 mice, Fig. 1C).

3.2. The effect of AZD6140 on human internal mammary artery

10 μ M 2-MeSADP induced prominent contractions in human internal mammary artery, as shown in an earlier study [8]. The contractions were significantly reduced by 1 μ M of



Fig. 1. (A) AZD6140 1 μ M significantly inhibits the contractile response in mouse aorta to a single dose of 2-MeSADP 10 μ M. DMSO 1:1000 was added in control ($n = 9$ vessel segments per group from 4 resp. 5 mice, see "Methods"). (B) Oral clopidogrel 50 mg/kg did not have any effect on contractile response in mouse aorta to a single dose of 2-MeSADP 10 μ M ($n = 5$ vessel segments per group, from 5 resp. 4 mice) (C) AZD6140 1 μ M significantly inhibits the contractile response in mouse aorta to a single dose of 2-MeSADP 10 μ M even in the presence of oral clopidogrel treatment 50 mg/kg. ($n = 17$ vessel segments per group from 5 resp. 4 mice) The contractions are expressed as percentage of an initial contraction induced by 60 mM K^+ . Data are shown as means \pm SEM, * $P < 0.015$, ** $P = 0.002$. The vessels segments were taken from 5 clopidogrel-treated and 4 untreated mice.

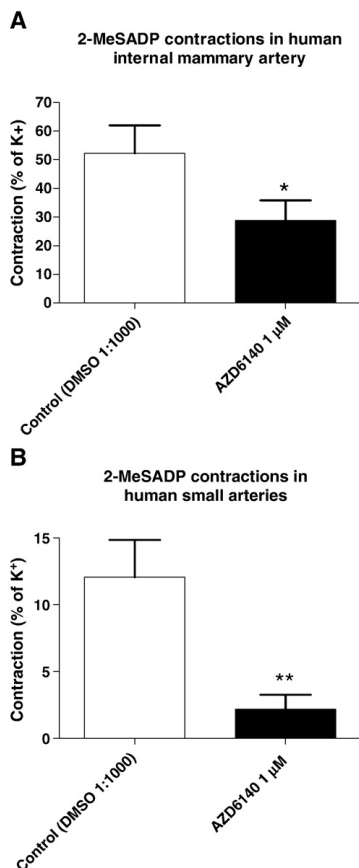


Fig. 2. AZD6140 inhibits ADP-induced contractions in both (A) human internal mammary arteries ($n=15$ vessel segments per group from 7 patients) and (B) small arteries ($n=10$ vessel segments per group from 7 patients). Contractile response in human internal mammary artery and small arteries to a single dose of $10 \mu\text{M}$ 2-MeSADP (see “Methods”). The contractions are expressed as percentage of an initial contraction induced by 60 mM K^+ . Data are shown as means \pm SEM. * $P<0.05$, ** $P<0.01$.

AZD6140, from $52 \pm 10\%$ to $29 \pm 7\%$ ($P<0.05$, $n=15$ vessel segments per group from 7 patients, Fig. 2A).

3.3. The effect of AZD6140 on human small pericardial fat arteries

2-MeSADP induced contractions in human small pericardial fat arteries. The contractions were significantly reduced by $1 \mu\text{M}$ of AZD6140, from $12 \pm 3\%$ to $2 \pm 1\%$ ($P<0.01$, $n=10$ vessel segments per group from 7 patients, Fig. 2B).

3.4. The effect of AZD6140 on contractions by other receptor agonists

$10 \mu\text{M}$ norepinephrine was added to produce a precontraction, which gave a maximum contraction of $30 \pm 5\%$ in the internal mammary artery and $10 \pm 3\%$ in pericardial fat arteries. AZD6140 $1 \mu\text{M}$ did not have any effect on the NE-induced contractions in either internal mammary artery or pericardial fat arteries (Fig. 3). Similarly, AZD6140 $1 \mu\text{M}$ did not have any effect on the $\alpha\beta\text{-MeATP}$ -induced contractions in either internal mammary artery or pericardial fat arteries (Fig. 4). A similar lack of effect of AZD6140 on NE and $\alpha\beta\text{-MeATP}$ stimulated contractions were found in mouse aorta (data not shown).

4. Discussion

We have previously shown that the ADP P2Y_{12} receptor mediates vasoconstriction in human internal mammary artery and subcutaneous vein [8]. Here we demonstrate ADP-stimulated, P2Y_{12} -mediated contractions in the mouse aorta. High doses of oral clopidogrel treatment had no inhibitory effect, while AZD6140 added *ex vivo* inhibited P2Y_{12} -mediated vasoconstriction in murine vessels regardless of clopidogrel

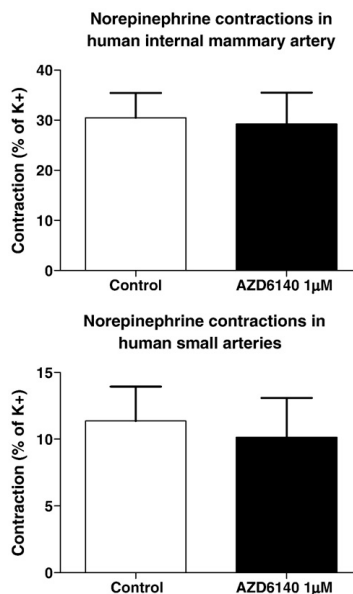


Fig. 3. Examination of specificity of AZD6140: contractile response in (A) human internal mammary artery ($n=15$ per group) and (B) small arteries ($n=11$ per group) to 10 nM norepinephrine (NE). There was no effect of AZD6140 on NE-induced vasoconstriction in either of the arteries. The contractions are expressed as percentage of an initial contraction induced by 60 mM K^+ . Data are shown as means \pm SEM.

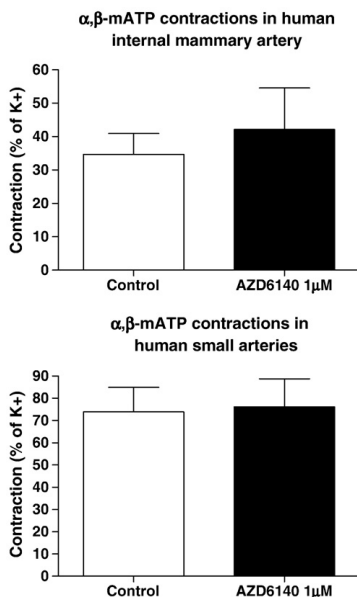


Fig. 4. Examination of specificity of AZD6140. Contractile response in (A) human internal mammary artery ($n=8$ per group) and (B) small arteries ($n=11$ per group) to 1 μ M AZD6140. There was no effect of AZD6140 on $\alpha\beta$ -MeATP-induced $P2Y_{12}$ -mediated vasoconstriction in either of the arteries. The contractions are expressed as percentage of an initial contraction induced by 60 mM K⁺. Data are shown as means \pm SEM.

pretreatment. Furthermore, AZD6140 blocked ADP-induced vasoconstrictions in both large and small human arteries.

The finding of an effect of ADP both on platelet aggregation and on arterial contraction is not surprising. Several other platelet aggregation factors are also stimulators of vasoconstriction, e.g., epinephrine, thromboxane, and serotonin. The combined effect could be an evolutionary adaptation important in stopping bleeding. Previously, a contractile effect of ADP acting on $P2Y_{12}$ receptors in arteries had only been demonstrated in human blood vessels [8]. Here we demonstrate $P2Y_{12}$ -mediated contractions in another species, the mouse. Thus, $P2Y_{12}$ -mediated vasoconstriction may be a general phenomenon.

The competitive $P2Y_{12}$ antagonist AZD6140 significantly inhibited the contractions in mouse aorta, demonstrating a potential new physiological effect of the drug, in addition to its inhibitory effects on platelet aggregation. As demonstrated in human blood vessels [8], oral clopidogrel treatment did not block $P2Y_{12}$ -receptor-mediated vasoconstriction. If the active metabolite of clopidogrel reaches the artery, it should remain irreversibly bound to the receptor during the short time between removal of the aorta and the contraction experiments. However, even in the present study, high oral clopidogrel doses given to mice did not result in an

inhibitory effect in murine vasculature (Fig. 2B). This is probably due to low systemic concentrations of the active metabolite of clopidogrel [9].

Clopidogrel could not be added *in vitro* because it is a prodrug. AZD6140 per os was not tested because it is a reversible drug and is expected to be washed away during dissection. AZD6140 blocked ADP-induced vasoconstriction mediated by $P2Y_{12}$ receptors in denuded mouse aortic rings regardless of oral pretreatment with clopidogrel. The effect was similar as in the absence of clopidogrel (Fig. 2).

In large arteries of patients with ACS, the high concentrations of ADP released in a platelet thrombus could contribute to the vasospasm often seen in a culprit lesion. The vasospasm worsens the ischemia caused by the thrombus. We therefore examined the effect of AZD6140 on the left internal mammary artery. AZD6140 significantly inhibited the ADP-mediated contractions in human left internal mammary artery. Since AZD6140 reaches therapeutic serum concentration *in vivo*, it is possible that it may have a dual anti-ischemic effect in patients by inhibiting both thrombus formation and vasospasm, although any clinically significant benefit would need to be validated in a prospective clinical trial.

We also examined the effect of ADP in small arteries, approximately 0.3–0.5 mm in internal diameter, which are involved in blood flow regulation. The inhibitory effects of AZD6140 were even more prominent in these smaller arteries, indicating a possible role in regulating tissue perfusion. The net effect of ADP is a balance between its vasodilatory effects via endothelial activation and contractile effects on the smooth muscle cells. The contractile effects of ADP have previously been masked by the vasodilatory effect of ADP mediated via activation of $P2Y_1$ receptors on the endothelium [14,15], and endothelial denudation was a prerequisite in our experiments to reveal the contractile effects. ADP mediated vasoconstriction is most likely of importance in situations of endothelial damage, such as in a ruptured plaque.

AZD6140 did not have any effect on NE-induced contractions (α_1 -receptors) or contractions mediated by the ATP receptor $P2X_1$ in either murine or human vessels, indicating a specific effect on $P2Y_{12}$ receptors. This is in agreement with previous studies in platelets [5].

Interestingly, nicotine has been shown to prominently upregulate $P2Y_{12}$ receptors in human VSMC [16]. This nicotinic upregulation of $P2Y_{12}$ is mediated by the nicotinic acetylcholine receptors. Studies in platelets have shown that the functional and active $P2Y_{12}$ receptor is organised as homooligomeric complexes [17], represented in cholesterol dependent microdomains called lipid rafts found in the cell membranes [18]. We speculate that high cholesterol and nicotine may have an upregulating effect on the $P2Y_{12}$ receptor, and hence the risk of atherothrombosis.

In conclusion, clopidogrel does not block ADP mediated contraction, not even when given in high doses. AZD6140 inhibits ADP-mediated vascular contraction both in murine

and human vasculature. The effects of AZD6140 could be beneficial in the management of conditions in which vaso-spasms may play a role.

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Conflict of interest

The authors received an institutional research grant from AstraZeneca to conduct this research.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [19].

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Paper III

Succinate independently stimulates full platelet activation via cAMP and PI3 β kinase signaling

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Summary. *Background:* The citric cycle intermediate succinate has recently been identified as ligand for the G-protein coupled receptor (GPCR) SUCNR1. We have previously found that this receptor is one of the most expressed GPCRs in human platelets. *Objective:* The aim of this study was to investigate the role of SUCNR1 in platelet aggregation and to explore the signalling pathways of this receptor in platelets. *Methods and Results:* Using RT-PCR, we could demonstrate that SUCNR1 is expressed in human platelets at a level corresponding to that of the P2Y₁ receptor. Light transmission aggregation experiments showed a dose-dependent aggregation induced by succinate reaching a maximum response at 0.5mM. The effect of succinate on platelet aggregation was confirmed with flow cytometry showing increased surface expression of activated GPIIb/IIIa, and P-selectin. Intracellular SUCNR1 signalling was found to result in decreased cAMP levels, Akt phosphorylation mediated by PI3K β activation and receptor desensitisation. Further, succinate-induced platelet aggregation was demonstrated to depend on Src, generation of thromboxane A₂ and ATP release. The platelet SUCNR1 is subject to desensitization through both homologous and heterologous mechanisms. In addition, the P2Y₁₂ receptor inhibitor ticagrelor completely prevented platelet aggregation induced by succinate. *Conclusions:* Our experiments show that succinate induces full aggregation of human platelets via SUCNR1. Succinate-induced platelet aggregation depends on thromboxane A₂ generation, ATP release and P2Y₁₂ activation.

Keywords: Succinate, SUCNR1, GPR91, platelet, PI3-Kinase, GPCR

Introduction

The dicarboxyl acid succinate is known to be a physiologically important intermediate in the citric acid cycle where it plays a crucial role in mitochondrial energy production [1]. In recent years it has become clear that succinate also functions as ligand for the membrane receptor SUCNR1 [2]. The

transcript for the human SUCNR1 receptor encodes a 330-amino acid protein that belongs to the genetic superfamily of G-protein coupled receptors (GPCR) [3]. SUCNR1 has been identified and quantified in many important organs throughout the human body particularly high levels of SUCNR1 are present in the spleen, liver, testis and kidneys [4-7]. When we

examined mRNA expression of GPCRs in platelets using microarray and realtime-PCR, the succinate receptor displayed the third highest expression of all GPCRs examined [7], indicating an important role in platelet regulation.

More than two decades ago Huang *et al.* [8] discovered that succinate had a potentiating effect on platelet activating substances like adenosine 5'-diphosphate (ADP), epinephrine and serotonin. Huang *et al.* [8] also concluded that high concentrations, 5 - 10 mM of succinate could induce primary platelet aggregation. The potentiating effect of succinate on ADP has also been described by Macaulay *et al.* [6]. Succinate is normally present systemically in concentrations that range between 1 – 20 μ M [9], but during pathophysiological conditions like ischemia and metabolic stress, plasma levels of succinate have been reported to reach millimolar concentrations [10-12]

Succinate, is a multi-functional molecule with a physiological potential that has not yet been fully evaluated. The aim of this study is to characterize the platelet succinate receptor SUCNR1 signaling pathways and to determine the role of this novel receptor in the aggregation process. This involves investigating regulation and function of SUCNR1 in signaling pathways with known importance platelet function. Our experiments demonstrate that succinate alone is capable of mediating full platelet aggregation by decreasing cAMP levels and activating the PI3K β /Akt pathway leading to P-selectin and GPIIb/IIIa activation. Furthermore, SUCNR1 is dependent on Src kinase signaling and is subject to homologous as well as heterologous desensitization.

Methods and material

Chemicals

Sodium succinate dibasic hexahydrate, 2'-Deoxy-N6-methyladenosine 3', 5'-bisphosphate tetrasodium salt (MRS2179), Ethylenediaminetetraacetic acid disodium salt solution (EDTA), prostaglandin E1 (PGE1), Apyras, Forskolin, prostaglandin I₂ (PGI₂), Rhodamine-6G and Src-Inhibitor-1 were bought from SigmaAldrich, USA. 3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione (IBMX), and trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y27632) were purchased from Tocris Bioscience. 5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione (AS-604850) and 7-methyl-2-(4-morpholinyl)-9-[1-(phenylamino)ethyl]-4H-pyrido[1,2-a]pyrimidin-4-one (TGX221) were purchased from Larodan Fine Chemicals AB, Malmö, Sweden. [³⁵S]GTP γ S were bought from Perkin Elmer Inc., USA. Ticagrelor (AZD6140) was a gift from AstraZeneca R&D Mölndal, Mölndal, Sweden.

Platelet preparation for Light Transmission Aggregation (LTA)

Whole blood was drawn from healthy volunteers through venous puncture (Vacutainer[®] tubes, 0.129 M Na-Citrate, BD, NJ, USA). Whole blood was centrifuged (140 rcf, 20 min, RT) and the supernatant containing platelet rich plasma (PRP) was collected. As a control, platelet poor plasma (PPP) was used and prepared from whole blood through centrifugation (2260 rcf, 10 min,

RT). Each PRP sample contained 250 μ l. The LTA measurements were performed using two serially connected Chronolog aggregometers, model 490 (Chronolog, PI, USA). Antagonists were pre-incubated with PRP for 5 minutes prior to addition to succinate. Aggregation values from LTA experiments are presented as percent of the mean of maximum platelet aggregation (MPA %). Washed platelets were prepared according to the protocol by Cazenave *et al.* [13] before aggregation studies done as described for the PRP.

Platelet qRT-PCR

(See supplement and reference [7].)

Western blotting on human platelet for SUCNR1.

(See supplementary material.)

Confocal immunofluorescence microscopy on platelets

(See supplementary material.)

Calciumflux assay on platelets

Ca²⁺-release, mediated through succinate stimulation, was measured as described earlier by Liu *et al.* [14], with one change in the protocol for the P2Y₁ assay: 0.3 U/ml apyrase was added to the assay buffer to exclude indirect signaling from P2Y₁. Fluorescence reading was done using a Victor3 1420 Multilabel Counter (Perkin Elmer, USA).

cAMP assay on platelets

(See supplementary material.)

Flow cytometric analysis of platelet activation

(See supplementary material.)

Platelet degranulation – ATP release

PRP was prepared as in the LTA-measurement and was incubated at 37°C in 10 min (1,200 rpm) before addition of the ligand. The released ATP was determined by luminometry in a GloMax[®] TD-20/20 luminometer (Promega, CA, USA), using the ATP SL kit (BioThema, Sweden).

Western blot assay for PKBa /Akt1 determination

(See supplement and reference [15].)

Platelet [³⁵S]GTPγS binding

PRP was prepared from ACD-treated whole blood through centrifugation (150 rcf, 20 min, RT) and passed carefully through a Pall Autostop[™] Leukocyte removal filter (Pall Medical, NY, USA). The filtered PRP was centrifuged (17,000 rcf, 15 min, RT). The pellet was resuspended in a roomtemperature membrane-buffer (20mM HEPES, 20mM EDTA, 150mM NaCl, pH 7.4, RT) and the centrifugation was repeated once. The pellet was resuspended into 3 mL ice-cold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4) homogenized for 30 seconds in a Polytron (setting 6), and diluted to 30 ml with ice-cold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4). The platelet homogenate was pelleted twice (35,000 rcf, 15 min, 4°C). The final pellet was resuspended in ice-cold membrane buffer (20mM HEPES,

0.6mM EDTA, 5mM MgCl₂, 0.1mM PMSF, pH 7.4) and stored at -80°C. Protein concentration was measured with a modified Lowry protein assay Kit (Thermo Scientific, USA).

The binding assay was performed essentially as described by Vasiljev *et al* [16]. Briefly: The final volume of the assay was 200µl and contained 20 µg platelet membranes in binding buffer (20mM HEPES, 100mM NaCl, 5mM MgCl₂, 10µg/mL saponin, pH 7.4 + 4nM [³⁵S]GTPγS, 20µM guanosine diphosphate (GDP)). The mixture was incubated (30 min, 30°C) and then filtered through a glass fiber filter (GF/C, Whatman, UK). The filters were washed with 3 x 500µl ice-cold binding buffer and then counted using OptiPhase Hisafe 3 (Perkin Elmer, USA) in a LS 6500 Multi-Purpose Scintillation Counter (Beckman-Coulter, USA).

Statistical analysis

Calculations and statistics were performed using GraphPad Prism 4.0 software. One-way analysis of variance (ANOVA) was used followed by a Bonferroni correction between groups if there was statistical significance. Student's *t* - test was used to compare means between two groups. Statistical significance was accepted when *P* < 0.05. Values are presented as means ± SEM, *n* = number of experiments.

Ethics

The project was approved by the Ethics Committee of Lund University, Sweden. All participants submitted written consent to take part in the study. The study conforms to US National Institutes of

Health guidelines, and the Declaration of Helsinki.

Results

Quantification of SUCNR1 in human platelets

The succinate receptor SUCNR1 is expressed in human platelet at approximately the same level as P2Y₁, (1.28. vs. 1.00, *n*=6). The expression level of P2Y₁₂ was about 12-times higher than the expression level of SUCNR1 (*n*=6) while the expression of A_{2a} was approximately 10-times lower (*n*=6) (Fig. 1a). The expression of SUCNR1 on human platelets was confirmed with western blot (Fig. 1b) and confocal immunofluorescence microscopy (Fig. 1c). The platelet expression of SUCNR1 was further evaluated using flow cytometry (21.3 % of platelets positive, data not shown) while CHO cells, a cell line previously used to characterize the SUCNR1 receptor, displayed negligible levels of SUCNR1 antibody binding (0.97 % positive, data not shown). CHO cells stained with the SUCNR1 antibody did not display any fluorescence as evaluated with confocal microscopy (see *supplementary material*).

Succinate stimulates platelet aggregation

Huang *et al.* [8] has previously demonstrated that succinate potentiates agonist-induced platelet aggregation. Using LTA, we tested whether succinate alone is sufficient to activate platelets. Our results clearly demonstrate that succinate independently activates platelets and cause platelet aggregation

in a dose-dependent manner. A typical succinate aggregation displays a biphasic aggregation kinetic. The effect of succinate is half-maximal at 0.3mM and maximal at 0.5mM. To exclude possible disturbances in the serum, we isolated washed platelets and repeated the experiment. This resulted in a displacement of the dose-response curve to the right (Fig. 2a). We could also confirm the potentiating effect of succinate on ADP mediated platelet activation that has been described earlier (Fig. 2b). The effect of succinate on platelet activation was further characterized by demonstrating that both the platelet activation marker for GPIIb/IIIa, PAC-1, and the degranulation marker, P-selectin, were activated (% positive cells) (23.0 ± 3.3 vs. 6.9 ± 2.0 , $^{**}=P<0.001$, $n=16$ and 7.6 ± 1.5 vs. 2.7 ± 0.5 , $^{*}=P<0.05$, $n=16$) in response to 3mM succinate (Fig. 3a and Fig. 3b). ADP was added as a positive marker both for PAC-1 binding, (58.5 ± 7.0 vs. 6.9 ± 2.0 , $^{***}=P<0.01$, $n=16$) and for P-selectin, (29.1 ± 5.1 vs. 2.7 ± 2.0 , $^{***}=P<0.001$, $n=16$).

Activation of SUCNR1 inhibits cAMP production in platelets but has no effect on calcium signaling

As the succinate receptor has been reported to be coupled to $G_{\alpha i}$ activation [9], and is thus expected to result in an attenuation of platelet cAMP production, we tested the effect of 3mM succinate on both forskolin and PGE1 stimulated cAMP production and also on the phosphorylation status of the cAMP/PKA marker VASP. We found that cAMP production in platelets pre-stimulated by forskolin was significantly reduced for platelets treated with

succinate ($2.6\mu\text{M} \pm 66\text{nM}$ vs. $1.0\mu\text{M} \pm 70\text{nM}$, $^{***}=P<0.001$, $n=4$). This reduction in cAMP also was seen in platelets pre-stimulated with PGE1 ($0.7\mu\text{M} \pm 30\text{nM}$ vs. $0.4\mu\text{M} \pm 7\text{nM}$, $^{***}=P<0.001$, $n=4$) (Fig. 3c). The decrease in cAMP production was confirmed in VASP phosphorylation (% positive cells) (75.4 ± 6.3 vs. 66.7 ± 7.0 , $^{**}=P=0.0025$, $n=8$) comparing to ADP (68.1 ± 7 vs. 23.1 ± 4.7 , $^{***}=P=0.0002$, $n=8$) (Fig. 3d).

Succinate (3mM) stimulation of platelets loaded with Fluo-4AM did not result in any change in Ca^{2+} mobilization compared to untreated platelets, ($5284 \text{ RFU} \pm 6.4 \text{ RFU}$ vs. $4963 \text{ RFU} \pm 6.2 \text{ RFU}$, ($n.s$)= $P>0.05$, $n=4$) (Fig. 3e). Thrombin (1U/mL) elicited a positive calcium response vs. control ($7563 \text{ RFU} \pm 185 \text{ RFU}$ vs. $4963 \text{ RFU} \pm 6.2 \text{ RFU}$, $^{***}=P>0.001$, $n=4$) thus demonstrating the functional integrity of the platelets. In order to avoid indirect ADP signaling, mediated through P2Y_1 or caused by ATP release, the experiment was conducted in the presence of apyrase. However, a minor difference could be detected between platelets stimulated with $10\mu\text{M}$ ADP vs. control ($5578 \text{ RFU} \pm 7.7 \text{ RFU}$ vs. $4963 \text{ RFU} \pm 6.2 \text{ RFU}$, $^{***}=P>0.001$, $n=4$).

Succinate stimulated aggregation is dependent on $\text{PI3K}\beta$ activation but not on the $G_{\alpha 12/13}$ pathway

As both the PI3K/Akt and the $G_{\alpha 12/13}$ pathway are involved in mediating platelet aggregation, we used the general phosphoinositide 3-kinase (PI3K) inhibitor wortmannin and the Rho-GEF/ROCK¹⁶⁰-inhibitor Y27632 to explore the involvement of these

pathways in succinate-stimulated aggregation. While 10 μ M of Y27632 had no effect, there was a significant decrease in succinate-stimulated aggregation after pre-incubating platelets with 10 μ M of wortmannin (75.7 ± 0.8 vs. 1.8 ± 0.7 , ***= $P < 0.0001$, $n=6$), indicating involvement of the PI3K/Akt pathway (Fig. 4a and Fig. 4b).

In order to identify which PI3K isoform is involved in succinate-induced platelet aggregation we employed TGX221 and AS-604850, which are specific inhibitors for PI3K β and PI3K γ respectively. 10 μ M MRS2179 was added in all experiments to avoid potential interference of the P2Y₁ receptor. Stimulating platelets with 3mM succinate in the presence of 1 μ M AS-604850 resulted in a small, but significant reduction in MPA (72.1 ± 1.1 vs. 77.1 ± 1.5 , *= $P < 0.05$, $n=8$). However, when AS-604850 was substituting for 1 μ M of the PI3K β specific TGX221 there was a 99 % reduction in MPA compared to the control (77.1 ± 1.5 vs. 0.6 ± 0.3 , ***= $P < 0.001$, $n=8$) (Fig. 4c). The involvement of PI3K β was confirmed by western blot analysis of Akt phosphorylation. Treatment of platelets with 3mM succinate resulted in Akt phosphorylation that was reversed when 1 μ M TGX221 was added (Fig. 4d).

The ability of succinate to achieve platelet aggregation in LTA was abolished in the presence of a Src-kinase inhibitor. A statistically significant difference was seen between platelets pre-incubated with 10 μ M Src inhibitor-1 vs. platelets stimulated with 3mM succinate only (74.7 ± 1.4 vs. 7.0 ± 1.2 , ***= $P < 0.0001$, $n=7$) (Fig. 4e).

Succinate-stimulated platelet aggregation is dependent on ATP release

Acetyl salicylic acid (ASA) inhibits COX-1 which converts arachidonic acid into thromboxane A₂ (TXA₂). Pretreatment with 1mM ASA abolished the stimulating effect of 3mM succinate in platelets (73.5 ± 5.2 vs. 6.9 ± 1.2 , *= $P < 0.05$, $n=14$) indicating a role for TXA₂ in succinate-mediated aggregation (Fig. 5a). Since the response of platelets to TXA₂ depends on ADP serving as a positive-feedback mediator, which is required for sustained activation, these results prompted us to investigate the effect of succinate on ATP release. We found that 3mM succinate elicited an approximately 1000-fold increase in ATP release ($0.3\text{nM} \pm 0.1\text{nM}$ vs. $0.4\mu\text{M} \pm 1.6\text{nM}$, ***= $P < 0.0001$, $n=6$), confirming the involvement of ATP release in succinate-mediated aggregation (Fig. 6). These results were further strengthened by demonstration that the pro-activating effect induced by 3mM succinate is absent in platelets pre-incubated with 10 μ M of the potent P2Y₁₂ antagonist ticagrelor (79.7 ± 0.9 vs. 4.0 ± 0.7 , *= $P < 0.05$, $n=12$) (Fig. 5b). Platelets pretreated with 10 μ M of the P2Y₁ antagonist MRS2179 did on the other hand not display any reduction in MPA (76.2 ± 1.0 vs. 74.4 ± 1.0 , (n.s) $P=0.25$, $n=14$), (Fig. 5c).

Succinate stimulates [³⁵S]GTP γ S binding to platelet cell membranes

Since demonstration of functional SUCNR1 receptors using standard radio-receptor binding techniques is unpractical due to the low affinity of succinate, we investigated the ability of

succinate to stimulate [35 S]GTP γ S binding in platelet plasma membranes. As shown in Fig. 7, increasing concentrations of succinate generated a dose-dependent binding of [35 S]GTP γ S with an EC $_{50}$ -value of 100 μ M (n=4). Desensitization of the receptor upon ligand stimulation is a well-studied negative regulation mechanism of GPCRs. In order to investigate if the platelet SUCNR1 is subject to homologous or to heterologous desensitization, we prepared plasma membranes from platelets that were pre-challenged with either 3mM succinate or 10 μ M 2MeSADP. The results show that in both cases, the ability of succinate to stimulate [35 S]GTP γ S binding was significantly reduced to 69.6% \pm 3.4%, (**=P<0.01, n=3) for homologous desensitization and 81.6% \pm 2.9%, (*=P<0.001, n=3) for heterologous desensitization (Fig. 7).

Discussion

In this study we identified succinate as an independent agonist of full platelet aggregation. Succinate-stimulated platelet aggregation involves activation of the platelet activation marker GPIIb/IIIa and the degranulation marker P-selectin. Succinate stimulation of platelets results in decreased cAMP levels and VASP activation but did not stimulate cytosolic calcium release. Furthermore, succinate-induced platelet aggregation depended on PI3K β /Akt1-induced signaling and on Src kinase but did not involve Rho-GEF or PI3K γ . The SUCNR1 is desensitized both homologously by succinate and heterologously by ADP. Finally, our data showed that succinate induces platelet aggregation via TXA $_2$ generation

and ATP/ADP release. Interestingly, we observed a complete abolishment of succinate-mediated platelet aggregation in the presence of the P2Y $_{12}$ receptor antagonist ticagrelor while the P2Y $_1$ antagonist MRS2179 had on the other hand no such effect.

The influence of the citric acid cycle metabolite succinate on platelets was first described by Huang *et al.* [8], who reported that succinate augments the actions of ADP and epinephrine and that this effect was related to attenuation of cAMP levels. In a previous study, using gene array screening, we identified the message of the recently deorphanized succinate receptor SUCNR1 in human platelet mRNA. In an effort to confirm these results we quantified the platelet message of SUCNR1. Interestingly, the level of SUCNR1 expression was the third highest expressed GPCR examined and equaled the levels of P2Y $_1$ receptor, a receptor with a demonstrated role in platelet activation, thus predicting functionality at the same level.

The succinate receptor is reported to be a low affinity receptor with an EC $_{50}$ value for succinate that, depending on the assay method, ranges from 28 μ M to 391 μ M. These results are well in line with our findings with an EC $_{50}$ of approximately 300 μ M based on LTA measurements and at 100 μ M for [35 S]GTP γ S binding. Succinate at 3mM activated the marker PAC-1, recognizing activated GPIIb/IIIa, and the degranulation marker P-selectin, thus supporting the picture of an independent platelet activator. Huang *et al.* [8] reported that succinate treatment resulted in a decrease in platelet cAMP levels. These results correspond well with our findings that show an inhibition of the

cAMP/PKA pathway resulting in decreased cAMP levels and, as a consequence, a decrease in PKA-mediated phosphorylation of the vasodilator-stimulating phosphoprotein (VASP). Phosphorylated VASP inhibits activation of the membrane bound integrin, GPIIb/IIIa [17] which is an important step for platelet-platelet adhesion, and hence for aggregation of platelets.

In contrast to the report by He *et al.* [4], we were unable to detect any effect on cytosolic Ca^{2+} mobilization. In this study SUCNR1 is described as a receptor capable of coupling not only to $G_{\alpha i}$, mediating inhibition of adenylate cyclase, but also to $G_{\alpha q}$ stimulating IP_3 turnover and Ca^{2+} release. However, only a part of the IP_3 signal could be attributed to $G_{\alpha q}$ activation as the other half was sensitive to pertussis toxin and thus likely caused by $G_{\beta\gamma}$ mediated activation of $\text{PLC}\beta$ [18]. Furthermore, in a recent report by Hakak *et al.* [9] where endogenous succinate receptors were studied in TF-1 cells, the pertussis toxin insensitive part of the IP_3 signal was absent. Those authors suggest that since the results by He *et al.* [4] were obtained in recombinant CHO cells, over-expressing SUCNR1, it is likely that the PTX-insensitive signal is an artifact, caused by promiscuous G-protein coupling. Our results support the view of SUCNR1 as a receptor primarily coupled to $G_{\alpha i}$ and we believe that the absence of a succinate stimulated Ca^{2+} signal in platelets can be explained by that the lack of $G_{\beta\gamma}$ -mediated activation of $\text{PLC}\beta$ in platelets. There are reports that support the absence of $G_{\beta\gamma}$ -mediated Ca^{2+} signaling in platelets: for example, it has been demonstrated that while stimulation of the P2Y_{12} receptor results

in $G_{\alpha i}$ -dependent activation of PI3K, it will not result in Ca^{2+} mobilization [19]. While it is currently not known which $G_{\beta\gamma}$ combinations are expressed in platelets it has been shown that the type of the G_{β} subunit determines whether PI3K or $\text{PLC}\beta$ activation is favored [20], [21].

Although we could detect no effect on calcium mobilization, we obtained a dose dependent increase in $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding upon stimulation with succinate, thus confirming the involvement of $G_{\alpha i}$. Furthermore, the importance of $G_{\beta\gamma}$ -mediated signaling was confirmed by a strong PI3 kinase activation resulting in Akt/PKB phosphorylation. The responsible isoform turned out to be $\text{PI3K}\beta$, which is somewhat surprising, as $\text{PI3K}\gamma$ is the isoform that usually is associated with stimulation by $G_{\beta\gamma}$. However, $\text{PI3K}\beta$ has previously been identified as the dominant PI3K isoform responsible for G_i mediated GPIIb/IIIa activation following ADP stimulation of the P2Y_{12} receptor [22]. The Src kinases have recently been reported by Nash *et al.* [23] to be involved in adrenergic G_i -coupled signaling in platelets. Nash *et al.* described that Src kinases acting downstream of the α_{2a} receptor mediate both the primary and secondary wave of aggregation. They also demonstrated that P2Y_{12} mediated aggregation is dependant on Src kinases and suggested this pathway to be common for G_i -coupled receptors.

This finding led us to investigate if Src kinases could be involved in SUCNR1 activation as well. Indeed, we found that platelet aggregation, induced by succinate, was nearly abolished, from approximately 75 to 7 % MPA, in the presence of a Src kinase inhibitor.

The importance of a positive feedback was demonstrated by the finding that succinate stimulated a massive ATP/ADP release and was confirmed both by the sensitivity to the P2Y₁₂ antagonist ticagrelor and by the increase of the vesicular-inner membrane bound protein P-selectin. Negative regulation of G-protein coupled receptors is triggered by phosphorylation leading to desensitisation and internalization of the receptor. Our results demonstrate that the platelet SUCNR1 is rapidly desensitized upon challenge with succinate. Previously Robben *et al.* [24] showed that SUCNR1 is similarly desensitized in Madin-Darby Canine Kidney cells.

Interestingly, the platelet SUCNR1 also seems to be subject to heterologous desensitization through activation of either P2Y₁₂ and/or P2Y₁. Hardy *et al* [25] have previously reported that the desensitization mechanism triggered by P2Y₁ and P2Y₁₂ differ. While P2Y₁₂ is desensitized through a homologous mechanism involving GRK phosphorylation and receptor internalization, P2Y₁ desensitization is to a great extent dependant on PKC which phosphorylate ligand-bound and inactive GPCRs in a heterologous manner [26]. Thus it seems likely that the observed cross-desensitization of SUCNR1 by 2MeSADP is caused by stimulation of P2Y₁ rather than P2Y₁₂.

When discussing the physiological implications of succinate-mediated platelet activation it is important to note that the succinate levels in serum generally are too low to permit activation of SUCNR1 [27]. However, under pathological conditions such as animal models of type 2 diabetes and during different forms of ischemia, succinate concentrations have been

reported to rise to well up to millimolar ranges [10, 11]. The potential importance of succinate/SUCNR1 in cardiovascular disease was highlighted in a recent publication showing that succinate, at concentrations equal to ischemic levels, modulate apoptosis in rat ventricular cardiomyocytes Aguiar *et al.* [28] and that this effect is mediated by SUCNR1. Moreover, succinate has been suggested as a myocardial marker of ischemia/reperfusion injuries by Kakinuma *et al.* [29]. Thus, succinate could be important during myocardial infarction, both via apoptotic effects on the cardiomyocyte and by causing platelet aggregation and thereby epicardial vessel occlusion or microembolism. Since succinate is ubiquitous in all cell types, the physiological role could potentially be very important. However, to prove this hypothesis, selective succinate antagonists are needed to perform in vivo experiments or even clinical studies.

As for many platelet activators, succinate activation is dependent on the positive feedback system thromboxane and ADP for full platelet aggregation. Succinate-induced platelet aggregation was inhibited by aspirin and interestingly, also by the potent P2Y₁₂ receptor antagonist ticagrelor. Ticagrelor was recently tested in the phase III study PLATO, where it was superior to clopidogrel and reduced both myocardial infarctions and mortality [30]. It is possible that some of the beneficial effects of aspirin and ticagrelor are due to an inhibition of succinate-induced platelet aggregation.

In conclusion, the succinate receptor is one of the most expressed receptors on

human platelets. It can stimulate platelet aggregation by itself and increase P-selectin and GPIIb/IIIa expression. The effects are mediated via the cAMP-PKA-VASP pathway, but also depend on Src kinase activation and PI3K β /Akt1-induced signaling. Succinate-induced platelet activation can be prevented with aspirin or ticagrelor, and this observation may explain some of their beneficial effects. It is possible that selective succinate receptor blockers could have further effects in preventing thrombosis and could have a place in the treatment of cardiovascular disease.

Addendum

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Supplementary material

Platelet qRT-PCR

Platelet extraction for RNA isolation, cDNA synthesis and qRT-PCR, followed the methodology described by Amisten *et al.* [7]. cDNA was synthesized using Fermentas RevertAidTM H MinUS First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturers instructions. qReal-Time PCR was performed in a LightCycler[®] (Roche, Switzerland) using Maxima TM SYBR Green qPCR (x2) mix (Fermentas). Primer sequences used for RT-PCR are listed in table 1. The data was normalized to β -actin and P2Y₁, essentially as previously described by Amisten *et al.* [7].

Western blotting on human platelet for SUCNR1.

Protein was extracted from isolated human platelets and chinese hamster ovary (CHO) cells with SDS sample buffer (62.5 mmol/l Tris-HCl, 2% SDS, and 10% glycerol). Caki-1 cells in loading buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Equal amounts of protein were separated by SDS-PAGE on 12.5% Tris-HCl gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat dry milk and subsequently incubated with primary antibody against SUCNR1 (goat polyclonal; 1:1000 dilution in 5% milk),

and secondary horse peroxidase-conjugated antibody (1:10,000 dilution in 5% milk). Both antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bands were detected using chemiluminescence (Supersignal West Femto, Pierce Biotechnology, Rockford, IL, USA).

Confocal immunofluorescence microscopy on platelets

Platelets were isolated as previously described by Liu *et al.* [14] and fixed in 0.75% paraformaldehyde for 15 minutes and subsequently washed in phosphate buffered saline (PBS). After 2 hours blocking in room temperature with 2% bovine serum albumin (BSA), platelets were incubated over night at 4°C with primary goat polyclonal anti-SUCNR1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:25. Several washing steps were performed and platelets were incubated for 2 hours at room temperature with a secondary donkey anti-goat Alexa 488 antibody diluted 1:500. Finally platelets were incubated with rhodamine-6G (0.02 g/dl) for 20 minutes. The platelets were let to adhere to coverslips over night in a humid chamber and were subsequently mounted with Polyscience Mounting Media (Aqua Poly Mount, Polyscience Inc.). Control of unspecific staining was performed by omitting the primary or secondary antibody from the protocol. Confocal images were acquired with a Zeiss LSM 510 laser scanning confocal microscope using a 100X objective.

Confocal immunofluorescence microscopy on CHO cells

CHO cells cultured on coverslips were fixed in 4% formaldehyde in PBS and blocked with 2% BSA in PBS. Primary antibody, goat anti-GPR91 (1:25, Santa Cruz Biotechnology) and secondary antibody, Cy5-anti-goat IgG (1:1000, Abcam) were used at excitation-emission wavelengths of 633 and >650 nm, respectively. The fluorescent nucleic acid dye SYTOX Green (1:3,000, Molecular Probes) was used for nuclear identification. Cells were examined at x40 in a Zeiss LSM 5 Pascal laser scanning confocal microscope. Result from CHO study is presented in Fig 1 supplementary material.

cAMP assay on platelets

PRP prepared as described earlier was incubated in the presence of 1mM acetylic salicylic acid (ASA), 0.3U/mL apyrase (15 min, RT). After incubation, the PRP was further incubated with 5mM EDTA and centrifuged (700 rcf, 10 min RT). The supernatant was discarded and the PRP-pellet was resuspended in HEPES-Tyrod buffer (145mM NaCl, 5mM KCl, 0.5mM Na₂HPO₄, 1mM MgSO₄, 10mM HEPES, 5mM glucose, 0.3U/mL apyrase, pH 7.4), and pelleted again. The supernatant was removed and the pellet was resuspended to give 1.65×10^8 cells/mL in modified HEPES-Tyrod buffer (145mM NaCl, 3mM KCl, 0.5mM Na₂HPO₄, 1mM MgSO₄, 10mM HEPES, 5mM glucose, 0.6U/mL apyrase, 1mM IBMX - phosphatase inhibitor, pH 7.4). 0.2×10^8 platelets were incubated for 15 min at 37°C before addition of stimulants. After 10 minutes platelets were pelleted by centrifugation

at 800rcf for 5min. The platelets were lysed in 100 μ L of 0.1M HCl and pelleted again (1200 rpm, 5 min, 4°C). The supernatant was stored in -80°C until analysis. For measuring the stimulated platelets a Cyclic AMP EIA assay (Cayman Chemical, MI, USA) was used following the manufacturers instructions. The readout of the results from the cAMP assay was performed in a Victor3 spectrophotometer, 1420 Multilabel Counter, (Perkin Elmer, USA).

Flow cytometric analysis of platelet activation

Whole blood was collected into 0.129M Na-Citrate, (Vacutainer®, BD, CA, USA). Succinate stimulation (3mM) of platelet vasodilator-stimulated phosphoprotein (VASP) was measured using the PLT VASP/P2Y12-kit (Biocytex, Marseille, France). For P-selectin and GPIIb/IIIa activity, whole blood was incubated in HEPES-saline buffer (145mM NaCl, 5 mM KCl, 10mM HEPES pH 7.4) with fluorescently labelled monoclonal antibodies against P-selectin (CD62P) and GPIIb/IIIa (PAC-1) or corresponding isotype control antibodies (BD, CA, USA). To induce platelet activation, 3 mM of succinate and 0.1 μ M ADP was added. After 15 minutes in the dark at room temperature 0.2 % paraformaldehyde was added to fix the cells. All samples were diluted 1:20 in PBS and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, CA, USA). The platelet population was gated based on forward and side scattered light. A threshold for non-specific fluorescence was set using isotype control antibodies and all events

above that value were considered positive. A total of 3000 events were collected in the platelet gate from each sample.

Western blot assay for PKBa /Akt1 determination

Whole blood was collected in ACD-A Vacutainer® tubes as described earlier. The acidified whole blood was centrifuged to extract the PRP (230 rcf, 20 min, RT). The PRP fraction in the supernatant was aspirated into a fresh tube. The PRP was incubated (45 min, 37°C) with 1mM ASA. After the incubation the PRP was centrifuged (980 rcf, 10 min, RT) and the platelet rich pellet was collected and resuspended in modified Tyrodes-HEPES buffer; (138mM NaCl, 2.7mM KCl, 2mM MgCl₂, 0.42mM NaH₂PO₄, 5mM glucose, 10mM HEPES, 0.2% BSA, 0.1U/ml apyrase, pH 7.4). Cell count was set to 2*10⁸ cells per mL. Cell suspension was aliquot in concentrations of 1*10⁸ cells per sample, and pre-incubated in 37°C before addition of specific inhibitors and further stimuli. Following western blot procedure and analysis were performed as previously described by Tan *et al.* [15]

Figure 1. (A, B and C).

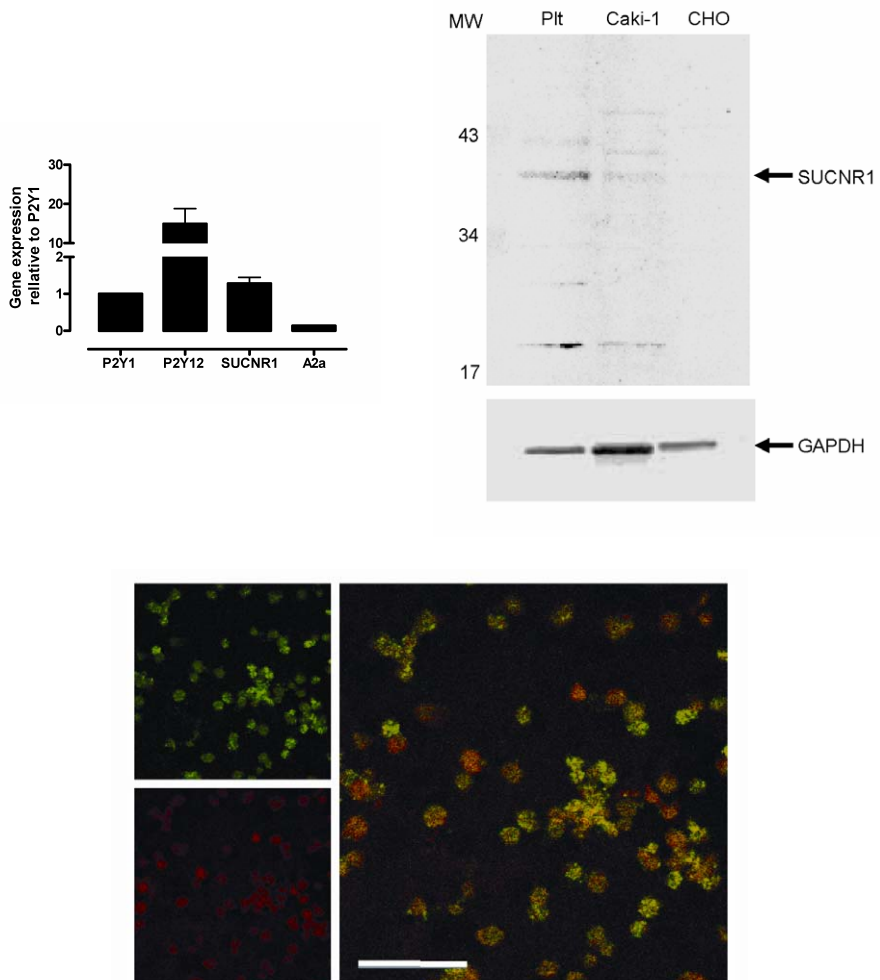


Fig. 1. (A) Upper left: Quantitative Real-Time PCR analysis of the SUCNR1, P2Y₁₂ and A_{2a} expression levels in platelets. P2Y₁ was used as reference gene. (B) Upper right: Expression of SUCNR1 protein in platelets (Plt). SUCNR1 protein (38 kDa) was detected in human platelets by Western blot. Caki-1 and CHO cells were used as positive and negative controls, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are representative for two experiments. (C) Lower centre: Representative confocal image of SUCNR1 protein expression (green) in human washed platelets stained with rhodamine-6G (red). Right panel; merged images. Bar 10 μ m.

Figure 2 (A and B).

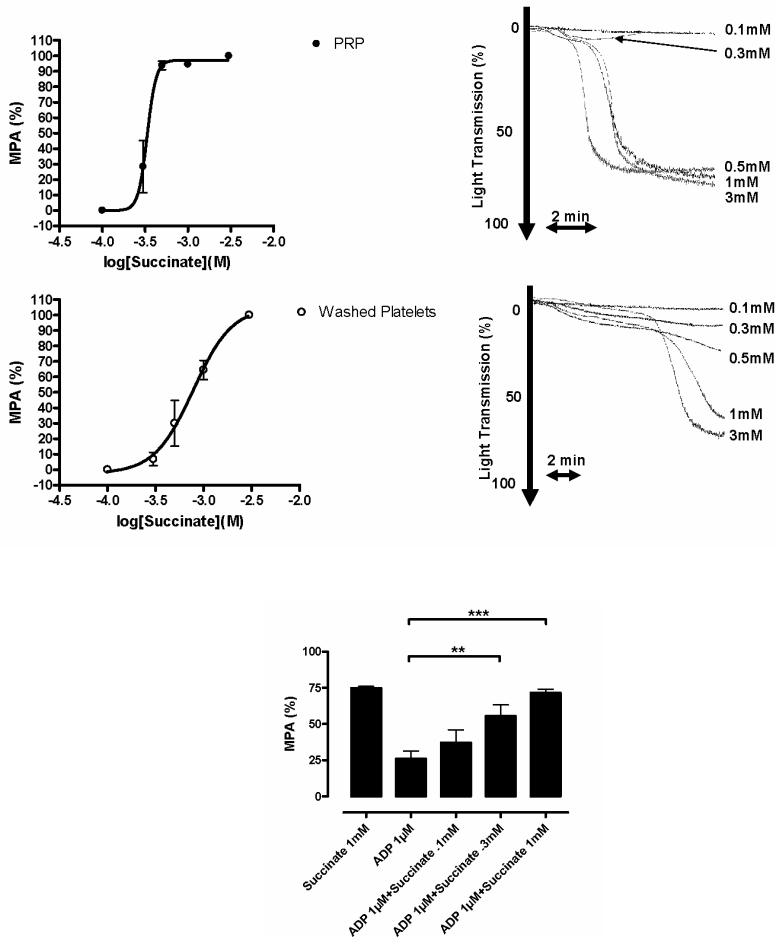


Fig. 2. (A) Upper left: Dose-response curve presenting platelet PRP aggregation mediated through elevated doses of succinate. Time for aggregation was 6 minutes; data were collected from six experiments. Upper right: Curve diagram with representative aggregation curves from each experiment. Lower left: Dose-response curve presenting aggregation of washed platelets mediated by elevated doses of succinate. Time for aggregation was delayed through the washing procedure, to 16 minutes; data were collected from six experiments. Lower right: Curve diagram illustrates representative aggregation curves from each experiment. **(B)** Lower bar graph: Potentiating effect of succinate on ADP-mediated platelet activation in washed platelets (ANOVA: **= $P < 0.05$, ***= $P < 0.001$, $n=5$).

Figure 3 (A, B and C).

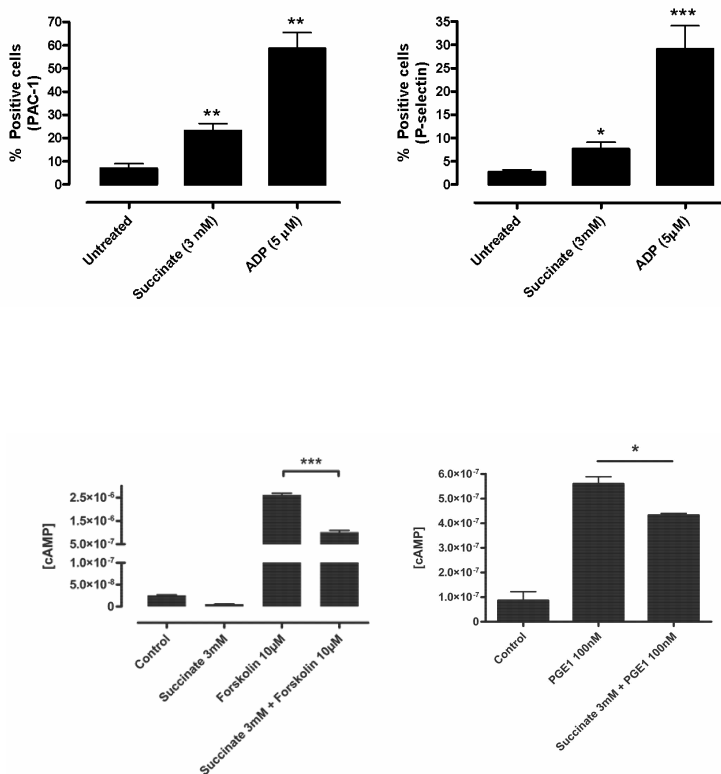


Fig. 3. (A) Results from a flow cytometry study on GPIIb/IIIa activation in platelets. Upper left: The PAC-1 antibody was used to detect platelet with activated GPIIb/IIIa. There was marked differences between un-stimulated platelets and platelets stimulated with 3mM succinate or 5μM ADP (ANOVA: **= $P < 0.01$, $n = 16$). (B) Upper right: Flow cytometry study on P-Selectin release from platelets. Vesicle secretion of α -granules mediated by succinate could be statistically confirmed between untreated platelets vs. platelets treated with 3mM succinate or 5μM ADP (ANOVA: *= $P < 0.05$, ***= $P < 0.001$, $n = 16$). (C) Lower centre: Effects of succinate on forskoline and PGE1 induced cAMP-production in platelets. 1×10^8 washed platelets were used per sample. Significant differences in cAMP production is observed between: platelets pretreated with 3mM succinate and 10μM forskolin vs. 10μM forskolin (ANOVA: ***= $P < 0.001$, $n = 4$) and platelets pretreated with 3mM succinate and 100nM PGE1 vs. 100nM PGE1 (ANOVA: *= $P < 0.05$, $n = 4$).

Figure 3 (D and E).

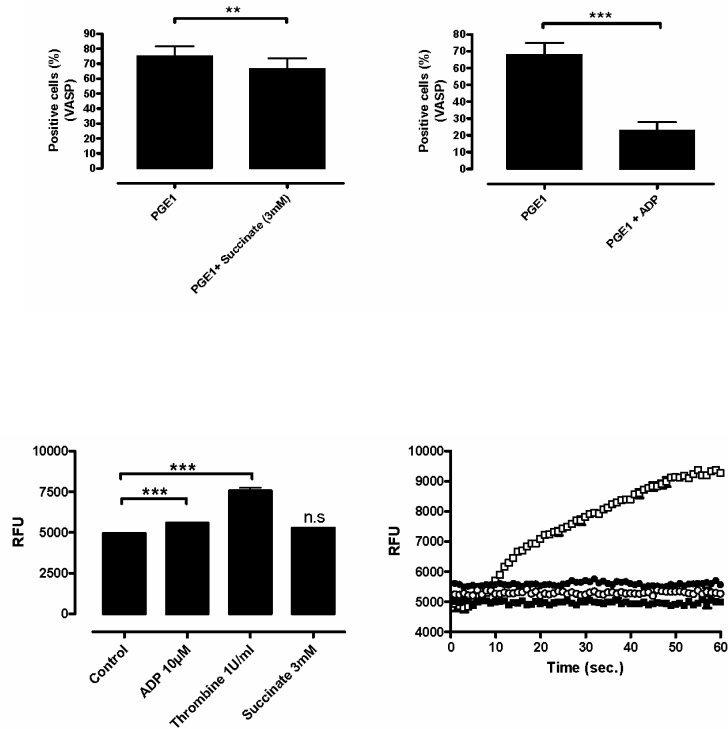


Fig 3. (D) Upper panel: Flow cytometry study on platelets measuring the ratio of phosphorylated/dephosphorylated vasodilator-stimulated phosphoprotein (VASP). Left bar graph represents the dephosphorylating effect of 3mM succinate on VASP on PGE1 stimulated platelets (Student's *t*-test: **= $P=0.0025$, $n=8$). Right bar graph represent the dephosphorylating effect of ADP on VASP on PGE1 stimulated platelets (Student's *t*-test: ***= $P=0.0002$, $n=8$). **(E)** Lower panel: Calcium signaling in platelets. Left bar graph: Data are presented as relative fluorescence units (RFU). No significant difference between platelets treated with succinate vs. control (ANOVA: (n.s)= $P>0.05$, $n=4$). Significance were seen between platelets stimulated with thrombin vs. control (ANOVA: ***= $P<0.001$, $n=4$). Difference were also seen in ADP vs. control (ANOVA: ***= $P<0.001$, $n=4$). Right graph illustrating peak values from experiments: (■) vehicle (control), (○) 3mM succinate, (●) 10μM ADP and (□) 1U/ml thrombin.

Figure 4 (A, B and C).

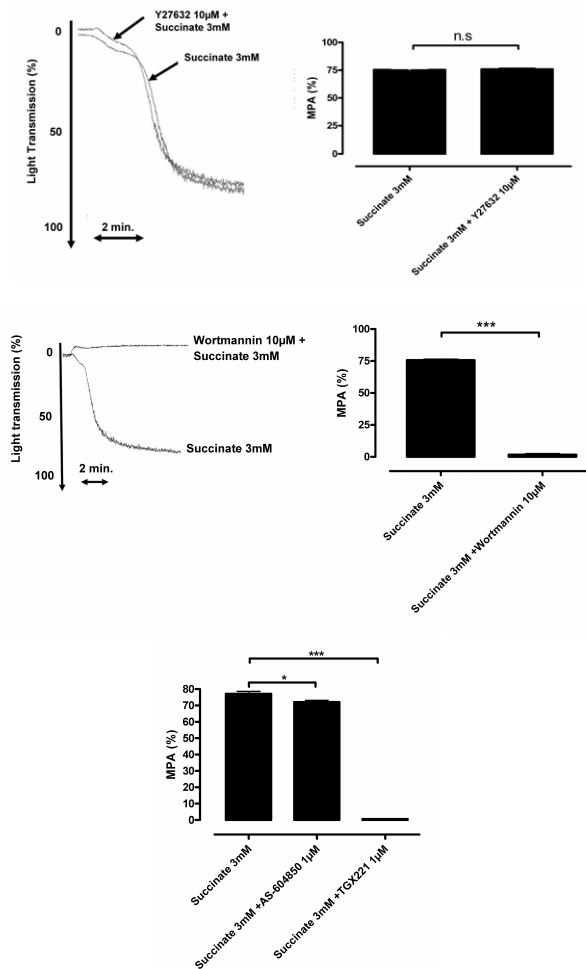


Fig. 4. (A) Upper panel: PRP pretreated with 10 μ M of the Rho-GEF/ROCK¹⁶⁰-inhibitor, Y27632 (blocking G_{12/13} -pathways) and stimulated with 3mM succinate vs. PRP stimulated with 3mM succinate only (Student's *t* - test: (n.s)=P=0.6, n=4). (B) Centre panel: PRP pretreated with 10 μ M wortmannin and stimulated with 3mM succinate vs PRP stimulated with 3mM succinate only, (Student's *t*-test: ***=P<0.001, n=6). The LTA-curves accompanying the bar charts represent the characteristic aggregation pattern from each of the experiments. (C) Lower panel: LTA on PRP pre-incubated with AS-604850 (PI3K γ -inhibitor) and TGX221 (PI3K β -inhibitor). Samples were further stimulated with 3mM succinate. Platelets pre-incubated with 1 μ M AS-604850 showed a moderate significance (ANOVA: *=P<0.05, n=8). However platelets pre-incubated with 1 μ M TGX221 displayed an augmenting statistical difference (ANOVA: ***=P<0.001, n=8).

Figure 4 (D and E).

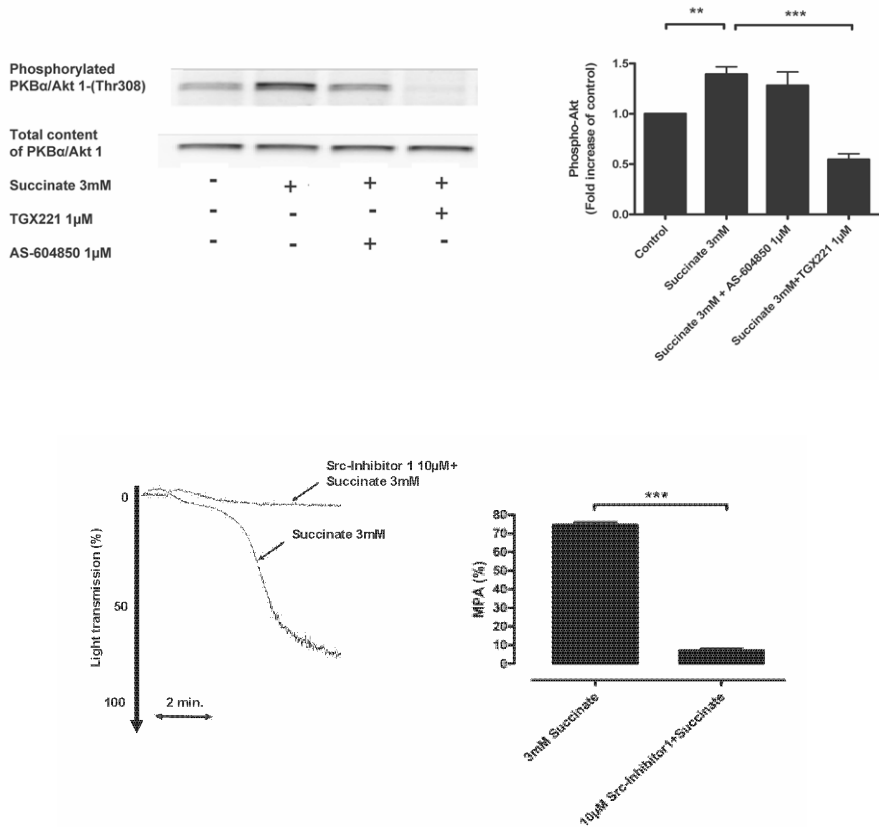


Fig 4. (D) Upper panel: Western blot (including bar chart) of PKBa/Akt 1 on washed platelets treated with 1μM of the PI3Kγ inhibitor AS-604850, or 1μM of the PI3Kβ inhibitor TGX221. All platelet samples were stimulated with 3mM succinate, except for un-stimulated platelets which represent the negative control. (ANOVA: ***=P<0.001, **=P<0.01, n=3). **(E)** Lower panel: Inhibition of Src-kinase by 10μM Src Inhibitor-1 on 3mM succinate stimulated PRP (Student's *t*-test: ***=P<0.0001, n=7).

Figure 5 (A, B and C).

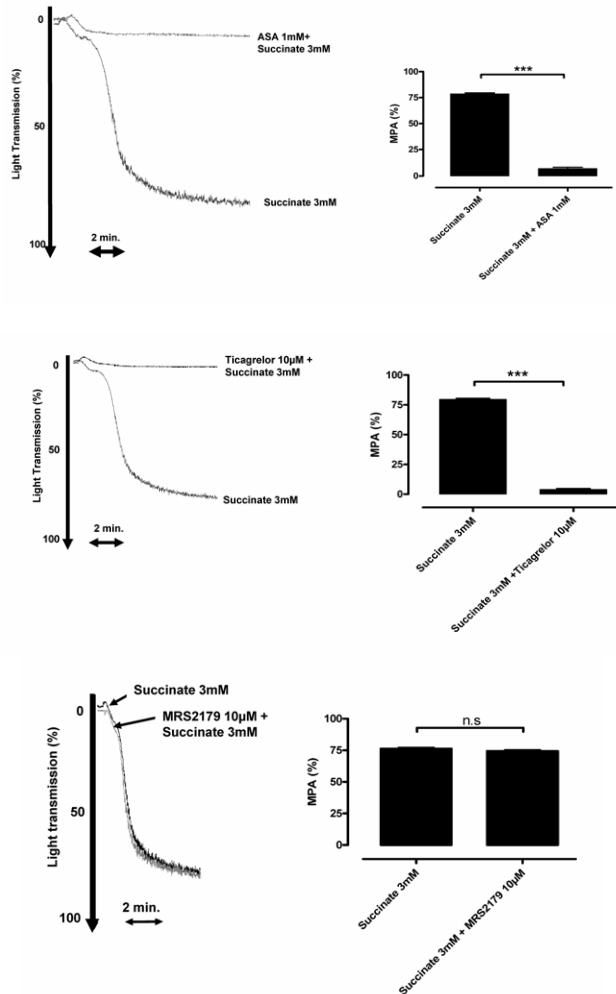


Fig. 5. (A) Upper panel: Succinat stimulated PRP vs. PRP pre-treated with 1mM ASA and further stimulated with succinate. There is a statistical significance between succinate stimulated platelet aggregation in the presence and absence of ASA (Student's *t*-test: ***= $P < 0.0001$, $n = 14$). (B) Centre panel: P2Y₁₂ dependence in succinate stimulated platelet aggregation. Significant difference was found between PRP stimulated with 3mM succinate vs. PRP pre-treated with 10μM of the P2Y₁₂ inhibitor ticagrelor and further stimulated with succinate (Student's *t*-test: ***= $P < 0.0001$, $n = 13$). (C) Lower panel: P2Y₁ dependence of succinate-stimulated platelet aggregation. No statistical difference was seen between untreated PRP vs PRP pre-treated with the P2Y₁ antagonist MRS2179 (Student's *t*-test: (n.s.)= $P = 0.25$, $n = 16$). The LTA-curves accompanying the bar charts represent the characteristic aggregation pattern from each of the experiments.

Figure 6.

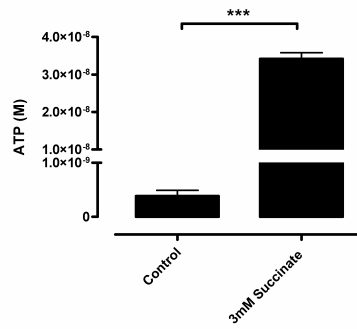


Fig. 6. Succinate mediated ATP release. Succinate is capable of stimulating ATP release in PRP. (Student's *t*-test: ***= $P < 0.0001$, $n=6$).

Figure 7.

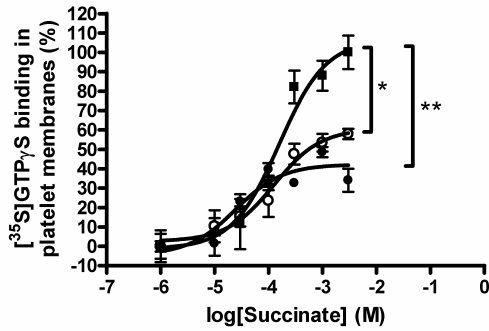


Fig. 7. Dose-response of succinate-stimulated [³⁵S]GTPγS-binding in platelet membranes. EC₅₀-value equals 110 μM. untreated platelet membranes (■), platelet membranes pre-treated with 10 μM 2MeSADP (○), platelet membranes pre-treated with 3 mM succinate (●). The maximal response, obtained for membranes pretreated with either 10 μM 2MeSADP or 3 mM succinate, was significantly reduced compared to untreated membranes (ANOVA: **= $P < 0.001$, *= $P < 0.01$, $n = 3$).

Supplementary material: Högberg *et al.*

Table 1. Primers used for RT-PCR assay (Amisten *et al.* [7]).

Gene name	Forward primers	Reverse primers	Annealing temp. °C
ACTB	5'-CTACCTCATGAAGATCCTCACCGA -3'	5'-CGCTCATTGCCAATGGTGAT -3'	60
P2Y1	5'-ATCTGTATCAGCGTGCTGGTGTG-3'	5'-GCCCAGAATCAGCACCAAGG-3'	60
P2Y12	5'-CTATCGATCGCTACCAGAAGAC-3'	5'-TCATGCCAGACTAGACCGAA-3'	60
SUCNR1	5'-TGTGTCTAACACTGTTGGGGTTCC-3'	5'-TCCTCACATTCCGCATGACG-3'	60
ADORA2A	5'-TGCTGGCTGCCCCTACACAT-3'	5'-TAAGCGTCGGTGCAGGACTC-3'	62

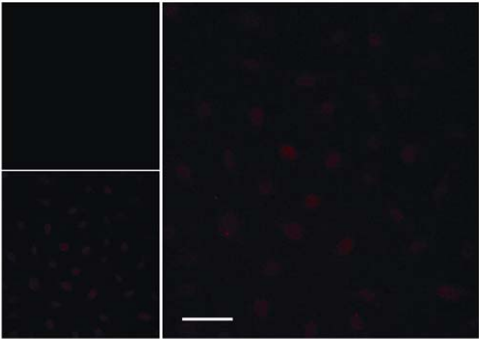


Fig. 1 Representative image of CHO cells stained for SUCNR1 (green). Upper left: SUCNR1 staining. Lower left: Nuclear staining (red). Right: Merged. Scale bar 50 μ m.

Paper IV

Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y₁₂ receptor mediated platelet aggregation

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(Manuscript)

Abstract

Background: Farnesyl pyrophosphate (FPP) is an intermediate in the mevalonate synthetic pathway, but it has also been reported to activate LPA platelet receptors. The aim of this study was to investigate the role of FPP in platelet aggregation.

Methods and Results: Human platelets were studied with light transmission aggregometry, flow cytometry and [³⁵S]GTPγS assays. As shown previously, FPP could potentiate LPA stimulated shape change. Surprisingly, FPP also acted as a selective non-competitive antagonist to ADP induced platelet aggregation. FPP blocked ADP-induced cAMP inhibition and [³⁵S]GTPγS binding in platelets. In CHO cells expressing P2Y₁₂ receptors FPP caused a rightward shift of the [³⁵S]GTPγS binding curve. Docking of FPP in a P2Y₁₂ receptor model revealed molecular similarities with ADP and a good fit into the binding pocket for ADP.

Conclusions: FPP is a noncompetitive antagonist of ADP induced P2Y₁₂ mediated platelet aggregation. It could be an endogenous antithrombotic factor modulating the strong platelet aggregating effects of ADP in a similar way as we use clopidogrel, prasugrel or ticagrelor in the treatment of ischemic heart disease.

Short title: Farnesyl pyrophosphate is an antagonist of P2Y₁₂ receptors.

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Introduction

Farnesyl pyrophosphate (FPP) is an intermediate in the mevalonate synthetic pathway, an important metabolic route controlling the cholesterol production, which is present in all eukaryotic animals, Goldstein *et al.* [1]. FPP is formed by sequential condensation of dimethylallyl pyrophosphate (DMPP) and 3-isopentenyl pyrophosphate (IPP). Besides the function as a precursor in the cholesterol synthesis FPP serves as a donor in post-translational isoprenylation of proteins both directly, and indirectly by being a intermediate for geranylgeranyl pyrophosphate (GGPP) McTaggart *et al.* [2].

Although FPP is synthesized in the cytoplasm, it can be detected in human plasma. The steady-state plasma level was reported to be 6.6 ng/ml (17 nM), but even the mild physiological alteration caused by eating a meal have been demonstrated to increase the plasma concentration 200 times. Very little is known about plasma levels of FPP during e.g. ischemia. Since HMG-CoA reductase is the main target for statins, the major group of cholesterol lowering medications, it is likely that this kind of therapy also affects the plasma FPP levels.

While the basic function of FPP seems to be structural, there have recently been several reports indicating a role also in signalling. FPP was shown to act as a natural antagonist on the LPA₂ and LPA₃ receptors while it was reported to be an agonist at the structurally divergent LPA₄ and LPA₅ receptors, Williams *et al.* [3]. In an earlier report we inventoried the different G-protein coupled receptors present in platelet cDNA and found high expression levels of LPA₅ and LPA₄ mRNA. As there have been conflicting reports regarding the function of FPP, on platelets, we decided to investigate the function. Somewhat surprisingly we discovered that the main function of FPP is

not as an agonist at the LPA₄ and LPA₅ receptors but rather as an antagonist at the structurally related and in the platelet activation process most important adenosine di-phosphate (ADP) receptor P2Y₁₂. Furthermore, the molecular structure of FPP is similar to ADP in having a di-phosphate side chain.

Here we demonstrate that FPP attenuates platelet aggregation by competing with 2-MeSADP, both at the receptor site and on the functional level.

Ethics

The Ethics Committee of Lund University approved the project. All volunteers submitted written consent to participate in the study. The study conforms to US National Institutes of Health guidelines, and the Declaration of Helsinki.

Calculations and statistics

Calculations and statistics were performed using GraphPad Prism 4.0 software. Statistical significance was accepted when $P < 0.05$, using One-way ANOVA or Student's t - test analysing the data. Values are presented as means \pm SEM. n = number of tests.

Chemicals

3,7,11-Trimethyl-2,6,10-dodecatrien-1-yl pyrophosphate ammonium salt (FPP), 3,7,11-Trimethyl-2,6,10-dodecatrien-1-yl monophosphate ammonium salt (FMP), all *trans*-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraenyl pyrophosphate ammonium salt (GGPP), *trans*-3,7-Dimethyl-2,6-octadienyl pyrophosphate ammonium salt (GPP), Isopentenyl

pyrophosphate triammonium salt (IPP), γ,γ -Dimethylallyl pyrophosphate triammonium salt (DMAPP), 2-Methylthio adenosine-5'-diphosphate (2-MeSADP), Thrombin Receptor Activator Peptide (TRAP) and Epinephrine was bought from SigmaAldrich, USA. [35 S]GTP γ S was bought from Perkin Elmer Inc., USA. Ticagrelor (AZD6140) was a gift from, AstraZeneca R&D Mölndal, Mölndal, Sweden. Fluo4-AM was from Invitrogen,

Methods and material

Platelet preparation for Light Transmission Aggregation (LTA)

Aggregation studies on human platelet poor plasma (PRP) were done as previously described by Högborg *et al.* [4]. Preparation for aggregation studies on washed platelets were performed as described by Cazenave *et al.* [5].

Flow cytometric analysis of platelet activation

Whole blood was collected into 0.129M Na-Citrate, (Vacutainer®, BD, CA, USA). For P-selectin and GPIIb/IIIa activity, whole blood was incubated in HEPES-saline buffer (145mM NaCl, 5mM KCl, 10 mM HEPES pH 7.4) with fluorescently labelled monoclonal antibodies against P-selectin (CD62P) and GPIIb/IIIa (PAC-1) or corresponding isotype control antibodies (BD, CA, USA). To induce platelet activation, 30 μ M of FPP and 0.1 μ M ADP was added. After 15 minutes in the dark at room temperature 0.2 % paraformaldehyde was added to fix the cells. All samples were diluted 1:20 in PBS and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, CA, USA). The platelet population was gated based on forward and

side scattered light. A threshold for non-specific fluorescence was set using the isotype control antibodies and all events above that value were considered positive. A total of 3.000 events were collected in the platelet gate from each sample.

Calciumflux assay on platelets

PRP for studies on internal Ca^{2+} -release mediated through succinate stimulation, was prepared as earlier described by Liu *et al* 2006 [6], with the following modifications: 5mM Fluo4-AM was used instead of 2mM. Fluorescence reading was done using a Victor3 spectrophotometer, 1420 Multilabel Counter, (Perking Elmer, USA).

Platelet [35 S]GTP γ S binding

PRP was prepared from ACD treated whole blood through centrifugation (150 rcf, 20 min, RT) and passed carefully through a Pall Autostop™ Leukocyte removal filter, (Pall Medical, NY, USA). The filtrated PRP was centrifuged (17,000 rcf, 15 min, RT). The pellet was resuspended in a room tempered membrane-buffer (20mM HEPES, 20mM EDTA, 150mM NaCl, pH 7.4, RT) and the centrifugation was repeated once. The pellet was first resuspended into 3mL ice-cold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4) then homogenized for 30 seconds in a Polytron (setting 6), and deluted to 30ml with ice-cold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4). The platelet homogenate was pelleted twice (35,000 rcf, 15 min, 4°C). The final pellet was resuspended in ice-cold membrane buffer (20mM HEPES, 0.6mM EDTA, 5mM MgCl_2 , 0.1mM PMSF, pH 7.4) and stored at -80°C. Protein concentration was measured with a

modified Lowry protein assay Kit (Thermo Scientific, USA).

The binding assay was performed essentially as described by Vasiljev *et al.* [7]. Briefly: The final volume of the assay was 200 μ l and contained 20 μ g platelet membranes in binding buffer (20mM HEPES, 100mM NaCl, 5mM $MgCl_2$, 10 μ g/mL saponin, pH 7.4 + 4nM [35 S]GTP γ S, 20 μ M guanosine diphosphate (GDP)). The mixture was incubated (30 min, 30°C) and then filtrated through glass fiber filter (GF/C, Whatman, UK). The filters were washed with 3 x 500 μ l ice cold binding buffer and then counted using OptiPhase Hisafe 3 (PerkingElmer Inc. USA) in a Liquid Scintillation Counter (Beckman Coulter Inc. USA).

[35 S]GTP γ S binding on P2Y $_{12}$ -transfected CHO-K1 cell membranes

pcDNA3 vector with cDNA for the human P2Y $_{12}$ receptor (Missouri S&T cDNA Resource Center, MI, USA) was transfected into Chinese Hamster Ovary Cells-K1 (CHO-K1) by using TransIT[®]-CHO Transfection kit (Mirus Bio, WI, USA). [35 S]GTP γ S binding studies on competent P2Y $_{12}$ -transfected CHO-K1 cell membrane was performed essentially as described by JJJ Van Giezen *et al.* [8].

Results

Farnesyl pyrophosphate potentiates LPA stimulated platelet shape change

FPP has previously have been reported to stimulate platelet shape change. However while our results show that FPP, 30 μ M, clearly potentiated LPA stimulated shape change ($^*P=0.0168$, $n=10$), we were unable to show an effect of FPP alone (fig 1).

Farnesyl pyrophosphate antagonizes 2-MeSADP stimulated platelet aggregation

While confirming the results by Williams *et al.* [3] we noticed that FPP, except for the documented effect on shape change, also was exerting an inhibitory effect on platelet aggregation. In order to investigate this phenomenon we stimulated platelets with 0.03 μ M

2-MeSADP in the presence of 30 μ M FPP. The results revealed a clear, dose dependent inhibition of aggregation ($^{**}P<0.01$, $n=15$) (fig 2A). However, the inhibitory effect was specific for ADP mediated aggregation as both TRAP (15 μ M), ($^{ns}P=0.14$, $n=7$) and epinephrine (1 μ M), ($^{ns}P=0.054$, $n=10$) stimulated aggregation were unaffected by 30 μ M FPP (fig 2B, C). In our next experiment we tested the other intermediates of the mevalonate pathway, related in chemical structure to FPP, for their ability to inhibit ADP stimulated aggregation only to find that FPP is the sole member with this kind of activity ($^{**}P=0.01$, $n=6$) (fig 3A). The FPP mediated inhibition of aggregation was dose-dependent resulting in 40 (± 5) % inhibition at 10 μ M ($^{**}P=0.01$, $n=15$) (fig 3B).

Farnesyl pyrophosphate decreases ADP stimulated up regulation of GPIIb/IIIa and P-selectin on platelets, hence inhibits ADP mediated attenuation of cAMP production, but has no effect on calcium mobilization.

In the next experiment we demonstrated that ADP stimulation of the platelet activation marker PAC-1 for GPIIb/IIIa activation was significant decreased in the presence of 30 μ M FPP (37 ± 5.3 vs. 16.1 ± 2.8 , $^{***}P=0.01$, $n=7$). Also, the degranulation marker, P-selectin, was significantly decreased in the presence of

30 μ M FPP (16.4 ± 2.7 vs. 8.6 ± 1.1 , $^{**}P=0.05$, $n=6$)

(fig 4A). These data correlates well with results on FPPs effect on ADP suppressed cAMP generation. The presence of 30 μ M FPP clearly reversed the ADP mediated attenuation of forskolin stimulated cAMP production in platelets ($^{**}P=0.01$, $^{*}P=0.05$ $n=4$) (fig 4B).

However when we loaded platelets with Fluo-4AM and tested the effect of FPP on ADP and thrombin stimulated calcium signalling, the results revealed no statistical significance in Ca^{2+} mobilization, neither between platelets stimulated with 5 μ M ADP ($^{n.s}P=0.24$, $n=6$) (fig 4 C:1) nor thrombin (0.5 U/ml) ($^{n.s}P=0.46$, $n=6$) in the presence or absence of 30 μ M FPP (fig 4 C:2).

2MeSADP stimulated [^{35}S]GTP platelet binding is antagonized by farnesyl pyrophosphate.

As the preceding results indicated the likely target to be P2Y₁₂ we isolated platelet plasma membranes and investigated the ability of FPP to interfere with 2-MeSADP stimulated [^{35}S]GTP γ S binding. The presence of 60 nM of the P2Y₁₂ specific antagonist ticagrelor right shifted the dose response curve thus confirming the identity of the target as P2Y₁₂ (2-MeSADP; $EC_{50}=17$ nM vs. 2-MeSADP+60 nM ticagrelor; $EC_{50}=0.42 \mu$ M) (fig 5A). FPP, at 6 μ M, shifted the dose-response curve slightly to the left while, at the same time, decreasing the efficacy of 2-MeSADP (2-MeSADP; $EC_{50}=16$ nM vs. 2-MeSADP+30 μ M FPP; $EC_{50}=6.8$ nM). The agonistic action of FPP was readily blocked by 10 μ M ticagrelor ($^{*}P=0.05$, $^{**}P=0.05$, $n=4$) (fig 5B). However, when increasing the concentration of FPP, the antagonist properties became more obvious by

simultaneously decreasing the efficacy and right shifting the dose-response curve of 2-MeSADP (fig 5C). EC_{20} values were calculated and analyzed, ($EC_{20(2-MeSADP)}$: 0.95 nM, $EC_{20([FPP] 1 \times 10^{-5} M)}$: 4.5 nM, $EC_{20([FPP] 5 \times 10^{-5} M)}$: 39 nM, $EC_{20([FPP] 1 \times 10^{-4} M)}$: no data). The inhibitory effect mediated by FPP vs. 2-MeSADP on the platelet P2Y₁₂ receptor yielded an IC_{50} value at 45 μ M. The results indicate that FPP is a non-competitive antagonist for the P2Y₁₂ receptor with partial agonistic properties.

The backbone structure of FPP is similar to the one of ADP. Both ligands consist of a hydrophobic and a hydrophilic part. The hydrophobic part presents a molecular variance. While FPPs hydrophobic part is made up of a 15-carbon chain, the corresponding part on the ADP nucleotide is composed of a ribose and adenine ring. However, the hydrophilic part is totally similar when comparing the two ligands as they both, structurally, are made up by negatively charged pyrophosphate groups (fig 6).

Farnesyl pyrophosphate is a ligand to the P2Y₁₂ receptor.

We next repeated the [^{35}S]GTP γ S binding experiment using CHO cells stably expressing the P2Y₁₂ receptor (fig 6). The results clearly confirms the identity of P2Y₁₂ as the target by showing a dose-dependent inhibition pattern, of 2-MeSADP stimulated [^{35}S]GTP γ S binding, that is very similar to that obtained with platelet membranes in the previous experiment and also confirms FPP as a non-competitive antagonist. The reason for the higher maximal response at 100 μ M FPP, is likely due to the higher receptor density of the CHO cells in combination with the partial agonist nature of FPP. We repeated the calculation and analyze of EC_{20} values from the CHOK1 binding assay, ($EC_{20(2-MeSADP)}$: 1 nM, $EC_{20([FPP] 1 \times 10^{-5} M)}$: 2.8 nM, $EC_{20([FPP] 5 \times 10^{-5} M)}$: 15 nM,

EC₂₀([FPP] 1×10^{-4} M): 21nM), and the result indicates the same thing, i.e increasing concentrations of FPP decreases the 2-MeSADP potency.

Docking of Farnesyl pyrophosphate to a P2Y₁₂ receptor model

Structure of farnesyl pyrophosphate (FPP)

The sequences of all of the human adenosine and P2Y receptors were retrieved from the SWISS-PROT and TrEMBL database of the ExPASy Molecular Biology Server Bairoch *et al.* [9] and aligned using the CLUSTALW program Thompson *et al.* [10]. To verify the correct alignment of the highly conserved residues in the helices and to remove gaps within the transmembrane (TM) domains the automated alignment was manually adjusted. Because of the lack of sequence similarity in the amino-terminal domains and the carboxyl-terminal domains, the first 13 residues and the last 25 residues of the P2Y₁₂ receptor sequence were excluded from the alignment and the modeling.

The crystallographic structure of the human A_{2A} adenosine receptor (A_{2A}AR), Jakkola *et al.* [11] was retrieved from the Protein Data Base, Berman *et al.* [12], (PDB ID: 3EML) and used as structural template for the construction of the P2Y₁₂ receptor model by means of the Prime package; (Prime, vers. 2.1; Schrödinger LLC, NY, USA). The chemical correctness of the A_{2A}AR structure was achieved with the “Protein Preparation Wizard” automated process in Maestro 9.0 (Maestro, vers. 9.0 Schrödinger LLC, NY, USA). The T4-lysozyme insertion replacing the third intracellular loop (IL3)

in the A_{2A}AR crystal structure was removed.

The highly conserved disulfide bridge between TM3 and the second extracellular loop (EL2) was built during the homology modelling stage based on the corresponding disulfide bridge in the A_{2A}AR structure. Instead, the disulfide bridge between the N-terminal and the third extracellular loop (EL3), conserved within the P2Y family but absent in the A_{2A}AR, was created afterward on the P2Y₁₂ receptor model by means of the refine loops tool in Prime. During this stage, a cooperative loop sampling of the N-terminal region and EL3 was applied with a harmonic distance constraint between the thiol groups of Cys17 in the N-terminal and Cys270 in the EL3, using a distance of 2.05 Å and a constant force of 350 kcal mol⁻¹ Å⁻². The top ranked model from the loop refinement procedure was further inspected to assess the stereochemical quality and chosen for the molecular docking study.

All the ligands structures were sketched in Maestro and minimized with the Polak-Ribiere conjugate gradient (PRCG) minimization consisting of 1000 steps of CG until a gradient of 0.01 kJ/(mol Å²).

The molecular docking of the ligands was performed by means of the Induced Fit Docking (IFD) protocol as in the Schrödinger package Sherman *et al.* [13]. The docking site was defined according to the site-directed mutagenesis data [14, 15] and previous modeling results [16, 17]. A 26 Å x 26 Å x 26 Å box was centred on the ligands in the pocket. Briefly, in the first stage a docking of the ligands was performed in a rigid binding site with Glide using the SP (standard precision) procedure, (Glide, version 5.5, Schrödinger LLC, NY, USA), [5] using a van der Waals scaling of 0.5 for both receptor and ligands non polar atoms. For each ligand, the top 20 docking conformations were retained and subjected to the receptor sampling by means of the Refinement module in Prime. The Prime side-chain sampling and energy

minimization were performed on all the residues within a 5 Å of the ligand in any of the 20 poses using the OPLS parameter and a surface Generalized Born implicit solvent model. The complexes within 30 kcal/mol of the minimum energy structure, ranked by Prime energy, were retained for the re-docking stage. In the final step, Glide with the XP (extra precision) scoring function and default parameters was used to re-dock all the ligands into their respective conformations produced by Prime. No constraints were used for all the docking calculations. The top scored model for each ligand was chosen as final binding conformation. The graphical pictures were made with the PYMOL program (Delano Scientific LLC, CA, USA).

FPP contains a hydrophobic 15-carbon chain and a negatively charged pyrophosphate group. It presents a very different scaffold from ADP or 2-MeSADP, typical agonists of the P2Y₁₂ receptor. FPP shares a negatively charged phosphate moiety with ADP and 2-MeSADP but it has a hydrophobic 15-carbon chain that is different from the either of the rings of the nucleotides. In order to understand the way FPP and 2-MeSADP bind to the same receptor and to find the common rational basis for the binding modes, a molecular modelling study was conducted. The three dimensional model of the P2Y₁₂ receptor was built, based on the crystallographic structure of A_{2A} adenosine receptor (PDB code: 3ELM), and the binding modes of 2-MeSADP and FPP were studied by means of flexible molecular docking.

The putative binding site of P2Y₁₂ receptor was identified, based on the available site-directed mutagenesis data Hoffmann *et al.* [15] and Cattaneo *et al.* [14] on previously published modeling studies by Costanzi *et al.* [16]. The recently published mutagenesis data confirmed the key role in the ligand binding of residues including

Arg256^{6,55} in the sixth transmembrane domain (TM6) and Lys280^{7,35} in TM7, as suggested by the docking model proposed by Costanzi *et al.* In our P2Y₁₂ receptor model with docked agonists these residues are directly involved in hydrogen bond interactions with the ligands. The binding pocket in our model is located in a pocket formed by residues of TM1, TM2, TM3, TM6, and TM7. In addition to the hydrophilic and charged residues like Arg256^{6,55} and Lys280^{7,35}, several aromatic residues including Tyr32^{1,39}, Phe77^{2,57}, Phe104^{3,32}, Tyr 105^{3,33}, Tyr259^{6,58}, and Phe177 (EL2), hydrophobic residues including Leu284^{7,39}, Ile81^{2,61}, and Pro78^{2,58}, and polar residues including Thr287^{7,42} and Ser288^{7,43} delineate the binding pocket of the P2Y₁₂ receptor. Residues of the second extracellular loop (EL2), such as Ser176 and Leu178, delimit the upper part of the pocket.

From the docking results of 2-MeSADP in the P2Y₁₂ receptor binding pocket, there is a crucial role of Arg256^{6,55} and Lys280^{7,35} in anchoring the diphosphate group of 2-MeSADP to the binding cavity. In addition, the hydroxyl group of Tyr259^{6,58} and the backbone amino group of Phe177 in EL2 are stabilizing the molecule from above with further hydrogen bond interactions with the diphosphate group. In the adenine ring of 2-MeSADP, the amino group at position N⁶ is stabilized by interactions with the side chain of Glu281^{7,36}, while the N¹ atom interacts with the hydroxyl group of Tyr32^{1,39}. Other favourable interactions occur between

2-MeSADP and the residues Phe177 in EL2, Phe104^{3,32}, Phe77^{2,57}, Ile81^{2,61}, Leu284^{7,39}, Ser288^{7,43}, and Thr287^{7,42} as shown in fig 9 A,B.

From the docking results of FPP in the P2Y₁₂ receptor binding site, the pyrophosphate group binds, as expected, in a similar way to the diphosphate moiety of

2-MeSADP (fig 8A,B,C). Strong electrostatic interactions occur between the diphosphate moiety of FPP and the side chains groups of Arg256^{6,55} and Lys280^{7,35}. The hydroxyl group of Ser176 and the backbone groups of Leu178 and Phe177, all in EL2, stabilize the pyrophosphate group of FPP in the pocket. The binding mode of FPP showed a conformation with an extended orientation of the flexible chain of FPP. The hydrophobic nature of the isoprenoid chain of FPP would be expected to lead to a more folded conformation. The 15-carbon flexible farnesyl group can assume both a *trans* or *gauche* disposition about the C₄-C₅ bond of the chain. The *trans* conformation leads to an extended form of the chain while the *gauche* conformation leads to a folded form of FPP. From the docking results of FPP in P2Y₁₂ binding pocket, the compound adopts a *trans* conformation about the C₄-C₅ bond with an extended isoprenoid chain. An extended conformation for the farnesyl chain of FPP both in solution and in a protein-bound conformation was already observed in several other experimental and computational studies on isoprenoid derivatives, Cui *et al* [18] and Zahn *et al*. [19, 20]. In the P2Y₁₂ receptor binding pocket, the aromatic and hydrophobic side chains of residues such as Tyr32^{1,39}, Phe77^{2,57}, Phe104^{3,32}, and Leu284^{7,39} make optimal contacts with the isoprenoid chain creating a favourable environment to embed the FPP.

Fig 9C and illustrates a superposition of the best docking poses of FPP and 2-MeSADP shows the steric and electronic overlap between the two molecules. The α -phosphate group of FPP is superimposed on the β -phosphate group of 2-MeSADP, but the diphosphate groups of both the compounds share the same strong electrostatic interactions with the residues in the top part of the P2Y₁₂ receptor binding pocket. Moreover, the farnesyl chain of FPP is well overlapped with the adenine ring and the thiomethyl group at

the 2 position of 2-MeSADP. The hydrophilic nitrogen atom N¹ of the adenine ring is reaching out for the hydroxyl group of Tyr32^{1,39} creating a hydrogen bond interaction, while the terminal of the isoprenoid chain of FPP is oriented deep under the aromatic ring of Tyr32^{1,39}. The possible involvement of Tyr32^{1,39} in the binding of nucleotide ligands was previously suggested by Costanzi *et al* [16]. The FPP chain is reaching the bottom of the pocket, close to the residues Leu35^{1,42}, Pro78^{2,58}, and Ser288^{7,43}. In order to maintain an extended conformation of the isoprenyl chain, the pyrophosphate group can not bind as deep as the diphosphate group of 2-MeSADP, as shown by the superposition between the docking poses of FPP and 2-MeSADP, even if the interaction between the diphosphate group and the positively charged residues in the binding pocket are conserved for both the molecules.

Discussion

In the present study we demonstrate that FPP, apart from being an intermediate in the mevalonate pathway, is an endogenous non-competitive antagonist of the P2Y₁₂ receptor acting as an inhibitor of ADP induced platelet aggregation.

The mevalonate pathway is a metabolic pathway of great importance to all eukaryotic cells. Although the isoprenoid metabolites of this pathway, FPP, GGPP, DMPP and IPP are mainly known as precursors in the biosynthetic route of cholesterol or to mediate the membrane association of certain GTPases, Goldstein *et al* [1] there have recently been several reports that indicates alternative functions. Farnesyl pyrophosphate was reported to serve as an antagonist at the classical LPA₂ and LPA₃ receptors while being an agonist on the atypical LPA₄, LPA₅ and LPA₆, Lee *et al*. [21]. Furthermore, FPP has also been described by Bang *et al*. [22] as an

agonist of the pain producing TRPV3 channel while GGPP is known to antagonize the liver X receptor (LXR), Gan *et al.* [23] In a previous study, using gene array screening, we identified the message of the recently deorphanized LPA receptors LPA₄ and LPA₅ in human platelet mRNA, Amisten *et al.* [24]. As these receptors both have been described as receptors for FPP by Williams *et al.* [3] we tested the effect of this compound on platelet activation. Although we were able to confirm the findings previous investigated by Williams *et al.* [3] who reported an effect of FPP on platelet shape change mediated by LPA₅, we noticed that the major effect was antagonistic rather than agonistic and specifically antagonistic to ADP stimulated aggregation, as both aggregation triggered by TRAP or by epinephrine were unaffected. FPP had no effect on 2-MeSADP stimulated calcium mobilisation in platelets thus ruling out the other ADP receptor, P2Y₁, as a target. However, both the degranulation marker P-selectin and the adhesion integrin, GPIIb/IIIa, was attenuated in the presence of FPP. These results correlates well with results from cAMP measurement which showed that FPP repressed ADP induced decrease in cAMP generation. A recent report states that between 60 % - 90 % of the platelet P2Y₁₂ receptor sites need to be blocked in order for the VASP phosphorylation attenuation to be detectable, HM Judge *et al* [25] and Schumacher *et al.* [26]. However, when we studied 2-MeSADP stimulated [³⁵S]GTP γ S binding in platelet membranes the action of FPP, above 10 μ M, was clearly antagonistic affecting both efficacy and potency thus showing the typical hallmarks of non-competitive antagonism. At concentrations below 6 μ M, FPP independently stimulated [³⁵S]GTP γ S binding rather than blocking it. This effect could be completely reversed by ticagrelor thus indicating P2Y₁₂ as the main target. In order to confirm the target identity we tested the ability of FPP to compete with 2-

MeSADP on P2Y₁₂ receptors expressed in CHO cells. The results substantiated the platelet results as FPP was able to antagonize the effects of 2-MeSADP stimulated [³⁵S]GTP γ S binding. The antagonistic response to FPP, in P2Y₁₂-CHO cells was slightly less than for platelet membranes and the most likely explanation for this is that FPP being a partial agonist, is more dependent on receptor density. Thus in the recombinant cell system with a high receptor density an affinity-driven partial agonist will activate a higher proportion of receptors.

The affinity of FPP for P2Y₁₂ is understandable, partly because of the structural similarity between ADP and FPP but also since the receptors LPA₅ and LPA₆, both phylogenetically related to P2Y₁₂, previously been demonstrated to bind FPP. As both LPA₅, LPA₆ and P2Y₁₂ belong to the purinergic cluster of GPCRs, Fredriksson *et al.* [27] and sharing a high degree of general structural similarity, they are also likely to have similarities in the receptor binding pocket. Indeed, a chemogenomic evaluation of the transmembrane ligand binding cavity of GPCRs placed LPA₅ (GPR92), LPA₆ (P2Y₅) and P2Y₁₂ in the same group, Surgand *et al.* [28]. To further test the validity of our results we evaluated the ability of farnesyl pyrophosphate to dock into the binding cavity of the human P2Y₁₂ receptor.

From the docking poses of 2-MeSADP and FPP, we could deduct that the positively charged residues in the upper part of the binding cavity of the P2Y₁₂ receptor are essential for the binding of the negatively charged diphosphate groups. The docking modes of FPP and

2-MeSADP nicely superpose with the side chain at position 2 of 2-MeSADP and the terminal part of the farnesyl chain of FPP pointing both toward a deep region of the P2Y₁₂ binding pocket formed by residues of TM1, TM2, and TM7. The aromatic and hydrophobic residues in the P2Y₁₂ cavity

create a good environment for both of the compounds. The side chains of the residues at the bottom of the binding pocket are well adjusting around the different side chains of the ligands. In particular, in the complex with 2-MeSADP the aromatic ring of Tyr32^{1,39} and the polar chain of Ser288^{7,43} are oriented more into the pocket closing around the short 2-methylthio chain at position 2 compared to the pocket of the complex with FPP, where Tyr32^{1,39} ring is slightly higher and the hydroxyl group of Ser288^{7,42} is pointing away from the pocket creating more room for the longer isoprenyl chain of FPP and also reducing the polarity of the cavity. To summarise: as the docking modes of FPP and ADP are superimposable there are reason to believe that they reflect the function meaning that, based on the docking pose, it is more likely to expect FPP to be a partial agonist than a pure antagonist. This is in agreement with our finding of agonistic effects in lower concentrations of FPP [³⁵S]GTPγS binding analysis in platelet membrane.

Although FPP have been reported by Taggart et al [2] to be present in the circulation only a single report describes physiological circumstances affecting FPP plasma levels showing that fasting and feeding cause different diurnal variations in do FPP plasma levels. To understand a possible physiological role for FPP as platelet regulator, we need to know the plasma concentrations in different physiological and patophysiological situations, such as coronary ischemia and inside a thrombus. In low concentrations FPP could have a prothrombotic effect both by stimulating LPA induced shape change via LPA receptors or by partial agonism at P2Y₁₂ receptors. However, at higher concentrations (micromolar), FPP is antithrombotic by blocking the fundamentally important positive feedback loop of ADP stimulating P2Y₁₂ receptors. P2Y₁₂ blockers are among the most used drugs for treatment of acute coronary

syndromes. Clopidogrel has a variable effect and patients with diabetes or CYP2C19 variants are commonly poor responders. The more potent P2Y₁₂ blockers prasugrel and ticagrelor has been shown to be superior to clopidogrel in preventing myocardial infarction, stent thrombosis and mortality, Michelson *et al.* [29] and Wallentin *et al* [30]. FPP could be seen as everyman's own endogenous P2Y₁₂ blocker that may balance the strong pro-aggregatory effects of ADP.

Another interesting aspect is the widely used statins, which are inhibitors of the mevalonate pathway and thereby FPP synthesis. Could this be one explanation for the pleiotrophic effects of statins? In the MIRACLE and ARMYDA-ACS studies, the incidence of myocardial infarction was reduced very early and it has been argued that this is to short time for cholesterol lowering to reduce plaque burden or even stabilising the plaque [31, 32]. Based on this pleiotrophic effects have been suggested to explain the early effects of statins [33]. Statins have been shown to reduce platelet aggregation. If changes in FPP synthesis contribute to these effects needs to be examined.

In conclusion, FPP is a competitive antagonist of ADP induced P2Y₁₂ mediated platelet aggregation. It could be an endogenous antithrombotic factor modulating the strong platelet aggregating effects of ADP in a similar way as we use clopidogrel, prasugrel or ticagrelor in ischemic heart disease treatment.

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Figure 1.

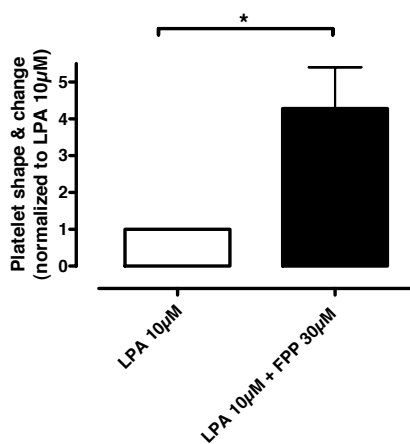


Fig 1 LTA with PRP. 30µM FPP has an additive effect on 10µM LPA mediated platelet shape/change activation. Student's *t*-test: *P<0.05, n=10.

Figure 2.

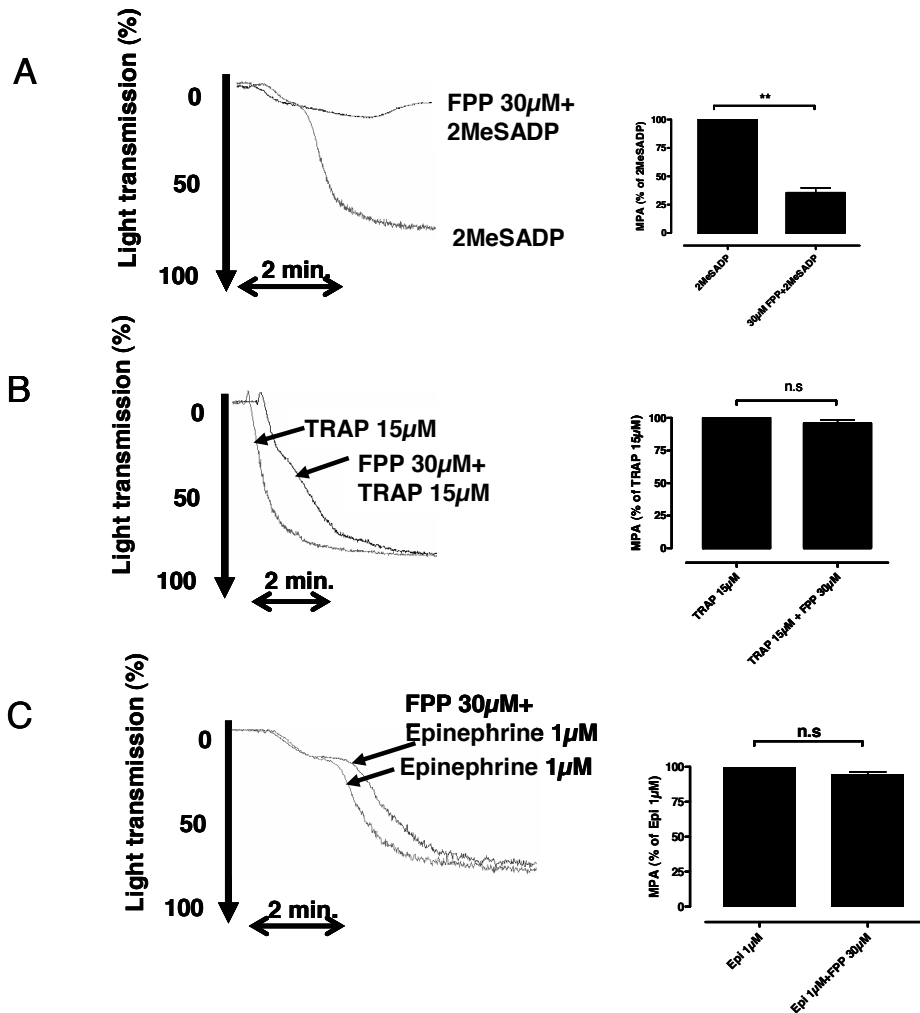


Fig 2. LTA with PRP. (A) FPP (30 μ M) shows an inhibitory effect on 2-MeSADP (0.03 μ M) mediated platelet activation, Student's *t*-test: $^{**}P<0.01$, $n=15$. (B) FPP (30 μ M) has no inhibitory effect on TRAP (10 μ M), Student's *t*-test: $^{n.s}P=0.077$, $n=7$. (C) FPP (30 μ M) has no inhibitory effect on epinephrine (1 μ M), Student's *t*-test: $^{n.s}P=0.054$, $n=15$. Each of bargraph accompanied by a representing tracer.

Figure 3A and 3B.

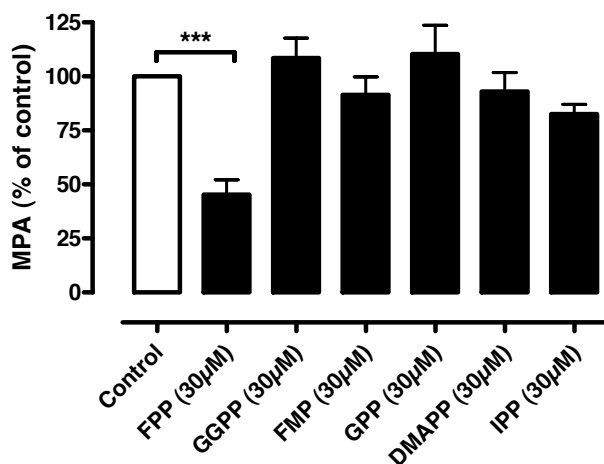


Fig 3A. LTA on washed platelets. Screening for the inhibitory capacity of intermediates present in the mevalonate pathway (30µM) vs. 2-MeSADP (0.04µM). Only FPP had statistical significance, One-way ANOVA analysis: **P<0.01, n=6.

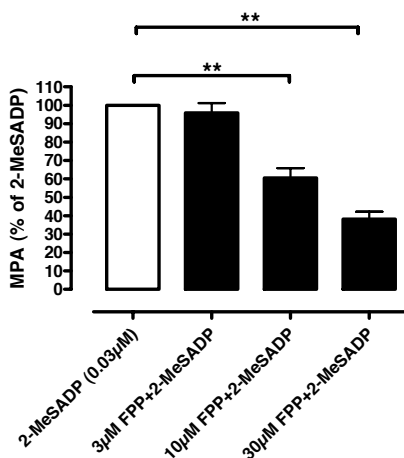


Fig 3B. LTA on PRP. Bar graph representing the antagonistic effect of FPP on P2Y₁₂ in a dose-responsive manner, One-way ANOVA analysis: **P<0.01, n=15.

Figure 4A and 4B.

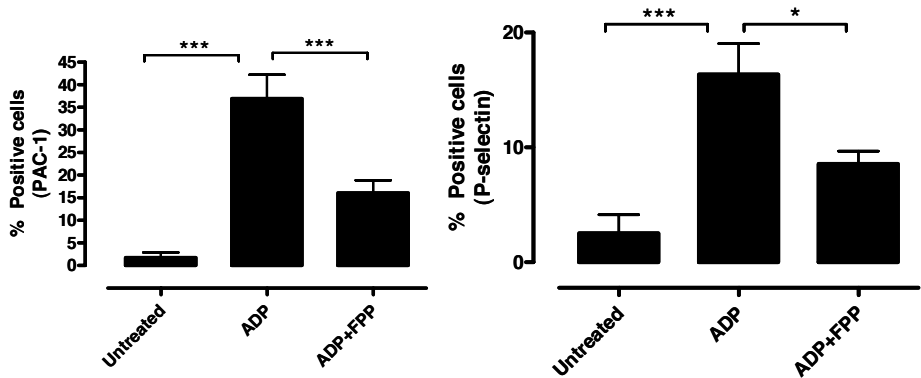


Fig 4A. Flow cytometric study on platelet GPIIb/IIIa and P-Selectin activation, showing the decreasing effect of ADP in the presence of FPP (30 μ M) compared to ADP. Student's *t*-test: ****P*=0.001, **P*=0.05, *n*=15).

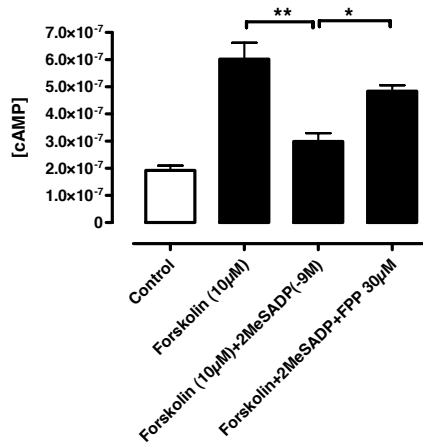


Fig 4B. Bar graph representing cAMP measuring on platelets. Statistical significance were seen between Forskolin (10 μ M) treated platelets vs. platelets incubated with 2-MeSADP (1 \times 10 $^{-9}$ M), One-way ANOVA analysis: **P*<0.01. A significance in cAMP production were also seen between platelets treated with 2-MeSADP and Forskoline vs. platelets also pre-treated with FPP (30 μ M), One-way ANOVA analysis: **P*<0.05, *n*=4.

Figure 4C

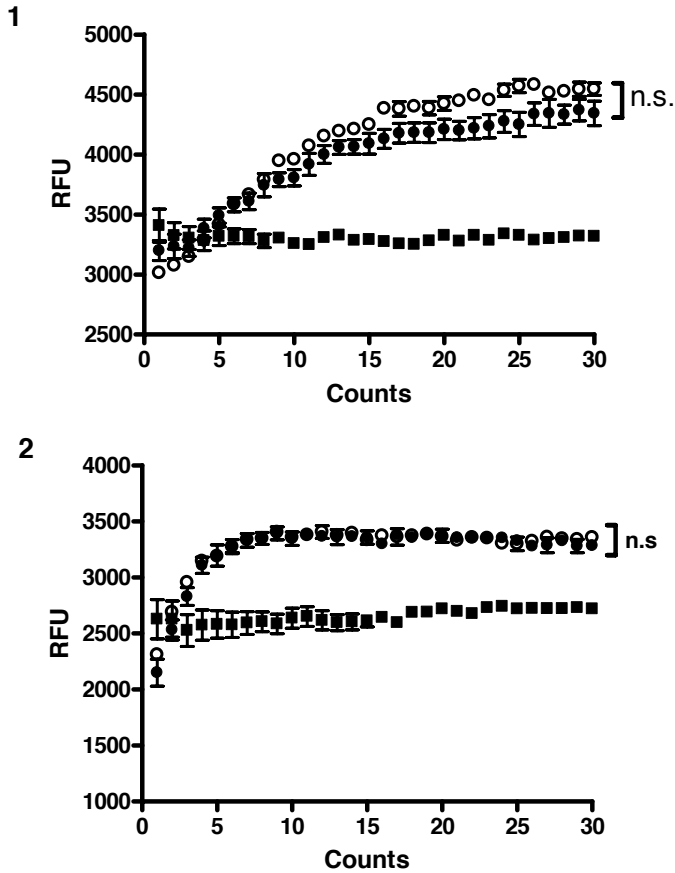


Fig 4C. Calcium flux on platelets. Tracer 1, representing the non-significant relationship between 2-MeSADP treated platelets (○) vs. platelets pre-treated with FPP (30 μM) (●), Student's *t*-test: $n.s.P=0.24$, $n=6$. Tracer 2, representing the non-significant relationship between platelets activated by thrombin (0.5U/ml) (○) vs. platelets, pre-treated with FPP (30 μM) (●), Student's *t*-test: $n.s.P=0.46$, $n=6$.

Figure 5A

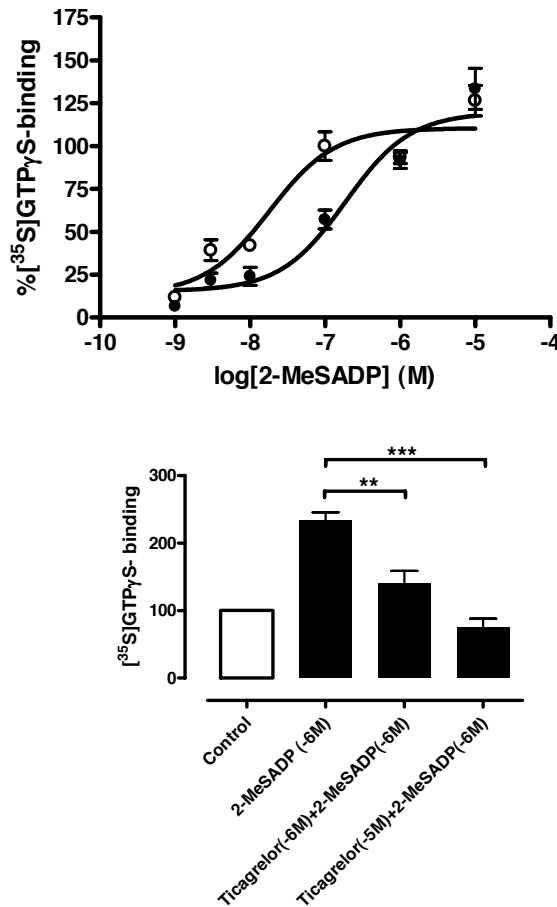


Fig 5A. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding on platelet membranes, identifying the P2Y_{12} receptor. When adding the P2Y_{12} -receptor blocker ticagrelor, there becomes a shift towards the right in the dos-response curve. Platelet membranes treated with increasing concentrations of 2-MeSADP, (○), receive an EC_{50} value at 17nM, whereas platelet membranes pre-incubated with ticagrelor (60nM), (●), increase the EC_{50} value to 0.4 μM . Bar graph representing the blocking effect of ticagrelor (1 resp 10 μM) on 2-MeSADP mediated P2Y_{12} receptor on platelet membranes (One-way ANOVA analysis: ** $P < 0.01$, *** $P < 0.001$, $n = 4$).

Figure 5B

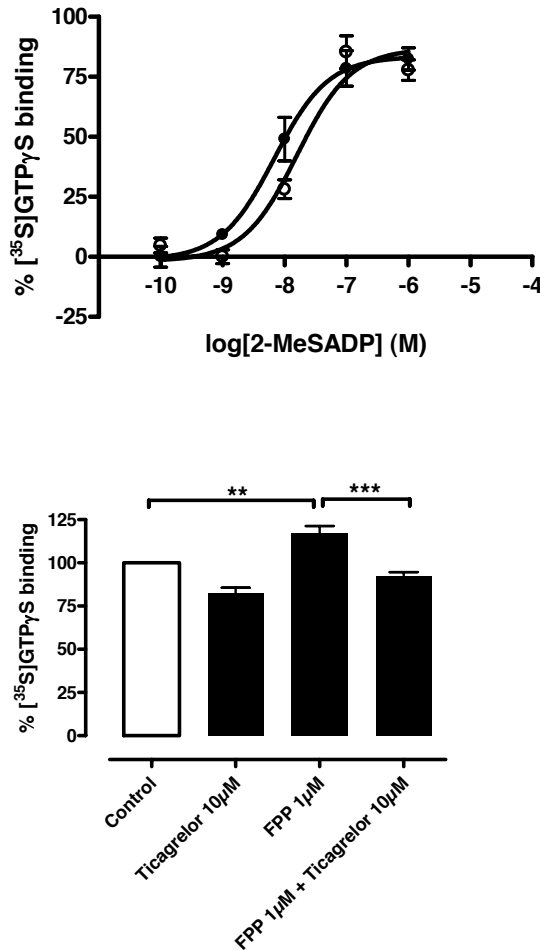


Fig 5B. Upper figure: $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding on platelet membranes identifying FPP ($6\mu\text{M}$) having an endogenous antagonist effect on the 2-MeSADP stimulated P2Y_{12} receptor. FPP shift the dose-response curve to the left, i.e. increase the potency of 2-MeSADP. Platelet membranes treated with increasing concentrations of 2-MeSADP, (\circ), receive an EC_{50} value at 16nM , whereas platelet membranes pre-incubated with FPP ($30\mu\text{M}$), (\bullet), increase the EC_{50} value to 7nM . Lower figure: Bar graph representing the attenuating effect of $1\mu\text{M}$ FPP on platelet membrane protein. This effect is suppressed by $10\mu\text{M}$ of the P2Y_{12} receptor blocker ticagrelor which indicates that FPP interacts with P2Y_{12} . (One-way ANOVA analysis: ** $P < 0.01$, *** $P < 0.001$, $n=4$).

Figure 5C

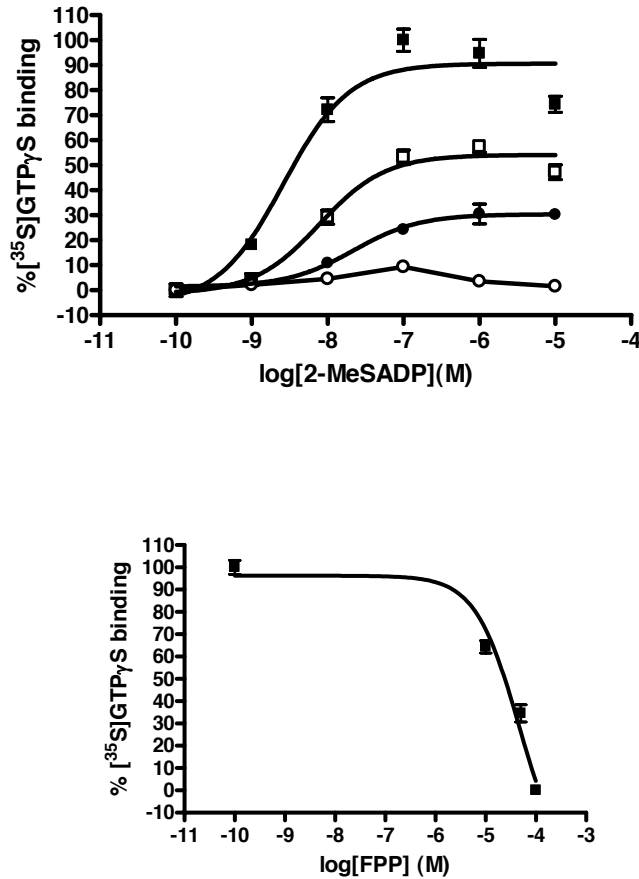
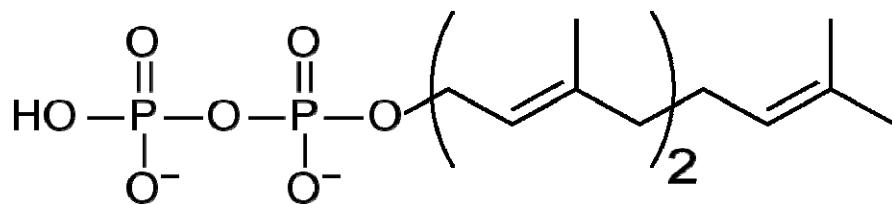


Fig 5C. Upper figure: [35 S]GTP γ S binding study on platelet membrane to investigate FPPs antagonistic/agonistic features in a dose-responder manner, in presence of increasing concentrations of 2-MeSADP. 2-MeSADP (control), $EC_{20}=0.95\text{nM}$ (■), 2-MeSADP with; [FPP] $10\mu\text{M}$, $EC_{20}=4.9\text{nM}$ (□), [FPP] $50\mu\text{M}$, $EC_{20}=39\text{nM}$ (●), and [FPP] $100\mu\text{M}$, $EC_{20}=\text{n.d}$ (○). There is a right shift in the curve unison with increasing concentrations of FPP. A right shift in the dose-response curve and a decrease in potency and efficacy relative to the increasing concentrations of FPP. Lower figure: The analyzed IC_{50} -value on $45\mu\text{M}$ is calculated from dose-response data at [2-MeSADP]= $1\mu\text{M}$.

Figure 6.

FPP



ADP

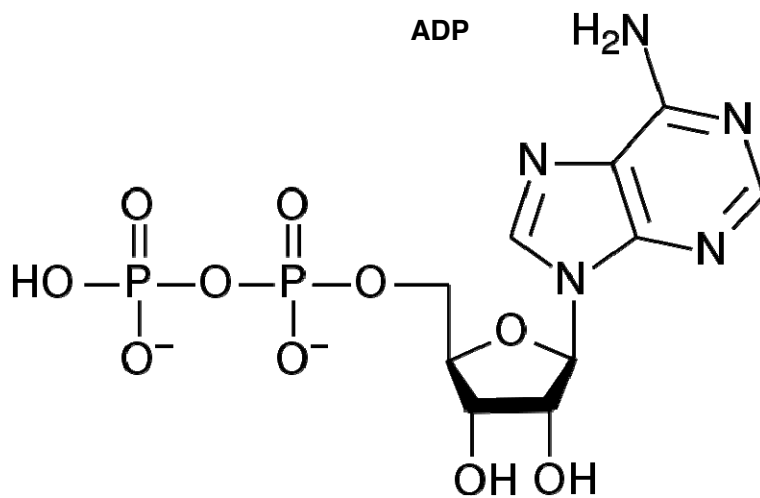


Fig 6. Molecular backbone structures of FPP and 2-MeSADP.

Figure 7.

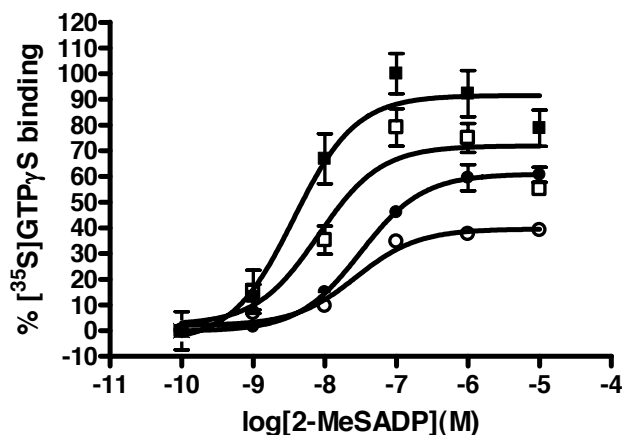


Fig 7. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding study on P2Y_{12} -transfected CHOK1-cells, to study FPPs antagonistic/agonistic features in an dose-responding manner, in presence of increasing concentrations of 2-MeSADP. 2-MeSADP (control), $\text{EC}_{20}=1\text{nM}$ (■), 2-MeSADP with; [FPP] $10\mu\text{M}$, $\text{EC}_{20}=2.8\text{nM}$ (□), [FPP] $50\mu\text{M}$, $\text{EC}_{20}=15\text{nM}$ (●), and [FPP] $100\mu\text{M}$, $\text{EC}_{20}=21\text{nM}$ (○). Like the case of platelet membrane protein, there is a right shift in the curve unison with increasing concentrations of FPP. A right shift in the dose-response curve and a decrease in potency and efficacy, relative to increasing concentrations of FPP.

Figure 8.

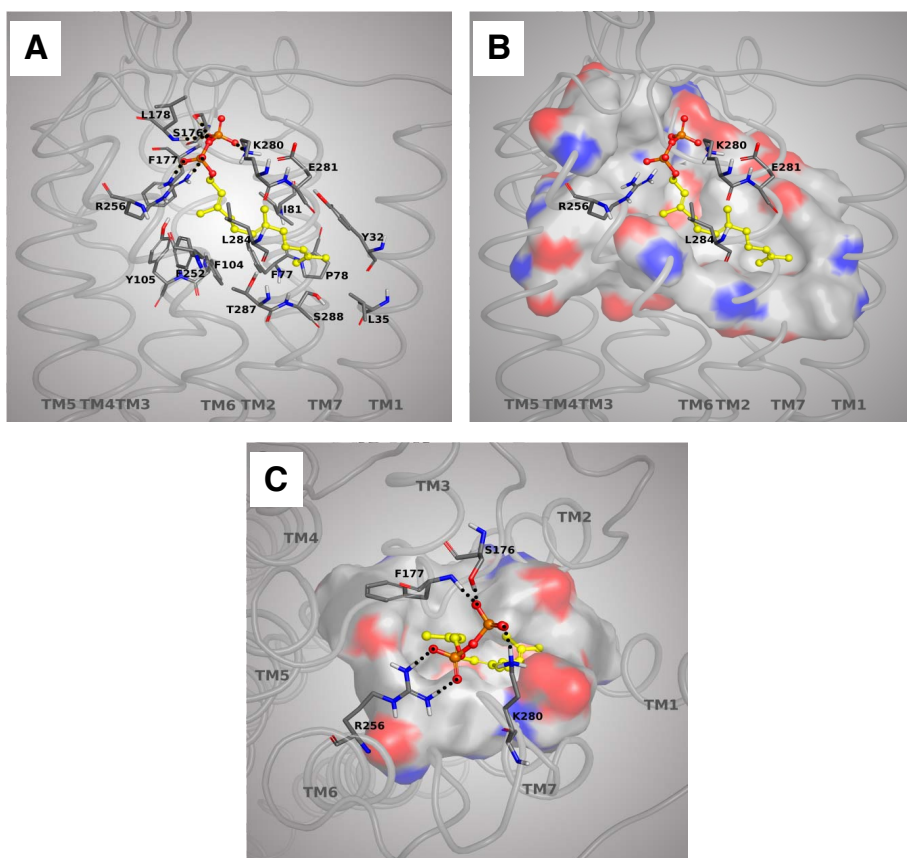


Fig 8. Fig 8A and 8B visualizes the computerized calculated docking pose of FPP in the binding cavity of the P2Y₁₂ receptor model. The FPP (depicted as ball and stick with yellow carbon atoms) is anchored in the pocket by strong electrostatic interactions between the charged and hydrophilic side chains and the negatively charged diphosphate group. The isoprenoid chain of FPP is favourably embedded by aromatic and hydrophobic residues of the P2Y₁₂ receptor. In Fig 8B, the residues at the back of the pocket of P2Y₁₂ is represented as surface colored by electrostatic potential (red for the partially negative charges, blue for the partially positive charges, and white for the neutral atoms). Fig 8C illustrates the complex shown from a top view, highlighting the orientation of the FPP chain deep in the pocket toward TM1 and TM2. For clarity only the residues interacting with the pyrophosphate are visible.

Figure 9.

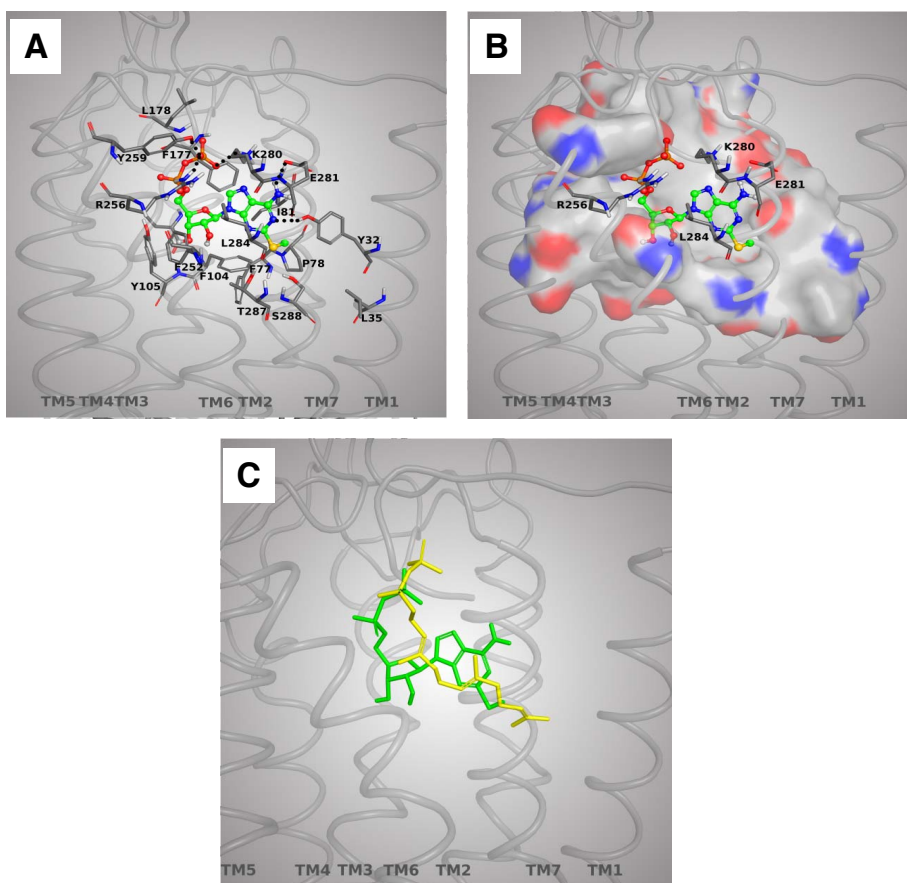


Fig 9. Fig 9A: binding mode of 2-MeSADP after docking in P2Y₁₂ receptor binding site. 2-MeSADP is shown in a ball and stick representation with green carbon atoms. The diphosphate group of 2-MeSADP has the same interactions with the residues of the P2Y₁₂ receptor shown for FPP in Fig. 8A. Differently from the hydrophobic chain of FPP, the more hydrophilic adenosine group of 2-MeSADP is anchored in the pocket by other electrostatic interactions with the residues in the pocket. The exocyclic amino group at the N⁶ position of the adenine ring is interacting with the carboxyl group of E281, and the ring N¹ atom is interacting with the hydroxyl group of Y32. In Fig 9B, the residues in the binding pocket are represented with a surface. Fig 9C visualizes the calculated superposition of the docking poses of FPP (in yellow) and 2-MeSADP (in green) in the binding pocket of the P2Y₁₂ receptor. Only the ribbon representing the backbone of the receptor is shown.