MEGA KARYOCYTE PURINERGIC RECEPTOR
ELECTROPHYSIOLOGY, CALCIUM DYNAMICS,
AND THE EFFECTS OF ACETYLSALICYLIC ACID

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by

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MEGAKARYOCYTE PURINERGIC RECEPTORS ELECTROPHYSIOLOGY, CALCIUM DYNAMICS AND ACETYLSALICYLIC ACID

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ABSTRACT

Purinergic receptor activation increases \([\text{Ca}^{2+}]_i\) in a fluctuating fashion, triggering oscillatory outward \(\text{Ca}^{2+}\) activated \(\text{K}^+\) currents in rat megakaryocytes (MKs). Whole cell and nystatin perforated patch clamp techniques were used to analyze changes in ionic conductance in MK with acetylsalicylic acid (ASA), an antithrombotic agent and COX-1 inhibitor. MKs are a model for platelet reactivity, particularly in ASA treatment failure (ASA resistance). Freshly isolated MKs were incubated 30 min in the absence or presence of 1mM ASA. Using a \(\text{K}^+\) rich internal solution, outward currents were recorded in response to 10 µM ATP, 10 µM ADP and 5 µM 2MeSADP in the voltage clamp mode. Agonist-induced currents decreased in amplitude over time, but this decline was attenuated by ASA in both continuous and repeated agonist challenge, indicating increased MK reactivity with ASA treatment. In separate experiments heterologous desensitization was observed when MKs were stimulated with ADP after exposure to a thromboxane receptor agonist (U46619) indicating cross-talk between thromboxane-purinergic pathways. Different cells, treated with ASA or MRS2179 (P2Y\(_1\) receptor antagonist), were stimulated with 2MeSADP. The dose response curve was shifted to the left in both cases, suggesting increased MK reactivity. ASA also caused an increased interval between currents (delay). ASA
attenuated desensitization of purinergic receptors and increased delay. In Ca^{2+} imaging studies of primary rat MKs, ASA enhanced [Ca^{2+}]_i elevation associated with, a central part of platelet activation, in two major pathways: inositol 1,4,5-triphosphate (IP_3)-mediated Ca^{2+} mobilization from intracellular stores and store-operated Ca^{2+} entry (SOCE) via STIM1-Orai1 interaction. Ca^{2+}-imaging studies indicated ASA-treated MKs had higher 2MeSADP-evoked (a specific agonist for the P2Y_1 and P2Y_{12} ADP receptors) [Ca^{2+}]_i, as well as enhanced store-operated Ca^{2+} entry through the plasma membrane following emptying of intracellular Ca^{2+} stores with thapsigargin. Immunohistochemistry revealed STIM1 puncta formation and subplasma membrane localization, which are required for SOCE, and are more prominent in ASA-treated MKs.

ASA treatment may potentiate Ca^{2+} release from internal stores through IP_3Rs, which may also enhance internal Ca^{2+} store depletion to trigger greater STIM1-Orai1-mediated SOCE. These findings may be relevant to ASA resistance, because individual variations in sensitivity to the multiple effects of ASA on signaling pathways could result in insensitivity to its antiplatelet effects in some patients.
ACKNOWLEDGEMENTS

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DEDICATED TO:

MY WIFE
AMADA
Whose support and understanding made this work possible

MY SONS
KEVIN ALEXANDER
&
EDGAR JOSUÉ
Who give meaning to my life, give me the energy to keep working, and make me proud
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<td>2MeSADP</td>
<td>2 methyl-thio-adenosine diphosphate</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ASA</td>
<td>acetylsalicylic acid or aspirin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CalDAG-GEFI</td>
<td>calcium and DAG regulated guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>CFU-EM</td>
<td>colony forming unit erythrocyte-megakaryocyte</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>colony forming unit–granulocyte-erythroid-macrophage-megakaryocyte</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CRAC</td>
<td>calcium release activated calcium current</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTS</td>
<td>dense tubular system</td>
</tr>
<tr>
<td>ES</td>
<td>external solution</td>
</tr>
<tr>
<td>F0</td>
<td>resting fluorescence value</td>
</tr>
<tr>
<td>F1</td>
<td>maximal fluorescence</td>
</tr>
<tr>
<td>GPIb</td>
<td>glycoprotein Ib</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GP VI</td>
<td>glycoprotein VI collagen receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>g protein–coupled receptor kinase</td>
</tr>
<tr>
<td>GPCR</td>
<td>g protein coupled receptor</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>I</td>
<td>current</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IS</td>
<td>internal solution</td>
</tr>
<tr>
<td>MEP</td>
<td>megakaryocyte-erythroid progenitor</td>
</tr>
<tr>
<td>MK</td>
<td>megakaryocyte</td>
</tr>
<tr>
<td>MPF</td>
<td>mitosis promoting factor</td>
</tr>
<tr>
<td>MRS2179</td>
<td>2′-deoxy-N6-methyladenosine 3′,5′-bisphosphate tetrasodium salt</td>
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<tr>
<td>Orai1</td>
<td>transmembrane protein 142A</td>
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<tr>
<td>P2</td>
<td>purinergic receptor</td>
</tr>
<tr>
<td>P2X</td>
<td>ATP receptor</td>
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<td>P2Y</td>
<td>ADP receptor</td>
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<tr>
<td>PECAM-1</td>
<td>that endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PGI2</td>
<td>prostaglandin I2 or prostacyclin</td>
</tr>
<tr>
<td>PIP2</td>
<td>inositol 4, 5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PLA₂</td>
<td>phospholipase A 2</td>
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<td>Definition</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol ester phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>R</td>
<td>resistance</td>
</tr>
<tr>
<td>Rab1b</td>
<td>ras-related small GTP-binding protein</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<td>SERCA</td>
<td>sarcoendoplasmic reticulum calcium ATPase</td>
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<td>SNARE</td>
<td>soluble NEM-sensitive attachment protein receptors</td>
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<td>SOCE</td>
<td>store operated calcium entry</td>
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<tr>
<td>STIM1</td>
<td>stromal interaction molecule 1</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TG</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>TP</td>
<td>thromboxane receptor</td>
</tr>
<tr>
<td>TPO</td>
<td>thrombopoietin</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A 2</td>
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<tr>
<td>U46619</td>
<td>15-hydroxy-11 alpha, 9 alpha-(epoxymethano)prosta-5,13-dienoic Acid</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt;</td>
<td>holding potential</td>
</tr>
<tr>
<td>V&lt;sub&gt;m&lt;/sub&gt;</td>
<td>transmembrane potential</td>
</tr>
<tr>
<td>X-C</td>
<td>xestospongin C</td>
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CHAPTER 1

INTRODUCTION
1. **INTRODUCTION**

Platelets play a pivotal role in the genesis and complications of heart and cerebrovascular diseases, leading causes of death in the United States (Heron et al 2009; Xu et al 2009). These anucleated formed elements of the blood, derived from bone marrow megakaryocytes, are in a delicate balance of reactivity to control bleeding through platelet plug formation. However, when platelets become too reactive, they generate potentially fatal clots or thrombi. That balance is maintained by a very complex interplay of inhibitory and stimulatory signals. Many drugs have been tested to control platelet reactivity and related thrombotic mechanisms. Cyclooxygenase blocker acetylsalicylic acid (aspirin) and vitamin K antagonists such as warfarin are the most widely used antithrombotics. In spite of the growing knowledge on platelet physiology, patient resistance to aspirin and the development of drug complications, especially bleeding, drive the search for new cellular and subcellular targets for prevention of thrombotic events and their secondary complications. The patch clamp technique, recently applied to the study of megakaryocytes offers promises to gain insights to the physiology of platelets.

1.1. **Bone marrow megakaryocytes.**

The megakaryocyte (MK) is predominantly found in the bone marrow, but it is also observed in the lung and sometimes in circulation. It has a kidney-shaped nucleus or lobed nucleus that becomes a multi-lobed as the cell matures. MKs show cycles of DNA replication without cell division or
cytokinesis, a unique process of endomitosis that usually gives haploid DNA content up to 64. The bone marrow MK is easily recognized due to its size (up to 150µm diameter) (Italiano & Hartwig, 2002; Wickramasinghe, 2007).

1.1.1. Development of megakaryocyte

Megakaryopoiesis, the process of MK production, starts with a master stem cell that populates the yolk sac during embryonic development as well as the liver and spleen (Kaushanski, 2008). Eventually, in the bone marrow, stem cells are committed to specific lineages for each blood cell. In the classical pathway, the hematopoietic stem cell (HSC) is the main precursor for all blood cells. It becomes a hematopoietic pluripotent cell that differentiates into a colony forming unit–granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM). Stem cell gene studies demonstrated in murine, primate, and human that thrombopoietin (TPO) is the main regulator of thrombopoiesis (Solar et al, 1998; Chuen et al, 2004; Wang et al, 2005). In coordinated action with specific cytokines (interleukins 3, 6, and 11) and sequential expression of genes like GATA-1, FOG-1 and NF-E2, stem cells develop towards CFU-GEMM progenitor cells which generate bipotential colony forming unit erythrocyte-megakaryocyte (CFU-EM), recently referred to as megakaryocyte-erythroid progenitor (MEP),
which can be generated directly from HSCs (Akashi et al., 2000; Weissman et al., 2001; Pang et al., 2005, Kaushansky, 2008). The colony forming unit megakaryocyte is entirely committed to generate diploid MKs. These cells pass through endomitosis A (“cells enter M phase but do not complete anaphase prior to re-entering the cell cycle” [Zimmet & Ravid, 2000]). TPO stimulates successive rounds of DNA duplication without cell division which gives rise to polyploid MKs with up to 128n DNA content (Zimmet & Ravid, 2000). Human bone marrow MK population normally is about 0.05-0.1 percent of total nucleated marrow cells (Stiff, 2002).

**Fig 1.1 Summary of classical pathway of megakaryopoiesis.** Large multinucleated mature MKs are generated by the coordinated action of several cytokines and chemokines, importantly, TPO. Different
transcription factors influence each stage of the maturation process standing out GATA1, FOG-1 and NF-E2

The exact mechanism regulating endomitosis in MKs is not completely understood. Mitosis promoting factor (MPF) and reduced activity of serine/threonine kinase AIM-1 (Kawasaki et al, 2001) in concert with the action of cyclin D3 have been demonstrated to be essential for megakaryopoiesis (Wang, 1995; Zimmet et al, 1997).

1.1.2. **Megakaryocyte structural classification**

MKs can be identified by their large size and better characterized using Wright staining or phase contrast microscopy. Morphological classification in this study is based on previous descriptions (Pisciotta et al, 1953; Levine et al 1982; Gewirtz & Schick, 1994; Mazur, 1994; Cramer & Vainchenker, 2006; Kaushansky, 2009). Four stages are identified using light or phase contrast microscopy:

- **Stage I megakaryocyte or megakaryoblast:** no pseudopodia formation or membrane blebbing. It has a large lobed, compacted nucleus with coarse chromatin organization. Cell size: up to 24 µm in diameter and “nuclear to cytoplasm ratio is high” (Cramer & Vainchenker, 2006). In electron micrographs, the demarcation membrane has been demonstrated to be present, few α granules and
many ribosomes are present. The endomitotic process is active.

–**Stage II megakaryocyte or promegakaryocyte**: pseudopodia formation and a nucleus: cytoplasm ratio decreases getting closer to 1:1. It has multilobulated nuclei arranged in a “horseshoe” shape. Cell size: up to 40 µm. In electron microscopy, more granules are observed and the demarcation membrane system is visible.

–**Stage III or granular megakaryocyte**: ruffling and blebbing are observed. The nucleus is lobulated, presenting a low nuclear to cytoplasmic ratio with dense chromatin. Cell size: >50µm. The demarcation membrane system is well developed with abundant granules and organelles seen in electron microscopy.

–**Stage IV or mature megakaryocyte**: the nucleus become more compact and very lobulated compared to stage II and III MKs. Sometimes, these cells are smaller than stage III. Cell size: >50-60µm. Proplatelet formation can be seen and the demarcation membrane system is homogenously distributed. In this stage, MKs are totally engaged in platelet generation.

1.2. Platelet formation

The discovery of MKs as a precursor for platelets was made in 1906 by James Wright, described as a process of budding off platelets from extensions of the
MKs (Patel et al., 2005). After polyploidization, MK cytoplasmic maturation takes place with the development of three important structures: the dense tubular system (DTS), granules (dense and alpha) and the demarcation membrane. Fully mature MKs are able to shed platelets. Each MK can release more than 5000 platelets with a 7-10 day lifespan in humans (Tavassoli, 1980; Kleiman et al, 2008; Jennings, 2009 [b]). and around 4-7 days in rats (Hjorn & Paputchis, 1960; Johnson et al, 1977).

The process of platelet formation is highly regulated, as described earlier (1.1.1.), and is the culmination of MK maturation. Although megakaryopoiesis is not been fully understood, the most prevalent models to explain the mechanism for platelet formation from MKs are the demarcation membrane and proplatelet formation.

1.2.1. Demarcation membrane

The rat bone marrow MK demarcation membrane was described as early as 1968 using electron microscopy (Behnke, 1968). This internal membranous system has contact with the plasma membrane and it has been proposed to be a membrane pool for proplatelet formation (Italiano & Shivdasani, 2003, Patel et al, 2005). Early electron microscopic evidence as well as acetylcholinesterase staining studies suggest a cell membrane origin for the demarcation membrane system (see below)
(Harven & Friend, 1960; Darzynkiewicz, 1967). The demarcation membrane develops through a fusion-fission process and changes from tubular to flattened structures that enclose a portion of MK cytoplasm (Tavassoli, 1980). By using Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) in CD34+ human megakaryocytic cells, the demarcation membrane system was identified as a series of flattened cistern-like structures with cell membrane continuity that create cytoplasmic portions, which eventually fragment into platelets (Falcieri et al, 2000; Kosaki, 2008). More recently, electrophysiological continuity of the demarcation system and surface membrane has been demonstrated using whole-cell patch clamp and fluorescent indicators in living MKs. Moreover, a possible membrane voltage dependent Ca^{2+} release mechanism in MKs, similar to the muscle cell T-tubule system, has been proposed based on membrane capacitance measurement (Mahaut-Smith, 2003).

1.2.2. Proplatelet

Cytoplasmic extensions were proposed in 1956 as an alternate mechanism for platelet formation (Thiery & Bessis, 1956). The term proplatelet for these cytoplasmic extensions did not appear until the 1970s with studies on passage of cells from bone marrow to vascular
space (Becker & De Bruyn, 1976). Lately, in vitro studies have demonstrated that TPO stimulated cytoplasmic extensions or proplatelets were formed via rearrangement of cytoskeletal components (Leven & Yee, 1987). Combined studies of video microscopy, immunofluorescence and flow cytometry have demonstrated that proplatelet formation is a dynamic process in which the MK cytoplasm shows bead-like particles after pseudopodia formation followed by β1-tubulin-supported round ended thin cytoplasmic extensions releasing platelets (Italiano et al, 1999) (Fig. 1.2). It has been estimated that each 2.5 x 120 micron elongated structure, or proplatelet, could generate around 1000 platelets (Tavassoli & Aoki, 1989). The actual mechanisms by which platelets are released into the blood stream remains to be elucidated. Nevertheless, MK cytoplasmic processes extending towards sinusoidal spaces in mouse bone marrow and organelle transport with microtubule assembling-based proplatelet elongation in MK cultures have been visualized with video-enhanced differential-interference-contrast microscopy (Scurfield & Radley, 1981; Patel et al 2005; Richardson et al 2005). This theory has been criticized for being derived mostly from cultured MKs studies.
Fig 1.2 Overview of platelet generation from megakaryocytes. As megakaryocytes transition from immature cells (A) to released platelets (E), a systematic series of events occurs. (B) The cells first undergo nuclear endomitosis, organelle synthesis, and dramatic cytoplasmic maturation and expansion, while a microtubule array, emanating from centrosomes, is established. (C) Prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. Proplatelet formation commences with the development of thick pseudopods. (D) Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked
into proplatelet ends, where nascent platelets assemble. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. (E) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. The nucleus is eventually extruded from the mass of proplatelets, and individual platelets are released from proplatelet ends (With permission from: Patel et al. 2005).

Despite all the experimental evidence, the mechanism of platelet formation remains controversial. Proplatelet formation as a requisite for platelet generation has been even consigned in favor of the global fragmentation theory of platelet formation. In fact, a new proposed concept of proplatelets (Kosaki, 2005) favoring the demarcation membrane theory has become prevalent. This theory proposes that when the MK completes its maturation, after centriole migration and centrosome formation, a putative cytoplasmic compartment is covered by a piece of demarcation membrane and the platelets are released after disruption of the MK (Kosaki, 2008).

1.3. Megakaryocyte and platelet signaling

1.3.1. Major stimulatory and inhibitory agonists.

Platelets show great diversity of inside-out and outside-in signals that
keep cell reactivity fine tuning. Since platelet have no nucleus, all receptors are generated during megakaryopoiesis, and in consequence, the megakaryocyte is a feasible model to study platelet physiology.

Throughout this thesis dissertation the terms platelet and megakaryocyte will be used interchangeably.

**Table 1. Summary of Platelets and MKs Signaling.**

<table>
<thead>
<tr>
<th>STIMULATORY SIGNALS</th>
<th>Natural agonist</th>
<th>RECEPTORS</th>
<th>Transduction Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP&gt;ATP</td>
<td>ADP&gt;ATP</td>
<td>P2Y1</td>
<td>GiQ-PLCβ/γ (1a, 1b)</td>
</tr>
<tr>
<td>ADP&gt;ATP</td>
<td>P2Y12</td>
<td>GiQ/γ (2a, 2b)</td>
<td></td>
</tr>
<tr>
<td>Adrenergic</td>
<td>α2A</td>
<td>*Gz-AC (3)</td>
<td></td>
</tr>
<tr>
<td>ATP&gt;ADP</td>
<td>P2X1</td>
<td>Ligand gated Ca²⁺/Na⁺ channel (4)</td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>Ig:GP VI</td>
<td>FcγRI-src-PLCγ complex (5a)</td>
<td></td>
</tr>
<tr>
<td>Ephrins</td>
<td>Eph kinases</td>
<td>Tyrosine phosphorylation (5b)</td>
<td></td>
</tr>
<tr>
<td>von Willebrand Factor</td>
<td>GPIb-IX-V complex ; GPIIb/IIIa</td>
<td>GPIb-Actin binding protein interaction (6a,b)</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen and collagen,</td>
<td>Integrin family: (αⅠbβ3 [unique to platelets], αⅡbβ1, α5β1, α6β1, αvβ3)</td>
<td>Tyrosine phosphorylation, protein complexes with FAK, talin, myosin, β3-endonexin, CIB1, Shc, Src and Syk, the PRP-1b tyrosine phosphatase (7a,b,c)</td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>5HT2A</td>
<td>Gq-PLC-IP3 (8)</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>protease-activated receptors PAR-1(human)</td>
<td>Gi-AC, Gq-PLCβ, G12/13 Rho-GEFs (9a)</td>
<td></td>
</tr>
<tr>
<td>Thromboxane A2</td>
<td>TPα and TPβ</td>
<td>GqG12/G13PLCβ/γ (10)</td>
<td></td>
</tr>
<tr>
<td>Vasopressin</td>
<td>V1a</td>
<td>Gq11 (11)</td>
<td></td>
</tr>
<tr>
<td>Platelet Activating Factor</td>
<td>PAF</td>
<td>Gq/G11 (12)</td>
<td></td>
</tr>
<tr>
<td>Prostaglandin E</td>
<td>EP3</td>
<td>G1 (13)</td>
<td></td>
</tr>
</tbody>
</table>

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Since platelet have no nucleus, all receptors are generated during megakaryopoiesis, and in consequence, the megakaryocyte is a feasible model to study platelet physiology.

Throughout this thesis dissertation the terms platelet and megakaryocyte will be used interchangeably.
Podoplanin C-type lectin receptor 2 (CLEC-2) | Src kinase phosphorylation (14a,b,c) |  
| INHIBITORY SIGNALS |  
| Adenosine | $A_{2a}$ | Gs-AC (16) |  
| Prostacyclin | PGI$_2$ | Gs-AC-cAMP (17) |  
| Nitric oxide | diffusible | cGMP (18) |  

**Note**: Table based on these references:

Since a comprehensive review of all receptors is beyond the scope of this work, this section will focus on the receptors and signaling systems examined in this thesis, specifically the transduction pathways involving purinergic receptors P2Y$_1$, P2Y$_{12}$, and P2X$_1$ and thromboxane receptor TP$\alpha$.

### 1.3.1.1. G protein-coupled receptors

#### 1.3.1.1.1. P2Y receptors in megakaryocytes

**P2Y$_1$ receptor**

The P2Y$_1$ receptor is a 373 amino acid, seven transmembrane domain protein mapped to chromosome 3E1 and 2q31 in mouse and rat, respectively (Tokuyama, 1995), and to human chromosome 3q25.2 (Ayyanathan et al., 1996). The P2Y$_1$
the receptor has been cloned and expressed in various mammalian cells and was the first purinergic receptor to be cloned (Webb et al, 1993). P2Y₁ mRNA was detected first in mice and rats (Tokuyama, 1995), in Jurkat cells and megakaryoblastic cell lines (Leon et al, 1996, 1997), and deficient P2Y₁ gene transcription has been detected in human platelets of patients with bleeding disorder (Oury et al, 1999) exhibiting a profile similar to that of the platelet P2T receptor (a collective term for P2Y₁ and P2Y₁₂ receptors before they were individually identified). This receptor has a wide distribution; P2Y₁ mRNA is reported to be expressed at its highest levels in placenta, prostate and brain. Areas in the brain with the highest mRNA expression are the nucleus accumbens and striatum. Interestingly, P2Y₁ mRNA is expressed at low levels in the liver, kidney, stomach, lymphocytes and bone marrow (Moore et al, 2000a, 2001). Platelets express 150 P2Y₁ receptors/platelet, relatively few compared to other receptors such as the thrombin receptor (up to 2000) (Hechler et al, 2005). The main function of this receptor is to initiate platelet activation upon binding ADP, characterized by changes in shape with filopodia formation and centralization of secretory granules (Eckly et al, 2003). Increased intracellular calcium concentration
and small reversible platelet aggregation have been reported in clopidogrel-treated (P2Y_{12} antagonist) rat platelets (Hechler et al, 1998a; Savi et al, 1998). It has been demonstrated in P2Y_{1} receptor knockout mice that this receptor participates in collagen-induced change in shape and platelet aggregation (Léon et al, 1999).

Besides the natural agonist ADP, several agonists bind to P2Y_{1} with different potency: 2-methylthio-ADP > 2-methylthio-ATP = ADP > ATP; \[[(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl\] diphosphoric acid mono ester trisodium salt (MRS2365). 2'-Deoxy-N6-methyladenosine 3', 5'-bisphosphate tetrasodium salt (MRS2179), (1R, 2S, 4S, 5S)-4-[2-Iodo-6-(methyl amino)-9H-purin-9-yl]- 2-(phosphonoxy) bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetra ammonium salt (MRS2500) is a selective antagonist has been developed as well (Burnstock, 2006).

**P2Y_{12} receptor**

ADP is the natural agonist of this 372 amino acid receptor that has the structure of a typical G-protein coupled receptor (GPCR) (Abbracchio et al, 2006). It was cloned, using a special strategy
to detect Gi-coupled receptors in *Xenopus* oocytes, from the rat-cDNA and human-cDNA platelet libraries. P2Y₁₂ was found in the central nervous system (amygdala, hippocampus, thalamus, substantia nigra) and in platelets (Hollopeter et al, 2001). ADP binding site studies in rats (Savi et al, 1994) and in human platelets, around 500 receptors /platelet have been estimated (Cattaneo et al, 1997). P2Y₁₂ genes are located in chromosomal region 3q24-q25 in human and in 2q31 in rat (Gachet, 2001). The P2Y₁₂ receptor has been recognized for its particular role activating platelets by inhibition of adenylyl cyclase and for its function completing the aggregation initiated by P2Y₁ receptor activation and amplifying the ADP action on platelets after thrombin or thromboxane stimulation (Hechler et al, 2005). This receptor, along with the P2Y₁ receptor, has been linked to collagen-induced restored platelet aggregation in Gαq deficient mice (Ohlmann et al, 2000). Two additional important actions have been ascribed to P2Y₁₂ activation: first, its role in potentiating TXA₂ independent secretion or thrombin-promoted aggregation and secretion (Cattaneo et al, 1997), and second, completing or consolidating thrombin-induced irreversible aggregation, the so-called aggregate stabilization (Trumel et al,
The P2Y$_{12}$ receptor is primarily activated by ADP. The order of agonist potency is as follows: 2-MeSADP $\geq$ ADP $>$ ADP$\beta$S $>$ ATP. Because of the previously outlined actions associated with activation of this receptor and the successful development of pharmacological blockers, attention has focused on the discovery of effective antagonists such as 2-methylthioadenosine 5’-monophosphate (2MeSAMP), CT50547, AR-C69931MX (a non competitive antagonist also known as cangrelor), R-138727 (active metabolite of prasugrel), INS49266, AZD6140, PSB0413.

### 1.3.1.1.2. Thromboxane A$_2$ receptors

The TXA$_2$ receptor is a typical seven transmembrane domain GPCR with two isoforms: TP$\alpha$ and TP$\beta$, both of which are coupled to Gq/G13 protein mechanism. Its natural ligand is the arachidonic acid derivative prostanoid, thromboxane A$_2$. The TP receptor in rat was isolated from the kidney cDNA library by homology screening (Abe et al, 1995) against the mouse lung cDNA library (Namba et al, 1992), and from human placenta and megakaryocytic leukemia. It is a 341 amino acid protein in both mouse and rat, while in human it is 343 amino acids long. The TP receptor gene is located in chromosomal
region 19p13.3 in humans (Hirata et al, 1991) and in the 7q11 and 10 C1 chromosomal regions in the rat and mouse genome, respectively (Namba et al, 1992). The TP receptor has a wide tissue distribution; it has been reported in endothelial cells (Raychowdhury et al, 1994), gastrointestinal tract smooth muscle cells (LeDuc & Needleham, 1979), brain, aorta, platelets (Borg et al, 1994), and megakaryocytic cells (Dorn, 1992). In rat hippocampal CA1 neurons, a TP agonist has been shown to affect neuronal signaling (Hsu et al, 1996).

The TP receptor mediates smooth muscle contraction (vascular, uterine, and gastrointestinal) and TP activation stimulates fibroblast growth factor release which promotes vascular smooth muscle hypertrophy (Ali et al, 1996). One of the most important actions of TXA$_2$ is on blood clotting. Immediately after platelet activation, TXA$_2$ is generated from arachidonic acid a membrane lipid, by the cyclooxygenase 1 enzyme. The unstable TXA$_2$ is able to stimulate platelet aggregation and secretion before being rapidly converted to TXB$_2$ (Samuelson, et al, 1978; Huang 2004). TXA$_2$ production is involved in a feedback mechanism for further platelet activation in the same cell and increases the clotting response of additional platelets.
after ADP, thrombin or collagen stimulation (Samuelson, et al, 1978; Paul et al, 1999). TXA$_2$ is the natural ligand for TP. 15-Hydroxy-11 alpha,9 alpha-(epoxymethano)prosta-5,13-dienoic acid (U46619) is a commercially available full TP agonist. Recently, the TXA$_2$ receptor has become a clinical target for oral, specific TP blocker drugs due to clopidogrel and aspirin resistance and bleeding complications (Chamorro, 2009).

1.3.1.2. Ligand-gated cation channels

1.3.1.2.1. P2X1 receptor

This is a 399 amino acid ATP-gated ionic channel with three subunits that must be associated to form a pore for inward Ca$^{2+}$ and Na$^+$ currents (Valera et al 1994; Ralevic & Burnstock, 1998; Gachet, 2001). A cDNA encoding this receptor was isolated from the human urinary bladder and the P2X gene was located in chromosome 17 (Valera et al, 1995). It has been reported to be present in the rat central nervous system (Kidd et al, 1995; Xiang et al, 1998) and vascular smooth muscle cells (Nori et al, 1998). Using degenerate reverse transcription and PCR, a P2X1-cDNA encoding a P2X$_1$ receptor was cloned from human platelets and megakaryoblastic cells (Vial et al, 1997; Sun et al, 1998). The use of real-time reverse transcription polymerase chain reaction
allowed the quantification of P2 in human platelets. It was found that P2X$_1$ mRNA was fourfold lower than P2Y$_{12}$ mRNA (Wang et al, 2003). The order of potency for agonists on P2X$_1$ is ATP $\geq$ 2MeSATP $> \alpha,\beta$ meATP (Valera et al, 1994; Evans et al 1995; Nörenberg & Illes, 2000). Antagonists for this receptor include 2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP), 8,8'-(carbonylbis(imino-3,1-phenylene-carbonylimino))bis-1,3,5-naphthalene-trisulphonic acid, hexasodium salt (NF023), 4,4',4'',4''''-[carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino)] tetrakis-1,3-benzenedisulfonic acid, octasodium salt (NF449), 8,8'-[carbonylbis[imino-3,1-phenylene-carbonylimino(4-methyl-3,1-phenylene) carbonylimino] bis-1,3,5-naphthalene trisulfonic acid hexasodium salt (suramin) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS).

1.3.2. Receptor desensitization

As GPCRs, in comparison to other GPCRs P2Y receptors do not desensitize rapidly; desensitization usually includes kinase activity and G protein uncoupling. Upon P2Y$_1$ receptor stimulation with ADP in HEK-293 cells, desensitization takes place by receptor internalization after phosphorylation of the intracellular loop and the proximal C
terminus (Reiner et al, 2009). The role of PKC in P2Y₁ desensitization remains controversial. Some models suggest P2Y₁ desensitization to be PKC and calcium independent (Galas & Harden 1995) while others indicate it is PKC dependent (Hardy et al, 2005). P2Y₁₂ receptor desensitization shows similarities to P2Y₁, but in contrast, in human platelets, the desensitization mechanism is dependent upon G protein–coupled receptor kinase (GRK) and P2Y₁₂R remains sensitive for longer time compared to P2Y₁R (Baurand et al, 2000). Platelets express GRK2 and GRK6 types (Hardy et al, 2005). Recently, a mechanism for P2Y receptor resensitization has been proposed in human platelets to be dynamin dependent receptor internalization, receptor dephosphorylation and recycling (Mundell et al, 2008). Similarly, as a GPCR, the TP receptor is desensitized by a PKC mediated GRK mechanism (Flannery & Spurney, 2002).

It is commonly agreed that P2X₁ receptor desensitizes rapidly in contrast to other members of the P2X group, which desensitize slowly. The mechanism for desensitization was studied using chimeric receptors generated by mutagenesis and in vitro transcription of P2X₁ and P2X₂ receptors, demonstrating that specific domains of the receptor molecule operate as functional units conferring desensitization properties while others do not (Werner et al, 1996).
1.3.3. P2Y1 and TP receptors signaling

P2Y1 and TP are G\alpha_q-coupled-receptors that activate PLC\beta, increasing inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG) (Knezevic et al, 1993).

1.3.3.1. Inositol triphosphate cascade in megakaryocytes

IP3 binds to its receptor in the dense tubule membrane, which allows calcium release into the cytosol. Protein kinase C (PKC), a calcium sensitive enzyme, becomes active and phosphorylates pleckstrin, a protein that triggers dense granule content secretion. Released ADP not only feeds back on P2Y receptors, amplifying the reaction, but also desensitizes the receptors over time (Fig. 1.3).

1.3.3.2. Diacylglycerol signaling

Calcium and DAG binding to CalDAG-GEFI is a key factor regulating Rab1b, a small GTPase that activates the enzyme ERK which phosphorylates PLA2 (Fig. 1.4) (Bergmeier & Stefanini, 2009). PLA2 activation leads to phospholipid hydrolysis, releasing arachidonic acid, which is then converted by prostaglandin endoperoxide H synthase-1 or PGH synthases (also known as COX1 and COX2) into several important prostanoids such as TXA2. (Kresge et al, 2008) MKs and
platelets release TXA\(_2\), which binds its TP\(\alpha\beta\) receptors in the plasma membrane as an amplifying system to stimulate platelet reactivity.

![Diagram](image)

Fig. 1.3 ADP-P2Y\(_1\) mediated signaling in megakaryocytes. (see text for details) TP\(\alpha\): Thromboxane A2 receptor alpha, PLC\(\beta\): phospholipase C beta; PI: phosphatidilinositol, PIP: phosphatidylinositol phosphate, PIP\(_2\): inositol 4,5-bisphosphate, IP\(_3\): inositol 1,4,5-trisphosphate, IP\(_3\)R: IP\(_3\)
1.3.3.3. Calcium mobilization

Calcium in platelets and megakaryocytes is mobilized from internal stores and from the external media. The increase in cytosolic calcium concentration after agonist stimulation has been demonstrated in platelets and the main source has been recognized as the dense tubular system (Rink et al, 1982). Calcium entry has been classified as capacitative and non capacitative (Rink & Sage, 1990).
1.3.3.3.1. Capacitative: store operated calcium entry

Changes in intracellular calcium concentration have multiple effects. In rat, mouse and guinea pig megakaryocytes and human platelets it has been shown that purinergic receptor stimulation increases cytosolic calcium, activating calcium sensitive potassium channels resulting in oscillatory outward ionic currents that correspond to variation in cytosolic calcium concentration (Kawa, 1990; 1996; Mahaut-Smith et al, 1992; Uneyama et al, 1993; Somasundaram & Mahaut-Smith, 1994; Vial et al, 2002). IP$_3$ generated in response to ADP and TXA$_2$ activates its DTS receptor, releasing calcium into the cytosol. Calcium is sequestered back into the DTS by a SERCA transporter and also transported from the cytosol to the extracellular space by a calcium ATPase.
**Fig. 1.4. Thromboxane A2 generation and aspirin action site.** PLA₂ cleaves arachidonic acid from membrane phospholipids and cyclooxygenase and specific synthases produce thromboxane and other prostanoids.

These calcium dynamics are complemented by calcium entry activated by the store operated calcium entry (SOCE) $I_{\text{CRAC}}$ currents (calcium release activated calcium current). The SOCE system is activated when DTS calcium concentration is reduced. In the aforesaid state, STIM1 (the calcium sensor in the DTS) is activated and translocates next to the plasma membrane making contact with Orai1 (pore forming protein in the plasma membrane) which is an $I$
CRAC-type calcium channel. However there is recent evidence that STIM1 translocation is not a requisite for Orai1 activation See Fig. 1.2 (Frischauf et al, 2008; Jardin et al 2008; Liao et al, 2008; Gwozdz et al, 2008; Bergmeier & Stefanini, 2009). A critical role for phosphoinositides in calcium entry has been demonstrated. In COS-7 cells, cytoplasmic calcium measurements, TIRF analysis, and electrophysiological recordings showed that phosphatidylinositol (4,5)-bisphosphate (PtdIns4P) is necessary for calcium entry via the Orai1 channel after P2Y receptor stimulation in the presence of a SERCA blocker (thapsigargin) (Korzeniowski et al, 2009).

1.3.3.3.2. Non-capacitative: transient receptor potential channels

Transient receptor potential (TRP), non selective cation channels have been reported in MKs, particularly, two groups: C and M. Type C TRP channel expression was found in megakaryocytic cell lines (TRPC1-C3) (Berg et al, 1997; den Dekker et al, 2001). TRPC1 and TRPC6 channels and Type M TRP channels (TRPM1, TRPM2, TRPM7) mRNA were detected using RT-PCR and were electrophysiologically characterized in the murine primary megakaryocyte (Carter et al, 2006). The physiology of these
Non-capacitative: TRP megakaryocyte (Carter et al, 2006). The physiology of these channels in platelets and megakaryocytes is under study and their contribution to calcium mobilization by agonist action as well as its regulation by second messenger systems remains to be fully understood. Several facts suggest that TRP signaling might be important in platelet physiology. Platelet IP$_3$ type II receptor coupling to hTRP1 has been demonstrated to activate calcium entry in thapsigargin treated human platelets suggesting that a SOCE mechanism is involved (Rosado & Sage, 2000; Authi, 2007). TRPC channel receptor mediated activation is PLC dependent (Estacion et al, 2001) and P2Y and TXA$_2$ receptors are coupled to PLC as well. The presence of TRPC1-C3 and TRPC6 in megakaryocytes suggests that this enzyme-receptor signaling system might be a primary mechanism for calcium entry in platelets and a not fully characterized pathway for intracellular calcium mobilization since TRPC1 protein has been found in intracellular membranes (Authi, 2007). TRPC1 has been shown to be present in lipid raft domains associated with the IP$_3$ receptor G$q$ and calcium
ATPase in the salivary gland, suggesting a more complex calcium system. (Lockwich et al, 2000).

1.3.4. **P2Y\textsubscript{12} receptor signaling**

P2Y\textsubscript{12} couples to G\textsubscript{i2} heterotrimeric protein with two different pathways associated with it. Following ADP activation, the G\(\beta\gamma\) dimer dissociates and act upon phosphoinositide 3-kinase (Kauffenstein et al, 2001) a ras-related small GTP-binding protein which via ERK can activate PLA\textsubscript{2}. Activation of PLA\textsubscript{2} generates prostanoids, importantly, TXA\textsubscript{2} independently of P2Y\textsubscript{1} activation. The G\(\beta\gamma\) complex has been linked to activation of G protein-gated inwardly rectifying potassium channels (Kahner et al, 2006). The mechanism behind that interaction P2Y\textsubscript{12} - G\(\alpha\iota2\) is controversial (Jin & Kunapuli, 1998). As a result of G\(\alpha\iota2\) activation, cAMP levels decrease owing to adenylyl cyclase inhibition (platelets are inhibited by
Fig. 1.5. Interaction between P2Y_{12} receptor signaling and P2Y_{1} and TXA_{2} pathways. See text for details. AC: adenylyl cyclase; DGK: DAG kinase; PA: phosphatidic acid; PI3K: phosphoinositide kinase 3; CalDAG-GEFI: calcium and DAG regulated guanine nucleotide exchange factor; Rab1b: ras-related small GTP-binding protein; ERK: extracellular signal-regulated kinase; PLA\(_2\): phospholipase A\(_2\); AA: arachidonic acid; COX1: cyclooxygenase 1; STIM1: Stromal interaction molecule 1, Orai1: transmembrane protein 142A; I CRAC: calcium release activated calcium current.
signaling through the P2Y\textsubscript{12} pathway now suggest a key role for DAG kinase and CalDAG-GEFI. It is proposed that upon activation of the P2Y\textsubscript{12} receptor, Gi-mediated decrease in cAMP levels inactivate DAG kinase, raising DAG concentration and thereby activating CalDAG-GEFI, which amplifies the signal further by increasing PKC activity, integrin activation, and release of TXA\textsubscript{2}, ADP, and ATP via pleckstrin phosphorylation (Fig. 1.5) (Guidetti et al, 2008).

1.3.5. **Cross talk between TXA\textsubscript{2} and P2Y signaling**

Purvis et al. proposed a model for calcium regulation and phosphoinositide metabolism in platelets which can be used to analyze how “signaling modules” interact with one another: 1- Ca\textsuperscript{2+} release and uptake, 2- phosphoinositide (PI) metabolism, 3- P2Y G-protein signaling, and 4- protein kinase C (PKC) regulation of phospholipase C\(\beta\) (PLC\(\beta\)). (Purvis et al, 2008). In the resting state, MK cytosolic calcium concentration has been measured to be near 600 nM (Ikeda, 1994) although Purvis et al in their platelet calcium balance model pointed out levels as low as 40-100nM. In this state TXA\textsubscript{2} production is not stimulated and ADP is not released from platelets or from other sources to a significant extent. For instance, once the levels of ADP rise due to ATP degradation or release from red cells, P2 receptors are activated. P2Y\textsubscript{1} exerts a priming effect on cytosolic calcium
concentration and P2Y_{12} carries out an amplification cascade increasing cytosolic [DAG] by inactivation of DGK, which activates PKC-pleckstrin and the CalDAG-GEFI protein (Stefanini et al, 2009). The signaling gradually fades out as PKC generated by either GPCR inactivates PLCβ by phosphorylation. ADP action on human platelets needs P2YR activation and integrin α_{IIb}β_{3} to generate TXA_{2} (Jin et al, 2002), which stimulates TP receptors and leads to PKC activation and further ADP and ATP release from dense granules. TP receptor activation has been shown to attenuate the P2Y-induced response in platelet aggregation studies, presumably by desensitization of the signaling cascade. (Barton et al 2008, Bynagari et al, 2009; Kim et al 2009) The extent of this relationship in MKs remains a matter of current biochemical research, but has yet to be studied electrophysiologically.

1.3.6. P2X_{1} signaling

Upon activation, the P2X_{1} receptor channel allows transient calcium and sodium entry (4:1 ratio) (Valera et al, 1994). Calcium influx promotes platelet shape change from discoid to ovoid and filopodia extension. Sodium seems to be unnecessary for these changes, since its replacement with other cations does not prevent shape change (Clifford et al, 1998). In platelets, P2X_{1}-mediated Ca^{2+}-calmodulin dependent Erk2 activation and myosin light chain (MLC) phosphorylation by MLC calcium dependent
kinase have been reported, which ultimately give rise to platelet shape change, granule centralization, and pseudopodia formation (Oury et al, 2002; Toth-Zsamboki et al, 2003). Electrophysiological evidence demonstrated a “priming” effect of P2X$_1$ activation on P2Y-induced currents. Besides potentiating P2Y-induced current amplitude (Vial et al, 2002), P2X$_1$ action accelerates the onset of P2Y mediated currents, probably by increasing [Ca$^{2+}$]i enough to activate mechanisms such as P2Y-PLC platelet activation (Tolhurst et al, 2005). Increased P2X$_1$-mediated platelet reactivity has been demonstrated in transgenic mice. Overexpression of the receptor leads to increased extracellular signal-regulated kinase 2 and increased platelet aggregation and secretion in response to U46619 and low doses of collagen indicating an important role of the P2X$_1$ pathway in platelet physiology (Oury et al, 2003).

1.4. Platelet secretion

Platelet granule content secretion is important for signaling amplification. There are three main types of granules with different content: alpha granules with protein-type molecules, dense granules with purines and smaller molecules, and lysosomal granules with enzymes (Reed et al, 2000). The steps between plasma membrane receptor activation and the exocytosis mechanism are not fully understood, but calcium and PKC have been identified as key mediators (Knight et al, 1982; Rink et al, 1982;
Konopatskaya, 2009). Recently, store operated channel-permeable (SOC-permeable) \( \text{Mn}^{2+}/\text{Na}^+ \) driven by a \( \text{Na}^+/\text{Ca}^{2+} \) exchanger activity has been shown to be involved in dense granule release in human platelets. (Harper et al, 2009). The secretion process is finely regulated by cytoskeleton components and vesicle-trafficking proteins such as soluble NEM-sensitive attachment protein receptors (SNARE) and the open canalicular system (Flaumenhaft, 2003).

1.4.1. Dense granules

These contain serotonin, histamine, epinephrine, ADP, ATP, calcium, magnesium, \( \alpha_{\text{IIb}}\beta_3 \); glycoprotein Ib (GPIb), P-selectin, granulophysin –a specific dense granule marker- (Holman et al, 1994; Youssefian & Cramer, 2000).

1.4.2. Alpha granules

Alpha granules contain platelet derived growth factor, epidermal growth factor, fibrinogen, plasminogen activator inhibitor-1, P-selectin and \( \alpha_{\text{IIb}}\beta_3 \) adhesion molecules (Youssefian, 1997; Reed et al, 2000)

1.4.3. Lysosomal granules

These granules are present in the MK before alpha and dense granules. Their content is heterogeneous. Lysosomal proteins such as lysosomal-associated membrane proteins (LAMP-1, or CD107a; LAMP-2 and LAMP3) (Stenberg, 1986; Israels et al, 1996) are found in these granules,
and cathepsins D and E hydrolases and hexosaminidase have been reported to be released from lysosomal stores (Ciferri et al, 2000; Ren et al, 2008).

1.5. Phases of platelet plug formation

The main goal of platelet function is to prevent bleeding through blood clotting. Platelets are in a delicately balanced state of reactivity, ready to be prompted to initiate a series of reactions inside the cell as well as to recruit new cells. Any condition with low or high reactivity will lead to platelet disorder. Once the signaling system is activated three main processes take place: initiation, extension and perpetuation.

1.5.1. Initiation

Integrins or glycoproteins (GP) work as adhesive ligands, not only causing cell-cell interaction, but also binding plasma proteins to the platelet surface. The initiation step comes about when platelet surface receptors, especially GP VI collagen receptor (the most potent signal) and integrin (α2β1) make contact with collagen, or GPIb-IX-V and integrin αIIIβ3 make contact with the von Willebrand Factor, enabling monolayered platelets to adhere to subendothelial surface. This phase is important for conversion of prothrombin to thrombin (Brendt et al, 2001; Michelson, 2004). When collagen binds to GP VI, it promotes
clustering of this receptor and PLC activation, generating DAG and IP₃, similar to the P2Y signaling depicted in Fig 1.2 and Fig. 1.4.

1.5.2. Extension

G protein coupled receptors are important in this phase. Released TXA₂ and ADP (via P2Y₁ receptor) increase cytosolic [Ca²⁺]; cAMP decreases due to Gi-coupled-P2Y₁₂ receptor action on adenylyl cyclase. This series of reactions amplify platelet reactivity, and importantly, bring together more platelets (aggregation) over the initial monolayer to actually control bleeding. GP VI has been demonstrated to have an important role in this phase, which is also called recruitment (Massberg et al, 2003).

1.5.3. Perpetuation

This phase refers to events after aggregation to consolidate plug stability; therefore it is also called the stabilization phase. A major part of the stability is achieved through outside-in signaling after binding of GPIb-IX-V to von Willebrand factor, GPVI to collagen, and αIbβ₃ to fibrinogen. The outside-in signaling going on in platelets after aggregation is not completely known, but it requires phosphorylation of Tyr residues of the αIbβ₃ complex (Law et al, 1996; Shattil, 1999). Recently, calcium and integrin binding 1 protein (Cib1) has been demonstrated to be an important regulator of αIbβ₃
(Naik et al 2009). Signals generated by collagen-activated \( \alpha_{IIb}\beta_3 \) complex promote further aggregation via ADP and TXA\(_2\) release. Locally produced tissue factor generates thrombin, a very strong platelet aggregating compound. Moreover, PKC and calcium act upon glycoproteins \( \alpha_2\beta_1 \) and \( \alpha_{IIb}\beta_3 \), the latter being the most abundant integrin in platelets (Jennings, 2009 [a]; Rivera et al, 2009).

Prévost et al demonstrated that “human platelets express Eph receptor kinases EphA4 and EphB1 and the Eph kinase ligand ephrinB1” (Prévost et al, 2005). This finding is important for a complete understanding of outside-in signaling in thrombus growth and stabilization because this study showed Eph/ephrin interaction facilitated Tyr residue phosphorylation.

1.6. Relevance of plug formation and cardiovascular disease

Platelets play an essential role in hemostasis and the development of heart disease and stroke, two leading causes of death in the US (Xu et al, 2009).

1.6.1. Platelets and atherosclerosis

Atherosclerosis has been thought about as a lipid accumulation in blood vessel walls, particularly arteries. An increased number of macrophages in the arterial intimal layer and fat filled macrophages (“foam cells”) characterize the initial vascular lesion which further gives rise to the so called “lipid streak”, recognized as a typically visible lesion (Stary et al, 1994). However,
there are many vascular and inflammation-associated factors involved in atherogenesis. The endothelium exhibits increased platelet adhesiveness and has pro-coagulant tendencies after injury (Ross, 1999). Platelet-endothelial cell interaction induces changes in both: platelets express CD40 protein, which activates antigen presenting cells and stimulates release of cytokines and chemokines from endothelial cells (particularly interleukin-1β, monocyte chemotactic protein-1, and macrophage inflammatory protein-1α) (Gawaz et al, 2000; Cha et al, 2000). These substances recruit cells with inflammatory actions on the injured vascular wall. Moreover, it has been shown that endothelial cell adhesion molecule (PECAM-1) is found in the plasma membrane and alpha granule membrane of platelets and MKs (Cramer et al, 1994) and that these molecules participate in platelet hyperactivity in patients with type-2 diabetes mellitus (Randriamboavonjy et al, 2008).

1.6.2. Thromboembolism, cerebral ischemia and cardiac disease

An abnormal particle (such as a blood clot or air bubble) circulating in the blood is an *embolus* while a *thrombus* is a blood clot formed within a vessel that remains attached to its place of origin. Since “almost all emboli represent some part of a dislodged thrombus, hence the term Thromboembolism” (Kumar et al, 2009). Platelet activation leading to occlusion of blood flow is potentially fatal, particularly when it occurs in the brain or coronary circulation. Brain tissue demands constant oxygen, glucose supply, and removal of CO₂,
accomplished by local metabolite-auto regulated continuous circulation. Therefore, decrease in blood flow or ischemia will result in low tissue perfusion and reduced availability of metabolically vital substances in the blood. Neurons have a low tolerance for glucose and oxygen deficit. Ischemia could trigger a glutamate-mediated increase in $[\text{Ca}^{2+}]_i$ with excitotoxic effects on neuronal function. Tissue damage spreads rapidly, with a high possibility of permanent motor and sensory system impairment as well as associated mood changes or even death. (Mark et al, 2001; Ruiz et al, 2009; Yu et al, 2009) Cerebrovascular disease was the third leading cause of death in the US in the years 2006 and 2007 (Xu et al, 2009). Cardiac disease is the number one cause of death (Xu et al, 2009) in the US. Coronary heart disease (CHD) “is the single largest killer of American males and females. About every 26 seconds an American will suffer a coronary event, and about every minute someone will die from one. About 40% of the people who experience a coronary attack in a given year will die from it” (Thom et al, 2006). Similar to the brain circulation, coronary circulation is autoregulated and controlled mostly by local metabolites, among which adenosine is important and local oxygen concentration (Constanzo, 2006). Physiologically, changes in coronary blood flow are caused by changes in coronary resistance regulated by the aforementioned metabolites: hypoxia due to reduced blood flow or increased oxygen demand (increased cardiac work) causes vasodilation via adenosine
Coronary thrombosis due to atherosclerotic plaque rupture leads to myocardial ischemia and infarction with potentially fatal consequences. The importance of platelet reactivity in coronary heart disease is exemplified by the clinical trials of $\alpha_{\text{IIb}}\beta_3$ and P2Y$_{12}$ receptor inhibitors and the common use of aspirin and thienopyridines, especially after myocardial infarction and coronary stenting (Lefkovits et al 1995; Mehta, 2002; Harding et al, 2001; Galla & Lincoff, 2007; Jennings, 2009 [c]).

1.7. Physiological basis of antiplatelet therapy

Platelets have been identified as key blood elements in the development of cardiovascular disease and its complications. However, controlling platelet reactivity is a matter of considerable difficulty given that many signals converge upon and come from platelets. Today, COX enzyme and P2Y$_{12}$ receptor blockade are used widely in preventing myocardial infarction and thrombotic complications after percutaneous coronary intervention (Sellers et al, 2009).

1.7.1. Cyclooxygenase inhibitor: aspirin or acetylsalicylic acid (ASA)

Aspirin (ASA) was first recognized for its antipyretic and analgesic effects, but it also has anti-inflammatory and anti-thrombotic actions. Probably the earliest large scale clinical evaluation of ASA, as an oral antithrombotic drug, was ISIS-2 (Second International Study of Infarct Survival) (ISIS-2, 1988). Since then, ASA efficacy on platelet
reactivity has been demonstrated in many studies. ASA is absorbed in the upper gastrointestinal tract with a manifest platelet inhibitory effect within 60 minutes and a plasma half-life of 15-20 min. Its bioavailability is around 50% and systemic circulation independent indicating a pre-hepatic platelet acetylating ASA action (Pedersen et al, 1984; Patrono et al, 2008).

1.7.1.1. Mechanism of action

ASA irreversibly inhibits COX 1 via acetylation of serine residues 530 and 516 of COX2 (Ser 530 and Ser 516). (Burke et al, 2006). Because Ser 530 is located in the enzyme hydrophobic channel, its acetylation does not allow substrate-enzyme catalytic site interaction and arachidonic acid does not interact with the catalytic site (Shimokawa & Smith 1992; Undas et al, 2007; Kresge et al, 2008). COX1 is the predominant form in platelets, but, interestingly, COX2 is detectable in newly formed platelets and is the predominant PGE2 generating enzyme in megakaryocytes (Rocca et al, 2002; Borgdoff et al, 2006). By blocking these two isoenzymes, ASA inhibits early steps in prostanoid synthesis; resulting in low levels of PGG2 synthesis and therefore inhibition of TXA2 generation (see Fig. 1.3.). Since platelets are anucleated formed elements, ASA blocking action on
COX, and therefore TXA$_2$ production, lasts throughout the platelet lifespan (Awtry & Loscalzo, 2002). Nevertheless, direct action of ASA on rat MKs has been shown to be 70% effective with stable recovery 36 hrs after ASA exposure, presumably due to regeneration of COX (Demers et al, 1980; Worthington & Nakeff, 1982). COX2 has less sensitivity to ASA, and as a key enzyme in MKs, could be relevant for this research project. In considering the effects of ASA in the whole organism, ASA effect on PGI$_2$ inhibition in vascular endothelial cells should be taken into consideration since PGI$_2$ inhibits platelets by elevating intraplatelet cAMP (Patrono et al, 2001). Elevation of cAMP stimulates DAG kinase activity, preventing increases in DAG (Guidetti et al, 2008), and decreases calcium release from intracellular stores by phosphorylation of the IP$_3$ receptor, attenuating its sensitivity (Cavallini et al, 1996).

1.7.1.2. Prevention of cardiovascular events

Aspirin is a commonly used antiplatelet drug with a cardioprotective effect demonstrated in clinical trials. (Meyer et al, 1989; SPRIT study group, 1997; Catella-Lawson et al, 2001; ESPRIT study group, 2007). An ASA daily single dose (325 mg) doubles bleeding time in a healthy person while doses ranging
from 75 to 81 mg/day are reported to achieve cardioprotection in patients after coronary complications or thrombotic stroke. At such doses irreversible platelet COX1 inhibition by ASA takes place in the portal circulation before deacytlation to salicylate is carried out by the liver (Pederson and FitzGerald, 1984; Burke et al, 2006). ASA produces 98% inhibition of COX1-generated TXA2/TXB2 production within 2 hours (Catella-Lawson et al, 2001). In terms of reduced vascular mortality, 11% reduction in patients with suspected acute ischemic stroke, 46% in patients with unstable angina, and 53% after coronary angioplasty have been reported (Antithrombotic TRIALISTS, 2002).

1.7.1.3. Aspirin resistance phenomenon

The main goal of antiplatelet therapy is to inhibit platelet aggregation by maintaining platelet reactivity at a normal level, thus reducing the occurrence of thrombotic events. Normal physiological platelet reactivity should be enough to prevent bleeding but at the same time not allow thrombus development with cardiovascular complications. However, platelet response to antithrombotic drugs is variable, and the term “variability of platelet response (VPR)” has been coined to describe responses to aspirin and purinergic receptor blockers (Jennings, 2009 [b]). As
part of the VPR, patients with low response (recurrence of cardiovascular events or lack of platelet inhibition to ASA treatment are said to be aspirin resistant, which is considered a treatment failure (Patrono et al, 2001; Patrono, 2003). On one hand, ASA resistance could be defined in terms of repeated thrombo-embolic episodes with decreased blood flow related to patient non-compliance, drug interaction, or subject pharmacodynamic variability. On the other hand ASA resistance can be measured as an in vitro/ex vivo phenomenon with increased platelet reactivity after antiplatelet drug exposure with prevalence between 8-51% depending to the applied experimental technique and ASA time exposure (Grotemeyer, 1991; Mueller et al, 1997; Machi et al, 2002; Coakley et al, 2005; Jennings (b), 2009). Interpretation of ASA resistance should take into consideration COX2 action, especially in experiments with MKs and young platelets where COX2 expression is higher. ASA action on COX2 is 170 fold less potent than on COX1 (Weber et al, 1999) and TXA2 levels must be suppressed by more than 90% for a clinical treatment to be successful (Udaya et al, 2009) Interestingly, some studies have proposed that ASA treatment eventually leads to increased risk of cardiovascular complications
associated with increased bleeding time (Ferraris et al, 2002; Gum et al, 2003, Coakley et al, 2005).

### 1.7.2. ADP receptor blockers

Besides the ASA sensitive COX1-TXA$_2$ pathway, purinergic receptors have become an important target for antithrombotic drugs. These two systems activate the expression of $\alpha_{\text{IIB}}\beta_3$ on the platelet surface as a final, common step towards platelet aggregation. Because the P2Y$_1$ receptor acts mostly as a primer complemented by the P2Y$_{12}$ activation for a full aggregating response, P2Y$_{12}$ blockers have been tested in clinical trials. In fact, the thiopyridine P2Y$_{12}$ blocker, clopidogrel, proved to have higher effectiveness in reducing cerebrovascular events compared to ASA in a three year study (CAPRIE, 1996). The main study favoring the P2Y$_{12}$ blocker clopidogrel as a first choice after myocardial infarction is the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial, which concluded a 2.1% reduction in absolute risk for death, stroke, or myocardial infarction (Yusuf et al, 2001). In a large comparative study, cited as number 1 in the top ten 2006-2008 studies on stroke (Hart, 2008), a comparison of the P2Y$_{12}$ blocker, COX1 inhibitor (ASA) and adenosine uptake inhibitor/cAMP stimulator (dipyridamole) showed no difference for recurrent stroke
rate among the three drugs tested, but less hemorrhage episodes in the clopidogrel treated group during the 2.3 years follow-up (Diener et al, 2007). In spite of these results there are some unsolved issues regarding the use of P2Y₁₂ blockers: side effects and non-responding patients, or clopidogrel resistant patients. After an ischemic episode and coronary artery stenting, 300 mg load dose and 75 mg daily do not reduce coronary occlusion, myocardial infarction or even death. (Steinhubl et al, 2002). A platelet aggregation study in patients with the standard aspirin (325 mg) and clopidogrel regimen (300 mg load/75 mg qd) reported 55% of patients with increased platelet reactivity with 5 and 20 μM ADP-induced aggregation 2hrs post-stenting (Gurbel et al, 2004). Consequently, “clopidogrel resistance” has been defined as an absolute difference between baseline aggregation and post treatment aggregation of 10% or less, when 5 μM ADP is used as stimulus of platelet aggregation (Gurbel et al, 2003; Gurbel & Tantry, 2007; Siller-Matula et al, 2007). This definition might be too restrictive since “no single receptor signaling pathway mediating platelet activation is responsible for all thrombotic complications” (Gurbel & Tantry, 2007).
1.8. Electrophysiological studies in megakaryocytes and platelets

The whole voltage clamp technique developed in the seventies (Neher et al., 1978), measure ionic currents through the cell. The development of single membrane patch, higher recording resolution, and direct control over a membrane patch voltage were achieved later by Hamill et al. with the single channel recording technique (Hamill et al., 1981). The first patch clamp studies in non-excitable cells were carried out on chromaffin cells of the adrenal medulla (Fenwick et al., 1982). Early studies showed that activation of platelets with ADP or U46619 did not change human platelet membrane potential (MacIntyre & Rink, 1982) but later reports in platelets (Mahaut-Smith et al., 1990) and megakaryocytes (Kawa, 1990; Mahaut-Smith et al., 1992; Unemaya et al., 1993) suggested a more complex cell signaling system where purinergic receptors might play a role. However, diverse and not completely understood biochemical signals, striking changes in cell shape, and a 2-3 µm cell size represent a challenge to study platelet physiology at a single cell level. Purinergic receptors are used currently as targets for drugs to prevent coronary obstruction and stroke. However, there are issues, like drug resistance and drug-related side effects that demand further studies. Current available techniques require the use of many cells to study platelet reactivity. In contrast, the patch clamp technique offers a unique opportunity to analyze,
at single cell level, changes in agonist-induced ionic currents using the megakaryocyte (30-150 µm cell size) model of platelet reactivity, overcoming a major constraint related to platelet size. Besides the size advantage of MKs over platelets, MKs, as progenitor cells, hold all metabolic components present in platelets.

1.8.1. Patch clamp technique

Cell membrane phospholipids keep charged molecules and ions effectively separated in two water compartments: intracellular and extracellular, with different concentration of these molecules and ions at each site. According to the fluid mosaic model of the cell membrane (Singer & Nicholson, 1972), this lipid bi-layered barrier is dynamically fluid and contains embedded proteins, which work as channels, allowing ion movement across the membrane according to the electrochemical gradient. There are striking differences between compartments in terms of the elevated sodium and calcium concentrations in the extracellular milieu and potassium in the intracellular fluid. The ionic gradients are kept in equilibrium by pumps (Na⁺/K⁺ ATPase), exchangers, and passive diffusion, generating a fairly constant and distinctive membrane potential. The resultant concentration gradient across the cell membrane can force ionic movement through the highly selective channels down the
electrochemical gradient. These channels are closed at resting state, but can be opened by chemical agonists (“ligand gated”), by changes in membrane voltage (“voltage gated”), by mechanical forces (“stretch activated”), by second messengers (calcium activated channels), etc. A matter of vital importance for every cell is to keep intracellular calcium ion concentration at a very low level (<100nM in MKs [Ikeda et al, 1994]), which is why intracellular calcium regulatory mechanisms have evolved through time.

The membrane potential can be measured. Since the lipid bilayer separates two water compartments (highly conductive) and protein channels allow ions to pass-through, it is a system where current flow can be measured. Two important components of this system are membrane resistance and capacitance. Membrane resistance depends directly on ion channel activity while capacitance, a property that results in charge storage, depends on the electromagnetic forces across the membrane (Molleman, 2003).

1.8.2. Biophysical principles

1.8.2.1. Basic circuitry

A glass pipette is attached to the cell membrane and by suction a tight seal is created with resistance in the order of one giga-ohm. If any ion
crosses through that patched section of the cell membrane, a current will be generated and recorded by an electrode inside the pipette connected to a head-stage amplifier while holding the membrane voltage constant. The signal will be sent to an amplifier and stored in a computer by the appropriate software.

**Fig. 1.6. Whole cell patch clamp - Nystatin perforated variant.** Nystatin in the internal solution (IS) inserts pores through the patched membrane, allowing ionic flow. Voltage is clamped at physiological value (holding potential) while changes in current are amplified and recorded.

As in any electrical circuit, the current flow depends on the resistance, which, in this case, is represented by the pipette and the membrane patch resistance. The latter is the most important because current is measured through this
resistor. The attachment between the glass pipette and the membrane patch is very tight, so any fluctuation in current would be attributable to ions crossing through the patch. It does not mean that some “leaky” resistance –between the pipette and the cell surface- is not present during the experiment. The advantage of the patch clamp technique is that specifically designed amplifiers clamp either voltage or current value for any given cell. Amplifiers usually generate command voltage internally, enabling changes in transmembrane potential (Vm); by clamping that value at the experimenter’s will, current is measured. By clamping the current value, the voltage membrane can be measured (Molleman, 2003).

1.8.2.2. Voltage, current and resistance – Ohm’s Law

Ohm’s law states that current (I) varies directly with changes in voltage (V) and inversely with changes in resistance (R).

\[ I = \frac{V}{R} \]

Since patch clamp amplifiers operate on voltage clamp or current clamp modes, in which that specific variable becomes constant. Therefore, in the voltage clamp mode, current changes are directly proportional to changes in resistance through the membrane patch and reflect ionic conductance. When current is clamped, changes in resistance directly affect voltage.
1.8.2.3. Patch clamp configurations

1.8.2.3.1. Conventional whole cell recording

Once the pipette makes contact with the cell surface, suction through the pipette ruptures the membrane, and the internal solution and the patch electrode in the pipette will be in direct contact with the intracellular compartment. A second electrode (ground electrode) is in the bath solution (external solution) see Fig. 1.6, allowing comparison of the potentials at each side of the cell membrane, thus measuring transmembrane potential ($V_m$).

1.8.2.3.2. Nystatin perforated whole cell recording

This is a modified version of the conventional whole cell recording that applies the nystatin pore-forming property which slowly makes pores in the patch underneath the pipette small enough for ions to cross through while maintaining most of the intracellular components in the cytosol. In our experiments $≥50\, MΩ$ access resistance value was acceptable for current recording. Whole cell patches do not allow single channel recordings but measure a mean current of a given channel population, sometimes called a “macroscopic current” (Schubert et al, 1987; Zhou & Lipsius, 1993; Standen et al, 1994)
1.8.2.4. Pipettes for patch and gigaohm seal.

The patch clamp technique owes its high resolution to the development of a gigaseal. This high resistance attachment effectively separates the extracellular compartment from the intracellular milieu so that all ionic movement through the patch will be recorded by the head-stage amplifier (Levis & Rae, 1993, 2007). Pipettes are pulled from glass capillaries usually <1.2 mm in diameter. By applying heat with a filament in a vertical puller, the glass capillary gets thinner and is separated into two micropipettes. Pipette resistance relies mostly on its size at the tip, and in most experiments the resistance varies between 2-8 mega-Ohms. Pipette resistance from 1 to 2 MΩ is recommended (Mahaut–Smith & Gibbins, 2004), but in our experiments a 3 to 6 MΩ resistance value offered easiness for seal formation and acceptable resolution.

**Gigaseal formation:** once the pipette is attached to the cell surface, suction through the internal solution-filled pipette develops a giga-Ohm seal. This is the cell-attached configuration. If nystatin is used in the internal solution (IS), then the cell is left in this configuration to allow pore formation, which causes a gradual decline in resistance. In conventional patch clamp, continuous suction finally ruptures the cell membrane, allowing electrical
access for current recording evidenced by a usually large capacitative transient. Once the capacitative transient appears, after nystatin action or suction, a whole-cell configuration is attained and recording is possible.

1.8.3. Current recording: purinergic-induced ionic currents

Soon after the whole cell configuration or a substantial decrease in access resistance is detected in the perforated recording mode, 5 mV hyperpolarizing/depolarizing steps are used to activate voltage gated potassium currents to ascertain voltage control and exclude the possibility of closure.

1.8.3.1. ATP-induced ionic currents

Human platelets and rat MKs have voltage-activated K⁺ currents (Maruyama, 1987; Romero & Sullivan 1997) as well as purinergic-induced currents. P2 receptors in platelets and MKs can be stimulated by ATP and ADP, elevating intracellular calcium and activating calcium dependent potassium outward currents.

The P2X₁ receptor, upon activation, opens up as a cationic channel mainly for Ca²⁺ and Na⁺ in human platelets and rat MKs (Clifford et al, 1998; Vial et al 2003). P2X₁ human receptor expressed in HEK293 cells have shown to be permeable to sodium and
potassium (Evans et al, 1996). The preferential agonist for this receptor is ATP, and to a lesser extent ADP (see section 1.3.1.2.1.) This fact has been questioned because of contamination of commercially available ATP with some ADP (Mahaut-Smith et al, 2000; Vial et al 2002). In whole cell recordings in human platelets, at a holding potential of -70 mV, it has been shown that 40 µM ATP induces a response with an initial 25-106 pA transient inward current, producing an intracellular calcium elevation to 138 nM in the presence of 1 mM external calcium (MacKenzie et al, 1996). The ATP-induced P2X$_1$ response desensitizes rapidly but synergizes with P2Y to elevate intracellular calcium and even cause platelet shape change. (Rolf et al, 2001, Vial et al 2002). In nystatin perforated patch clamp experiments, the ATP-induced response has been characterized as biphasic: after the calcium-sodium inward transient followed by calcium activated potassium currents. P2X$_1$R stimulation, in 2mM [Ca$^{2+}$]e, with 10µM αβ–methylene has been shown to increase [Ca$^{2+}$]i from 90 to 350 nM (Fung et al, 2007). ATP 1 µM generated concentration dependent oscillatory outward currents in rat megakaryocytes (Uneyama et al, 1999). The amplitude and frequency of the calcium oscillations depend on the pumping action of plasma
membrane and SERCA Ca\(^{2+}\) ATPases after IP\(_3\), DAG and cGMP generation (Uneyama et al, 1993a, b; Somasundaram & Mahaut-Smith, 1994; Uneyama et al, 1995; Uneyama et al 1998).

Oscillatory ATP-induced currents decrease during the first minute not only in amplitude but also in duration. The time between peaks has a tendency to increase during this period (Uneyama et al, 1993b). ATP-induced K\(^+\) currents disappear as the agonist application continues, suggesting desensitization. After washing the MKs and a period of recovery in the absence of the agonist, it is possible to evoke outward oscillatory currents again. The oscillatory outward current pattern changes to plateau-like with elevated calcium concentration such as that induced by calcium ionophore A23187. The same effect is observed when intracellular calcium dynamics is disrupted by blocking the SERCA transporter with thapsigargin or blocking the IP\(_3\) receptor with xestospongin C, suggesting a critical calcium concentration threshold to sustain oscillatory currents. (Uneyama et al, 1993b). It has been shown that ATP-induced oscillatory currents are inhibited by PKC activation, but the exact mechanism for this effect is not understood. The first possible mechanism for desensitization in ATP and ADP stimulated MKs was analyzed with phorbol ester
phorbol 12-myristate 13-acetate (PMA) treatment showing a decreased outward current amplitude with P2Y receptor internalization and purinergic receptor phosphorylation expressed in 1321N1 cells (Mundel et al, 2006). The second possibility is that in MKs, a PKC isoform stimulates a Ca$^{2+}$ pump, expelling this ion from the cells, decreasing the amplitude of the outward currents as has been demonstrated in Jurkat cells (Balasubramanyam & Gardner, 1995). The role of P2X$_1$-mediated signaling in thrombus formation has been clearly demonstrated with reduced collagen induced aggregation and secretion in P2X$_1^{-/-}$ receptor deficient mouse platelets. It is noteworthy that these mice do not exhibit spontaneous bleeding and the platelet count is normal (Hechler et al, 2003).

1.8.3.2. ADP-induced ionic currents

In the guinea pig MK, ADP induces >400pA outward calcium activated K$^+$ currents at -43mV voltage clamped potential (Kawa, 1990, 1996). Similar to the ATP-induced currents, the ADP-induced response decreases over time, presumably due to desensitized receptors, and shows recovery after desensitization. An important observation is that subsequent response amplitude is
always lower than initial, even after increasing the rest period before
the next ADP application (Kawa, 1990). In rat megakaryocytes,
oscillatory calcium activated K\(^+\) currents are also generated by ADP
stimulation with a frequency of 3-10 spikes/min, preceded by a
small inward transient. These oscillations perfectly match
intracellular calcium variations. After 10 µM ADP application in
guinea pig and rat MKs the initial intracellular calcium
concentration changes from 50 nM to >200 nM (Kawa, 1990;
P2Y receptor-mediated mechanism in rat MKs can be potentiated by
membrane voltage changes with an increase in intracellular calcium
concentration dependent on the depolarizing step amplitude
(Martinez-Pinna et al, 2004). However, the voltage-induced K\(^+\)
currents are sensitive to TEA, quinine and 4-amino-pyridine,
suggesting a delayed rectifier K\(^+\) channel in the MK. In contrast to
the previous observation, the ADP induced K\(^+\) currents are nearly
insensitive to those blockers (Kawa, 1990). ADP and 2methyl-thio-
ADP stimulate both P2Y\(_{1}\) and P2Y\(_{12}\) receptors, generating a very
similar inward transient followed by an outward oscillatory current
pattern with great cell-to-cell diversity. Moreover, specific P2Y\(_{1}\)
agonist MRS2365 also induces oscillatory outward currents in rat
Importantly, there is a P2Y1 – P2Y12 interaction demonstrated in P2Y1+/− mouse as a requisite for further ADP current activation and that interaction extends to P2X1. Therefore, for a full platelet aggregatory response, P2 receptor co-activation is required; with P2Y1 exerting platelet shape change and P2Y12 further amplifying calcium elevation and the release of dense granule content (Hecheler et al, 1998; Jin & Kunapuli, 1998; Tolhurst et al, 2005).

Purinergic receptor activation triggers calcium dependent mechanisms and potentiates response to other platelet agonists. Changes in ionic currents, pseudopodia formation, and blebbing are frequently observed after exposure to ADP and ATP. Purinergic signaling might play a role even in proplatelet formation (Leven 1983, Leven & Yee, 1987).

1.9. Summary of platelet regulation.

Platelets are regulated by many different factors coming from the endothelium, the vascular wall, leukocytes, red cells, and from the platelets themselves, creating a complicated series of chemical reactions that keep these cells at the appropriate level of responsiveness (Michelson, 2002). The importance of evaluating platelet responses to agonists such as arachidonic acid, ADP, thrombin,
thromboxane, and collagen has long been recognized. Therefore, laboratory tests using large numbers of cells, such as aggregometry and PF-100, have been developed to evaluate platelet reactivity to those agonists. Recently, recognition of the importance of purinergic agonists in platelet physiology has been regained, due to the identification of two groups of purinergic receptors in megakaryocytes (MKs): the ATP receptor P2X$_1$ has been characterized in mouse and rat (Ikeda 2007; Mahaut-Smith et al 2000; Somasundaram & Mahaut-Smith, 1994; Tolhurst et al, 2005; Weber et al, 1999) while two distinct types of P2Y receptors have been found in these species: P2Y$_1$ and P2Y$_{12}$ (Somasundaram & Mahaut-Smith, 1994; Tolhurst et al, 2005; Martinez-Pinna et al, 2005). ADP receptors have been identified in human platelets (Hollopeter et al, 2001). Cross-talk between P2Y$_1$ and P2Y$_{12}$ receptors (Hardy et al, 2004) and, rapid receptor resensitization (Mundell et al, 2008) has been demonstrated. ADP induces repetitive outward potassium currents characterized by a time-dependent rapid decay in amplitude explained by receptor desensitization (Kawa K 1990, 1996; Somasundaram & Mahaut-Smith 1994; Tolhurst et al 2005). In addition ADP-induced inward transient currents have also been reported in voltage-clamp studies of mouse MKs and human platelets using KCl internal solution at a holding
potential \((V_h)\) of \(-42\) mV (Kawa 1996; Mahaut-Smith et al 1992). ATP-induced inward transient currents in rat and mouse MKs are sensitive to P2X blockers which reveal \(\text{Ca}^{2+}\) dependent \(K^+\) outward current (Ikeda 2007; Somasundaram & Mahaut-Smith 1994; Weber et al, 1999). Few studies have been performed on the electrophysiological characteristics of these anucleated formed elements due to methodological limitations primarily related to platelet size. In this dissertation thesis there is a unique opportunity to analyze platelet signaling at a single cell level using the megakaryocyte. This large cellular precursor for platelets in the bone marrow contains all the metabolic components present in platelets and has similar sensitivity to drugs, and can, therefore, be used as platelet surrogate to address changes in ionic conductance after cell membrane receptor activation applying the patch clamp technique (Carter et al 2006, Cazenave & Gachet 1997; Mason et al 2005; Tolhurst et al 2005; Uneyama et al 1993a, b).

We hypothesize that ASA, in addition to being a COX inhibitor, attenuates purinergic-induced \(\text{Ca}^{2+}\)-activated \(K^+\) currents through a mechanism related to \(\text{Ca}^{2+}\) mobilization into the platelet cytosol. This novel action alters the expected anti-aggregatory effects of ASA which are reduced in the ASA resistant population. Because 1)

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thromboembolism-related diseases including ischemic heart disease and cerebrovascular disease are the number one and two causes of death worldwide and 2) ASA is widely used to treat these diseases, our work has considerable translational relevance.
CHAPTER 2

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1. Drugs

- 2-methyl-thio-ADP (2MeSADP)
- 2, 2, 2-tribromoethanol
- 4-aminopiridine (4-AP)
- 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F\textsubscript{2α} (U46619)
- Acetylsalicylic acid (ASA)
- Adenosine diphosphate (ADP)
- Adenosine triphosphate (ATP)
- Anti-Orai1 primary antibody (rabbit polyclonal)
- Anti-STIM1 primary antibody (mouse monoclonal from BD Biosciences)
- Fluo4-AM from Invitrogen (Carlsbad, CA, USA).
- Inositol 1,4,5-triphosphate (IP\textsubscript{3})
- Ionomycin
- Normal Donkey Serum, Dylight GREEN 488-conjugated AffiniPure
  AntimouseIgG (H+L) (Jackson Immuno Research. PA, USA)
- Nystatin
- Thapsigargin
- Normal Donkey Serum Dylight RED 549-conjugated AffiniPure
  AntirabbitIgG (H+L) (Jackson Immuno Research. PA, USA)
- Trypan blue
- Xestospongin C

All drugs above were from Sigma Aldrich, (St Louis, MO) or otherwise stated.

- 2'-deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS2179) (from TOCRIS Bioscience Inc. Ballwin MO)

- For most drugs stock solutions were prepared in water and diluted in ES as needed. Ionomycin, nystatin, xestospongin C and MRS2179 were prepared fresh and diluted in dimethylsulfoxide (DMSO). Fluo-4AM was dissolved in DMSO 0.1% for a final concentration of 1 nM and stored at -20 °C in aliquots

2.2. Solutions

2.2.1. Electrophysiological recordings

2.2.1.1. Outward current recordings

2.2.1.1.1. Internal pipette solution (IS) to record outward currents:

IS was modeled from (Uneyama et al, 1993a) and had the following composition (in mM): 150 KCl, 10 HEPES, pH 7.2-7.3 adjusted with KOH 5N.

2.2.1.1.2. External solution (ES):

This solution was used to suspend the megakaryocytes during the isolation procedure as well as for an extracellular recording solution. It contained (in mM) 145
NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES 10 D-glucose, 10 µM D-serine. pH 7.2-7.3 with NaOH 5N.

2.2.2. Calcium imaging recordings

Cells were placed in calcium free ES. Aspirin (1mM) and 2MeSADP (5µM) were dissolved in ES. Thapsigargin stock solution was dissolved in DMSO and in ES for a 2µM final concentration. Fluo-4 1 nM final concentration was dissolved in 0.1% DMSO and stored at -20°C in aliquots.

2.3. Animals: 6 week old male Sprague-Dawley rats (from Harlan IN, USA).

2.4. Cell preparations

2.4.1. Megakaryocyte isolation

Cells were collected from rats, decapitated after complete anesthesia with 2, 2, 2-tribromoethanol, 240 mg/kg body weight i.p. in accordance with Georgetown University Animal Care and Use Committee policies. Marrow containing MKs was isolated from the femoral bones (Somasundaram & Mahaut-Smith, 1994) into extracellular recording solution with a previously described composition (2.2.1.1.2). Experimental cells were incubated in 1 mM ASA which is within the range of therapeutic concentration (US Pharmacopeial Conv, 2000) (diluted in ES) for at least 30 min. and
control cells were incubated in ES. An aliquot (100 µl) of cell sample suspension was plated directly onto the recording chamber allowing 5 - 10 min. for cell adhesion.

2.4.2. Trypan blue exclusion test of cell viability

A sample of marrow tissue was suspended in 1 ml extracellular solution and cells were dispersed by repetitive pipetting. One part of the cell suspension was mixed with one part of 0.4% trypan blue allowing 3 min. for incubation. Living cells were unstained and appeared clear while dead cells were darkly stained (Fig 1A) (Leven & Yee, 1987).

2.4.3. Megakaryocyte identification

MKs were observed with a Nikon upright or inverted phase contrast microscope (Tokyo, Japan) and photomicrographs were obtained with a digital camera system using Scion software (Frederick, MD, USA). Identification was based on cell size, the presence of multilobulated nucleus, and bright appearance with phase-contrast illumination (see Fig. 3.1 for examples).

2.5. Electrophysiological studies

2.5.1. Patch clamp amplifier and computer based acquisition system

Axopatch 1-D amplifier (Molecular Devices, Co. Sunnyvale CA, USA)
and a digitizer Digidata 1322A (Axon Instruments, Inc. Foster City, CA) were used for data recording.

2.5.2. Pipettes

Recording electrodes were pulled on a vertical pipette puller (Narishige, PP-83, Tokyo, Japan) from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA) and had resistance from 3 to 5 MΩ.

2.5.3. Perfusion system and tissue chamber

2.5.3.1. Drug application. Drug application was accomplished using the so-called Y tube system (Murase et al, 1989). A fine (50 µm i.d.) tube was inserted into a U-shaped polyethylene tube, and then the fine tube was positioned directly above the cell being patched. Suction pulled the solution through the U and when the suction was turned off, the solution flowed onto the cell by gravity. Applications were done soon after the whole cell configuration, or when substantial decreases in access resistance were detected in the perforated recording mode and recording continued through the period of oscillatory response currents. After 1, 3, and 5 min. of recovery while perfusing ES locally via Y-tube to remove the agonist, this procedure was repeated.
2.5.4. Recording protocols

2.5.4.1. The 30 second protocol

To evaluate decay in current amplitude, recording of oscillatory outward currents induced by 10 \(\mu\)M ADP, 10 \(\mu\)M ATP, and 5 \(\mu\)M 2MeSADP were analyzed during 30 seconds after the initial application of the agonist. Each peak was measured in the presence of the agonist during that time.

![Graph showing current evoked with the 30 second protocol](image)

Fig. 2.1 Example of current evoked with the 30 second protocol. After access resistance decreased to \(\leq 50\text{M}\Omega\), cells were stimulated with ADP 10 \(\mu\)M, ATP 10 \(\mu\)M or 2MeSADP 5 \(\mu\)M. Control group was incubated in ES while experimental cells were exposed to ASA 1mM for 30 minutes.

2.5.4.2. The U46619 and xestospongin C protocols

To study heterologous desensitization between TXA\(_2\) and ADP pathways, MKs were exposed to U46619 (2 \(\mu\)M) for 30 s
immediately before purinergic agonist application. U46619 was present during the 10 µM ADP application and both were removed by washing with ES. In a different set of cells, MKs were exposed for 30 s to 300nM xestospongin C solution and then challenged with 30µM ADP. This procedure was repeated after 1, 3 and 5 minutes recovery in both studies.

**Fig. 2.2 Example of current evoked with ADP in the 0,1,3,5 min protocol to test heterologous desensitization.** Experimental (Exp) group was exposed to U46619 2 µM for 30 sec and then ADP 10 µM was added. Control group was exposed to ES. Traces shown are actual single peak experimental responses to ADP 10 µM -initial, after 1 min and 3 min recovery in the U46619 pretreated group. (IS: internal solution; ES: external solution)

**2.5.4.3. The 0, 1, 3, 5 min. protocol.** Since recovery of MK responses have been reported, this protocol was designed to study current
amplitude over a longer period of time. Drugs were applied right after whole cell configuration was achieved with decrease in access resistance $\geq 50 \text{M} \Omega$ (time zero) and after 1, 3, and 5 min of recovery while perfusing ES locally. The same procedure was applied to experimental cells after 30 min incubation in ASA 1 mM.

![Diagram](image)

**Fig. 2.3 Example of current evoked with the 0,1,3,5 min protocol to test aspirin effect.** Experimental (Exp) group was incubated in ASA 1 mM for at least 30 min and then ADP 10 $\mu$M or ATP 10 $\mu$M was added. Control group was not exposed to ASA. The depicted actual traces are for ADP 10 $\mu$M control group. (IS: internal solution; ES: external solution)

**2.5.4.3.1. Calcium free external solution.** Experiments were carried out in calcium free media to address calcium activated potassium currents generated by calcium released from DTS. This solution had
the same composition as that described in 2.2.1.1.2 with no calcium included.

2.5.4.4. Dose response protocol

Dose response curves for 2MeSADP were produced applying increasing agonist concentrations with 30 s recovery between each application, in the presence of either 1 mM ASA, 10 μM MRS2179, or control solution.

2.5.4.5. Depolarizing steps to activate voltage-dependent K⁺ channels

Voltage gated potassium currents were generated with 10 mV depolarizing steps starting from -80 to 0 and +80 mV at -40 mV holding potential in whole cell patch-clamp configuration. This protocol was used primarily to ascertain voltage control during whole cell recordings.

2.6. Studies on calcium dynamics

2.6.1. Calcium measurement as change in fluorescence

2.6.1.1. Control vs. ASA treated MKs calcium variation

Fluorescent dye preparation: 50 mg freeze-dried aliquots of Fluo-4 (Invitrogen, Cat. No. F14201) were dissolved in 100 ml DMSO. An aliquot of 100-150 μL of MK suspension in ES (TG experiment, 30 s protocol, 0, 1, 3, 5 min protocol) or Ca²⁺-free ES (resting and 2MeSADP-evoked Ca²⁺ measurements) or 1 mM ASA diluted in ES
or Ca\textsuperscript{2+}-free ES were loaded for 1 hour with Fluo-4AM (3–5 µM) dye for 1 hr at 25°C for a final concentration of 1.0 nM. Cells were washed with several volumes of bathing solution before recording. The dye was excited at 480±15nm. Emitted fluorescence was filtered with a 535±25 bandpass filter captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and read into a computer. Fluo-4 was excited at 488±15 nm, and emitted fluorescence was filtered with a 535 ± 25 nm band pass filter. Cells were exposed to 5 µM 2MeSADP for 30 s following the 0,1,3,5 min protocol to compare with electrophysiological recordings. Absolute [Ca\textsuperscript{2+}]\textsubscript{i} was measured by applying 10µM ionomycin at the end of the experimental protocols to obtain the maximal fluorescence and converted to nanomolar concentration with equation described in 2.8.3. Images were recorded at 1 frame/s by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI), and read into a computer. Analysis was performed using Simple PCI software (Compix Inc., Sewickley, PA). Once the data was imported, the values obtained in each frame were copied into a spreadsheet for further analysis with Clampfit 10 (Molecular Devices) or Microsoft Excel (Redmond, WA) and graphs were generated.
2.6.1.2. Thapsigargin-induced and SOCE-induced intracellular calcium values

As a standard procedure in calcium studies, cells were exposed to thapsigargin (2 µM) in Ca\(^{2+}\)-free ES for 5 minutes and immediately afterwards to Ca\(^{2+}\) using a buffer containing 1mM calcium. The objective of using thapsigargin is to induce calcium elevation through activation of \(I_{\text{CRAC}}\) (Mercer et al, 2006) or TRPC\(_1\) (Carter et al, 2006) calcium channels.

2.7. Studies of STIM1 and Orai1 in megakaryocytes

2.7.1. STIM1 and Orai1 immunohistochemical staining

- MKs were harvested from bone marrow into external solution.
- Samples were homogenated by repetitive pipetting to make a suspension
- 100 µL cell suspension was added in each 12 well dish with a cover slip at room temperature
- Control cells were placed in ES
- ASA was added at final concentration of 1 mM
- Cells were incubated for 30 minutes at room temperature
- To examine the aggregation of STIM1 and Orai1 cells were incubated with thapsigargin (TG) 5 µM final concentration for 10 min.
After TG stimulation cells were washed with HBSS immediately and fixed with 3% PFA in phosphate buffered saline (PBS) overnight at 4°C. Cells were further washed with 100mM glycine in PBS for 25 min at room temperature: previous mix was removed and 200 μL ES added

PFA was removed followed by two washes with PBS (200-300 μL). PBS was used from a separate container to avoid direct use of main stock

After PBS washing, MKs were permeabilized with 0.5% TritonX-100 (mixed into PBS to make a 0.5% solution) during 5 minutes at room temperature.

PBS-glycine solution was removed. Samples were then washed twice with PBS.

Normal donkey serum (NDS - product #017-000-121 Jackson Immuno Research, PA, USA) was added as a blocker

Blocking: cells were blocked with 5% normal donkey serum in PBS with BSA (1%) at room temperature for 45 mins. The appropriate volume was removed to add Ab solution

The primary Ab, anti-STIM1 or Anti-Orai1, dissolved in PBS/BSA at 0.5%. Final concentration was added to each well. Anti-STIM1 (mouse monoclonal, BD Biosciences) or anti-
Orai1 (Rabbit polyclonal, Sigma Chemicals) were used at 1:1000 dilutions. For single staining, 200 µL of an antibody solution was added. For double staining, 100 µL of each antibody solution was added to each of the wells. Incubation was performed for 2 hrs at room temperature or overnight at 4°C.

- Primary Ab was removed
- Secondary Ab was added at 1:200 dilutions (Dyilight GREEN 488-conjugated and or Dyilight Red 549 conjugated for 20 min to stain STIM1 green and Orai1 red. Code #715-505-150 from Jackson Immuno Research, PA, USA). Samples were incubated at 4°C overnight
- Dyilight Green was used for STIM1 and Dyilight Red for Orai1
- Samples were incubated 15 min at room temperature
- Coverslips were mounted onto glass slides.
- Confocal microscopic observation: as single section at the middle of the cell in the XY planes with an Olympus Fluoview-FV300 Laser Scanning Confocal System (supported by Lombardi Comprehensive Cancer Center Microscopy and Imaging Shared Resource, U.S. Public Health Service Grant
2P30-CA-51008 and 1S10 RR15768-01). All images were taken at 60X. Bars represent 10 μm.

2.8. Data analysis

2.8.1. Data normalization

Since current oscillations exhibited a wide range of variation in amplitude in both control and experimental MKs, currents were normalized by setting the initial peak amplitude as 100% for the 0, 1, 3, 5 min protocol. Dose response currents were normalized by setting the greatest amplitude current as 100%. Statistical significance was assumed at $P \leq 0.05$ comparing control VS ASA at each time point.

2.8.2. Analysis of $I$ period and delay

$I$ period was defined and measured as duration of each current peak. Delay was measured as the time from peak to peak; interval 1 = between peaks #1 and #2, interval 2 = between peaks #2 and #3, etc. Average values for each cell group was calculated and plot as delay time vs. number of intervals (Uneyama et al, 1993).
Fig. 2.4 Delay and \( I \) period analysis. Example current traces recorded with the nystatin perforated patch-clamp configuration from a control cell stimulated with 5 \( \mu \)M 2MeSADP. Dashed lines indicate the \( I \) period: duration of the outward current and double arrowed segment define the “delay” as the time occurring from one peak to the next.

2.8.3. Intracellular calcium analysis

Calcium signals recorded were analyzed using Simple PCI software (Compix Inc., Sewickley, PA). Once the data was imported the values obtained per frame were copied into a spreadsheet for further analysis with Clampfit or MS Excel. To analyze images Fluoview acquisition software including Timelapse and Ratiometric modules were used. Calcium changes were measured as changes in fluorescence (\( \Delta F \)). The maximal fluorescence value \( (F_1) \) over the basal calcium value \( (F_0) \) was
calculated as $F_1/F_0$ and plotted vs time. For calcium concentration measurement maximal fluorescence was attained with 10µM ionomycin application and converted to nanomolar concentration with this equation: 

$$[Ca^{2+}] = K_D \times \frac{F_X}{(F-F_{MIN})/(F_{MAX}-F)}$$

where Fluo-4 $K_D(Ca^{2+}) = \sim 350 \text{ nM}$; $F_{MAX}$ and $F_{MIN}$: are the maximal and minimal fluorescence, respectively. $F_{MIN}$ is assumed to be negligible. (Hsu et al, 2001).

2.8.4. **Orai1 and STIM1 intensity analysis.** Confocal images of Orai1 and STIM1 immunostaining signal was quantified using ImageJ Software (ImageJ 1.43m - National Institutes of Health, USA). To analyze the intensities a two-dimensional graph was displayed with the ImageJ analysis option. Intensity of pixels was measured along vertical lines spanning the x-axis. Each cell was measured individually and an average for each group was calculated. The averaged value was plotted as intensity vs. distance using Excel Microsoft Office (Redmond, WA, USA).

2.8.5. **Software and statistical tests**

The Clampex 9.2 software (Molecular Devices) was used for electrophysiological data acquisition following junction potential correction. All recordings were made at -40 mV holding potential. Clampfit 9.2 (Molecular Devices) was used to measure and plot the
amplitude, I period and delay and to fit the dose response curve applying the 4 parameters Hill’s equation:

\[ f(x) = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + (C_{50}/x)^h} \]

Microsoft Office Excel (Redmond, WA, USA) was used to plot the raw data and normalize changes over time. Values are expressed as mean ±SEM and groups were compared using a two tails unpaired T test followed by Bonferroni correction for multiple comparisons as needed. Statistical significance was assumed below 0.05 or 0.01 probabilities as illustrated in the results. Z value was calculated using the Statistical Test for Significant Difference between Two Proportions/Percentages.

\[ z = \left| p_1 - p_2 \right| / s \]

where: \( p_1 \) = proportion 1; \( p_2 \) = proportion 2; \( s = \sqrt{p(1-p)/n_1 + p(1-p)/n_2} \); \( n_1 \) = sample size 1; \( n_2 \) = sample size 2; \( p = (p_1n_1 + p_2n_2)/(n_1 + n_2) \). Significance test: 1.96 for 95% level of confidence.
CHAPTER 3

RESULTS
3. RESULTS

Cell identification

**AIM:** To identify different megakaryocyte maturation stages.

**Morphological characteristics of megakaryocytes**

By measuring the megakaryocyte longest axis under phase-contrast microscopy, the range in diameter was 30 to 54 µm and the average diameter was 43±7 µm, similar to previously reported observations (Somasundaram & Mahaut Smith, 1994). Large, round, brilliant stages II and III MKs were selected for patch-clamp recordings. (Fig. 3-1A-2 and 3-1B).

Megakaryocytes were identified based on morphological features:

A- Stage I
B- Stage II
C- Stage III
D- Stage IV
Fig. 3.1. Sample MKs studied based on morphology.

A-1 & A-2. Stage I megakaryocyte. Several nuclei can be observed in A-1

B. Stage II MK ruffling and starting protrusions.

C. Stage III MK ruffling and blebbing.

D. Stage IV MK with demarcation membrane.

E & F. Stage IV MK with proplatelet formation. Cells were in ES at room temperature. Photomicrographs were obtained with a Cohu 4912 CCD camera (Cohu, Inc. Poway, CA) using Scion software (Frederick, MD, USA) in Nikon upright or inverted phase contrast microscopes (Tokyo, Japan).

All images at 60X, calibration bars represent 10 µm.
Fig 3-1 A-1 Stage I

Fig 3-1 A-2 Stage I

Fig 3-1 B Stage II
Fig 3-1 C Stage III

Fig 3-1 D Stage IV
Fig 3-1 E Stage IV

Fig 3-1 F Stage IV
Electrophysiological studies

Calcium-activated potassium currents

AIMS

1. To demonstrate that purinergic-induced ionic currents in the MK can be altered by ASA pretreatment.

2. To investigate potential roles of P2Y1 and P2Y12 in outward K$^+$ current amplitude and how they relate to ASA treatment effects.

The 30 second protocol in calcium 1mM ES

Three different groups of cells were distinguished in response to ADP or ATP (both at 10 µM) application: those with several repetitive outward currents (oscillations), a single sustained outward current, or lack of response to the agonist (Fig. 3.2). The majority of MKs studied displayed multiple oscillations and some responded with a single outward current (Table 2). The remaining cells were unresponsive. MKs were studied using the nystatin perforated patch-clamp configuration. In a sample of cells studied with conventional whole cell recordings as opposed to nystatin perforated patch clamp, we did not observe significant differences, except that responses were considerably less stable. In each cell studied we used large depolarizing steps to activate voltage gated potassium current to ascertain voltage control. (Fig. 3.3)
Fig 3.2 Representative traces illustrating variable responses of distinct MKs to ATP recorded with nystatin perforated patch-clamp.

A. Traces shown are from three different experiments in which 10 μM ATP was applied during the time indicated by the horizontal bar at the top. The internal solution contained (mM): 150 KCl; 10 HEPES, pH 7.2-7.3; $V_{it} = -40\text{mV}$. (a). ATP-induced oscillatory outward currents displaying gradual decrease in amplitude (desensitization). (b). Single current response in another MK. (c). Single sustained response. (d). Lack of response in a third example cell.

B. Examples of current recordings obtained with 2MeSADP (5μM) in nystatin perforated patch-clamp. External solution was calcium free and the internal was the same as in the ATP experiment in A. (a) Oscillatory currents (OSC). (b). Transient (SP). (c). No response (NR).
A. 10 µM ATP

(a) 10 sec
100 pA

(b) 10 µM ATP

(c) 10 sec
100 pA

(d) 10 sec
100 pA

B. 2MeSADP (5 µM)

(a) 10 sec
100 pA

(b) 10 sec
100 pA

(c) 10 sec
100 pA

(d) 10 sec
100 pA
Table 2. Percentage of MK displaying activity under control and experimental conditions in response to ADP, ATP, and 2MeSADP stimulation.

<table>
<thead>
<tr>
<th></th>
<th>ADP (10μM)</th>
<th>ATP (10μM)</th>
<th>2MeSADP (5μM)</th>
<th>2MeSADP (5μM) Ca free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OSC</td>
<td>SP</td>
<td>NR</td>
<td>OSC</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>27</td>
<td>13</td>
<td>62</td>
</tr>
<tr>
<td>ASA (1mM)</td>
<td>83</td>
<td>11</td>
<td>6</td>
<td>53</td>
</tr>
</tbody>
</table>

MKs were studied using the nystatin perforated patch-clamp configuration, $V_H = -40$ mV, control and experimental cells were stimulated with 10 μM ADP (control n= 9, ASA n = 15), 10 μM ATP (control n= 8, ASA n = 9) or 5 μM 2MeSADP in 1mM calcium media (control n= 14, ASA n =12) and calcium free solution (control n= 15, ASA n= 11). Cells were grouped according to agonist and response: **OSC**: oscillatory currents; **SP**: single peak currents; **NR**: no response. Values represent percent of total N cells.
Fig 3.3 Traces illustrating voltage gated potassium currents generated with large depolarizing steps. MKs were at -40 holding potential in whole cell patch-clamp configuration. Traces shown are representative from all experiments carried out to ascertain voltage control.
Oscillations of outward currents decreased gradually in amplitude over time revealing desensitization and lasted from several sec. up to 20 min. using the nystatin perforated patch clamp (Fig. 3.4).

The desensitization of currents produced by the purinergic (P2) receptor activation in MKs has been reported to be fast followed by a time-dependent recovery (Baurand et al, 2005; Hardy et al, 2005; Barton et al, 2008; Ikeda, 2007). Therefore, we examined current amplitude during the first 30 sec. of agonist applications.

Amplitude of the initial ATP-induced current was significantly higher in the ASA-treated MKs (Control $137 \pm 57$ pA, n=11; ASA $289 \pm 103$ pA, n=10; $p<0.05$). In contrast, ADP-induced currents were not different between control and ASA treated MKs (control $316 \pm 262$ pA, n=13; ASA $328 \pm 317$ pA, n=13). The normalized average amplitude of ADP and ATP-induced outward currents were plotted over time (Fig. 3.5). The amplitude of the currents in the control group (not exposed to ASA) exhibited a natural decay over time in many cells, as illustrated (Fig. 3.4A and 3.4B left traces) and previously reported. In contrast, in the ATP stimulated cells, MKs previously incubated with ASA, displayed a trend to lower decay (Fig. 3.5B) but not in those stimulated with ADP (Fig. 3.5A). Current amplitude was significantly higher in the ASA-treated MKs.
after ATP stimulation at both the 10 and 20 second time points ($P<0.05$). Nevertheless, no difference in current amplitude decline was observed between control and ASA-treated cells after ADP stimulation. We proceeded to use a highly specific P2Y agonist - 2MeSADP - to study in more detail the role of GPCRs in MKs signaling. 2MeSADP 5µM continuous application during 30 seconds induced outward currents showing progressive decay in amplitude in controls that was attenuated in ASA-treated MKs (Fig 3.5C). Results in C were obtained with nystatin perforated patch preventing rapid current decay as observed in conventional patch-clamp (A&B). All experiments were carried out in calcium 1mM ES.
Fig 3.4. Progressive decay of outward current amplitude during the first 30s of purinergic agonist exposure. Representative conventional patch-clamp recordings of oscillation in MKs showing desensitization during continuous agonist exposure in control cells (left traces) and cells exposed to 1 mM ASA (right traces). A. Agonist: 10µM ATP. B. Agonist: 10 µM ADP
A

10 μM ATP

---

100 pA

10 sec

10 μM ATP + 1 mM ASA

---

100 pA

10 sec

B

10 μM ADP

---

100 pA

10 s

10 μM ADP + ASA 1 mM

---

100 pA

10 s
Fig 3.5  Progressive decay of outward current amplitude during the first 30s of purinergic agonist exposure.

A & B. Effects of ASA on the progressive decay of outward current amplitude at distinct time points during the first 30s of continuous application of ADP (A), ATP (B) in 1mM ES. Since ADP and ATP could activate P2X1 and P2Y receptors en MKs we used 2MeSADP as agonist for P2Y receptors. ADP: Control \( n \geq 8 \); ASA \( n \geq 9 \). 10 µM ATP: control \( n = 9 \); ASA \( n = 8 \). *\( P: \leq 0.05 \)

C & D. Summary of 2MeSADP 5µM induced outward current showing progressive decay in amplitude. Results in C were obtained with nystatin perforated patch and in D with conventional whole cell patch C: Control \( n= 11 \); ASA n=10. D. control \( n =12 \); ASA \( n =13 \). *\( P: \leq 0.05 \)
A +ADP 10 μM

B +ATP 10 μM
Fig 3.6. Effects of ASA on the progressive decay of outward current amplitude at distinct time points during the first 30s of continuous application 2MeSADP in $Ca^{2+}$ free media

A. Trace in the left is control and in the right is from ASA-treated MK.

B. Summary of normalized current amplitude in the 30 s protocol in $Ca^{2+}$ free media. Current amplitude decay was observed after the first agonist application in controls but it was attenuated in ASA-treated cells. Each value represents the percent change of normalized current amplitude, setting the first peak amplitude as 100%. Values are means ± SE for 5 µM 2MeSADP: control $n = 15$; ASA $n = 11$. *$P: \leq 0.05$)
A

2MeSADP (5µM)  
ASA (1mM)

B

+2MeSADP 5 µM

Maximal current (%)  
control  ASA

Time (s)
3.2.0.0.1. Calcium free media 30 s protocol. When the experiment was carried out in calcium free external solution we were able to establish a Gigaseal (see section 1.8.2.4 page 56) and record oscillatory outward currents (Fig 3.6A). Oscillatory currents were recorded in 67% of control and 73% of ASA-treated MKs (Table 1). Current amplitude decayed in the 30 seconds after first 5μM 2MeSADP application as in calcium 1mM media experiment and this decay was also attenuated in ASA-treated MKs with significance at the 10 and 30 seconds points. (Fig 3.6B)

The 0,1,3,5 min protocol for outward currents.

3.2.0.1.1. Calcium 1mM external solution

We also studied the changes in 10 μM ATP or 10 μM ADP evoked outward currents by repeating the 30 s exposures at 1, 3, and 5 minute intervals after the initial application in 1mM calcium ES (Fig. 3.7A). The amplitude of the first response of the oscillatory currents showed some decline with time in control conditions. In the presence of 1 mM ASA, however, the outward current was larger at each interval considered and increased progressively with time. As illustrated in Fig. 3.7B&C, the amplitude of each subsequent response at the 1, 3,
5 min. intervals normalized to that of the initial current became significantly different between control and ASA treatments at both the 3 and 5 min. periods in the ADP experiment ($P < 0.01$, $P < 0.05$ respectively) and after 5 min. recovery time in the ATP experiment ($P < 0.05$).

To further investigate the individual roles of the P2Y and P2X pathways, the P2Y$_1$ and P2Y$_{12}$ agonist, 2MeSADP, was used in the same protocol as that used in Fig. 4. With this agonist, using conventional whole-cell recordings, the decay in the outward oscillating current amplitude was remarkable in the control but it was considerably less or in some occasions being completely absent after ASA incubation or indomethacin treatment. Significantly less desensitization in the ASA and indomethacin groups was observed at 5 min. interval (Fig. 3.8) ($P \leq 0.05$).

### 3.2.0.1.2. Calcium-free external solution

As in the 30 second protocols, we repeated the 0,1,3,5 min protocol in calcium free media. When MKs in calcium free media were stimulated with 5µM 2MeSADP outward potassium currents were generated. (Fig 3.2 panel B). However no decay in current amplitude was observed in control cells even at the 5 minutes point as it was
previously observed in calcium 1mM ES. Moreover, ASA-treated MKs outward currents became higher over time with significance in the 5 min point (Fig. 3.9)
Fig 3.7. ASA reverses time-dependent degradation of purinergic receptor agonist-induced outward current in MKs.

A. Example of conventional whole cell patch clamp recordings ($V_h = -40\text{mV}$) of 10 $\mu$M ATP-induced outward current comparing the action of 1 mM ASA (*right*) to control (*left*) in the first oscillatory response to that of subsequent application at distinct time intervals in minutes as indicated below each trace.

B. ADP summary data. Each value represents current amplitude observed with each repeated application at subsequent times (1, 3, 5 min) normalized to that of the initial application of 10 $\mu$M ADP. Values are means ± SE for control $n=9$; ASA treated $n=15$ MKs. **$P: \leq 0.01$ at 3 min. *$P<0.05$ at 5 min.

C. ATP summary data. Results obtained with a similar protocol as in B. Values are means ± SE for control $n=8$; ASA treated $n=9$ MKs *$P<0.05$ at 5 min.
Fig. 3.8. ASA and Indomethacin treatment prevents decline of outward current amplitude in MKs induced by a P2Y receptor selective agonist.

A. Example of recordings of 5µM 2MeSADP-induced outward current comparing in distinct MKs the action of 1 mM ASA (right) to control (left) on the first peak of the oscillatory response obtained with the 0, 1, 3, 5 min. protocol.

B. 2MeSADP summary data. Each value represents the current amplitude observed with each repeated application of 5 µM 2MeSADP at subsequent times (1, 3, 5 min) normalized to that of the initial application. Values are means ± SE for control n=15; ASA 1µM treated n= 7; indomethacin 10 µM treated n= 9 MKs. *P<0.01 compared to control.
A

5µM 2-Methyl-S-ADP

1mM ASA

B

+2MeSADP 5 µM
Fig. 3.9. ASA treatment increases P2Y receptor-mediated outward current amplitude in MKs induced by a selective agonist in calcium free media. 5 µM 2MeSADP summary data in calcium free external solution.

Control n =15; ASA n= 11. *P: ≤0.05
Maximal current (%)

Time (min)

+2MeSADP 5 µM

control

ASA

0 1 3 5
**AIM:** To demonstrate cross talk desensitization between ADP-TXA$_2$ pathways

The TXA$_2$ pathway is sensitive to COX-1 inhibition by ASA and we observed effects of ASA on P2-induced currents. Thus we investigated a possible heterologous desensitization between TXA receptor activation and subsequent P2 responses in an additional set of cells. In 58% of MKs ($n = 19$) exposed to the TXA$_2$ agonist U46619 at a concentration of 2 µM for 30 s oscillatory outward currents were observed upon subsequent exposure to ADP. The remaining cells exhibited single peak responses. These results were not different from those obtained in the control group in this cell set exposed to ES, of which 57% ($n = 7$) of cells responded to ADP with oscillatory currents.

The initial response was selected to study changes in the current amplitude over time. The time dependent decrease in the P2-induced outward current amplitude was significantly greater in the U46619 treated cells compared to control (Fig. 3.10A). Strikingly, after exposure to 2 µM U46619 and 10 µM ADP, significant differences were observed compared to control cells. Furthermore, even after a thorough washout for a period of 5 min., none of the
11 cells tested were able to respond to ADP whereas the control group responded \((n = 7)\). In contrast, MKs stimulated with 10 \(\mu\)M ATP (Fig 3.10B) did not show significant differences between control and experimental cells \((n = 4\) and 3 respectively), with most MKs responding even 5 min. after the last stimulation with ATP. These results after exposure to the selective agonist of the TXA\(_2\) pathway suggest that this pathway is heterologously related to that activated by ADP but not by ATP.
Fig. 3.10. A thromboxane agonist enhances desensitization of ADP-activated current in MKs with no effect on ATP-induced outward currents. Purinergic-induced outward current amplitude decays after 30 s. exposure to U46619 measured by patch clamp recordings ($V_H = -40\text{mV}$). Values represent the current amplitude observed with the 0, 1, 3, 5 min. protocol normalized to that of the initial application of the P2 receptor agonist.

A. 10 μM ADP summary responses after exposure to 2 μM U46619 demonstrate heterologous desensitization of the P2Y and TXA$_2$ receptor (TP) pathways. Values are means ± SE for control: n= 7; U46619 2 μM treated n= 19 MKs. *$P\leq 0.05$.

B. TXA$_2$ receptor stimulation with U46619 did not affect ATP evoked outward currents in MKs. 10 μM ATP summary response after the exposure to 2 μM U46619. The lack of significant changes in the response after exposure to 2 μM U46619 indicates that the P2X pathway is not affected by TP receptor stimulation. Values are means ± SE for control: n= 7; U46619 2 μM treated n= 5MKs. $P$: NS
2MeSADP dose response studies: effect of ASA and P2Y₁ blocker.

**AIM:** To demonstrate changes in MK sensitivity to 2MeSADP after ASA treatment and P2Y₁R blockade

The increased amplitude of the 2MeSADP-induced outward currents indicates a possible change in sensitivity of MKs to P2Y agonist after exposure to ASA. We therefore investigated the dose-dependency of this effect. As shown in Fig. 3.11, the maximal 2MeADP-induced current amplitude increased in those cells incubated in 1 mM ASA or exposed to 10 μM MRS2179 compared to control (Fig 3.11A). To further understand the individual role of the distinct P2Y receptors reported in MKs (Famaey & Paulus, 1992; Rocca et al, 2002) we performed a dose response experiment in the presence of 10 μM MRS2179 to block the P2Y₁ receptors while stimulating the P2Y₁₂ receptors with the non-selective P2Y agonist 2MeSADP. We observed a shift to the left very similar to that seen with ASA treatment (Fig 3.11B) indicating increased sensitivity to this agonist.
Fig 3.11  ASA increases maximal response and shifts to the left the dose response to the P2Y receptor agonist 2-methyl-thio-ADP.

A. Summary of outward current recorded with nystatin perforated patch clamp in MKs upon application of increasing doses of 2MeSADP. A. Maximal currents observed in response to 5 µM 2MeSADP. Values are means ± SE for control: n≥10; 1 mM ASA treated n≥6, and 10 µM MRS2179 treated n≥9 MKs; *P = 0.05.

B. Dose response curves with current values normalized to the highest outward current amplitude. Values are means ± SE. Squares represent values for control n≥10 cells in ES (EC$_{50}$= 0.20µM). Black circles represent values for n≥6 MKs incubated in 1 mM ASA for at least 30 min (EC$_{50}$= 0.033µM). Triangles represent values for n≥9 cells stimulated with 2MeSADP in the presence of 10 µM MRS2179 (EC$_{50}$= 0.029µM).
Delay and period analysis.

To further characterize the effect of ASA, we investigated in more detail the shape of each repetitive oscillatory outward current. We defined the duration of each peak as $I_{\text{period}}$ and the interval between peaks as delay as has been previously done. (Uneyama et al, 1993) (Fig. 2.4 section 2.5.5.2 Data analysis). $I_{\text{period}}$ and delay were analyzed during the first 30s of 2MeSADP application. While there was not a statistically significant difference between the control and ASA and indomethacin $I_{\text{period}}$ values, the delay under ASA and indomethacin treatment was significantly longer ($P \leq 0.05$) suggesting changes in the [Ca]$^\text{i}$ dynamics with COX-1 inhibitors treatment (Fig. 3.12 A). However, when the same protocol was applied in calcium free media, ASA-treated MKs did show a trend toward higher delay values but with no statistical significance (Fig 3.12B)
Fig. 3.12 Changes in delay and $I_{\text{period}}$ during the first 30s 5 µM 2MeSADP exposure recorded with the nystatin perforated patch-clamp configuration.

Delay and $I_{\text{period}}$ were analyzed as described in section 2.5.5.2 Data analysis.

A. Summary data illustrating values for delay in control cells compared to MKs incubated for 30 min. in 1 mM ASA or exposed to 10 µM indomethacin. Values are means ± SE for control $n \geq 14$; ASA $n \geq 17$ cells; indomethacin $n = 9$ cells; *$P:\leq 0.05$

B. Summary data illustrating values for delay in control cells compared to ASA-treated MKs in calcium free media. Control $n=15$; ASA $n=11$.

C. Summary of values for $I_{\text{period}}$ in control cells and in cells incubated with 1 mM ASA for 30 min. Values are means ± SE for control: $n \geq 10$; ASA: $n \geq 9$; Indomethacin: $n \geq 5$; $P$: NS

D. Summary of values for $I_{\text{period}}$ in control cells and ASA-treated MKs in calcium free media. Values are means ± SE for control $n=15$; ASA $n=11$. $P$: NS
Increased cytosolic IP₃ concentration and its effect on outward current amplitude.

**AIM:** To investigate the steps affected by ASA treatment in MK signaling that could explain increased platelet reactivity

One of the possible actions of ASA could be on the IP₃ system. Therefore, experiments with elevated IP₃ in the internal solution were carried out to uncover any difference in ASA treated cells compared to control. Conventional patch clamp with 30 µM IP₃ in the internal solution generated outward currents immediately after breaking the MK membrane. (Fig 3.13A). These currents were significantly higher in the ASA-treated MKs compared to untreated control MKs (Fig 3.13B). Using 2MeSADP 5µM as agonist the amplitude of the outward current was higher and greater than IP₃ generated outward currents.

We compared cells without extra IP₃ in the external solution as controls vs. those with 30µM IP₃ in the external solution in the 0,1,3,5 protocol. As expected, in control cells outward current amplitude decayed over time as desensitization took place. However those cells with additional cytosolic IP₃ had faster decay in current amplitude compared to control group (Fig 3.13C). Several mechanisms could be involved in this current amplitude decay that will be discussed in the discussion section.
Fig. 3.13. Changes in 5 µM 2MeSADP-induced outward current amplitude during the first 30s of agonist exposure with elevated intracellular IP\textsubscript{3} concentration.

A. Examples of traces recorded with conventional patch clamp in control cells (left) and ASA-treated MKs (right) both with 30µM IP\textsubscript{3} in the internal solution. The initial current recording before addition of 2MeSADP was higher in ASA-treated cells and, when 2MeSADP was added current amplitude increased and stayed elevated for longer time in ASA-treated MKs suggesting increased in reactivity compared to control cells.

B. Summary of IP\textsubscript{3}-induced current amplitude in control cells vs. ASA treated MKs. ASA-treated MKs show higher IP\textsubscript{3}-induced current amplitude compared to control cells. Only those cells with oscillations from the beginning were included. Control n=8; ASA n=6. Values are mean±SEM *P: 0.0339.

C. Summary of 0,1,3,5 minute protocol with IP\textsubscript{3} (30µM) in IS. In this condition addition of IP\textsubscript{3} promoted earlier decay in 5µM 2MeSADP-induced current amplitude and to a greater extent with significance at the 1 min point. Normalized data were obtained with the 0,1,3,5 protocol recorded with conventional patch clamp. Control: n≤ 10; IP\textsubscript{3}: n ≤ 12. *P≤0.01
Since ASA treatment attenuated current amplitude in previous experiments, and IP$_3$ promoted greater current decay, we treated MKs with ASA 1 mM for 30 min and added IP$_3$ 30µM to determine if ASA treatment could be preventing the maximal current amplitude decay under these conditions. Indeed, with additional cytosolic IP$_3$, cells treated with ASA showed attenuated decay in current amplitude in the 0,1,3,5 min protocol (Fig 3.14A). In summary, in whole cell patch clamp recording, 2MeSADP-induced response was lower over time in controls cells, additional cytosolic IP$_3$ increased current amplitude decay and aspirin treatment attenuated this effect (Fig 3.14B). These results suggest that aspirin action could be related to calcium-IP$_3$ dynamics via a possible IP$_3$-ubiquitin/proteasome pathway as it has previously been proposed (Oberdorf et al, 1999). To further analyse calcium dynamics as a possible aspirin effect, we exposed MKs to xestospongins C.
Fig 3.14. ASA (1mM) treatment attenuated the decay in 5μM 2MeSADP-induced current amplitude over time. In this experiment the two groups had IP3 30μM in the IS. Control cells (control IP3) refer to non-ASA treated MKs.

A. Normalized data in the 0,1,3,5 protocol recorded with conventional patch clamp. ASA-treated MKs showed attenuated decay in current amplitude over time compared to non-treated cells. Control IP3: n= 10; IP3-ASA: n=9. *P≤0.05

B. Summary of all experiments with IP3 in the IS. ASA 1 mM treatment prevented the decay in 5μM 2MeSADP-induced current amplitude compared to control MKs with no additional IP3 and to non-ASA MKs with additional IP3 in the 0,1,3,5 protocol recorded with conventional patch. Significance was observed at the 5 min period. Control: n≤ 10; IP3: n ≤ 12; IP3-ASA: n=9 *P≤0.05
A.

![Graph A](image)

B.

![Graph B](image)

**Notes:**

- Graph A: +2MeSADP 5 μM
  - Max current (%)
  - Time (min)
  - IP3
  - ASA+IP3

- Graph B: +2MeSADP 5 μM
  - Max current (%)
  - Time (min)
  - control
  - IP3
  - IP3+ASA

**Additional Text:**

A. +2MeSADP 5 μM

B. +2MeSADP 5 μM

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**IP\(_3\)R blockade with xestospongin C**

**AIM:** To compare changes in response type and current amplitude with IP\(_3\)R blockade in ASA treated cells

The experiment with additional IP\(_3\) in the intracellular milieu suggested that aspirin pretreatment preserves the sensitivity of the IP\(_3\) signaling cascade in MKs. To confirm IP\(_3\)R role in P2Y-mediated calcium mobilization and a possible ASA-treatment action we exposed MKs to xestospongin C (X-C). X-C is a membrane permeable highly specific IP\(_3\)R blocker with a 358 nM IC\(_{50}\) (Gafni et al, 1997; Miyamoto et al, 2000). X-C has been demonstrated to completely block IP\(_3\)R\(_{III-hTrp1}\) in platelets (Rosado et al, 2000; 2002) and at this point we hypothesized that a possible target for ASA-generated AA products could be calcium entry mechanisms. We used a low X-C concentration near IC\(_{50}\) to test ASA-treatment attenuation of X-C action. When we tested our MKs preparation with 2-5\(\mu\)M X-C, as in previous studies (Miyamoto et al, 2000; Rosado et al, 2000; Kwan et al, 2001) no recording was possible.

The response to 2 MeSADP 5\(\mu\)M after 30 seconds exposure to xestospongin C 300nM was variable (Fig 3.15 A-C). Calcium activated potassium currents, as readout of intracellular calcium oscillation, were blocked in 63% of control cells exposed to X-C. In contrast 22% of
ASA-treated MKs outward current were blocked (Fig 3.15D). 22% of ASA-treated cells exhibited a plateau-like sustained outward current and only 22% of control cells showed this pattern. These results strongly suggest that ASA treatment maintains sensitivity of the IP$_3$ cascade.
Fig 3.15 Representative traces of 2MeSADP-induced calcium-activated potassium outward currents in MKs exposed to Xestospongin C (X-C).

A. Control cell not exposed to X-C

B. Control cell exposed to 300 nM X-C for 30 seconds

C. ASA-treated MK exposed to 300 nM X-C for 30 s. Currents recorded in whole cell patch clamp at -42 mV

D. Percent distribution of 2MeSADP 5 µM-induced outward currents after 30 s exposure to 300 nM xestospongin C in control and ASA-treated MKs. ASA-treated MKs showed a greater proportion of oscillatory and plateau response compared to controls cells. Calcium-activated potassium outward currents recorded in whole cell patch clamp at -42 mV HP. Control n= 11; ASA n= 9. z = 2.335 *P: 0.0454 for difference between two proportions with actual population size.
A 2MeSADP 5 µM

B 2MeSADP 5 µM

C XC 300nM 2MeSADP 5 µM

D +X-C+2MeSADP 5 µM

- control
- ctrl+XC
- ASA+XC

osc  blocked  plateau

100 pA

10 sec

5s

%
Changes in current amplitude with X-C treatment were analyzed over time in the 0,1,3,5 min protocol. Control cells not exposed to X-C showed the expected decay in current amplitude over time as previously observed in the P2YR homologous desensitization studies, however control-X-C cells (cells with no ASA treatment but exposed to X-C) showed the same decay pattern but in a greater extent compared to control cells without X-C treatment. Unlike control cells, when ASA-treated MKs were exposed to X-C for 30 seconds the current amplitude decrease was importantly attenuated with statistical significance at the 5 min point. (Fig 3.16)
Fig 3.16 Normalized current amplitude of 2MeSADP 5 µM-induced outward currents after 30 s exposure to 300nM Xestospongin C in the 0,1,3,5 min protocol. Control refers to MKs not exposed to XC, control+XC cells exposed to XC and ASA+XC ASA treated MKs exposed to XC. Control n= 6; control+XC n= 4; ASA n= 5. Values are mean±SEM, *P :<0.05. Calcium-activated potassium outward currents recorded in whole cell patch clamp at -42 mV HP
Intracellular calcium imaging and immunostaining

AIMS

1. To measure [Ca$^{2+}$]$_i$, at resting level and with 2MeSADP stimulation in control and ASA-treated MKs

2. To demonstrate 2MeSADP induced [Ca$^{2+}$]$_i$ oscillation in ASA-treated MKs

Cytosolic calcium measurement

To confirm electrophysiological results on potassium currents, intracellular calcium concentration was measured using Fluo-4 as calcium indicator and maximal fluorescence was attained with 10µM ionomycin, as previously described in Methods 2.8.3 (Fig 3.17A, B). Fluorescence value was converted to calcium concentration applying the appropriate equation (page 77). Calcium values at the resting state were: Control: 225±60nM; ASA-treated MKs: 508±123nM. When MKs were stimulated with 5µM2MeSADP, as expected, intracellular calcium concentration increased. [Ca$^{2+}$]$_i$ in control: 381±82nM; ASA-treated MKs: 965±196nM. This difference was statistically significant (control n = 12; ASA n = 21 cells; $P$: 0.0497) (Fig 3.17C). Cytosolic calcium elevation took place through calcium released from internal
stores since this experiment was carried out in calcium free media.

**Intracellular calcium oscillations.**

Oscillatory outward currents recorded with whole-cell patch clamp in megakaryocytes have been previously reported to be calcium activated potassium currents (Uneyama et al, 1993a,b; Hussain & Mahaut-Smith, 1998; Thomas et al, 2001). In section 3.2 Ch 3 we analyzed those currents induced by ATP, ADP and 2MeSADP. In this section we describe intracellular calcium concentration changes in MKs at resting state and stimulated with the specific P2Y agonist 2MeSADP using Fluo-4 dye as indicated in Ch 2 2.5.4.6. In our experiments control and ASA treated MKs were compared applying the agonist continuously (30 s protocol) and repetitively (0, 1, 3, 5 min protocol).

**Changes during continuous agonist application.** Cells with calcium oscillations for at least 30 seconds were selected for 30 s protocol analysis. Intracellular calcium oscillations were observed in both, control and ASA-treated MKs in calcium 1mM ES (Fig 3.18A). In the 30 s protocol, the same pattern, as that in electrophysiological recording, was observed with the first calcium peak \( F_1/F_0 \) mirroring the first peak of the 2MeSADP-induced outward current with subsequent decay during 30 seconds of drug application in control cells. However, in ASA-treated cells \( F_1/F_0 \) ratio did not decline,
resembling the effects of ASA on outward currents recorded with nystatin perforated patch (Fig 3.5C and 3.6B). These results suggest that indeed the nystatin perforated technique keeps the intracellular milieu near the physiological cytosolic calcium dynamics. When fluorescence ratio was normalized considering the first peak as 100%, a summary histogram was plotted as maximal $F_1/F_0$ vs. time to compare control and ASA-treated MKs in calcium free media as well as in calcium 1mM (Fig 3.18 A, B). In calcium 1mM control cells showed the expected decay in fluorescence while those control in calcium free media showed a small decay in $[Ca^{2+}]_i$. In contrast, ASA-treated cells exhibited attenuated decay or even increased $F_1/F_0$ ratio, and presumably, elevated cytosolic calcium. When we compare these results to those obtained with nystatin perforated patch-clamp, having the same experimental conditions, control cells exhibited more decay in current amplitude and ASA-treated MKs showed attenuated decay in current amplitude (Figs 3.5C and 3.6B). This comparison suggests that ASA treatment keeps $[Ca^{2+}]_i$ elevated for longer time preventing decrease in current amplitude. The same experiment was carried out in 1mM calcium ES (Fig 3.18C). $[Ca^{2+}]_i$ in control cells decreased through continuous exposure to 2MeSADP as it has been observed in our previous experiments, however, ASA-treated MK
showed significantly higher levels of $[\text{Ca}^{2+}]_i$ during agonist exposure. The same pattern was observed in patch clamp experiments in calcium 1mM using the 30 seconds protocol (Fig 3.5C&D) in the control group. Using nystatin perforated patch no significant difference was observed but with conventional whole cell recording there was statistical significance between control and ASA-treated cells in the 20 and 30 s time points (Fig 3.5 D).
Fig. 3.17 Intracellular calcium concentration in control and ASA treated megakaryocytes.

A. Examples of 5µM 2MeSADP-induced change in Fluo-4AM fluorescence (F) and maximal fluorescence value obtained with 10 mM ionomycin application after 2MeSADP stimulation. (1): control, (2): ASA-treated MK

B. Summary data of resting $[\text{Ca}^{2+}]_i$ value and 2MeSADP-induced $\text{Ca}^{2+}$ elevation. $[\text{Ca}^{2+}]_i$ was calculated with this equation: $[\text{Ca}^{2+}]_i = K_d \cdot F_X / (F_{\text{MAX}} - F_X)$ where Fluo-4 $K_d(\text{Ca}^{2+}) = 350$ nM; $F_X$: resting or induced fluorescence value; $F_{\text{MAX}}$: maximal fluorescence obtained with ionomycin. Values are means ± SE for control $n = 12$; ASA $n = 24$ cells; $P: 0.0497$
Fig 3.18. Changes in fluorescence (ΔF) generated by intracellular calcium concentration variation in 2MeSADP stimulated megakaryocytes. As in the patch clamp experiments oscillatory pattern was observed.

A. Representative traces of calcium oscillations. Control (left), ASA treated MK (right).

B. Summary of calcium variation through continuous agonist application in control and ASA-treated cells in calcium free media.

C. Summary of calcium variation through continuous agonist application in control and ASA-treated cells in calcium 1mM ES. Control n= 10; ASA n= 10. **P<0.01; *P<0.05

Cells were loaded with Fluo-4 for 45 min and then exposed to 2MeSADP 5 µM. Control cells were incubated in ES solution and experimental cells were incubated in ASA 1mM for 30 min. Data was recorded with SPOT, analyzed with SPCI and transferred to Microsoft Excel to plot fluorescence variation over time. Traces are representative of three different experiments.
A

B

C

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Changes during repetitive agonist application. The experiment was developed to correlate with the patch clamp studies, following the 0,1,3,5 min protocol 1mM Ca$^{2+}$ ES measuring changes in [Ca$^{2+}$]i in both groups; control and ASA treated MKs (Fig 3.19A&B). The intensity of agonist-induced fluorescence in this protocol had a pattern similar to that observed in the patch clamp recordings. The first [Ca$^{2+}$]i peak, expressed as F$_1$/F$_0$ ratio (maximal 2MeSADP-induced fluorescence to resting fluorescence), at 0 min was significantly higher in ASA-treated MKs compared to control (Fig 3.19C). F$_1$/F$_0$ ratio through repetitive stimulation exhibited the expected decline; ASA-treated MKs showed attenuated normalized fluorescence ratio (F$_1$/F$_0$) decay compared to control (Fig 3.19 C). This result is comparable to that obtained in patch clamp studies with 2MeSADP in the 0,1,3,5 min protocol (Fig 3.8B) where current amplitude decay attenuation by ASA was observed and being significant in the 3 and 5 min points although in calcium imaging results these time points were not (P: 0.0833). It is interesting to point out that agonist induced intracellular calcium increase came back to resting state values in the 5 minute point suggesting that ASA-treated cells have higher sensitivity to the agonist but the mechanisms for cytosolic calcium removal are capable to keep calcium within normal levels in the cytoplasm. (Fig 3.19C)
Fig 3.19 Fluorescence ratio ($F_1/F_0$) representing intracellular calcium oscillations generated by repetitive application of 5µM 2MeSADP in megakaryocytes. $F_1$ is the maximal fluorescence obtained with the agonist and $F_0$ is the resting fluorescence value in the 0,1,3,5 min protocol.

A. Representative traces of intracellular calcium oscillations in the 0,1,3,5 min protocol as A-0, A-1, A-3 and A-5 respectively in control cell.

B. Same protocol as in A but in ASA-treated MKs. B-0, B-1, B-3 and B-5

C. Summary of percent $F_1/F_0$ variation normalized to the first peak in the 0,1,3,5 min protocol in control vs. ASA-treated MKs. Cells were loaded with Fluo-4 for 45 min and then exposed to 2MeSADP 5 µM. Control cells were incubated in calcium 1mM ES solution and experimental cells were incubated for 30 min in ASA 1mM diluted in calcium 1 mM ES. Values are means ± SEM; $P$: <0.08 Control: n= 9 cells; ASA n= 12 cells.
A. Control

B. ASA treated

C. 2MeSADP 5 µM
Thapsigargin-induced SOCE.

**AIM**

To demonstrate ASA enhancement of thapsigargin-induced \([\text{Ca}^{++}]_i\) elevation in MKs via SOCE.

Cytosolic calcium concentration in MKs is elevated by P2X\(_1\) receptor activation and release from intracellular stores as well as flux through, TRP channels (TRPC and TRPM) and Orai1 activated channels \((I_{\text{CRAC}})\). One possibility for the elevated \([\text{Ca}^{2+}]_i\) found in the 30 s and 0,1,3,5 min protocols (Figs 3.18 and 3.19) could be \(\text{Ca}^{2+}\) entry via SOCE. Therefore, we used the SERCA blocker thapsigargin to explore the possibility of TG-induced SOCE changes in ASA-treated cells comparing control to ASA-treated MKs. ASA-treated cells showed higher calcium elevation compared to control in calcium free medium as well as in ES 1mM calcium (Fig 3.20A&B). We measured the percent increase in calcium in control cells compared to ASA-treated MKs. Cells incubated in ASA 1mM for 30 min showed higher percentage in calcium elevation; however this difference was not statistically significant (Fig 3.20C).
Fig 3.20 Thapsigargin-induced calcium increase in megakaryocytes. ASA 1mM treatment promoted a greater increase in cytosolic calcium in response to thapsigargin. Cells were in calcium free media when thapsigargin was added. The second elevation in calcium is the result of DTS-released calcium and calcium entry via Orai1 and other channels.

A. Control

B. ASA treated MKs.

C. Summary of $F_1/F_0$ value after thapsigargin (TG) treatment and addition of calcium to ES. Control cells were incubated in normal buffer and ASA MKs were incubated with ASA 1 mM for 30 min. Both groups were loaded with Fluo-4 for 45 min and were stimulated with TG 2µM or calcium 1 mM buffer for 1 min. Images were recorded at 1 frame/sec by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Analysis was performed using Simple PCI software (Compix Inc., Sewickley, PA). Control: n= 9 cells; ASA n= 12 cells. Values are $F_1/F_0$ average ±SEM. *$P$: ≤0.05
**STIM1 and Orai1 immunohistochemical imaging**

**AIM**

To demonstrate higher SOCE response in ASA treated cells is due to enhanced Orai1 and STIM1 coupling.

Thapsigargin-induced calcium increase and the difference in resting calcium value in ASA-treated MKs suggested a possible direct or indirect ASA action on calcium entry modifying cytosolic calcium concentration. MKs express the calcium sensor protein STIM1 in the DTS membrane and the calcium channel Orai1 in the plasma membrane. It has been postulated that P2Y receptor stimulation generates IP$_3$ which in turn promotes calcium release from DTS. Calcium reuptake is carried on by SERCA; therefore thapsigargin treated cells will not restore calcium levels to normal values triggering STIM1 activation and punctae formation with Orai1. We examined STIM1/Orai1 response in ASA-treated cells and control cells using antibodies for these proteins to compare visual morphological changes (Figs 3.21 and 3.22). In most cells STIM1 is distributed in close proximity to the plasma membrane as more nuclear material pushes the DTS towards the periphery. In a few cells STIM1 signal was detected throughout the cell on the DTS membrane in the cytoplasm (Fig 3.21A [a]). Thapsigargin (TG)
treatment induced SOCE and punctae formation (Fig 3.21A [b]) with a stronger signal in ASA treated MKs (Fig 3.21A [c]). Puncta formation requires STIM1 and Orai1 interaction, therefore Orai1 distribution pattern was visualized using anti-Orai1 antibody. A similar distribution pattern was observed compared to that obtained with anti-STIM1 antibody images (Fig 3.22B). In control cells Orai1 was faint in control cells (Fig 3.22B[a]). As SOCE was induced by TG treatment, a stronger signal was evidenced (Fig 3.22B[b]) and interestingly in ASA treated MKs the brightness was even stronger (Fig 3.22B[c]). These preliminary immunohistochemical observations suggest that ASA treatment might contribute to STIM mobilization either mobilizing from internal stores activating the calcium sensor STIM1 or generating arachidonic acid metabolites that cause STIM1 to aggregate.
Fig. 3.21  STIM1 immunostaining confocal micrographs

**A. STIM1 distribution in MKs.** Anti-STIM1 antibody concentration was 1:1000. Control cells were in ES and ASA treated MKs were incubated 30 min in 1 mM ASA. Images were analyzed with Olympus Fluoview-FV300 Laser Scanning Confocal System. All images at 60X; Scale bars represent 10µm.

(1). Control Anti-STIM1

(2). Control cell stimulated with TG 5µM. Anti-STIM1 signal appeared throughout the cell in the middle plane and is suggestive of puncta formation.

(3). ASA treated MK stimulated with TG 5µM. Stronger anti-STIM1 signal is observed mostly next to the plasma membrane. Brilliant dots indicate punctae.

**B. STIM1 staining intensity.** Intensity was measured to compare control cells, thapsigargin stimulated cells and ASA-treated MKs stimulated with thapsigargin 5µM. TG-induced STIM1 signal was enhanced in ASA-treated MKs compared to TG action on control cells \( (P < 0.001) \) as well as compared to control cell treated with TG \( (P: 0.045) \). Intensity was analyzed with Image J. Control n= 5; control +TG n= 5; ASA-treated MKs+TG n = 4 cells.
Fig. 3.22. Orai1 immunostaining confocal micrographs

A. Orai1 staining intensity.

(1). Control Anti-Orai1

(2). Control cell stimulated with TG 5µM. Anti-Orai1 signal is restricted to
the plasma membrane.

(3). ASA treated MK stimulated with TG 5µM. Stronger anti Orai1 signal is
observed mostly next to the plasma membrane. Brilliant dots indicate pucta.
Primary anti-Orai1 and anti-STIM1 antibody concentration 1:1000. Control
cells were in ES and ASA treated MKs were incubated 30 min in 1 mM ASA.
Images were analyzed with Olympus Fluoview-FV300 Laser Scanning
Confocal System. All images at 60X magnification; bar represents 20µm

B. Orai1 distribution in MKs.

Intensity was measured to compare control cells, thapsigargin stimulated
cells and ASA-treated MKs stimulated with thapsigargin 5µM. TG-induced
Orai1 signal was enhanced in ASA-treated MKs compared to TG action on
control cells as well as compared to control cell treated with TG. Intensity
was analyzed with Image J. Control n= 5; control +TG n= 4; ASA-treated
MKs+TG n = 6 cells.
A

(1) 

(2) 

(3) 

B

gray value

distance (µm)

control

control + TG

ASA + TG

1  6  11  16  21  26  31  36  41
Orai1 and STIM1 analyzed gray values in MKs confocal images showed that TG stimulated cells have higher intensity probably due to a close interaction between STIM1-Orai1. Moreover, TG action in ASA-treated MKs was even higher demonstrated in several preparations. Since ADP can stimulate Orai1-STIM1 interaction, and in our previous experiments we used that agonist, we looked for changes in intensity after ADP stimulation in MKs. Anti-STIM1 and Anti-Orai1 double stained MKs were compared using TG and ADP as agonists in separate experiments. In control cells 10 µM TG stimulated punctae formation was clearly seen with anti-STIM1 (Fig 3.23[a]) and anti-Orai1 staining (Fig 3.23[b]). When the STIM1 and Orai1 pictures were overlaid puncta formation was clearly evidenced with a predominant distribution towards the cell membrane (Fig 3.23[c]).
Fig. 3.23. Thapsigargin-induced Orai1-STIM1 interaction. Panel (a) shows STIM1 reactivity with little puncta staining as compared to a more stronger puncta signal in Orai1 staining (b). The overlaid image (c) shows clearly puncta formation with 2 μM TG treatment. Arrows show puncta and bar is 10 μm long. Images captured with Olympus Fluoview-FV300 Laser Scanning Confocal System at 60X magnification.
After 10 µM ADP application cells exhibited punctae formation although with some difference compared to TG-induced punctae. In the ADP experiment, Orai1 signal was not stronger throughout the cell, in contrast it was more intense in the periphery. (Fig 3.24B) The overlaid STIM1-Orai1 signals showing puncta formation upon ADP addition confirmed a distribution towards the periphery suggesting more STIM1 mobilization in the DTS to nearby Orai1 located in the plasma membrane (Fig 3.24C). These findings suggest that puncta formation is part of the ADP mediated signal cascade leading to calcium mobilization from the external milieu to the cytoplasm via CRAC channels. Whether ADP operates on puncta formation mostly indirectly through SOCE or through direct stimulation via DAG remains to be elucidated.

Next step was to compare TG and ADP induced puncta formation in ASA-treated MKs. Cells were incubated in 1mM ASA for 30 min and then stained for STIM1 and Orai1 after application of 2µM TG or 10 µM ADP.
Fig. 3.24. ADP-induced Orai1-STIM1 interaction.

A. **Control.** Panels (a) and (b) show STIM1 and Orai1 signal after 10µM ADP application. The overlaid image (c) shows clearly punctae formation with 10µM ADP. Bar is 10 µm long. Images captured with Olympus Fluoview-FV300 Laser Scanning Confocal System at 60X magnification.

B. **Orai1 and STIM1 immunostaining in ASA-treated MKs.** STIM1 signal (a), Orai1 (b), overlaid image of Orai1-STIM1 signals after 10µM ADP addition (c). The overlaid images show that MK ASA treatment favors punctae formation restricted to plasma membrane. Arrows show puncta and bar is 10 µm long. Images captured with Olympus Fluoview-FV300 Laser Scanning Confocal System at 60X magnification.
CHAPTER 4

DISCUSSION
4. DISCUSSION

Cardiovascular disease and stroke are the most prevalent causes of death worldwide. Platelet activation and aggregation play a key role in the development of thromboembolism, a major component in the pathophysiology of cerebrovascular and coronary heart disease (Xu et al, 2009). The standard pharmacological treatment for these diseases is based on drugs such as aspirin, an irreversible cyclooxygenase inhibitor, and clopidogrel, a P2Y$_{12}$R blocker. However, controlling platelet reactivity has been a challenge due to the complexity of signaling in platelets with two relatively common adverse outcomes: increased bleeding time and resistance to aspirin or clopidogrel. For this reason, better understanding of intraplatelet signaling pathways is an important goal.

Physiological studies on purinergic signaling, cyclooxygenase pathway and calcium dynamics in platelets will lead to a better understanding of the adverse events associated with drugs intended to inhibit platelet activation. This present study combines the patch clamp technique and intracellular calcium imaging to analyze cellular events in the megakaryocyte as a model of platelet reactivity. We have demonstrated attenuated decay in current amplitude of calcium activated potassium currents and increased $[\text{Ca}^{2+}]_i$ after aspirin treatment. Furthermore, the electrophysiological evidence and the results obtained with SERCA blockers indicate enhanced IP$_3$-mediated calcium release and extracellular calcium influx as possible mechanisms responsible for increased platelet reactivity after aspirin treatment.
**Electrophysiologic studies in megakaryocytes**

To begin to investigate the effect of ASA on purinergic P2X and P2Y receptor mediated K\(^+\) currents, oscillatory outward currents were studied in rat MKs. These outward currents are consistent with those previously reported for oscillatory outward K\(^+\) currents in rat MKs (Kawa, 1990; Uneyama et al, 1993) and have been shown to be produced by the opening of Ca\(^{2+}\) activated K\(^+\) channels reflecting variations in [Ca\(^{2+}\)]\(_i\) (Kawa, 1996; Martinez-Pinna et al 2005). The majority (65% controls and 73% ASA-treated cells) of MKs displayed repetitive oscillations that decreased in amplitude over time but could be evoked again upon subsequent reapplication of agonists. The time-dependent decrease in amplitude that we observed could in part result from desensitization (Figs. 3.4; 3.5; 3.6) (Somasundaram & Mahaut-Smith, 1994; Weber et al, 1999). Outward currents exhibited a wide variation in amplitude in both control and experimental groups. The variability could be accounted for by the diversity in the degree of maturation of the cells studied or differing degrees of Ca\(^{2+}\) entry from extracellular sources via store-operated Ca\(^{2+}\) entry (SOCE or CRAC) or TRP currents. Ca\(^{2+}\) leak currents through SOCE have been recently reported in MKs to occur by Orai1 plasma membrane channel activated by the DTS calcium sensor, stromal interaction molecule 1 (STIM1) (Bergmeier & Stefanini, 2009; Tolhurst et al, 2008; Várnai et al, 2007). TRPC\(_{1,6}\) channels have also been reported to be present in MKs (Tolhurst et al, 2008). Because normal regulation of [Ca\(^{2+}\)]\(_i\) in
MKs occurs through several mechanisms, ASA treatment potentially alters $[\text{Ca}^{2+}]_i$ and extra-cellular Ca$^{2+}$ sources.

Our results show that pretreatment with ASA decreases desensitization during continuous exposure to the agonist and subsequent purinergic challenge following recovery (Fig. 3.7; Fig 3.8; Fig 3.9) (Young, et al, 2010). Initial studies using the 30 s protocol of continuous agonist exposure, in Ca$^{2+}$ 1mM containing medium (Fig 3.5A, B), demonstrated that ASA treatment results in significantly higher ATP-induced currents than in ASA-free control MK. There was a similar trend for ADP-induced currents, although not significant at any time point. With the same 30 s approach, using the specific P2Y agonist 2MeSADP there was a significant decay in control current amplitude, which was prevented in ASA-treated MKs. The inhibitory effect of ASA on oscillations in the 2MeSADP treated MK cells was significant in Ca$^{2+}$ 1mM media (Fig 3.5D) as well as in Ca$^{2+}$ free media (Fig 3.6), supporting the hypothesis that ASA acts on the P2Y pathway. The possible significance of this observation involves the proposed model of ASA action as a platelet antiaggregant (Werner et al, 1991). This model proposes that direct activation of P2 receptors causes a priming effect, resulting in increased intracellular Ca$^{2+}$. However, this is not sufficient to induce secondary aggregation and secretion. The critical factor for aggregation and secretion is the production of TXA$_2$, which independently activates PLC and DAG production. This, in turn, activates a PKC-dependent aggregation and secretion. Thus, the elevation of intracellular Ca$^{2+}$ and IP$_3$-dependent signaling are
postulated to stimulate platelet aggregation. ASA treatment of platelets inhibits aggregation and secretion by mechanisms that include blocking TXA₂ production. We speculate that in MKs, this secondary step involving TXA₂ may lead to a physiological feedback that decreases priming Ca²⁺ oscillations, resulting in the current patterns seen in control cells. Our findings support the notion that ASA inhibition of TXA₂ production may allow [Ca²⁺]ᵢ to remain in the priming oscillatory range. Whether these signaling pathways also are relevant to MK platelet production is unknown, but a subject of further study.

An alternative possibility to explain the decreasing amplitude during oscillation (desensitization) of outward currents and the effects of ASA is that the release of ATP and ADP from dense granules, which results from activation, leads to excessive purine exposure and homologous desensitization. Thus, ASA inhibition of ATP and ADP release from dense granules would prevent P2 receptor desensitization. Our results showed that ATP-induced currents were significantly higher under ASA treatment while ADP-induced currents were not in the first 30 s of exposure (Fig. 3.5). This difference is not likely to be indicative of differences in ASA action between the P2X and P2Y receptors as the ATP and ADP solutions used were not pure and usually contain both compounds (Coller, 2002). More probable is that the agonistic profile of the ATP solution is such that it elicits a greater response from the P2Y pathway than the ADP solution, at least with the 30 s continuous application
paradigm. This idea is supported by results obtained with the specific P2Y agonist 2MeSADP (Fig 3.6), which was used along with ADP and ATP in subsequent studies.

Desensitization was also observed with purinergic agonists using the repetitive agonist application (0, 1, 3, and 5 min) protocol, and as observed with the 30 s protocol, ASA treatment also significantly reduced the deterioration of the amplitude of agonist-induced oscillations. However, in these studies the ADP-evoked current was more strongly affected by ASA treatment than the ATP-induced currents (Fig. 3.7). Because cross activation between the P2 agonists used and the receptors studied has been shown (Tolhurst et al, 2005), and because of possible impurity in the ATP and ADP solutions, more specific agonists were used to study the individual roles of P2X and P2Y receptors. As expected, effects of ASA observed using the P2Y non-specific agonist 2MeSADP were very similar to those using ADP (Figs. 3.7; 3.8; 3.9), strongly supporting a role for P2Y receptors in the mechanism for ASA on the oscillation pattern. The decline of the response with repeated application may be due to previously discussed mechanisms. However, desensitization at longer time points may be also caused by intracellular changes such as temporary depletion of Ca\(^{2+}\) stores, uncoupling of the receptor from signaling molecules, PKC mediated receptor internalization (Mundell et al, 2006), or K\(^+\) current-related changes. Although these pathways may be active, our results would support the hypothesis for a major role of TXA\(_2\) in decline of P2Y-induced current, since similar results were observed with indomethacin treatment (Fig 3.8).
As indicated above, TXA$_2$ appears to have an inhibitory effect on the P2Y pathway because blocking the production of TXA$_2$ with ASA increased sensitivity to P2Y receptor activation in dose response studies of outward currents in MKs (Fig 3.10). To better understand how the TXA$_2$ pathway heterologously affects the P2 pathway, we used the TP receptor specific agonist U46619. We found that MKs exhibited significantly lower ADP-induced currents after 30 s exposure to U46619 when compared to the response in control cells (Fig. 3.10). This is consistent with recent platelet aggregometry studies, which have demonstrated that TP stimulation results in desensitization of the P2 pathway (Barton et al, 2008, Bynagari et al, 2009; Kim et al, 2009). This supports the idea that TXA$_2$ regulates P2-induced K$^+$ currents, specifically at the P2Y receptor activated pathway.

We also observed that in the presence of U46619 the desensitization to ADP was striking while ATP-induced currents were not affected. At both the 1 and 3 min time periods, ADP-induced currents were significantly lower in amplitude after U46619 exposure and most notably, no cells responded at the 5 min time point (Fig. 3.10). These results in combination with those shown in Figure 3.8 indicate that the TP pathway heterologously desensitizes the P2Y pathway. Thus, we conclude that by using ASA and indomethacin to block COX-1 and COX-2, which are highly expressed in MKs (Borgdorff et al, 2006, Rocca et al, 2002), and therefore production of TXA$_2$, desensitization of the P2Y pathway is attenuated. The difference between ATP and ADP response further supports the hypothesis for a distinct profile for ATP
and ADP on the P2Y pathway. TP activation leads to increased [Ca\(^{2+}\)]i, activating PKC, which has a direct negative feedback on P2Y\(_1\) but not P2X\(_1\) receptors, possibly explaining the difference in MK response to ATP and ADP (Baurand et al, 2000).

To better understand the individual roles of the P2Y\(_1\) and P2Y\(_{12}\) pathways in the activation of outward K\(^+\) currents in MK cells, as well as the mechanisms of ASA effects, dose response curves for 2MeSADP were obtained for control, for ASA treated cells, and in the presence of the P2Y\(_1\) receptor blocker MRS179 (to isolate P2Y\(_{12}\) signal) (Fig 3.11B). The maximal 2MeSADP-induced currents recorded from MKs incubated in ASA and those exposed to MRS2179 were significantly higher than control (Fig 3.11A). These three curves together indicate that the P2Y\(_1\) receptor activation in control conditions decreases the sensitivity to the P2Y agonist and that, interestingly, a similar effect is seen with ASA treatment. Furthermore, the potency of 2MeSADP was enhanced in the presence of MRS2179 (Fig 3.11B), indicating that blockade of the P2Y\(_1\) receptor enhances the activation of the P2Y pathway by 2MeSADP.

Nevertheless, when the P2Y\(_1\) receptor was blocked, the maximal current amplitude was larger than control, suggesting that selective activation of P2Y\(_1\) receptor has an inhibitory feedback, perhaps via PKC-mediated homologous desensitization. Importantly, we observed that the decay in current following 2MeSADP application was similar in both ASA and P2Y\(_1\) antagonist-treated MKs. This leads us to speculate that the mechanisms involved may be similar and may
relate to desensitization occurring when P2Y$_1$ and P2Y$_{12}$ pathways are both active. This hypothesis is supported by previous reports on homologous desensitization of the P2Y$_1$ receptor in platelets caused by PLC$\beta$ inhibition by PKC (Crisanti et al, 2005; Mundell et al, 2006). The similarity between the MRS2179 and the ASA treated maximal current amplitude values (Fig. 3.11A) suggests that ASA treatment might be preventing a PKC-regulated P2Y$_1$ inhibition, increasing the amplitude of maximal currents similarly to MRS2179. However, the interplay between P2Y$_1$, P2Y$_{12}$ and P2X$_1$ receptors remains highly speculative and requires further studies.

In addition to current amplitude, the delay and $I_{\text{period}}$ of purine-induced outward currents were also studied. Although $I_{\text{period}}$ did not change significantly after ASA and indomethacin treatment, 2MeSADP-induced oscillatory currents displayed a significantly higher average delay after ASA and indomethacin treatment (Fig. 3.12A). This increase in delay seems consistent with lower reactivity related to the “priming” mode of MK activation. At the same time, because ASA and indomethacin treatment did not affect the $I_{\text{period}}$, it is unlikely that Ca$^{2+}$ exit from the cytosol is affected. $I_{\text{period}}$ is indicative of the time that [Ca$^{2+}$]$_{i}$ is increased from baseline levels because outward K$^{+}$ channels are open (Sumasundaram & Mahaut-Smith, 1994). Therefore, our data suggest that the way Ca$^{2+}$ exits the cytosol, via SERCA and plasma membrane Ca$^{2+}$ ATPases, is not likely affected by COX inhibition. Instead, our results suggest an effect on the mobilization of Ca$^{2+}$, as indicated by the increased delay under ASA and indomethacin treatment. Ca$^{2+}$
increases in the cytosol from two sources: extracellular fluid and the intracellular dense tubular system (DTS). One or both of these systems may be altered by ASA in such a way that they act less frequently, but when they do act, they produce a higher concentration of \( \text{Ca}^{2+} \) in the cytosol as indicated by the increased current amplitude and delay (Figs 3.5; 3.7; 3.8; 3.12). Longer delay between currents during the oscillation implies more time to replenish \( \text{Ca}^{2+} \) in intracellular stores. Thus, by increasing the delay, ASA treatment prevents the temporary depletion of intracellular \( \text{Ca}^{2+} \) stores that might represent an additional cause for the decrease of outward current amplitude observed in control conditions.

In MKs, \( \text{Ca}^{2+} \) mobilization from these intracellular stores depends on IP\(_3\)R stimulation (Rink & Sage, 1990; Jin et al, 1998; Jardin et al, 2008). In the present studies, a greater decay in current amplitude was observed with elevated cytosolic [IP\(_3\)], when compared with control conditions (Fig 3.13). This was attenuated by ASA-treatment (Fig 3.14), suggesting that ASA might be providing cell signaling conditions that prevent IP\(_3\)R desensitization. It is known that the IP\(_3\)R desensitizes due to continuous IP\(_3\)-\( \text{Ca}^{2+}\)-G\(_{\alpha q}\) signaling (Kume et al, 2000) and it can be down-regulated through the ubiquitin/proteasome pathway where increased IP\(_3\) concentration promotes ubiquitination (Oberdorf et al, 1999). While it is unclear whether or not IP\(_3\)-\( \text{Ca}^{2+}\)-G\(_{\alpha q}\) signaling plays a role in the desensitization attenuated by ASA, ASA has been shown to inhibit proteasome function, possibly preventing IP\(_3\)R desensitization (Dikshit et al, 2006).
ASA has also been reported to acetylate the intracellular membranes in human platelets (Hack et al, 1984). In MK, this could affect the IP$_3$R and contribute to the change in delay seen in figure 3.12A. To study this potential effect of ASA on IP$_3$ signaling, we used the IP$_3$R antagonist xestospongin C (X-C), hypothesizing that if ASA treatment altered Ca$^{2+}$ mobilization dynamics through an effect on the IP$_3$Rs, cells treated with X-C would remain more sensitive to 2MeSADP with ASA treatment than in control conditions. In the 30 seconds protocol with XC + 2MeSADP (Fig 3.15D), outward currents were blocked in 63% of control cells compared to 22% of ASA-treated MKs. ASA-treatment attenuated current amplitude decay significantly at the 3 and 5 min points when we tested MK response to repetitive agonist application, while blocking the IP$_3$R (Fig 3.16). Thus, ASA treatment attenuates IP$_3$R inhibition by xestospongin C in both short and long timeframes, suggesting an effect of ASA on the IP$_3$R signaling cascade.

**Calcium Imaging Studies**

The variations in Ca$^{2+}$ activated K$^+$ currents in the electrophysiology studies indicate that there are agonist-induced changes in intracellular Ca$^{2+}$ dynamics that are altered by ASA exposure. To strengthen these findings, we directly measured cytosolic [Ca$^{2+}$] through fluorescence. In 1 mM external Ca$^{2+}$, resting and 2MeSADP stimulated Ca$^{2+}$ concentrations were higher in ASA-treated cells than control cells. The addition of the P2Y agonist to ASA treated cells elevated cytosolic Ca$^{2+}$ to a level
2.5 higher than 2MeSADP-stimulated control cells. (Fig. 3.17B; \( P: 0.058 \)). Calcium variation over time, in the 0,1,3,5 min protocol (Fig 3.19) closely matched our patch clamp results in the same protocol (Fig. 3.8) (Young et al, 2010), confirming previous studies on intracellular calcium variation and calcium activated potassium currents in rat MKs (Martinez-Pinna et al, 2004; Mason et al, 2005).

Cytosolic Ca\(^{2+}\) oscillations were observed with a similar pattern to that of the Ca\(^{2+}\) activated K\(^{+}\) currents (Fig 3.18). Using the 30 seconds protocol, ASA-treated cells had a higher maximal fluorescence ratio (\( F_1/F_0 \)) than control cells in medium containing 1mM Ca\(^{2+}\) experiments, demonstrating that ASA treatment increases cytosolic [Ca\(^{2+}\)] during this time (Fig 3.18C). It has been previously reported that the Ca\(^{2+}\) activated K\(^{+}\) currents occur even in Ca\(^{2+}\) free extracellular solution, which is supported by our results (Fig. 3.6) (Kawa, 1996; Kawa, 2004; Martinez-Pinna et al, 2005). As previously stated, patch clamp experiments using the 30 seconds protocol in nominally Ca\(^{2+}\) free conditions showed ASA-treated cells with significantly higher current amplitude with statistically significant difference at the 10 and 30 seconds time points (Fig 3.6), indicating that ASA increases current amplitude even in calcium free conditions. Extracellular Ca\(^{2+}\) has previously been reported to facilitate Ca\(^{2+}\) store refilling (Uneyama et al, 1993; Hofer et al 1998), an effect that may be stronger in the presence of ASA as suggested by our findings. Other investigators have was demonstrated that extracellular Ca\(^{2+}\) entry is through a Ni\(^{2+}\) sensitive Ca\(^{2+}\) channel (Ikeda et al, 1992) as well as CRAC (Galán et al, 2009) and TRPC\(_6\) (Hassock,
While patch-clamp measurements could not confirm if ASA affected these channels, outward $K^+$ channels, or both; these $Ca^{2+}$ imaging studies have confirmed the effects of ASA on $Ca^{2+}$ dynamics, and support the results of the patch clamp studies.

Repetitive application of 2MeSADP was performed in $Ca^{2+}$ imaging studies and consistent with the patch-clamp recordings, we found the same response decay pattern in controls and the attenuation of this decay in ASA-treated MKs (Fig. 3.19). These results suggest that ASA interferes with $Ca^{2+}$ dynamics in such a way that ASA contributes to the purinergic-mediated priming effect on cytosolic $[Ca^{2+}]_i$ increase over time.

We also demonstrated that blocking intracellular $Ca^{2+}$ release with xestospongin C eliminated oscillatory $Ca^{2+}$-activated $K^+$ currents in control cells. However, in ASA-treated MKs, the oscillatory current prevailed with significantly attenuated decay (Fig 3.15). Moreover, a plateau response was observed in the ASA-treated MKs with 2MeSADP following XC exposure, indicating $[Ca^{2+}]_i$ is elevated above the threshold for the opening of $Ca^{2+}$-activated $K^+$ channels for an extended period. This would suggest either the mechanisms of $Ca^{2+}$ exit from the cytosol were altered under these conditions or more $Ca^{2+}$ entered the cytosol, requiring more time to remove it. X-C has been reported to have an inhibitory effect on SERCA (De Semet et al, 1999), which may explain this plateau response and attenuated current amplitude decay from the $Ca^{2+}$ removal perspective. However, there is also reason to
believe increased Ca\textsuperscript{2+} entry may play a role in this observation because a greater number of ASA-treated MKs had this response (Figures 3.15; 3.16). We have demonstrated higher [Ca\textsuperscript{2+}]\textsubscript{i} elevation with ASA treatment and Ca\textsuperscript{2+} mobilization was greatly inhibited in control cells following X-C exposure as evidenced by the high number of control cells with no response (Fig 3.16). Thus, ASA-enhanced [Ca\textsuperscript{2+}]\textsubscript{i} elevation and SERCA2b inhibition by X-C may together account for the greater prevalence of the plateau response in ASA-treated MKs.

These two results confirm that Ca\textsuperscript{2+} from intracellular stores is necessary for Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents while entry of extracellular Ca\textsuperscript{2+} to the cytosol contributes much less to Ca\textsuperscript{2+}-activated K\textsuperscript{+} current oscillation. However, extracellular Ca\textsuperscript{2+} does contribute to purine-induced Ca\textsuperscript{2+} elevation (Uneyama et al, 1993). In human platelets TRPC\textsubscript{1}-STIM1-IP\textsubscript{3}R interaction has been demonstrated suggesting a more complex system where ASA could play a role (Lopez et al, 2006). The increased Ca\textsuperscript{2+} levels in response to thapsigargin observed in our experiments (Fig 3.20) and a stronger signal for STIM1 and Orai1 in cells treated with thapsigargin and ASA (Figs. 3.21; 3.22) suggest that ASA treatment could play a role in the interaction between these two proteins, thereby modifying Ca\textsuperscript{2+} entry. Immunohistochemical staining for the major platelet and MK SOCE proteins Orai1 and STIM1 (Tolhurst et al, 2008) was used to investigate this potential mechanism. Orai1 and STIM1 were found to be localized near the plasma membrane region to a greater extent in ASA-treated MKs than in control and TG-stimulated MKs. STIM1 puncta formation and mobilization
for interaction with Orai1 in the plasma membrane is required for $I_{\text{CRAC}}$. Thus, our results are consistent with previous reports that Ca\textsuperscript{2+} store depletion can elevate STIM1 expression at the plasma membrane region (Ong et al., 2007; Woodard et al., 2008). The increased punctae formation and plasma membrane localization combined with enhanced SOCE (the latter demonstrated with Ca\textsuperscript{2+} imaging) suggests that there was greater activation of STIM1; leading us to hypothesize that ASA treatment enhanced store depletion. On one hand, the strong STIM1 and Orai1 signals observed in the immunostaining open the possibility, yet to be demonstrated in platelets, for noncapacitative arachidonate-regulated Ca\textsuperscript{2+} (ARC) channels via STIM1-ARC interaction as has been shown in pancreatic cells (Shuttleworth et al., 2007; Yeung-Yam-Wah et al., 2010). On the other hand, Orai1 has been suggested to play a key role in ARC channel activation based on studies with HEK293 cell line stably transfected with the human m3 muscarinic receptor. Co-expression of Orai1 and STIM1 in this cell line resulted in increased inward currents through the ARC channels stimulated with exogenous 2\mu M arachidonic acid (Mignen et al., 2008). The present findings provide an intriguing area for additional studies.

The possibility that ASA might prevent decay of P2-induced currents and, by inference, decay of oscillatory elevation of [Ca\textsuperscript{2+}]i by blocking TXA\textsubscript{2} production, is discussed above. ASA enhancement of Ca\textsuperscript{2+} mobilization is consistent with previous findings related to ASA-triggered lipoxin (ATL). In platelets, ASA doubles lipoxin formation (Romano et al., 1993). Furthermore, ASA-treated angioplasty patients have
higher intracoronary blood lipoxin A₄ (LXA₄) concentration than those without ASA treatment (Brezinski et al, 1992). Activation of the g-protein coupled ATL receptor (ALXR) with specific agonists and LXA₄ induces [Ca^{2+}] elevation through a PLC and store-dependent mechanism, likely IP₃-mediated Ca^{2+} mobilization (Perretti et al, 2002; Li et al, 2008).

Platelets have a secondary intracellular Ca^{2+} store: the acidic organelle, which has the SERCA3b isoform and IP₃R with affinity for thapsigargin. This Ca^{2+} pool may contribute to the cytosolic [Ca^{2+}] to trigger the priming effect on Ca^{2+} oscillations, but the identity and importance of this store needs to be clarified (Jardín et al, 2008; Varga-Zsabo et al, 2009). Interestingly, type III IP₃Rs have been identified in the platelet plasma membrane and type II IP₃Rs have been identified in both internal store membranes as well as the plasma membrane contributing to both store emptying and direct Ca^{2+} entry through TRP₁ (El-Daher et al, 2000; Rosado & Sage, 2000).

Ca^{2+} can enter the cytosol through an array of intra- and extra-cellular mechanisms, any one of which may be affected by ASA treatment. Intracellular mechanisms include agonist-dependent IP₃-induced DTS or acidic organelle Ca^{2+} release while extracellular mechanisms include SOCE, TRPC₁,₆, and P2X₁. These interactions under ASA treatment need to be further analyzed.

Despite the use of antiplatelet drugs, morbidity and mortality rates in cardiovascular disease remain high (Heron et al 2009; Xu et al 2009). The standard
therapy to control thrombotic episodes leading to ischemic heart disease or recurrent stroke is not enough to ensure patient survival without risk of complications such as bleeding and especially periprocedural myocardial infarction (Hildemann & Bode, 2009). A better understanding of platelet physiology will improve the development of new strategies to control platelet reactivity. Our results have given an incremental contribution in elucidating ASA mechanisms, combining calcium imaging and electrophysiological approaches. However, it is difficult at the present to make firm conclusions as to ASA effects and resistance as further studies are required.

In summary, using rat MKs as a model of platelet reactivity, our results provide significant insight into the actions of ASA on purinergic-induced platelet reactivity which may contribute to future understanding of ASA control of clot formation and the ASA resistance phenomenon.
CHAPTER 5

CONCLUSIONS
5. Conclusions

- Rat megakaryocytes respond to purinergic receptor activation with oscillatory, 
  \(\text{Ca}^{2+}\)-activated, outward \(\text{K}^+\) currents as previously reported. These currents can 
  be recorded using conventional and nystatin-perforated patch clamp and are 
  observed in the majority of megakaryocytes tested.

- The oscillatory \(\text{K}^+\) currents reflect oscillations in intracellular free \([\text{Ca}^{++}]\) through 
  an \(\text{IP}_3\)-dependent mechanism. The oscillatory elevation of intracellular free 
  \([\text{Ca}^{++}]\) is believed to represent a “primed” state of activation of megakaryocytes, 
  and by inference, platelets, in response to purinergic stimulation.

- The oscillatory currents are subject to homologous desensitization, decaying 
  over time during 30 sec continuous purinergic exposure or upon restimulation 
  within a few minutes.

- Cyclooxygenase inhibition attenuates this desensitization. Treatment of 
  megakaryocytes with ASA or indomethacin attenuates the decay in oscillatory 
  current amplitude under conditions of both continuous and repetitive purinergic 
  agonist application. ASA increases delay between peaks in the oscillation 
  without affecting \(I\) period in \(\text{Ca}^{2+} 1\) mM and in \(\text{Ca}^{2+}\) free extracellular medium. 
  Based on the proposed priming effect of purinergic agonists in 
  platelet/megakaryocyte function, the effect of ASA appears to be one mechanism 
  whereby the drug might have a stimulatory, as opposed to inhibitory, effect on 
  megakaryocyte and platelet function.
• These oscillatory K\(^+\) currents in response to P2YR activation, associated with fluctuations in intracellular [Ca\(^{2+}\)], occur in both calcium-containing and calcium-free extracellular media, indicating no requirement for influx of extracellular Ca\(^{++}\) through the SOCE system. Furthermore, the purinergic agonist induced electrophysiological response is subject to heterologous desensitization when MKs are stimulated by a thromboxane A\(_2\) agonist.

• ASA appears to affect Ca\(^{2+}\) dynamics, as opposed to directly acting on K\(^+\) dynamics, based on the results of Ca\(^{2+}\) imaging studies performed in presence and absence of aspirin and various agents affecting Ca\(^{2+}\) release from intracellular stores or influx from the extracellular environment.

• Although effects of ASA are not dependent upon entry of extracellular Ca\(^{2+}\), Orai1 and STIM1 immunostaining experiments suggest that ASA may also affect SOCE.

• Our findings contribute to the knowledge of platelet physiology and signaling mechanisms, and may be relevant to the understanding the clinically important ASA resistance phenomenon. We hypothesize that individual variation in sensitivity to the multiple effects of aspirin on signaling pathways could result in insensitivity to its antiplatelet effects in some patients.
Fig. 5.1 Ca\textsuperscript{2+} signaling and the proposed mechanism of ASA treatment action.

Summary of P2Y, thromboxane, lipoxin, and SOCE signaling in platelets. The proposed effect of increased ATL production by ASA treatment, enhanced IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release from the DTS, and the downstream effect on SOCE are marked with red lines limited by dash-lined rectangles. Arrows indicate products or a positive effect. “T” indicates inhibition or a negative effect. PIP\textsubscript{2} = Phosphatidylinositol 4,5-bisphosphate, PI3K = Phosphatidylinositol 3-kinase, DAG = 1,2-diacylglycerol, Ca\textsuperscript{2+} = cytosolic calcium, Ca\textsuperscript{2+}\textsubscript{DTS} = dense tubular system calcium, AA = Arachidonic acid, ATL = ASA-triggered lipoxin, ALXR = ASA-triggered lipoxin receptor, Ac-COX = acetylated COX.
Future Studies

As is usually the case in studies of complex biological systems, the experiments described above have led to further questions, and have suggested studies for the future. Among others, some of these additional studies include:

1. Further studies of the effects of ASA on electrophysiology and Ca\(^{2+}\) dynamics in MKs from cardiovascular disease animal
2. Additional Ca\(^{2+}\) imaging, dose response, and electrophysiological studies with P2Y\(_{12}\)R blockers
3. Recording of ADP-induced currents with LTA4 or BML-111 treatment, to explore the role of aspirin-triggered lipoxins in the effects of aspirin on MK electrophysiology and calcium dynamics
4. Dose response studies of IP\(_3\) blockade with X-C and heparin, another IP\(_3\) blocker.
5. Testing of IP\(_3\)R sensitivity and expression in MKs
6. Examination of IP\(_3\)-mediated [Ca\(^{2+}\)]\(_i\) elevation with additional platelet agonists in ASA-treated MKs.
Bibliography:


2000. Desensitization of the platelet aggregation response to ADP:  
Differential down-regulation of the P2Y_1 and P2cyc receptors. *Thromb Haemost*  
84: 484–91

platelet P2Y receptors after activation: a way to avoid loss of hemostatic  

Becker RP, De Bruyn PP. 1976. The transmural passage of blood cells into  
myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a  

Behnke O. 1968. An electron microscope study of the megacaryocyte of the rat  
bone marrow. I. The development of the demarcation membrane system and the  

Expression of human TRPC genes in the megakaryocytic cell lines MEG01,  
DAMI and HEL. *FEBS Lett* 10;403(1):83-6


**Chuen CK, Li K, Yang M, Fok TF, Li CK, Chui CM, Yuen PM.** 2004. Interleukin-1β up-regulates the expression of thrombopoietin and transcription factors c-Jun, c-fos, GATA-1, and nf-E2 in megakaryocytic cells. *J Lab Clin Med* 143 (2): 75-78

Clifford EE, Parker K, Humphreys BD, Kertesy SB, Dubyak GR. 1998. The P2X₁ receptor, an adenosine triphosphate–gated cation channel, is expressed in human platelets but not in human blood leukocytes. *Blood* 91: 3172-3181


Cramer EM, Vainchenker W.  Platelet Production: Cellular and Molecular Regulation Ch 25 in Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Lippincott Williams & Wilkins. USA. 2006


Strokes (PRoFESS) trial: a double-blind, active and placebo-controlled study. 

*Lancet Neurol.* 7(11):985

Aspirin Induces Apoptosis through the Inhibition of Proteasome Function. *J Biol Chem* 281 (39): 29228-29235

**Dorland, WA Newman.** Dorland’s Illustrated Medical Dictionary. Saunders 31st ed. 2007


Differential involvement of the P2Y1 and P2YT receptors in the morphological changes of platelet aggregation. *Thromb Haemost* 85:694–701

193


ATP-gated cation channels (P2X receptors) expressed in mammalian cells. *J Physiol* 497.2, pp.413-422


Arterioscler Thromb Vasc Biol 23:1152

The STIM/Orai1 coupling machinery. Channels 2 (4): 1-8


Galán C, Zbidib H, Bartegib A, Salido GM, Rosado JA. 2009. STIM1, Orai1 and hTRPC1 are important for thrombin- and ADP-induced aggregation in human platelets. Arch Biochem Biophys 15; 490(2):137-44


Hack N, Carey F, Crawford N. 1984. The inhibition of platelet cyclooxygenase by aspirin is associated with the acetylation of a 72kDa polypeptide in the intracellular membranes. *Biochem J* 223, 105-111


Hardy A, Jones ML, Mundell SJ, Poole A. 2004. Reciprocal cross-talk between P2Y$_1$ and P2Y$_{12}$ receptors at the level of calcium signaling in human platelets. *Blood* 104 (6): 1745-1752


Hofer AM, Fasolato C, Pozzan T. 1998. Capacitative Ca\textsuperscript{2+} entry is closely linked to the filling state of internal Ca\textsuperscript{2+} stores: a study using simultaneous measurements of $I_{\text{CRAC}}$ and intraluminal [Ca\textsuperscript{2+}]. *JCB* 140:2325-334


mechanisms in mouse dendritic cells: CRAC is the major Ca\(^{2+}\) entry pathway.

*Journal Immunol* 166: 6126-6133


203


**Jennings L.** 2009 (c). Role of Platelets in Atherothrombosis. *Am J Cardiol* 103 (3), Supplement 1:4A-10A


through weak activation of the $\alpha_{IIb}\beta_3$ integrin - a phosphoinositide 3-kinase-dependent mechanism. *FEBS Letters* 505 (2001) 281-290


**Kaushansky K.** Part II Chapter 18 Megakaryocytes in Wintrobe’s Hematology. Lippincott, Williams & Wilkins, USA. 2009


210


Massberg S, Gawaz M, Grün S, Schulte V, Schulte I, Zöllnhöfer D,
Heinzmann U, Nieswandt B. 2003. A crucial role of glycoprotein VI for

Mazur EM. in Thrombosis and Hemorrhage edited by Loscalzo J, Schafer A.

Opin Cardiol* 17(5):552-558


Mercer JC, Dehaven WI, Smyth JT, Wedel B, Boyles RR, Bird GS, Putney
JW Jr. 2006. Large store-operated calcium selective currents due to co-
expression of Orai1 or Orai2 with the intracellular calcium sensor, STIM1. *J Biol
Chem* 281: 24979-24990

Michelson AD. Platelets. Academic Press an imprint of Elsevier Science, San


Neurocis Lett. 103, 1:56-63


Nori S, Fumagalli L, Boa X, Bogdanova Y, Burnstock G. 1998. Coexpression of mRNAs for P2X\textsubscript{1}, P2X\textsubscript{2} and P2X\textsubscript{4} receptors in rat vascular smooth muscle: an


by the depletion of subplasma membrane endoplasmic reticulum Ca\textsuperscript{2+} store. *J Biol Chem* 282:12176-12185


**Oury C, Lenaerts T, Peerlinck K, Vermylen J, Hoylaerts MF.** 1999. Congenital deficiency of the phospholipase C coupled platelet P2Y\textsubscript{1} receptor leads to a mild bleeding disorder. *Thromb Haemost* 85(suppl):20 (abst)

**Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF.** 2002. P2X\textsubscript{1}-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. *Blood* 100 (7): 2499


224


Rosado JA, Sage SO. 2000. Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca\(^{2+}\) stores are depleted. *Biochem J* 350, 631–635


Rolf MG, Brearly CH, Mahaut-Smith MP. 2001. Platelet shape change evoked by selective activation of P2X\(_1\) purinoceptors with α,β-methylene ATP. *Thromb Haemost* 85: 303–8


Shattil SJ, Leavitt AD. 2001. All in the family: primary megakaryocytes for studies of platelet $\alpha_{\text{IIb}\beta_3}$ signaling. *Thromb Haemost* 86: 259–65


Heart Association. *Circulation* 89:2462-78


Tertyshnikova S, Fein A. 1997. \([Ca^{2+}]_i\) oscillations and \([Ca^{2+}]_i\) waves in rat megakaryocytes. *Cell Calcium* 21, 331-344


232


**Wickramasinghe SN.** In Histology for Pathologists. 3rd ed./editor, Stacey E. Mills. Chapter 33. Lippincott Williams & Wilkins. Philadelphia. 2007


Web sites