Kirk Allen Taylor - Pannexin-1 and other anion channels in platelets and megakaryocytes.

Abstract:
Platelet ion channels are essential for Ca\textsuperscript{2+}-influx, maintaining the resting membrane potential and cell volume regulation. Cation channels have been widely studied but few reports of platelet anion channels exist. A recent ‘channelome’ screen has suggested that human platelets express several anion-permeable channels of unknown function. This thesis explores the function of two such channels, Pannexin-1 and TMEM16F, in platelets, primary megakaryocytes (MKs) and related cell lines.

Using pannexin-1 inhibitors, these channels were shown to open in response to stimulation by thrombin, contribute to Ca\textsuperscript{2+}-influx and release cytosolic ATP following stimulation by threshold concentrations of platelet agonists. Experiments also suggested that ATP release by pannexin-1 channels contributes to Ca\textsuperscript{2+}-influx via stimulation of ATP-gated P2X1 receptors.

Anion-selective TMEM16F channels have been recorded in a variety of cell types and activate in response to sustained elevation of [Ca\textsuperscript{2+}]\text{c} to 100 µM. Controversially, these channels were reported to be cation-selective in mouse MKs. Thus, whole cell patch clamp recordings were performed to assess the biophysical properties of TMEM16F channels in HEL cells and primary mouse and rat MKs. Elevating [Ca\textsuperscript{2+}]\text{c} to 100 µM in HEL cells and rat MKs induced a Ca\textsuperscript{2+}-dependent, outwardly rectifying anion-permeable conductance, which was blocked by the TMEM16F inhibitor A01. Recordings of mouse MKs identified an equally Ca\textsuperscript{2+}-dependent, outwardly rectifying and A01-sensitive conductance, however this was predominantly permeable to cations. Thus, a major interspecies difference exists in the ionic selectivity of MK TMEM16F channels; possible explanations for this difference, such as mutations within the pore region, are discussed.

In summary, this thesis has explored the biophysical properties and function of platelet and MK anion channels using in vitro assays. These studies have relied heavily upon pharmacological tools and future studies of platelet function would benefit from the use of transgenic models.
Publications:

Articles


Oral Communication


Poster Communications


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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>α,β-meATP</td>
<td>α,β-methylated adenosine triphosphate</td>
</tr>
<tr>
<td>A01</td>
<td>Calcium-activated chloride channel inhibitor (CaCCinh-A01)</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular Ca^{2+} concentration</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>Cbx</td>
<td>Carbenoxolone</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>Dith</td>
<td>Dithionite</td>
</tr>
<tr>
<td>DMS</td>
<td>Demarcation membrane system</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTS</td>
<td>Dense tubular system</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>E_{rev}</td>
<td>Reversal potential</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney-293 cell line</td>
</tr>
<tr>
<td>HEL</td>
<td>Human erythroleukaemic cell line</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>I-V</td>
<td>Current-voltage (relationship)</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MK</td>
<td>Megakaryocyte</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-Methyl-D-glucamine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSCC</td>
<td>Non-selective cation channel</td>
</tr>
<tr>
<td>Panx1</td>
<td>Pannexin-1</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease-activated receptor</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Prb</td>
<td>Probenecid</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated Ca²⁺ entry</td>
</tr>
<tr>
<td>TG</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>Thr</td>
<td>Thrombin</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>U4</td>
<td>Stable thromboxane A2 analogue, U46619</td>
</tr>
<tr>
<td>Vₘ</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WC</td>
<td>Whole cell</td>
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Chapter 1

1.0 General introduction

Platelets play a vital role in maintaining the integrity of the circulatory system in response to vascular injuries. Upon contact with components of the sub-endothelial matrix, these small and anucleate cells undergo a dynamic shape change, adhere and aggregate to prevent blood loss. Regulated activation of platelets to form a thrombus is haemostasis, whilst inappropriate platelet activation is thrombosis. Formation of thrombi within a coronary or carotid artery restricts blood flow to the heart and brain, respectively. A substantial reduction of blood supply to the heart causes a myocardial infarction, whilst reduced oxygenation of brain tissue manifests as an ischaemic stroke. Cardiovascular diseases (CVD) include stroke (ischaemic and haemorrhagic), myocardial infarctions, and coronary heart disease. Increased public awareness and cardiovascular research have aided a 19% reduction (272,000 deaths) in the mortality rate of CVD in the UK over 30 years (Heartstats, 2011). However, CVD remains the leading cause of mortality in the UK and further understanding of how platelets function is required to manage and treat such conditions. Furthermore, platelets are known to contribute to angiogenesis, leukocyte infiltration and other inflammatory responses that play a role in the development of cancers and respiratory diseases (Ho-Tin-Noé et al., 2009). This chapter provides an overview of the role of platelets and their precursor cells in health and disease and the proposed contribution of ion channels to these events.

1.1 Haemostasis and thrombosis

Trauma-induced blood loss can prove fatal without an adequate haemostatic response. A critical event within haemostasis is the interaction between platelets and the exposed vasculature, which promotes thrombus formation and procoagulant activity. However, excessive stimulation of these processes in response to thrombogenic material from a ruptured atherosclerotic plaque can cause vessel occlusion, leading to myocardial infarction, stroke and pulmonary embolism. Platelet activation can be triggered by exposure to sub-endothelial matrix proteins (e.g. collagen) (Barnes et al., 1998), exogenous agonists (e.g. ADP, ATP) (Born and Cross, 1964) or by mechanical deformation (e.g.
Figure 1.1 Cartoon representation of haemostasis in response to vascular injury. Circulating platelets are maintained in a quiescent state by the action of endothelial-derived NO and PGI2 (1). Injury to the blood vessel exposes the sub-endothelial matrix, which contains VWF and collagen. 2) Passing platelets interact with sub-endothelial VWF through transient interactions with the GPIb-V-IX complex. 3) Tethering to VWF initiates platelet rolling along the sub-endothelium where collagen binds to GPVI receptors on the platelet surface. 4) Platelet activation by collagen triggers intracellular signalling pathways, shape change, exposure of PS and release of α and dense granules (5). 6) ADP and ATP released from dense granules activate passing platelets, which are recruited into the platelet plug. 7) Inside-out activation of integrin α_{IIb}β_{3} receptors mediates fibrinogen cross-linking of adjacent platelets across the wound surface. 8) Coagulation factors assemble on the negatively charged, PS-enriched, platelet surface to promote thrombin formation. Once generated, thrombin cleaves fibrinogen to fibrin, creating an insoluble mesh across the platelet plug (not depicted). Covalent interactions between fibrin monomers serve to further stabilise the platelet plug.
shear stress) (Goncalves et al., 2005). Nitric oxide (NO) and prostacyclin (PGI₂) are released from healthy endothelial cells and act on platelets to maintain quiescence (Chapter 1.4) (Schwarz et al., 2001). Damage to the endothelium exposes von Willebrand factor (VWF) and the matrix proteins collagen, laminin and fibronectin that bind to platelets, initiating intracellular signalling pathways (Grüner et al., 2003, Gibbins, 2004, Schaff et al., 2013). Platelet activation is further stimulated by thrombin, ADP and thromboxane A₂ (TxA₂), which are generated or released by platelets. These signalling pathways culminate in inside-out activation of integrin αⅡbβ₃ receptors, which bind fibrinogen to cross-link adjacent platelets, forming an aggregate.

1.1.1 Formation of a stable platelet plug

Platelets bind to the sub-endothelium via interactions between VWF and the glycoprotein (GP) Ib-V-IX complex, which serves to transiently tether platelets (Gibbins, 2004) (Fig. 1.1). Platelets then bind collagen via GPVI and integrin α₂β₁ receptors on the platelet surface (Clemetson and Clemetson, 2001). Downstream signalling by GPVI triggers Ca²⁺-release, degranulation, shape change and amplifies platelet activation (Fig. 1.1, Chapter 1.3) (Nieswandt et al., 2001). Platelets contain alpha (α) and dense granules with distinct cargos. α-granules contain coagulation factors, cytokines, fibrinogen and adhesion molecules (Frojmovic and Milton, 1982), whilst dense granules contain ADP, ATP, serotonin and Ca²⁺ (King and Reed, 2002). ADP-evoked platelet activation promotes the synthesis and release of TxA₂ (Samuelsson et al., 1978). ADP, ATP and TxA₂ are secondary agonists that further promote platelet activation and aggregation. Signalling by collagen, ADP, ATP and TxA₂ receptors are discussed below (Chapter 1.3 and 1.6). Agonist-evoked reorganisation of the actin cytoskeleton stimulates shape change, causing discoid platelets to become rounded and extend pseudopodia; thereby increasing their surface area and facilitating the recruitment of passing platelets into the thrombus (Paul et al., 1999a) (Fig. 1.1). Interactions between VWF and GPIb-V-IX are relatively weak and therefore insufficient to mediate platelet adhesion. Stronger interactions are formed between integrin α₂β₁, α₅β₁ and α₉β₁ receptors and collagen, fibronectin and laminin in the sub-endothelial matrix (Grüner et al., 2003, Takagi et al., 2003, Gibbins, 2004, Inoue et al., 2006, Nieswandt et al.,
2009). In vivo studies have shown that interactions between platelets and several endothelial matrix proteins are required to mediate stable adhesion and subsequent thrombus formation (Kuijpers et al., 2007, Schaff et al., 2013). Intraplatelet adhesion is mediated by fibrinogen binding to integrin $\alpha_{\text{IIb}}\beta_3$. Furthermore, subsets of platelets expose phosphatidylserine (PS), a negatively charged phospholipid that provides a surface for thrombin generation (Munnix et al., 2007). Thrombin activates other platelets and coagulation factors (see below) and cleaves fibrinogen to fibrin, generating an insoluble mesh that serves to further stabilise the thrombus. These processes are summarised in Fig. 1.1.

1.1.2 Thrombin generation and platelet coagulation

Conversion of fibrinogen to fibrin, by the protease thrombin, is a critical step in haemostasis that serves to stabilise the thrombus. Coagulation is a highly regulated process, requiring several coagulation factors and cofactors; summarised in Fig. 1.2. In its classical form coagulation may be broken down into three major components; the intrinsic, extrinsic and common pathways (Furie and Furie, 2008). Under this scheme the intrinsic pathway is more complex and relatively slow-acting, whilst the extrinsic pathway gives a rapid response to vascular injury. The intrinsic and extrinsic pathways are initiated by vascular injury and tissue factor (TF), respectively (Fig. 1.2) (Furie and Furie, 2008). These pathways converge with the activation of coagulation factor (F)X on the platelet surface, marking the start of the common pathway. More recently, a revised model has been proposed (Monroe and Hoffman, 2006).

**Intrinsic pathway**

Interaction of FXII with sub-endothelial collagen activates FXII (FXIIa) and triggers the intrinsic pathway. FXIIa is a serine protease and cleaves pre-kallikrein to kallikrein and FXI to FXIa. Kallikrein and FXIa are anchored to the sub-endothelium by high molecular weight kininogen. FXIa-mediated cleavage of kininogen releases FXIa into the plasma (Mauron et al., 2000). Within the plasma, FXIa activates circulating FIX in the presence of Ca$^{2+}$; FIXa then forms a complex with FVIIIa (generated by cleavage of FVIII by thrombin) and Ca$^{2+}$. Known as the ‘tenase complex’ these components assemble on the PS-
Figure 1.2 Thrombin generation. Schematic representation of the intrinsic, extrinsic and common pathways of the coagulation cascade. The intrinsic pathway is triggered by vascular damage and exposure of sub-endothelial proteins; promoting the sequential activation of coagulation factors (F)XII, FXI and FIX. FIXa, FVIIIa and Ca$^{2+}$ assemble on the PS-enriched surface of activated platelets to form the tenase complex. The extrinsic pathway concerns Ca$^{2+}$-dependent activation of FVII by tissue factor (TF). The common pathway starts with formation of FXa by either the tenase complex or by FVIIa in the presence of Ca$^{2+}$. FXa, in the presence of the co-factors Ca$^{2+}$ and FVa, converts prothrombin to thrombin. Thrombin cleaves fibrinogen to fibrin and activates FXIII, FV, FVIII and Protein C. FXIIIa forms covalent bonds between fibrin monomers to stabilise the platelet aggregate. The coagulation cascade is regulated by Protein C, Protein S, antithrombin III and TFPI. Activation of FV and FVIII are negatively regulated by Protein C and Protein S, respectively. Antithrombin III neutralises thrombin and FXa outside of the thrombus. Finally, TFPI directly inhibits the extrinsic pathway by binding to FXa and the FVIIa-TF complex.
enriched surface of activated platelets and recruit FX to initiate the common pathway (Fig. 1.2).

**Extrinsic pathway**

TF is constitutively expressed on the surface of sub-endothelial fibroblasts (Wilcox et al., 1989, Mackman and Taubman, 2009), and on monocytes and endothelial cells exposed to pro-inflammatory stimuli (Semeraro et al., 1983, Bevilacqua et al., 1984). TF is also enriched on the surface of microparticles, which are recruited into a thrombus by binding to platelets via P-selectin (Falati et al., 2003). Ca²⁺-dependent binding of TF to FVII generates FVIIa, marking the end of the extrinsic pathway (Fig. 1.2).

**Common pathway**

The common pathway is where the extrinsic and intrinsic pathways converge. Here, FX is activated by either the tenase complex or FVIIa in a Ca²⁺-dependent manner. Together with FVa and Ca²⁺, FXa converts prothrombin to thrombin (Fig. 1.2). In its active form thrombin serves multiple functions, including platelet activation via surface PAR receptors (Chapter 1.3), cleavage of fibrinogen to fibrin and activation of FXIII, FVIII and FV. Thrombin cleaves fibrinogen present in the plasma creating a fibrin mesh that traps erythrocytes and other blood cells within the thrombus. Subsequently, FXIIIa (transglutaminase) introduces covalent bonds between fibrin monomers to stabilise the thrombus (Fig. 1.2) (Bereczky et al., 2003).

**Regulation of the coagulation pathways**

Thrombin is a potent platelet agonist and its generation is regulated accordingly. The intricate nature of the coagulation pathways allows inactive coagulation factors to circulate and quickly activate in response to injury. Furthermore, antithrombin III, protein C, and tissue factor pathway inhibitor (TFPI) inhibit FVII, FVIII, FX and FV (Fig. 1.2). Low concentrations of TFPI are present within the plasma and potently inhibit the extrinsic pathway by interacting with FXa and the FVIIa-TF complex (Broze, 1995, Baugh et al., 1998). Antithrombin III is a protease inhibitor within the plasma that neutralises thrombin and FXa downstream of the thrombus (Damus et al., 1973, Furie and Furie, 2008). Finally, thrombin binds to thrombomodulin on healthy endothelial cells to
activate Protein C and Protein S, which inactivate thrombin and degrade FVa and FVIIIa (Esmon, 1992, Dahlbäck, 1994) (Fig. 1.2).

Refinements of the coagulation cascade

The classical view of coagulation described above has been challenged by a recently proposed model (Monroe and Hoffman, 2006, Versteeg et al., 2013). Controversy exists regarding the relative importance of the intrinsic vs extrinsic pathways for thrombin generation, highlighting inherent redundancies within this scheme. In isolation, TF-dependent activation of FVIIa is capable of activating FX and initiating the common pathway (Furie and Furie, 1992). Interestingly, patients with defects in FXII, high molecular weight kininogen or pre-kallikrein have significantly impaired intrinsic pathways but do not present with haemorrhage (Furie and Furie, 1992). The extrinsic pathway is relatively inefficient and generates a small amount of thrombin, which feeds back to activate FV and FVIII to amplify thrombin production (Furie and Furie, 2008). However, the intrinsic pathway is not completely redundant as deficiencies of FVIII or FIX cause haemophilia A and B, respectively (Versteeg et al., 2013).

Taking these controversies into account, the revised model of coagulation encompasses 3 steps; initiation, amplification and propagation (Monroe and Hoffman, 2006). Initiation begins with TF-dependent activation of the extrinsic pathway and activation of FVII. FVIIa then cleaves small amounts of FIX and FX present in the plasma. Subsequent assembly of FIXa, FX and FVa on TF-expressing cells (i.e. fibroblasts) facilitates low level conversion of prothrombin to thrombin (Monroe and Hoffman, 2006). Amplification concerns the downstream activation of adherent platelets, FV and FVIIIa by thrombin. Stimulation of platelets adhering to collagen by thrombin causes a sustained rise of [Ca$^{2+}$]i, which is required for PS exposure (Zwaal and Schroit, 1997, de Witt et al., 2014). The underlying mechanisms governing PS exposure and thus procoagulant activity in platelets is incompletely understood. At present it is known to involve ATP-dependent flippase and floppase proteins and Ca$^{2+}$-dependent phospholipid scramblases in the plasma membrane. TMEM16F ion channels possess phospholipid scramblase activity and have recently been identified in platelets (Chapter 1.7) (Suzuki et al., 2010, Yang et al., 2012). At rest, flippase and floppase proteins maintain an asymmetric distribution of
membrane phospholipids, such that the inner leaflet is rich in negatively charged phospholipids (e.g. PS) and the outer leaflet contains positively charged phospholipids (e.g. phosphatidylcholine) (Lhermusier et al., 2011). Flippases translocate PS and PE (phosphatidylethanolamine) from the outer to the inner leaflet of the plasma membrane, whilst floppases transport internal phospholipids to the outer leaflet (Zwaal and Schroit, 1997, Clark, 2011). Elevation of [Ca^{2+}] inhibits the flippase and activates the scramblase, promoting rapid PS exposure (Zwaal and Schroit, 1997, Suzuki et al., 2010). This procoagulant activity enables assembly of the tenase complex on the platelet surface, leading to a burst of thrombin generation (Versteeg et al., 2013). Interestingly, it has been shown in flow experiments that PS exposure only occurs within a sub-population of platelets and coincides with the inactivation of integrin αIIbβ3 (Munnix et al., 2007, Mattheij et al., 2013). Furthermore, the core of a thrombus contains tightly packed aggregated platelets, which is overlaid with a loose shell of discoid platelets (Stalker et al., 2013). In this model, patches of procoagulant platelets promote the synthesis of fibrin to stabilise and compact cells within the core (Munnix et al., 2007, Stalker et al., 2013).

1.1.3 Atherogenesis

Atherosclerosis is a chronic inflammatory condition whereby atheromas (fatty and fibrotic lesions) develop within the arterial intima (Weber and Noels, 2011). Current hypotheses regarding the pathogenesis of atherosclerosis build upon the ‘response-to-injury’ hypothesis (Ross et al., 1977). Under this hypothesis, damage to endothelial cells recruits platelets and immune cells to the site, where monocytes migrate into the arterial wall. Monocytes later differentiate into macrophages that phagocytose surrounding low density lipoprotein (LDL). Oxidation of LDL (ox-LDL) in the lysosome initiates foam cell formation and release of reactive oxygen species (ROS); further damaging the endothelium (Weber and Noels, 2011). Foam cells undergoing apoptosis release ox-LDL and ROS, further extending the damage to the arterial wall. This iterative cycle of foam cell formation and release of ox-LDL and ROS continues, leading to the formation of a necrotic core within the arterial wall. In tandem, a fibrous cap enriched with collagen is overlaid. Furthermore, release of growth factors (i.e. PDGF, TG-α and TGF-β) into the smooth muscle layer induces phenotypic
changes that reduce arterial contractility (Weber and Noels, 2011). Narrowing of
the arterial lumen increases local shear rates that, combined with collagen
exposure, enhance the propensity for platelet activation at these sites
(Goncalves et al., 2003, Weber and Noels, 2011). This is particularly pertinent
when atherosclerotic lesions develop within the narrow coronary vasculature,
where vessel occlusion causes myocardial infarction.

1.2 Megakaryopoiesis and thrombopoiesis
Platelets are continually produced at a rate of \( \approx 1,000,000 \) platelets s\(^{-1}\) through
thrombopoiesis (Battinelli et al., 2007). This mammoth task is mediated by
progenitor megakaryocyte (MK) cells, which comprise less than 1% of bone
marrow cells. Platelet counts in humans can vary greatly from 150,000 to
400,000 per microliter without significantly affecting haemostatic function.
However, platelet counts below 50,000 µl\(^{-1}\) or above 450,000 µl\(^{-1}\) are indicative
of thrombocytopenia and thrombocythemia, respectively (Beer and Green,
2009).

Haematopoietic stem cells (HSCs) are a self-renewing pool of cells within the
bone marrow, which differentiate into either lymphoid or myeloid progenitor
cells. Differentiation of myeloid progenitor cells into MK colony forming units
commits them to the MK lineage, where they mature into MKs (Kaushansky,
2005). MKs can be distinguished from other bone marrow cells by their distinct
morphology and expression of GPIb. Maturing MKs undergo multiple rounds of
endoreplication, which increases their ploidy without undergoing cytokinesis
(Machlus et al., 2014). Endoreplication enables MKs to generate sufficient
mRNA transcripts to synthesise and pack platelets with proteins required to
function independently of the MK. Another feature of the maturing MK is its
extensive demarcation membrane system (DMS) (Behnke, 1968, Radley and
Haller, 1982). This network of surface connected membrane invaginations was
first described from electron micrographs of MKs using electron dense
membrane-impermeant stains (Behnke, 1968). More recent studies using
lipophilic dyes and membrane capacitance measurements of living MKs have
demonstrated that the DMS is continuous with the peripheral plasma membrane
(Mahaut-Smith et al., 2003). More recently, it has been proposed that the DMS
originates at the peripheral membrane and invaginates toward the nucleus
(Eckly et al., 2014). This network grows during endoreplication and is facilitated by close interactions with the endoplasmic reticulum. This membrane is thought to act as a reserve for platelet production (Radley and Haller, 1982, Schulze et al., 2006).

MK maturation and development is mediated by cytokines and growth factors, namely thrombopoietin (TPO), stromal-derived factor-1 (SDF-1) and fibroblast growth factor-4 (FGF-4) (Avecilla et al., 2004, Hitchcock and Kaushansky, 2014). Of these, TPO is the most potent MK growth factor and binds to its cognate receptor c-Mpl expressed by cells of haematopoietic origin (Hitchcock and Kaushansky, 2014). TPO binding to c-Mpl triggers complex downstream signalling cascades via Janus kinase (JAK)2, signal transducer and activator of transcriptase (STAT)3, STAT5, Akt, phosphoinositide-3 kinase (PI3K), Ras, ERK1/2 and mitogen-activated protein kinases (MAPK), which regulate gene transcription and promote MK development (Kaushansky, 2005). HSCs and mature MKs are found within the osteoblastic and vascular niches of the bone marrow, respectively (Yin and Li, 2006). Thus, maturing MKs migrate toward the vascular niche during development, in order to release platelets into the vasculature. This process involves SDF-1, S1P (sphingosine 1-phosphate) and FGF-4 (Yin and Li, 2006, Zhang et al., 2013, Niswander et al., 2014). In an elegant study, it was demonstrated that MKs migrate toward the vascular niche along a chemotactic gradient of SDF-1 (Niswander et al., 2014). In the vascular niche, MKs adhere to the bone marrow endothelial cells through interactions with FGF-4 (Yin and Li, 2006). This process aligns MKs with the venous sinusoid in preparation for thrombopoiesis. At the sinusoid, MKs extend pseudopodia that elongate into thin, branching tubes that contain maturing platelets (Junt et al., 2007). Projection of proplatelet extensions into the sinusoid requires interactions with the lipid S1P and its cognate receptor on the MK (Zhang et al., 2012). Subsequent signalling events, mediated by Src family kinases, and shear forces within the vasculature trigger platelet shedding (Junt et al., 2007, Zhang et al., 2013). Furthermore, large MK clumps and preplatelets also bud off into the circulation; where they split into final size platelets in a shear-dependent manner (Italiano et al., 1999, Junt et al., 2007, Thon et al., 2012).
1.3 Signalling events

Platelet activation is a coordinated process whereby primary agonists initiate signalling pathways that, through the action of second messengers and release of secondary agonists, act to prime and recruit platelets into a thrombus. Here, an overview of signalling by primary and secondary agonist receptors and the activation of integrin α_{IIb}β_{3} receptors is given.

1.3.1 Collagen receptors

Damage to the endothelium exposes the collagen-rich sub-endothelial matrix (Barnes et al., 1998). Platelets directly interact with these collagens primarily via GPVI and, to a lesser extent, through integrin α_{2}β_{1} (Gibbins, 2004). Patients with reduced GPVI expression and GPVI-deficient mice both have increased bleeding times, highlighting the role of this receptor in platelet activation (Nieswandt et al., 2001, Gibbins, 2004). GPVI has a simple structure consisting of two extracellular immunoglobulin (Ig) domains, a single transmembrane domain (TMD) and a short intracellular C-terminus (Nieswandt and Watson, 2003, Horii et al., 2006). GPVI couples to the ITAM-bearing (immunoreceptor tyrosine-based activation motif) Fc receptor (FcR) γ-chain homodimer via a salt bridge (Nieswandt and Watson, 2003). Collagen binds to the Ig domains, causing receptor dimerisation and downstream FcR γ-chain-dependent signalling (Gibbins et al., 1997, Horii et al., 2006). GPVI stimulation leads to PLCγ2-mediated Ca^{2+} mobilisation, shape change, degranulation, TxA_{2} synthesis and promotes integrin α_{IIb}β_{3} activation and platelet aggregation.

Briefly, collagen binding triggers GPVI phosphorylation by Src-family tyrosine kinases (TKs), which promote the docking of Src homology (SH) 3 domains. Fyn and Lyn TKs are recruited by interaction with the SH3 domains and phosphorylate tyrosine residues within the ITAM motif of the FcR γ-chain. Syk TK is recruited to the ITAM via tandem SH2 domains and subsequently undergoes autophosphorylation. At this point, the pre-assembled linker for activated T-cells (LAT) signalosome interacts with the GPVI complex to mediate downstream MAPK, PI3K, PKC and PLCγ2 signalling (Watson et al., 2005).

Integrins are a family of cell surface adhesion receptors that form heterodimers comprising α and β subunits that interact with the actin cytoskeleton (Hynes,
Integrins transduce signals in response to extracellular and intracellular cues via outside-in and inside-out signalling, respectively. Inside-out signalling concerns cytoskeletal rearrangements that increase the affinity of the receptor for its ligand, whereas outside-in signalling coordinates cellular responses to ligand binding. The principal platelet integrins are α\text{IIb}β\text{3} (≈80,000 per platelet (Wagner et al., 1996)) and α\text{2}β\text{1} (2,000-4,000 per platelet (Nuyttens et al., 2011)), which bind fibrinogen and collagen, respectively. Integrin α\text{IIb}β\text{3} signalling is discussed in Chapter 1.3.3. Evidence suggests that platelet signalling responses to collagen are primarily dependent upon GPVI, which activates integrin α\text{2}β\text{1} via inside-out signalling to promote collagen binding and thrombus stabilisation (Kuijpers et al., 2003, Gibbins, 2004, Watson et al., 2005, Pugh et al., 2010, Nuyttens et al., 2011).

1.3.2 G protein coupled receptors

G protein coupled receptors (GPCRs) possess 7 TMDs with an extracellular N-terminus and intracellular C-terminus. Platelet GPCRs include thrombin-activated protease-activated receptors (PARs), TxA\text{2}-activated TP receptors and ADP-binding P2Y receptors (Offermanns, 2006). G\text{q}-coupled receptors activate PLCβ-mediated Ca\text{2+} mobilisation, whilst G\text{i}-coupled receptors inhibit cAMP production by adenylate cyclase and G\text{12/13}-coupled receptors signal via the RhoGEF pathway (Rhee and Bae, 1997, Offermanns, 2006).

PAR receptors

Thrombin, generated by the coagulation pathway (Chapter 1.1.2, Fig. 1.2), is a potent platelet agonist that induces Ca\text{2+}-mobilisation, degranulation, shape change, aggregation and clot formation (Niewiarowski and Thomas, 1966, Brass, 2003). Platelets express two PARs that are coupled to both G\text{q} and G\text{12/13} regulatory G proteins (Offermanns, 2006). In human platelets, thrombin activates PAR1 and PAR4 (Vu et al., 1991), whereas mouse platelets are activated by PAR3 and PAR4 (Kahn et al., 1998). Serine protease activity of thrombin cleaves the PAR N-terminus revealing a new N-terminus, which binds to the receptor as a tethered ligand (Vu et al., 1991). PAR1 is 10-100x more sensitive to thrombin than PAR4, however, PAR4 stimulation gives a more sustained response (Brass, 2003). Interestingly, PAR3 does not mediate
thrombin-evoked signalling, rather it facilitates cleavage of PAR4 by thrombin (Sambrano et al., 2001). PAR activation is irreversible; therefore responses are regulated by rapid uncoupling, receptor internalisation and lysosomal degradation (Coughlin, 2005).

**Thromboxane A₂ receptor**

TxA₂ is a weak secondary agonist that is synthesised in response to stimulation by collagen, thrombin and ADP. These agonists release arachidonic acid from the plasma membrane so that it may be converted to TxA₂ by cyclooxygenase-1 (COX-1) and thromboxane synthase (Samuelsson et al., 1978). TxA₂ has an extremely short half-life of 30-40s, during which time it diffuses across the membrane to activate surface G₁q- and G₁₂/₁₃-coupled TP receptors (Samuelsson et al., 1978, Hourani and Cusack, 1991, Paul et al., 1999b, Offermanns, 2006). Direct stimulation of platelets by TxA₂ mobilises [Ca²⁺], shape change and degranulation, but aggregation responses require secondary P2Y receptor activation (Paul et al., 1999b). This is achieved through Ca²⁺- (Varga-Szabo et al., 2009) and PKC-dependent (Hashimoto et al., 1994) dense granule secretion.

**P2Y receptors**

The nucleotides ATP and ADP are pre-packaged in platelet dense granules at a ratio of 2:1 and are released in response to platelet stimulation (Weiss et al., 1979, Hashimoto et al., 1994, Varga-Szabo et al., 2009). ADP responses are coordinated by G₁q-coupled P2Y1 and Gᵢ-coupled P2Y12 receptors (Hechler and Gachet, 2011). Full platelet aggregation in response to stimulation by ADP requires stimulation of both P2Y1 and P2Y12 receptors (Offermanns, 2006, Jones et al., 2011). P2Y1 receptor-deficient mice lack ADP-evoked Ca²⁺ mobilisation but have residual aggregation responses (Fabre et al., 1999). On the other hand, P2Y12-deficient mice have normal Ca²⁺ responses but fail to aggregate (Foster et al., 2001). Furthermore, *in vivo* studies show that knockout of either receptor prolongs bleeding times (Fabre et al., 1999, Foster et al., 2001). Thus, P2Y1 and P2Y12 work in concert to elicit a complete response to ADP stimulation. This is likely due to concomitant activation of the small GTPase Rap1b, PKC and CalDAG-GEF1 (Ca²⁺ and diacylglycerol-regulated...
guanine nucleotide exchange factor 1) signalling pathways by these receptors
(Bergmeier et al., 2007, Hechler and Gachet, 2011). Rap1b activation is a
critical step for inside-out activation of integrin αIIbβ3 and is discussed below.

1.3.3 Integrin αIIbβ3

Integrin αIIbβ3 is the most abundantly expressed receptor on the platelet surface
and binds fibrinogen to cross-link between adjacent platelets, forming an
aggregate (Wagner et al., 1996). The essential role of integrin αIIbβ3 receptors in
platelet aggregation was clearly demonstrated using knockout mice for either
subunit (Hodivala-Dilke et al., 1999, Tronik-Le Roux et al., 2000). In these
studies, agonist-evoked aggregation was absent and bleeding times were
significantly prolonged. Fibrinogen, located within the plasma and α-granules, is
the primary ligand for integrin αIIbβ3 receptors. In quiescent platelets the integrin
αIIbβ3 receptor is expressed in its low affinity state at the plasma membrane.
The affinity for fibrinogen increases in response to agonist stimulation via
inside-out signalling; fibrinogen binding then initiates outside-in signalling events
(Qin et al., 2004). Inside-out activation of integrin αIIbβ3 has been shown in
response to Gq, Gi, G13 and GPVI-mediated signalling cascades. These
pathways converge at Rap1b activation and mice deficient in this protein have a
bleeding defect and impaired aggregation responses (Chrzanowska-Wodnicka
et al., 2005). Rap1b activation occurs in a Ca2+- and DAG-dependent and
independent manner through the actions of CalDAG-GEF1 and PI3K
(Chrzanowska-Wodnicka et al., 2005, Bergmeier et al., 2007, Stefanini et al.,
2009). Rap1b cycles between active (GTP-bound), and inactive (GDP-bound)
states, mediated by CalDAG-GEF1 (Lee et al., 2009). Active Rap1b forms a
complex with RIAM (Rap1-GTP-interacting adapter molecule) and talin (Lee et
al., 2009), which activate integrin αIIbβ3 via interactions with the cytosolic domain
of the β3 subunit (Petrich et al., 2007). This increases the affinity of integrin
αIIbβ3 for fibrinogen, promoting fibrinogen cross-linking of activated platelets,
triggering outside-in signalling. Outside-in signalling is associated with tyrosine
phosphorylation within the cytoplasmic tail of the β3 subunit (Law et al., 1996,
Law et al., 1999) and is mediated by receptor clustering and recruitment of Syk
and focal adhesion kinase (Watson et al., 2005). Downstream signalling serves
to further amplify platelet activation via PKC activation, Ca2+ mobilisation and
cytoskeletal rearrangements (Law et al., 1999).

1.4 Negative regulation of platelet activity

Release of soluble mediators from the healthy endothelium and the action of ectonucleotidases maintain platelets in a quiescent state. Negative regulation of platelet activity is achieved through a combination of endothelial-derived mediators, platelet surface receptors and inhibitory intracellular signalling pathways. In addition, negative feedback through these pathways down-regulate thrombus progression.

1.4.1 Endothelial-derived inhibitors

Healthy endothelial cells release PG12 and NO into the vasculature, where they act on platelets (Schwarz et al., 2001). PG12 and NO are potent platelet inhibitors and function primarily by raising [cAMP]i and [cGMP]i, respectively. PG12 is synthesised by COX-1 whilst NO is produced by nitric oxide synthase; these molecules then diffuse into the blood (Schwarz et al., 2001). PG12 binds to its cognate receptor on the platelet surface and NO diffuses into the platelet to bind soluble guanylate cyclase (Schwarz et al., 2001). PG12 receptors are Gs-coupled GPCRs and upon activation stimulate adenylate cyclase and induce cAMP synthesis (Gorman et al., 1977, Tanaka et al., 2004). Likewise, NO binding to guanylate cyclase leads to cGMP production (Gambaryan et al., 2004). cAMP and cGMP have an antiaggregatory effect, which are mediated by cAMP- and cGMP-dependent protein kinases A (PKA) and G (PKG), respectively (Schwarz et al., 2001, Anfossi et al., 2004, Gambaryan et al., 2004). PKA and PKG regulate several processes including; release of [Ca2+]i, cytoskeletal rearrangements and vasodilator stimulated phosphoprotein (VASP) phosphorylation. VASP is a 46 kDa protein that is associated with the actin cytoskeleton and has 3 phosphorylation sites; Ser157, Ser239 and Thr238. The principal PKA and PKG phosphorylation sites are Ser157 and Ser239, respectively (Butt et al., 1994, Smolenski et al., 1998). Platelet aggregation requires cytoskeletal rearrangements to achieve inside-out activation of integrin αIIbβ3 (Chapter 1.3.5). VASP phosphorylation blocks this process and VASP-deficient mice have been shown to have quicker aggregation responses (Horstrup et al., 1994, Aszódi et al., 1999); highlighting the role of this pathway in the negative
regulation of platelet activation. In addition, phosphorylation of IP$_3$ receptors by PKA and PKG prevents Ca$^{2+}$ release from the dense tubular system (DTS). PKG-dependent inhibition of IP$_3$ receptors requires IRAG but the underlying mechanism governing PKA-mediated phosphorylation remains unclear (Quinton and Dean, 1992, Quinton et al., 1996, Antl et al., 2007, Schlossmann and Desch, 2011).

Cyclic nucleotides evoke potent inhibition therefore endogenous regulatory mechanisms exist to allow efficient platelet activation. P2Y12 receptors are G$_i$-coupled and activation of these receptors inhibits AC activity; thus reducing [cAMP]$_i$ (Schwarz et al., 2001). Phosphodiesterase (PDE) activity represents a second regulatory mechanism. PDEs inactivate cAMP and cGMP by hydrolysis of their 3’-phosphoester bond (Haslam et al., 1999). Platelets express PDE2, PDE3 and PDE5 (Gresele et al., 2011). PDE2 hydrolyses both cAMP and cGMP, whilst PDE3 preferentially acts on cAMP and PDE5 is specific to cGMP (Haslam et al., 1999). Importantly, damage to the endothelium removes PGI$_2$- and NO-producing cells (Fig. 1.1), thus reducing the local concentration of these antagonists.

1.4.2 Ectonucleotidase activity

Platelet P2Y and P2X1 receptors are activated by the nucleotides ADP and ATP, respectively. These nucleotides are often spontaneously released in the bloodstream. Thus, regulation of extracellular nucleotide concentration is required to prevent inappropriate platelet activation through these receptors. To this end, extracellular ATP and ADP are sequentially hydrolysed to AMP and adenosine by the ectonucleotidases CD39 and CD73, which are expressed on the surface of leukocytes and endothelial cells (Zimmermann, 1992, Marcus et al., 1997, Fung et al., 2009).

1.4.3 Platelet-endothelial cell adhesion molecule-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is expressed on the surface of several cell types, including platelets, endothelial cells and T-cells (Muller et al., 1993, Berman et al., 1996, Falati et al., 2006). PECAM-1 receptors are activated by homophilic ligand binding of the Ig domains on apposite receptors (Albelda et al., 1991). Signalling by PECAM-1 is mediated by
a paired immunoreceptor tyrosine-based inhibitory motif (ITIM) within its C-terminus, which activates tyrosine phosphatases to inhibit TK signalling (Newman, 1999, Daeron et al., 2008). Studies have shown that PECAM-1 signalling inhibits platelet activation and thrombus formation in response to multiple agonists (Jones et al., 2001, Cicmil et al., 2002, Falati et al., 2006, Jones et al., 2009). However, some controversy regarding the relative importance of PECAM-1 exists (Dhanjal et al., 2007). Overall, evidence suggests that stimulation of PECAM-1 receptors serves to fine-tune platelet activation and down-regulate thrombus growth.

1.5 Antiplatelet therapies

The most recently available statistics indicate that the prevalence of CVD (e.g. myocardial infarction and stroke) in Great Britain is 11.7% for men and 10.1% for women. This reflects a modest increase from 2009 where prevalence rates were 11.4% and 9.5% for men and women, respectively (Heartstats, 2012). In 2009, management of these conditions cost in excess of £10.4 billion, of which £2.0 billion was spent on prescription medications (Heartstats, 2012). Arterial thrombosis is a major precipitating factor in the pathogenesis of CVD, and antiplatelet drugs play a pivotal role in the management of these conditions (table 1.1). The key challenges facing current antithrombotics are limitations of efficacy and safety (i.e. problem bleeding) (Angiolillo and Suryadevara, 2009, Kumar and Kao, 2009). For example, aspirin is the gold standard antithrombotic agent yet between 5%-40% of the population are aspirin-resistant (Gum et al., 2001). Currently available therapies are designed to inhibit agonist receptors (i.e. P2Y receptor antagonists), enhance inhibitory pathways (i.e. PGI2 analogues) or block adhesion receptors (i.e. the integrin αIIBβ3 blockers).

Aspirin was the first antiplatelet drug to be identified and has been used for over 100 years (Weiss and Aledort, 1967). Aspirin works by irreversibly acetyling COX-1 enzymes, present in platelets and the endothelium (Roth and Majerus, 1975), thus inhibiting TxA2 synthesis. Unlike platelets, endothelial cells can compensate for the effects of aspirin by de novo synthesis of COX enzymes. This is advantageous as the effects of aspirin last for the lifespan of the platelet and smaller maintenance doses are required to maintain efficacy (Holmsen, 1989, Awtry and Loscalzo, 2000). The irreversible P2Y12 antagonist clopidogrel
is another widely used antiplatelet drug (Michelson, 2004). Clopidogrel is a thienopyridine prodrug, which requires metabolism by the liver before it is biologically active (Michelson, 2004). Daily treatment with clopidogrel reduces ADP-evoked aggregation by up to 60% (Thebault et al., 1999).

As discussed in Chapter 1.4, platelets are potently inhibited by PGI2– and NO-evoked elevation of cyclic nucleotides. Furthermore, regulation of intracellular cyclic nucleotide concentration is primarily by the actions of PDEs. Blockade of PDE5 by dipyridamole increases intraplatelet cGMP levels but does not significantly affect platelet activation (Feijge et al., 2004). However, cilostazol, a PDE3 inhibitor, reduces platelet Ca2+ responses (Feijge et al., 2004) and decreases thrombus formation in vivo (Sim et al., 2004). Clinical trials of PDE inhibitors reported a modest benefit of treatment with cilostazol and dipyridamole (Reilly and Mohler, 2001).

**Table 1.1 Anti-platelet drugs for the management of arterial thrombosis.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abciximab</td>
<td>Blocker</td>
<td>Integrin αIIbβ3</td>
<td>(Popma and Satler, 1994)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Inhibitor</td>
<td>Cyclooxygenase</td>
<td>(Weiss and Aledort, 1967)</td>
</tr>
<tr>
<td>Cilostazol</td>
<td>Inhibitor</td>
<td>PDE3</td>
<td>(Sim et al., 2004)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>Antagonist</td>
<td>P2Y12</td>
<td>(Herbert et al., 1993)</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>Inhibitor</td>
<td>PDE5</td>
<td>(Feijge et al., 2004)</td>
</tr>
</tbody>
</table>

Targeting the integrin αIIbβ3 receptor is arguably the most effective method of preventing platelet aggregation but carries a significant bleeding risk (Jackson and Schoenwaelder, 2003). This is because fibrinogen binding to integrin αIIbβ3 is the critical end-point of agonist-evoked signalling pathways in the platelet (Fig. 1.1). Abciximab is a Fab fragment of a monoclonal antibody raised against integrin αIIbβ3 that has been used in high risk patients undergoing coronary interventions (Popma and Satler, 1994, Scarborough et al., 1999). There are ≈80,000 integrin αIIbβ3 receptors per platelet therefore high concentrations of abciximab are required to achieve ≈80% receptor occupancy (Wagner et al., 1996, Scarborough et al., 1999).
Advances in platelet research continue to identify novel antiplatelet targets and strategies. Future candidate receptors showing promise include GPVI, P2X1 and TPα. The development and progress of these therapies is beyond the scope of this thesis. However, several excellent reviews on this topic are available; (Jackson and Schoenwaelder, 2003, Barrett et al., 2008, Michelson, 2010, Yeung and Holinstat, 2012).

1.6 Ca^{2+} signalling in platelets

Mobilisation of Ca^{2+} is a common downstream response to platelet activation by a range of platelet agonists. Elevation of [Ca^{2+}]_i amplifies platelet activation via stimulation of several target proteins. For example, Ca^{2+}-dependent activation of myosin light chain kinase and protein kinase C mediate cytoskeletal rearrangements associated with shape change and platelet granule release, respectively (Varga-Szabo et al., 2009). Thus, regulation of [Ca^{2+}]_i is required to prevent spontaneous platelet activation. The primary Ca^{2+} store in platelets is the DTS (Gerrard et al., 1978). The DTS provides a limited source of Ca^{2+} in response to agonist stimulation, particularly compared to [Ca^{2+}]_o. Thus, contributions to Ca^{2+} influx across the plasma membrane represent an important means for elevating [Ca^{2+}]_i. In platelets, [Ca^{2+}]_i elevation occurs via store operated Ca^{2+} entry (SOCE) and non-SOCE pathways (Varga-Szabo et al., 2009). Non-SOCE can be classified further into second messenger operated and ligand-gated Ca^{2+} entry.

1.6.1 [Ca^{2+}]_i homeostasis in platelets

Stimulation of platelets by collagen and GPCR agonists (ADP, thrombin, TxA$_2$) activates the γ2 and β isoforms of PLC, respectively (Fig. 1.3) (Varga-Szabo et al., 2009). Activated PLC isoforms hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP2), present in the plasma membrane, to generate inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). DAG contributes to non-SOCE and is discussed below. IP$_3$ binds to IP$_3$ receptors, expressed in membranes of the DTS, to induce Ca^{2+} release and elevate [Ca^{2+}]_i to several hundred nanomolar (Fig. 1.1) (Varga-Szabo et al., 2009). Resting [Ca^{2+}]_i in platelets has been estimated at ≈100 nM (Tan et al., 1995), however, Ca^{2+} is lost from the DTS through a constitutive leak channel; the molecular identity of
which is unclear. Thus, [Ca$^{2+}$]$_i$ is regulated by the actions of both cell surface PMCA (plasma membrane Ca$^{2+}$ ATPase) and Na$^+$/Ca$^{2+}$ exchangers and SERCA (sarcoplasmic/endoplasmic reticulum Ca$^{2+}$ ATPase) pumps expressed along the DTS (Varga-Szabo et al., 2009). SERCA 2b is the predominant isoform present in platelets and mediates uptake of cytosolic Ca$^{2+}$ into the DTS (Enyedi et al., 1986). PMCA represent the primary route for the removal of [Ca$^{2+}$]$_i$ from platelets and have been shown to blocked by TK phosphorylation (Rosado and Sage, 2000). Ca$^{2+}$ transport by NCX provides an additional route for expedient removal of excess [Ca$^{2+}$]$_i$ (Valant et al., 1992). Interestingly, NCX has also been shown to function in a reverse mode following agonist-evoked Na$^+$ entry via TRPC channels, promoting the platelet procoagulant response (Harper et al., 2013).

1.6.2 Store operated Ca$^{2+}$ entry

Depletion of Ca$^{2+}$ from the DTS in the presence of extracellular Ca$^{2+}$ results in a substantial and rapid influx of Ca$^{2+}$ across the plasma membrane. This mechanism is termed SOCE and in many cell types, including platelets, has been demonstrated to be mediated by stromal interaction molecule 1 (STIM1) and Orai1 (Fig. 1.3) (Varga-Szabo et al., 2009). STIM1, present in the DTS membrane, is a Ca$^{2+}$ sensor that binds Ca$^{2+}$ within the DTS through its EF hand domain (Grosse et al., 2007). IP$_3$-evoked Ca$^{2+}$ release from the DTS causes Ca$^{2+}$ to dissociate from these domains and STIM1 translocates to the plasma membrane to interact with Orai1 (Zhang et al., 2005). Orai1 proteins have four TMDs and the crystal structure studies suggest that STIM1 interacts with the fourth TMD (Hou et al., 2012). Increased mortality (≤ 70%) and retarded development of Orai1$^{-/-}$ or STIM1$^{-/-}$ mice, required the authors to develop radiation chimeras whereby deletion of either protein was restricted to bone marrow derived cells (Varga-Szabo et al., 2008b, Braun et al., 2009). Using platelets derived from these mice, studies show defective SOCE and significantly impaired functional responses, thus confirming the central role of SOCE in Ca$^{2+}$ homeostasis and platelet functional responses.

1.6.3 Second messenger-operated Ca$^{2+}$ entry
Figure 1.3 Calcium signalling in platelets. Cartoon of the major routes for elevation of $[\text{Ca}^{2+}]_i$ and downstream signalling events in platelets. Stimulation of platelets via GPVI receptors (collagen) or GPCRs (ADP/Thr/TxA2) activate the γ and β isoforms of phospholipase C (PLC), respectively. Activated PLCβ/γ hydrolysates phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane, generating inositol-1,4,5-triphosphate ($\text{IP}_3$) and diacylglycerol (DAG). Non store-operated $\text{Ca}^{2+}$ entry (non-SOCE) is mediated by direct interaction of DAG with TRPC6 channels in the plasma membrane. Whilst binding of $\text{IP}_3$ to its cognate receptor on the dense tubular system (DTS) induces $\text{Ca}^{2+}$ release. Depletion of $\text{Ca}^{2+}$ from the DTS is sensed by STIM1, which subsequently translocates to the plasma membrane and binds to Orai1, triggering store-operated $\text{Ca}^{2+}$ entry (SOCE). Elevation of $[\text{Ca}^{2+}]_i$ facilitates several platelet functional responses including; dense granule release, shape change and aggregation. Dense granules contain the nucleotides ATP and ADP (red circles). Binding of ATP to P2X1 receptors triggers $\text{Ca}^{2+}$ influx through the receptor-operated $\text{Ca}^{2+}$ entry pathway (ROCE). Elevation of $[\text{Ca}^{2+}]_i$ also serves to activate $\text{K}_{\text{Ca3.1}}$, TRPM2 and TMEM16F channels present on the plasma membrane. In addition, Panx1 channels may be activated by a rise of $[\text{Ca}^{2+}]_i$. 
Non-store operated Ca\(^{2+}\) entry concerns Ca\(^{2+}\) influx pathways triggered either directly by ligand binding (e.g. ATP gating of P2X1 receptors) or by second messengers downstream of receptor activation. The latter pathway includes TRPC6 channels, which can be activated by interactions with DAG and PIP2 depletion (Hassock et al., 2002, Tolhurst et al., 2005, Ramanathan et al., 2012). Patch clamp recordings in a heterologous expression system demonstrated that TRPC6 channel conductances can be induced by application of membrane permeant DAG analogues, OAG and DOG (Hofmann et al., 1999). Studies of TRP channel mRNA expression by MKs and platelets has highlighted expression of TRPC1, TRPC6, TRPM2 and TRPM7 (den Dekker et al., 2001, Hassock et al., 2002, Carter et al., 2006). Besides TRPC6, other non-SOCE channels have been proposed, such as TRPC1 but studies of platelets from TRPC1-deficient mice do not show altered Ca\(^{2+}\) or functional responses (Varga-Szabo et al., 2008a). However a clear role for TRPC6 has been shown and TRPC6\(^{-}\) mice have an associated mild bleeding phenotype, thus implying a role for this channel in haemostasis and thrombosis (Paez Espinosa et al., 2012). Furthermore, these channels have been shown to contribute to Ca\(^{2+}\) responses to thrombin (Hassock et al., 2002) and dual stimulation by thrombin and collagen (Harper et al., 2013).

1.6.4 Ligand-gated Ca\(^{2+}\)-entry

ATP-gated P2X1 receptors represent the fastest route whereby a platelet agonist can generate Ca\(^{2+}\) entry across the platelet plasma membrane since the receptor contains both the ATP binding site and a Ca\(^{2+}\)-permeable pore that is activated within milliseconds by the ligand (Mahaut-Smith et al., 2011). The P2X receptor family comprises 7 members that each has two TMDs, intracellular N- and C-termini and a cysteine-rich head region. P2X subunits assemble into trimers, of one or more subtype, prior to insertion into the plasma membrane (Evans, 2010). P2X receptors form non-selective cation channels (NSCCs), with a slightly higher permeability to Ca\(^{2+}\) compared to physiological monovalent cations (North, 2002). For example, P2X1 receptors are \(=\)4 times more permeable to Ca\(^{2+}\) than Na\(^+\) (\(P_{Ca}/P_{Na}= 3.9\)) (Evans et al., 1996). ATP binding within the head region induces a conformational change of the TMDs, allowing ions to permeate the lateral pore (Kawate et al., 2009, Roberts et al., 2012).
Each receptor has distinct biophysical properties and recovers from desensitisation at different rates (North, 2002). Of the 7 receptor subtypes, P2X1 is the only member expressed by platelets and MKs (Sun et al., 1998, Wang et al., 2003). The first report of P2X1 receptors came from patch clamp studies of human platelets, when stimulation by ADP induced ‘flickery’ single channel events (Mahaut-Smith et al., 1990b). However, it was later shown that these currents were induced by contaminating ATP present in commercial ADP stocks (Mahaut-Smith et al., 2000). Neither MKs nor platelets from P2X1 receptor knockout mice respond to α,β-meATP, a non-hydrolysable ATP analogue and agonist of P2X1 receptors (Vial et al., 2002, Hechler et al., 2003). MKs from these mice also lack rapid ATP-evoked cation current and Ca^{2+} responses (Vial et al., 2002), thus together with the lack of detection of other P2X receptors, P2X1 appears to be solely responsible for the ATP-gated Ca^{2+} influx in platelets. P2X1 receptors have been shown to amplify platelet Ca^{2+} responses to threshold concentrations of both primary and secondary agonists (Fung et al., 2005, Fung et al., 2007). Furthermore, P2X1⁻/⁻ mice are protected against arterial thrombosis without significant impairment of haemostatic function (Hechler et al., 2003). These data suggest that P2X1 receptors could represent a novel antiplatelet target. Indeed, treatment of wild type mice with the concentration of the antagonist NF449 that is selective for P2X1 receptors also conferred protection against experimentally-induced thrombosis (Hechler et al., 2005).

### 1.7 Ca^{2+}-activated ion channels

Ca^{2+} is a ubiquitous second messenger that regulates many platelet responses, including degranulation, shape change, PS exposure and aggregation responses (Varga-Szabo et al., 2009). Amongst its numerous targets, Ca^{2+} has been shown to directly modulate ion channels permeable to both cations (i.e. K⁺ and Ca^{2+}) and anions (i.e. Cl⁻ and ATP^4⁻). Ca^{2+}-activated ion channels have also been implicated in platelet procoagulant activity (Suzuki et al., 2010). The activity of ion channels previously reported in platelets or MKs can be regulated by a variety of gating mechanisms, which include Ca^{2+} (i.e. K_{Ca3.1}) (Gardos, 1958, Grygorczyk et al., 1984), ligand binding (i.e. P2X1 receptors) (North, 2002), mechanical stimulation (i.e. TRPM7) (Runnels et al., 2001), temperature
Gating by these mechanisms allow cells to ‘sense’ their local environment and respond to changes accordingly. For example, ATP released from damaged endothelial contributes to the activation of passing platelets, via P2X1 receptors, initiating a haemostatic response (Mahaut-Smith et al., 2011). To date, clear roles have been identified for ATP-gated P2X1 receptors, voltage-gated Kv1.3 channels, Ca²⁺-activated K⁺ channels (Kᵥ3.1) and Ca²⁺-activated TMEM16F channels in platelet and MK function. Furthermore, mechanosensitive TRPM7 channels have been reported in MKs (Carter et al., 2006).

Gating of ion channels by [Ca²⁺]ᵢ can be by either a calmodulin- (CaM) dependent or CaM-independent mechanism. CaM-independent gating involves direct association of Ca²⁺ with intracellular domains of the channel, which induce conformational changes to open the ion pore. For example, Kᵥ1.1 (BK, see below) channels are activated in response to Ca²⁺ binding within their C-terminus (Berkefeld et al., 2010). CaM is a highly conserved Ca²⁺ binding protein, ubiquitously expressed amongst eukaryotic organisms, which consists of two globular EF motif-containing domains connected by a central linker domain (Mirzoeva et al., 1999, Berkefeld et al., 2010). Ca²⁺-binding to the EF hand domains induces conformational changes and bending within the linker region, which enables CaM to interact with target proteins (Mirzoeva et al., 1999). Interestingly, the Ca²⁺-binding affinity of the EF hand domain in the N-terminus is far greater (1-3 µM) than in the C-terminus (10-20 µM) (Berkefeld et al., 2010). Thus, presenting a mechanism whereby CaM can interact with various cellular targets in response to either small or large fluxes of [Ca²⁺]ᵢ.

Advanced techniques for the isolation, purification and quantification of platelet mRNA have identified transcripts for many previously unreported, ion channels in this cell type (Amisten, 2012, Wright et al., 2013). The latter study identified transcripts for pannexin-1 (Panx1) channels within platelets, which were later proposed to open in response to elevated [Ca²⁺] (this thesis Chapters 3 and 4; (Taylor et al., 2014)). The role of platelet and MK Ca²⁺-activated ion channels and their respective gating mechanisms are discussed in the following sections (1.7.1-2).
1.7.1 Ca\textsuperscript{2+}-activated cation channels

Platelets and MKs have been shown to express both intermediate conductance Ca\textsuperscript{2+}-gated K\textsuperscript{+} channels (likely K\textsubscript{Ca}3.1; (Mahaut-Smith, 1995)) and TRPM2 (Carter et al., 2006) channels, which display preferential permeability to K\textsuperscript{+} and Ca\textsuperscript{2+}, respectively. These channels are equally Ca\textsuperscript{2+}-dependent yet they participate in different functional events. Activation of K\textsubscript{Ca} channels is likely involved in contributing to the membrane potential (V\textsubscript{m}) of platelets during Ca\textsuperscript{2+} signalling and thereby modulates agonist-evoked Ca\textsuperscript{2+} influx (Mahaut-Smith, 2012). By contrast, TRPM2 channels are activated in response to ROS-induced cellular damage (McHugh et al., 2003).

**K\textsubscript{Ca}3.1**

There are three families of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels and they can be divided according to their biophysical properties into; small-conductance (SK; K\textsubscript{Ca}2.1, K\textsubscript{Ca}2.2 and K\textsubscript{Ca}2.3), intermediate conductance (IK; K\textsubscript{Ca}3.1) and large-conductance channels (BK; K\textsubscript{Ca}1.1) (Vergara et al., 1998, Wei et al., 2005). Both SK and IK channels are activated by small elevations (< 1 µM) of [Ca\textsuperscript{2+}]\textsubscript{i} (Mahaut-Smith and Schlichter, 1989) independently of V\textsubscript{m} (Wei et al., 2005). However, BK channels are influenced by both V\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i} (Wei et al., 2005). K\textsubscript{Ca}3.1 currents have been recorded from erythrocytes, leukocytes and platelets (Gardos, 1958, Mahaut-Smith and Schlichter, 1989, Grissmer et al., 1993, Mahaut-Smith, 1995). Patch clamp recordings demonstrated that human platelets express channels with properties indistinguishable from K\textsubscript{Ca}3.1, but not SK or BK, channels (Fig. 1.3) (Mahaut-Smith, 1995). K\textsubscript{Ca}3.1 channels are inactive at basal [Ca\textsuperscript{2+}]\textsubscript{i} (100-200 nM) but show steep Ca\textsuperscript{2+}-dependence above 300 nM and are maximally activated by 1 µM [Ca\textsuperscript{2+}]\textsubscript{i} (Mahaut-Smith and Schlichter, 1989, Grissmer et al., 1993). Activation of K\textsubscript{Ca}3.1 hyperpolarises the cell by increasing the relative permeability to K\textsuperscript{+}, thereby moving the cell membrane potential closer to the equilibrium potential for K\textsuperscript{+} (approx. -90mV under normal ionic conditions), and thus increases the driving force for Ca\textsuperscript{2+}-entry across the plasma membrane (Rink and Deutsch, 1983, Grinstein and Dixon, 1989, Mahaut-Smith and Schlichter, 1989).

Gating of IK and SK channels by Ca\textsuperscript{2+} is distinctly different to the mechanism
described for BK channels. BK channels contain a ‘Ca²⁺-bowl’ domain within the C-terminus that directly binds Ca²⁺, inducing conformational changes and opening of the channel pore (Berkefeld et al., 2010). However, a more complex, CaM-dependent, mechanism has been described for SK and IK channels (Xia et al., 1998, Fanger et al., 1999). CaM is constitutively bound to the proximal C-terminus of SK and IK channels (Xia et al., 1998, Fanger et al., 1999). The gating mechanism works by Ca²⁺ binding to the EF hand domain at the N-terminus of CaM, causing the linker domain to bend, which splays apart the TMDs of SK and IK channels to open ion pore (Berkefeld et al., 2010). Interestingly, SK- and IK-bound CaM is unable to bind Ca²⁺ at its C-terminus and instead Ca²⁺ binds solely to its N-terminus (Berkefeld et al., 2010). This likely represents an evolutionary mechanism to ensure efficient activation of these channels by smaller elevations of [Ca²⁺]. Once activated, Kᵥ3.1 channels remain open for up to 100ms and do not desensitise (Xia et al., 1998, Berkefeld et al., 2010). Thus, few channels are likely required to significantly influence the V_m and thereby Ca²⁺ influx. Indeed, electrophysiological recordings indicate that only 5-7 Kᵥ3.1 channels are expressed per platelet (Mahaut-Smith, 1995).

TRPM2

The transient receptor potential (TRP) channel superfamily can be sub-divided into TRPC, TRPM, TRPV, TRPP and TRPML families (Clapham, 2003). RT-PCR analysis of primary MK cDNA identified expression of TRPC1, TRPC6, TRPM1, TRPM2 and TRPM7 transcripts (Carter et al., 2006). Of these, only TRPM2 has been reported to be activated by elevated [Ca²⁺]. (Perraud et al., 2001). TRPM2 channels possess an ADP-ribose (ADPR) pyrophosphatase domain within the C-terminus (Perraud et al., 2001). ADPR is synthesised in response to release of ROS, which damages cells and initiates apoptotic pathways (Zhang et al., 2003, Circu and Aw, 2010). Interestingly, both a rise of [Ca⁺]; and ADPR binding to the C-terminus are required to activate TRPM2 currents (McHugh et al., 2003). These channels are inactive at basal [Ca²⁺]; and become active when [Ca²⁺]; rises above 100 nM, with maximal activation observed at 600nM (Fig. 1.3) (McHugh et al., 2003). Ca²⁺-dependent gating of TRPM2 channels involves interactions with CaM at conserved binding sites in
the N-terminus (Tong et al., 2006, Du et al., 2009). Unlike $K_{Ca}3.1$, CaM is not constitutively bound to TRPM2 and interactions between these proteins is $Ca^{2+}$-dependent (Tong et al., 2006). TRPM2 is a NSCC with principal permeability to $Ca^{2+}$, however $K^+$, $Mg^{2+}$, $Na^+$ and $Cs^+$ conductances have also been reported (Perraud et al., 2001, Sano et al., 2001). In primary MKs, activation of TRPM2 induces large $Ca^{2+}$- and ADPR-dependent whole cell currents with a linear I-V relationship (Carter et al., 2006). To date, this study is the only report of functional TRPM2 channels in MKs. The dependence of these channels on ADPR production suggests that they may represent a mechanism to respond to cellular damage by mediating $Ca^{2+}$-influx and triggering apoptotic pathways. Furthermore, ROS released from damaged endothelial cells may activate passing platelets to initiate wound healing.

1.7.2 $Ca^{2+}$-activated anion channels

$Ca^{2+}$-activated $Cl^-$ channels are expressed by a variety of cells and perform diverse functions by contributing to regulatory volume decrease (RVD) (Livne et al., 1987), PS exposure (Suzuki et al., 2010) and ATP release (Bao et al., 2004). Platelet shape change is a $Ca^{2+}$-dependent process (Hathaway and Adelstein, 1979), requiring dynamic changes in cell volume. Regulation of platelet volume is through a $Ca^{2+}$- and CaM-dependent process, which causes $Cl^-$ efflux that is followed by movement of $Na^+$ and $H_2O$ (Livne et al., 1987, Fine et al., 1994). In many cell types, the molecular identity of $Ca^{2+}$-activated anion channels are unknown. This is, in part, due to a lack of specific channel blockers available to study the biophysical and pharmacological properties of these channels. The gating and function of $Ca^{2+}$-activated anion channels, recently identified in human platelets (Wright et al., 2013), is discussed below.

Pannexin-1

The family of three pannexins were identified as relatives of the invertebrate innexins with whom they share 20% sequence homology (Panchin et al., 2000). Expression profiling indicates that pannexin-1 (Panx1) is ubiquitously expressed whereas Panx2 is found in the brain and central nervous system and Panx3 is restricted to the bone and skin (Bruzzone et al., 2003). Panx1 channels are expressed at the plasma membrane and have four TMDs with two extracellular loops and intracellular N- and C-termini (Scemes et al., 2009). Panx1 has been
shown to be extensively glycosylated at Asn254 on its second extracellular loop, which is a pre-requisite for plasma membrane insertion (Boassa et al., 2007, Boassa et al., 2008). Glycosylation prevents apposite Panx1 channels from docking to form gap junctions and they instead form bona fide channels (Sosinsky et al., 2011). Panx1 channel opening has been reported in response to oxygen-glucose deprivation, caspase cleavage, mechanical stimulation and elevation of [Ca$^{2+}$]; (Bao et al., 2004, Locovei et al., 2006b, Thompson et al., 2006, Sandilos et al., 2012). Open Panx1 channels are anion-selective with permeability up to 1kDa (Bao et al., 2004, Ma et al., 2012).

ATP release through Panx1 (Bao et al., 2004) provides a non-lytic and non-vesicular mechanism for the regulated release of cytosolic ATP. Several studies have reported interactions between Panx1-dependent ATP release and activation of P2 receptors (Locovei et al., 2006b, Garre et al., 2010, Pelegrin and Surprenant, 2006, Romanov et al., 2007, Woehrle et al., 2010, Poornima et al., 2011, Vessey et al., 2011, Xia et al., 2012). As discussed above (Chapter 1.6.4), ATP-gated P2X1 receptors have an established role in platelet activation and thrombus formation. Interactions between P2X1 receptors and Panx1 are explored within Chapters 3 and 4 of this thesis. Ca$^{2+}$-dependent gating of Panx1 channels has been proposed by Locovei and colleagues (Locovei et al., 2006b). In these experiments using a heterologous expression system Panx1 channel opening was shown in response P2Y1 receptor-evoked Ca$^{2+}$ mobilisation. This mechanism is incompletely understood and could be mediated either in a CaM-dependent or CaM-independent manner. Given that the original report recorded Ca$^{2+}$-dependent Panx1 currents in excised patches, the gating mechanism is most likely CaM-independent (Locovei et al., 2006b). Interestingly, attempts to recapitulate these findings in a heterologous expression system were unsuccessful as neither Ca$^{2+}$ elevation or PLC inhibitors affected Panx1 currents (Ma et al., 2009). Further investigation of the Ca$^{2+}$-dependent gating mechanism of Panx1 channels using primary cells, which possess physiologically relevant signalling pathways and interacting molecules, are therefore required.

Anion-selective Panx1 channels preferentially permeate larger anions (I$^-$ > Br$^-$ > Cl$^-$) (Ma et al., 2012). This opens the possibility that these channels may be
permeable to ADP$^3$-; however, this is yet to be demonstrated. ADP may also be generated locally by ectonucleotidase-mediated (e.g. CD39) hydrolysis of released ATP (Marcus et al., 1997). Contributions by Panx1 to ADP release through either of these pathways may represent a further mechanism for amplification of platelet activation.

**TMEM16F**

The TMEM16 protein family contains 10 members named TMEM16A-K (excluding I) (Schreiber et al., 2010). TMEM16A, TMEM16B, TMEM16F are the most widely studied members and function as Ca$^{2+}$-activated Cl$^-$ channels (Hartzell et al., 2009, Martins et al., 2011). TMEM16F channels are particularly interesting given that they function as both Ca$^{2+}$-activated Cl$^-$ channels and phospholipid scramblases (Suzuki et al., 2010). The relationship between these processes is unclear and PS exposure has been shown under conditions where the Cl$^-$ channel activity is blocked (Kmit et al., 2013, Harper and Poole, 2013). Missense mutations of the TMEM16F gene give rise to Scott syndrome, a rare congenital bleeding disorder (Suzuki et al., 2010, van Kruchten et al., 2013). At present, debate surrounds the ion selectivity of TMEM16F channels as currents from primary MKs indicated that they are cation- rather than anion-selective (Yang et al., 2012). The work of Yang et al. (Yang et al., 2012) recorded from membrane patches of murine MKs; the controversy surrounding the ionic selectivity of TMEM16F in platelets is explored further in Chapters 5 and 6 using whole-cell patch clamp recordings from native MKs. The work of Yang and colleagues (Yang et al., 2012) is the only account of cation-selective TMEM16F currents and may be due to mutations restricted to the MK lineage in mice (see Chapter 6 discussion). Interestingly, Scott syndrome is phenocopied by TMEM16F-deficient mice highlighting the crucial role of the protein in haemostasis in mouse and man (Yang et al., 2012, Kmit et al., 2013).

Activation of TMEM16F currents requires sustained elevation of [Ca$^{2+}$] with reported EC$_{50}$ values between 10-100 µM (Fig. 1.3) (Grubb et al., 2013, Pedemonte and Galietta, 2014). By comparison, TMEM16A channels are activated by [Ca$^{2+}$] with an EC$_{50}$ value close to 1µM (Tien et al., 2014). To date, there have been no structural studies of TMEM16F. However, structural studies of related TMEM16A channels (Gokhale et al., 2012, Yu et al., 2012) provide an
excellent template to understand how TMEM16F channels are regulated. For example, Ca^{2+}-dependent activation of TMEM16A has been proposed to be via a CaM-independent mechanism, as both inhibition of CaM and disrupting Ca^{2+} binding to CaM had no effect on TMEM16A currents (Tien et al., 2014). In addition, structural studies have shown that Ca^{2+}-binding domains proximal to the ion pore (Yu et al., 2012, Tien et al., 2014) and within the transmembrane helices (Lee et al., 2014) regulate TMEM16A channel activity. Of particular interest is the highly conserved motif: [E/D]-[Y/F]-[L/M/Q]-E-[L/M/Q/T], which is E-Y-M-E-M and E-Y-L-E-M for TMEM16A and TMEM16F, respectively. The Ca^{2+}-dependence of TMEM16A increased 100-fold upon substitution of glutamic acid to glutamine at positions 702 (E702Q) and E705Q; indicating that channel function requires Ca^{2+} binding at these sites (Yu et al., 2012). Additional acidic residues, E654, E734, D738, have since been identified that coordinate Ca^{2+} binding to TMEM16A (Tien et al., 2014). Interestingly, the reciprocal E702Q mutant in TMEM16F (E667Q) reduced Ca^{2+} sensitivity 2000-fold (Yang et al., 2012). These data suggest that wild type TMEM16F channels bind Ca^{2+} with lower affinity than TMEM16A channels, possibly explaining the variation in EC_{50} values for Ca^{2+} at these channels. Further studies are required however to determine other residues that coordinate Ca^{2+} binding to TMEM16F.

Other Ca^{2+}-activated Cl^{-} channels

Patch clamp recordings of human platelets have reported Ca^{2+}-dependent Cl^{-} channels of unknown identity (Mahaut-Smith, 1990, MacKenzie and Mahaut-Smith, 1996). These studies described Cl^{-} conductances in response to elevating [Ca^{2+}] in intact cells and excised patches. These currents were reversibly blocked by the Cl^{-} channel inhibitor NPPB (Mahaut-Smith, 1990, MacKenzie and Mahaut-Smith, 1996). At the time of these studies, identification of the underlying channel in these studies was hindered by both a lack of Cl^{-} channel blockers and molecularly characterized Ca^{2+}-activated Cl^{-} channels. However, the I-V relationships and Ca^{2+} dependence of these currents suggest that they may be carried by TMEM16 family members (Schreiber et al., 2010, Yu et al., 2012).

1.7.3 Conclusions and future directions
Sections 1.7.1-2 above have summarised the gating mechanisms of several Ca\(^{2+}\)-activated ion channels; highlighting roles for both CaM-dependent pathways and direct interaction of Ca\(^{2+}\) with the channel. Panx1 and TMEM16F have only been investigated relatively recently and it is not fully understood how these channels are regulated. However, important residues for Ca\(^{2+}\) binding to TMEM16F channels have been identified using data reported for related family members (Yu et al., 2012, Yang et al., 2012, Tien et al., 2014). In addition, the molecular identity of previously reported Ca\(^{2+}\)-activated Cl\(^{-}\) channels requires further investigation (Mahaut-Smith, 1990, MacKenzie and Mahaut-Smith, 1996). The biophysical properties and Ca\(^{2+}\) sensitivity of these currents indicates that they may be carried by TMEM16A or TMEM16B channels (Scudieri et al., 2012).

Further studies are required to fully understand the cohort of Ca\(^{2+}\)-activated ion channels expressed by both platelets and MKs and the mechanisms by which they are regulated. Further dissection of these pathways and specific contributions by platelet anion channels remains hindered by a lack of specific pharmacological inhibitors and the difficulties associated with genetic modification of these terminally differentiated and anucleate cells.

1.8 Aims and objectives

Recent advances in the purification and analysis of human platelet mRNA has led to the conclusion that this blood cell expresses several ion channels with as yet unknown roles in haemostasis or thrombosis (Amisten, 2012, Wright et al., 2013). Of these channels, several have been shown to open in a Ca\(^{2+}\)-dependent manner. This thesis focusses on the expression, functional role and properties of Ca\(^{2+}\)-activated Panx1 and TMEM16F ion channels in platelets and MKs. The specific questions addressed by this thesis are:

1. Is Panx1 expressed by human platelets? If so, where are these channels localised?
2. Do platelet Panx1 channels form bona fide channels at the plasma membrane and how are they activated?
3. Which platelet functions (i.e. aggregation, ATP release and Ca\(^{2+}\) influx) do Panx1 channels contribute to? How does ATP release through Panx1
channels contribute to platelet activation?

4. Which Ca\textsuperscript{2+}-activated channels are expressed by the erythroblastic/megakaryocytic cell line HEL and by primary MKs?

5. What are the biophysical properties and ionic selectivity of these channels?

6. Are there differences between TMEM16F channels expressed by MKs/megakaryocytic cells from human, mouse and rat?
Chapter 2

2.0 Materials and Methods

2.1 Reagents

2.1.1 Materials

Collagen type I, from bovine tendon, was a gift from the Ethicon Corporation (New Jersey, USA) and horm collagen, from equine tendon, was purchased from Nycomed (Linz, Austria). CaCCinh-A01 (A01), ionomycin, thapsigargin and U46619 were purchased from Calbiochem (Nottinghamshire, UK), while α,β meATP, ATP, carbenoxolone (Cbx), DMSO, probenecid (Prb), sodium dithionite and thrombin were purchased from Sigma (Dorset, UK). Prb was prepared in standard external saline (SES; see 2.1.2 for composition) at pH 8.3, which was titrated to pH 7.35 as described previously (Niles and Smith, 1982). Unless indicated, all other reagents were from Sigma.

2.1.2 Salines and buffers

SES (in mM; 145 NaCl, 5 KCl, 1 MgCl$_2$, 10 glucose, 10 HEPES, titrated to pH 7.35 with NaOH) was used for preparation of washed platelets and bone marrow isolation protocols. 1 mM CaCl$_2$ was added to SES for bone marrow isolation and electrophysiological recordings. For electrophysiological recordings, external solutions were based on SES; KCl and NaCl were exchanged by equimolar substitution, where indicated. Furthermore, NaCl was substituted by equimolar Na-gluconate or NMDG-gluconate for studies where external Cl$^-$ and Na$^+$ were removed, respectively. Pipette filling solutions contained, in mM; 150 NaCl, 1 MgCl$_2$, 10 glucose, 10 HEPES, 0.05 Na$_2$-GTP titrated to pH 7.2 with NaOH; NaCl was substituted by equimolar KCl where indicated in Chapter 5. Ca$^{2+}$ in the pipette solution was buffered with 0.2 mM Na$_4$-BAPTA (Invitrogen, Paisley, UK) or 1 mM Na$_4$-EGTA, for studies investigating Ca$^{2+}$-activated currents induced by ionomycin or by setting [Ca$^{2+}$], respectively. For the latter experiments, internal Ca$^{2+}$ was set at 1 or 100 µM Ca$^{2+}$ by addition of CaCl$_2$ to the pipette solution, calculated by the programme Maxchelator (http://web.stanford.edu/~cpatton/webmaxcS.htm).
2.2 Isolation of primary cells

2.2.1 Preparation of washed platelet suspensions from human blood

The study was approved by the University of Leicester Committee for Research Ethics concerning human subjects (non-NHS). Blood was collected into acid citrate dextrose anticoagulant (ACD; 85mM trisodium citrate, 78mM citric acid and 111mM glucose) from informed, consenting donors in accordance with the Declaration of Helsinki. Blood was drawn into 50 cm$^3$ sterile syringes using a 21 G butterfly® winged infusion set (Southern Syringe, Leicestershire, UK) from a forearm vein by standard phlebotomy techniques. The blood : ACD mix (6:1 v/v) was centrifuged at 700 x g for 5 min. Platelet-rich plasma (PRP) was removed and treated with aspirin (100µM, to inhibit TxA2 synthesis) and type VII apyrase (0.32 U ml$^{-1}$ to degrade spontaneously released ADP and ATP) (Jones et al., 2011). For [Ca$^{2+}$]$_i$ or dye efflux studies PRP was loaded by incubation with 2 µM Fura-2AM (Invitrogen) or 0.5 µM calcein-AM (Invitrogen) for 45 min at 37$^\circ$C. Washed platelets were then prepared by centrifugation at 350 x g for 20 min and resuspended in SES supplemented with 0.32 U ml$^{-1}$ apyrase. CaCl$_2$ or MgCl$_2$ (2mM) was added to individual cuvettes for studies in the presence or nominal absence of extracellular Ca$^{2+}$, respectively.

2.2.2 Preparation of primary megakaryocytes from bone marrow

Male Wistar rats were culled by stunning and cervical dislocation, whilst male C57/bl mice were killed by cervical dislocation and death was confirmed by exsanguination after cutting the femoral artery. All procedures were conducted in accordance with Home Office regulations and authorised by the University of Leicester Home Office Certificate of Designation. For some studies, bone marrow was obtained from mice after blood had been collected by exsanguination under terminal anaesthesia (covered by PPL 60/4452). Bone marrow suspensions were prepared by flushing femoral and tibial bones with SES (with 1mM CaCl$_2$). Briefly, hind legs were amputated at the pubis and subsequent dislocation of the patella and tarsals enabled the dissection of tibial and femoral bones. Remaining muscle and connective tissue was removed and bones were washed with SES before removing the epiphyses with either bone rongeurs (rat) or dissection scissors (mouse). Bone marrow was flushed with
SES using a pipette tip affixed to a 1 ml Pasteur pipette (P200 and P10 pipette tips were used for rat and mouse bones, respectively). Marrow was transferred to 1.5 ml Eppendorf tubes and fixed immediately for immunocytochemistry (ICC) experiments (Chapter 2.4.8); otherwise suspensions were left to disperse for 3-4 hours on a rotator prior to patch clamp experiments (Chapter 2.7).

2.3 Cell culture

2.3.1 Culture of the human embryonic kidney-293 cell line

Human embryonic kidney-293 (HEK-293) cells were cultured in minimum essential growth medium with 2 mM glutaMAX, supplemented with 10% foetal bovine serum and 1% non-essential amino acids (Invitrogen, Paisley, UK). Cells were kept in a humidified incubator (37°C, 5% CO₂; Panasonic Biomedical, Leicestershire, UK) and were passaged when they reached ≈80% confluency. Briefly, culture media was removed and cells were washed with phosphate buffered saline, which was followed by addition of trypsin-EDTA cell dissociation buffer (2 min, 37°C; Invitrogen). Cells were resuspended in fresh culture medium and centrifuged (200 x g, 5 min). The supernatant was discarded and cells were resuspended in media and diluted 1:50 for continuous culture; otherwise, cells were lysed for Western blot experiments (section 2.4.1). For HEK-293 cells expressing the hPanx1 construct (Chapter 2.9), 600 µg ml⁻¹ Geneticin (Invitrogen) was added to the media.

2.3.2 Transfection of HEK-293 cells with Lipofectamine

Native HEK-293 cells were seeded onto a 6 well plate and incubated overnight. 4 µg of hPanx1 construct and 10-µl of Lipofectamine 2000 (Invitrogen) were added to 250µl aliquots of Opti-mem I medium (Invitrogen), before mixing together and incubating (room temperature, 20 min). 100µl of the mixture was then added to each well and cells were incubated at 37°C for 4-h before changing the media. Successful transfection was assessed by Western blotting using an α-FLAG antibody (Chapter 2.9).

2.3.4 Culture of suspension cell lines

Myeloid cell lines (CHRF, human erythroleukaemia [HEL] and Meg-01) were cultured in RPMI-1640 supplemented with 10% foetal bovine serum and 500 U
36 ml⁻¹ penicillin/streptomycin (Invitrogen) and kept in a humidified incubator (37°C, 5% CO₂). Cells were pelleted (200 x g, 5 min) and diluted (1:20) into fresh media after three to four days. For patch clamp studies HEL cells were found to be optimal between passages 3-12.

2.4 Antibody techniques

2.4.1 Preparation of whole cell lysates

Cells were pelleted by centrifugation (350 x g, 20 min or 200 x g, 5 min for platelets and cell lines, respectively), resuspended in 200-300 µl SES and placed on ice. Cell suspensions were lysed 1:1 with ice cold 2 x RIPA buffer (150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0 with NaOH) plus protease inhibitor cocktail (1 mM PMSF, 2 mM Na₃VO₄, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ aprotinin and 1 µg ml⁻¹ pepstatin-A) for 10 min. Insoluble genetic material and debris was pelleted by centrifugation (15,700 x g, 10 min) and the supernatant was retained for protein studies.

2.4.2 Bradford Assay

![Figure 2.1 Bradford assay standard curve.](image)

Representative standard curve of \( A_{595} \) plotted against the protein concentrations of known standards. The protein concentration of cell lysates was then interpolated according to their \( A_{595} \) value.
Total protein concentration of cell lysates was determined using the Bradford assay. Protein binding to coomassie brilliant blue causes an absorbance shift from 465 nm to 595 nm, which can be detected using a spectrophotometer (Bradford, 1976). Thus, protein concentration of a sample can be determined by interpolation using a standard curve of absorbance at 595 nm (A<sub>595</sub>). Bovine serum albumin (BSA; Calbiochem) was used to generate a standard curve for each assay (0.1 to 2.0 mg ml<sup>-1</sup>). 5µl of either protein standard or sample was added to a 96 well plate in duplicate. 250µl of Bradford reagent (Sigma) was added before reading A<sub>595</sub> on an Infinite® 200 NanoQuant plate reader (Tecan, Männedorf, Switzerland). Standard curves were plotted within GraphPad Prism 6 (Fig. 2.1) and were used to calculate the protein concentration. Samples were then diluted with ddH<sub>2</sub>O to give a final concentration of 1 mg ml<sup>-1</sup>.

2.4.3 Immunoprecipitation

Whole platelet lysates (1 mg ml<sup>-1</sup>) were centrifuged to pellet the actin cytoskeleton (15,700 x g, 10 min, 4⁰C). The retained supernatant was precleared for 1h at 4⁰C with protein-A (P2X1) or protein-G (Panx1) agarose beads. Agarose beads were pelleted (15,700 x g, 2 min, 4⁰C) and the supernatant retained. 1 µg ml<sup>-1</sup> of α-P2X1 or α-Panx1 antibody was added to the samples (table 2.1; 2h at 4⁰C) prior to addition of protein-A/G agarose beads (1h, 4⁰C). After incubation, the beads were washed three times (15,700 x g, 2 min, 4⁰C) with RIPA buffer and once with TRIS-buffered saline, tween-20 (TBS-T; 0.1% (v/v) tween-20, 137 mM NaCl, 20 mM tris base, pH 7.6 with HCl) before resuspending in sample buffer (0.125M Tris HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue) for analysis by 12% SDS-PAGE (Chapter 2.4.7).

2.4.4 Biotinylation

Washed platelets were counted by a Z2 particle counter (Beckman coulter, Pasadena, CA, USA) and adjusted to a final concentration of 8x10<sup>8</sup> ml<sup>-1</sup>. Surface proteins were labelled with 0.5 mg ml<sup>-1</sup> sulfo-NHS-LC biotin (Pierce, Rockford, IL, USA; room temperature, 30 min). Excess biotin was quenched by 40 mM glycine before washing twice in SES. Platelet pellets were lysed in 150µl RIPA buffer, of which an aliquot (20µl) was taken for total protein, whilst the
remainder was incubated overnight at 4°C with streptavidin-agarose beads (Invitrogen; 75µl). Beads were washed four times with SES (15,700 x g, 5 min) and resuspended in 30-µl of sample buffer. Total and streptavidin IP samples were resolved by 10% SDS-PAGE and detection of Panx1 and ERK 1/2 was performed by Western blot (Chapter 2.4.7). Surface expression of Panx1 was quantified relative to the cytosolic protein ERK1/2 by densitometry (Image-J v1.45s, National Institutes of Health, Maryland, USA).

2.4.5 Removal of N-linked glycans by PNGaseF

For deglycosylation experiments, protein lysates were treated with 750 units of PNGaseF (New England Biolabs [NEB], Hertfordshire, UK) for 1h at 37°C, according to the manufacturer’s instructions, prior to Western blot analysis.

2.4.6 Stimulation of washed platelets for Western blot analysis

Washed platelets (Chapter 2.2.1) were incubated at 37°C in a model 400 lumi-aggregometer (Chronolog, Manchester, UK). Platelet suspensions were stimulated by agonists for up to 15 min as indicated in Chapters 3 and 4. Experiments were terminated by addition of equal volumes of 2x RIPA buffer and lysates were prepared as described above (Chapter 2.4.1-2).

2.4.7 Western Blotting

Cell lysates were diluted 1:1 with 2 x sample buffer, boiled (100°C, 10 min) and centrifuged (15,700 x g, 10 min) before loading 10µg of sample per lane. Pre-stained molecular weight marker (Bio-Rad laboratories, Hertfordshire, UK; range 10-250 kDa) was loaded into the first lane of each gel. Gels were run at 100V for 1.5h and transferred to polyvinylidene fluoride membrane (Fisher, Loughborough, UK). The membrane and gel were sandwiched between sheets of filter paper, assembled into transfer cassettes and immersed in transfer buffer (192 mM glycine, 25 mM tris base, 10% (v/v) methanol); transfers were performed at 100V for 1h. Membranes were then blocked (1h, room temperature) with 5% (w/v) skimmed milk (Premier Foods, Hertfordshire, UK) in TBS-T. Primary antibodies were diluted in blocking buffer and applied to membranes (table 2.1; overnight, 4°C). Excess antibody was washed away (three 5 min washes in TBS-T) and membranes were incubated with HRP-conjugated secondary antibodies (1h, room temperature). Membranes were
washed a further three times, treated with Enhanced Chemiluminescence reagent (Pierce; 1 min) and mounted into a hyperfilm cassette (GE Healthcare, Buckinghamshire, UK). In a dark room, hyperfilm was exposed to the membrane and processed by a hyperfilm processor (GE Healthcare). For studies of TMEM16F protein expression, optimal antibody binding was achieved when 5% milk was substituted by 5% BSA (Fisher) for membrane blocking and antibody incubation steps.

Table 2.1 Antibodies used for Western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panx1</td>
<td>Goat</td>
<td>1:500</td>
<td>Santa Cruz, California, USA</td>
</tr>
<tr>
<td>Panx1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>TMEM16F (extracellular epitope)</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>TMEM16F (intracellular epitope)</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Santa Cruz</td>
</tr>
<tr>
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<td>Rabbit</td>
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<td>Alomone, Jerusalem, Israel</td>
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<td>Rabbit</td>
<td>1:1000</td>
<td>Cell Signalling Technology, Boston, USA</td>
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<td>Dako, Ely, Cambridge</td>
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<td>Dako</td>
</tr>
<tr>
<td>α-Mouse HRP</td>
<td>Goat</td>
<td>1:5000</td>
<td>Dako</td>
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</tbody>
</table>

2.4.8 Immunocytochemistry

Cells were fixed in cacodylate buffer (100 mM sodium cacodylate, 2% (w/v) sucrose, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.3) containing 3% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde (Agar Scientific, Essex, UK; 1h at room temperature). Background fluorescence was quenched with sodium borohydride (BDH lab supplies, Poole, Dorset; 15 min, room temperature). Cells were permeabilised with 0.1% (v/v) Triton X-100 and blocked with 0.3% (w/v) BSA for 30 min. Cells were then pelleted by centrifugation (5 min at 200 x g for
cell lines and bone marrow, whilst platelets were pelleted at 400 x g) and washed three times in cacodylate buffer. Primary antibodies were added to the respective samples and incubated overnight at 4°C (table 2.2). Excess antibodies were removed by three washes in cacodylate buffer. Alexafluor-647 conjugated secondary antibodies were then added to samples for 30 min at room temperature (table 2.2). Following three further washes, fluorescence was assessed at 635 nm excitation (650-750 nm emission) using an Olympus FV1000 confocal microscope (Olympus, Essex, UK). HEK-293 cells were fixed onto coverslips (30 mm, #1; VWR, Leicestershire, UK), whilst all other cells were fixed in suspension and allowed to settle on uncoated glass coverslips prior to imaging.

Table 2.2 Antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
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<td>Panx1</td>
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<td>Panx1</td>
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<td>AbCam</td>
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<tr>
<td>TMEM16F (extracellular epitope)</td>
<td>Rabbit</td>
<td>1:50</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>TMEM16F (intracellular epitope)</td>
<td>Rabbit</td>
<td>1:50</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>α-Goat – Alexa Fluor® 647</td>
<td>Donkey</td>
<td>1:1000</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>α-Rabbit – Alexa Fluor® 647</td>
<td>Goat</td>
<td>1:1000</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>α-Rabbit – FITC</td>
<td>Swine</td>
<td>1:1000</td>
<td>Dako</td>
</tr>
</tbody>
</table>

2.5 Intracellular calcium measurements in washed platelet suspensions with the ratiometric calcium dye FURA-2

Agonist evoked-[Ca²⁺] responses were recorded from washed platelets using Fura-2, a ratiometric Ca²⁺ indicator. PRP was loaded by incubation with 2 µM Fura-2AM (45 min, 37°C) and washed platelets were prepared (section 2.2.1). Washed platelet suspensions (1 ml) were diluted 1:1 by addition of SES containing apyrase (0.32 U ml⁻¹) in a cuvette. Cuvettes were placed into heated chambers with magnetic stirrers and samples were warmed to 37°C for three minutes. For studies of Panx1 function the inhibitors Prb and Cbx or their respective vehicle control was applied during this warm-up period. Excitation
light was provided by a xenon arc lamp, which passed through a rotating (45 rpm) six-position filter wheel containing two 340 nm and four 380 nm excitation filters. Excitation light travelled along a liquid light guide into the cuvette holder and emitted light was detected at 90° to the light source by a photomultiplier tube (PMT), after passing through a 480 nm long pass filter. Signals were converted by an analogue to digital converter (A/D converter) and recorded by a computer using Cairn proprietary software (Cairn Research, Kent, UK). The Fura-2 signal in the presence of high and low Ca\(^{2+}\) was calibrated each experimental day in order to calculate \([\text{Ca}^{2+}]_i\). Platelets were lysed (50 \(\mu\)M digitonin) to release Fura-2 dye, which was quenched by MnCl\(_2\) (10 mM) to assess background fluorescence at 340 and 380 nm. In another cuvette, platelets were lysed and 1 mM CaCl\(_2\) was added to saturate the dye, followed by addition of 10 mM EGTA to chelate Ca\(^{2+}\), giving values for 340 and 380 nm signals in the presence of high and low Ca\(^{2+}\), respectively. Background-corrected 340 and 380 nm values were used to calculate \(R_{\text{min}}\), \(R_{\text{max}}\) and the proportionality constant; where \(R_{\text{min}}\) is the minimum 340/380nm ratio, \(R_{\text{max}}\) is the maximum 340/380 ratio and the proportionality constant is the ratio of the 380 signal at low vs high Ca\(^{2+}\). \(R_{\text{min}}\) and \(R_{\text{max}}\) were multiplied by a viscosity correction factor of 0.85 (Poenie, 1990). Background-corrected 340/380nm ratios (R) were then converted to \([\text{Ca}^{2+}]_i\) using equation 2.1, where the dissociation constant for Fura-2 (Kd) = 224 nM (Gryniewicz et al., 1985).

\[
[\text{Ca}^{2+}]_i = \text{Kd} \times \frac{F_{380 \text{ max}}}{F_{380 \text{ min}}} \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}
\]

2.6 Platelet aggregation and ATP secretion

Washed platelet suspensions (225\(\mu\)l) were diluted 1:1 with SES containing apyrase (0.32 U ml\(^{-1}\)) in a cuvette with a stir bar. Samples were heated to 37\(^{0}\)C for three minutes in the warming wells of a model 400 lumi-aggregometer. 500\(\mu\)l of SES was placed in the reference well for the duration of the experiment. Samples were placed in the recording well and 100 \(\mu\)g ml\(^{-1}\) fibrinogen (from bovine plasma), 20\(\mu\)l CHRONO-LUME\textsuperscript{®} luciferin:luciferase (Chronolog) and 2 mM CaCl\(_2\) were added; aggregation baselines were set 30s prior to agonist
addition. ATP was measured simultaneously using the luciferin:luciferase assay kit according to the manufacturer’s guidelines. Luminescence values were converted from an ATP standard curve (30-1000 nM), which showed a linear relationship with gradient $y = 0.3438x$ (Fig. 2.2). Luminescence values were not affected by the presence of Prb or Cbx (97.6 ± 8.6% and 95.5 ± 7.1% of control, respectively, $P > 0.05$, n=3). Data were sampled at an acquisition rate of 10 Hz, filtered through a low pass Bessel filter and converted by a Mini Digi 1A A/D converter (Molecular Devices, Berkshire, UK) and recorded in AxoScope V9.0 (Molecular Devices).

![Figure 2.2 ATP concentration standard curve.](image)

**Figure 2.2 ATP concentration standard curve.** A) Representative luminescence output traces for ATP concentrations between 30-1000 nM. B) Standard curve of ATP luminescence output (Y-axis) plotted against the ATP standard concentration (nm; X-axis). Values were fitted by slopes with gradients of $y = 0.36x$ (circle), $y = 0.37x$ (triangle) and $y = 0.37x$ (square) under control, Cbx-treated and Prb-treated conditions, respectively.

Data files were exported from Axoscope and opened within Microsoft Excel for processing and conversion of aggregation and ATP responses to percentage transmission and nM values, respectively. Aggregation and luminescence and data points were background-corrected by subtraction of the average signal from the first 10s of the recording. Background-corrected data points were then expressed as a percentage change from baseline. Corrected luminescence values were interpolated using a standard curve to give nanomolar ATP release values (Fig. 2.2).
2.7 Calcein Dye Efflux

Platelets were loaded with the fluorophore calcein by addition of 0.5µM calcein-AM to PRP (Invitrogen; 30 min, 37°C) prior to preparation of washed suspensions (Chapter 2.2.1). Two-millilitre aliquots of washed platelets (4 x 10^8 ml^-1) were stirred at 37°C and 5µl samples taken at 1 or 5 min intervals were diluted into 0.2% formyl saline (0.85% [w/v] NaCl, 0.2% [v/v] formaldehyde), for analysis by a BD FACSCANTO flow cytometer (488 nm excitation, 530 ± 30 nm emission). The platelet population was selected based on forward and side scatter properties and included >90% of the 10,000 events counted. Dye efflux was calculated as the percentage of events that moved out of fluorescence gate F1 (Figs. 3.7-8).

2.8 Electrophysiology

2.8.1 Microelectrode manufacture

Microelectrodes were pulled from thick-walled borosilicate glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter; Harvard apparatus, Kent, UK) with a Narishige PP-3 two stage pipette puller (Narishige, Tokyo, Japan). Pipette tip diameter was assessed using the “bubble” technique (Bowman and Ruknudin, 1999). Briefly, pipettes were attached to a 10 ml syringe (filled with 10 ml air) by a short silicon tube and the tip was submerged in ethanol. The plunger was depressed and the bubble number corresponds to the volume of air within the syringe when bubbles first appear. Pipettes with bubble numbers of 7.0 to 7.5 were used for HEL cells, whilst larger pipettes with bubble numbers 7.5-8.0 were used for MK studies. These bubble numbers corresponded to pipette resistances of 1.5 to 4.0 MΩ when filled with standard internal saline (see above). Pipettes were lightly fire polished in a microforge immediately before use to improve formation of high resistance glass-membrane seals.

2.8.2 Whole cell recording

Perspex electrophysiological chambers were manufactured by the Biomedical Joint Workshop (University of Leicester). The recording chamber (20 mm x 8 mm) was drilled into the centre of a 3 mm thick Perspex plate. Lateral holes were drilled for gravity-fed solution inflow and the ground AgCl pellet on the right and left sides, respectively. A glass coverslip (30 mm, #1) affixed with
silicone grease (Dow Corning, Missouri, USA) formed the base of the chamber. Solution inflow passed through a Perspex block (six inlets to one outlet) connected to the bath by 1.5 mm silicone tubing, whilst outflow to a waste flask was carried by 1.5 mm silicone tubing connected to a motorised pump. Solution flow was controlled by manually switching three-way taps on the outside of the cage. The chamber was mounted on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) and cells visualised at 400x magnification (40x oil immersion objective lens and 10x eyepiece lenses).

Whole cell recordings under voltage clamp were performed using an Axopatch 200B headstage and amplifier (Molecular Devices) connected to a computer running Clampex (V6.0, Molecular Devices). Data from the amplifier were low-pass filtered (1kHz) to prevent aliasing. 30-50µl of cell suspension (HEL or bone marrow) was added to the bath solution and allowed to settle for 2-3 min, after which excess cells were removed by perfusion of external solution. Meanwhile, pipettes were backfilled with the required internal solution (Chapter 2.2) and air bubbles expelled by filipping of the glass. The pipette was assembled into the headstage-mounted pipette holder so that the AgCl-coated Ag wire within the holder contacted the solution. Slight positive pressure was applied to the pipette by mouth with a 1 ml syringe connected to the pipette holder by 1.5 mm silicone tubing. The pipette was then lowered into the bath solution and moved towards the cell of interest using an electronically-controlled micro-manipulator (Luigs & Neumann, Ratingen, Germany). A square waveform pulse of 5mV was constantly applied to the pipette under voltage clamp conditions and the level of the current at 0mV was set to zero using the amplifier pipette offset dial. Positive pressure was released when the pipette was within a few µm of the cell. After moving the pipette tip to lightly touch the cell surface, gentle negative pressure was applied to facilitate the formation of a multi giga-Ohm resistance seal between the cell and pipette. A holding potential of -60mV (HEL) or -80mV (MKs) was applied and the slow and fast capacitive transients were removed using the pipette capacitance facility of the amplifier at 1 and 5 kHz, respectively. Further suction ruptured the membrane patch and gave a larger capacitance transient, which was cancelled out using the amplifier’s whole-cell capacitance and series resistance compensation facility. Typical series
resistance values were in the range 4.2-10.9 mΩ; where possible, series resistance was compensated by 70-75%. Whole cell currents were recorded using either voltage ramp or step protocols described in Chapters 5 and 6. Liquid junction potential offset values were calculated using JP Calc within Clampfit (V 10.3, Molecular devices) and were -12.8 and -7.5mV for Na-glucunate and NMDG-gluconate bath solutions, respectively. These offsets were cancelled out by a priori correction on the patch clamp amplifier.

2.8.3 Data analysis

Data files were opened in Clampfit and traces were exported to Microsoft Excel 2010 for analysis. Presented figures were generated using Graph Pad Prism 6. Within Excel, currents were normalised to whole cell capacitance to give current densities (pA pF-1). Voltage ramps were performed for 10 min immediately following transition to the whole cell configuration; data were averaged at 1 min intervals for analysis and summary data are shown for t=0, t=5 and t=10 min. Voltage steps were performed after completion of voltage ramps (10+ min) and I-V relationships generated for averaged peak currents from 3-5 cells at each 20 mV increment. Theoretical equilibrium potentials stated in Chapters 5 and 6 were calculated from the Nernst equation (equation 2.2). Experimental $E_{\text{rev}}$ values for Ca$^{2+}$-activated currents were calculated from the point of intersection with background currents measured with 1mM EGTA (no added Ca$^{2+}$) in the pipette. These values were used to calculate the relative permeability of K$^{+}$, Na$^{+}$ and Cl$^{-}$ in Chapter 5 using the Goldman-Hodgkin-Katz equation (equation 2.3).

**Equation 2.2 Nernst equation**

$$E_{\text{rev}} = \frac{RT}{zF} \ln \left( \frac{[I^+]_i + [I^-]_i}{[I^+]_o + [I^-]_o} \right)$$

at 21°C

$$E_{\text{rev}} = 58 \log_{10} \left( \frac{[I^+]_o + [I^-]_i}{[I^+]_i + [I^-]_o} \right)$$

Where $E_{\text{rev}} =$ equilibrium potential in mV; $R =$ Universal gas constant; $T =$ temperature (°K); $z =$ valency; $F =$ Faraday’s constant; $[I^+]_o$, $[I^+]_i$, $[I^-]_o$, and $[I^-]_i =$ concentration of monovalent cations ($I^+$) or monovalent anions ($I^-$) present in the intracellular (i) and extracellular (o) solutions.
Equation 2.3 Goldman-Hodgkin-Katz equation

Modification of Nernst equation:

\[ E_{rev} = 58 \log_{10} \frac{P_x[X^+]_o + P_y[Y^-]_i}{P_x[X^+]_i + P_y[Y^-]_o} \]

Where \( E_{rev} \) = experimental reversal potential; \( P_x \) and \( P_y \) = relative permeability of ions \( X \) and \( Y \) respectively.

2.9 Cloning of hPANX1 and generation of a stable hPANX1-HEK-293 cell line

Whole blood was centrifuged (700 x g) to separate the PRP, buffy coat and erythrocyte fractions. The buffy coat was removed and centrifuged (200 x g, 5 minutes) to pellet white blood cells. Cells were resuspended in SES, lysed in TriZol® reagent (Invitrogen) and mRNA was extracted according to the manufacturer’s instructions. This extraction yielded 9 µg mRNA, which was quantified using a nanodrop (Infinite® 200 NanoQuant plate reader). Contaminating genomic DNA was degraded by DNase I (NEB; 15 min). mRNA was reverse transcribed to cDNA by cloned AMV reverse transcriptase (Invitrogen), according to the manufacturer’s instructions.

Table 2.3 Standard PCR conditions

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</tbody>
</table>

PCR was then used to amplify cDNA encoding human Panx1 (transcript ID ENST00000227638) for insertion into the mammalian expression vector, pcDNA3 (Invitrogen, Fig. 2.3A). Standard PCR conditions are given in table 2.3 and specific annealing temperatures are referred to in the text. Full length hPanx1 cDNA, plus an 85 base pair (bp) region upstream of the start codon (ATG), was amplified during the first round of PCR using the tot-hPanx1 primer set, listed in table 2.4. This reaction yielded a single product at the expected
size, 1600bp, for each annealing temperature (57-59°C; Fig. 2.3B). Bands were excised and cDNA extracted using a gel extraction kit, according to the manufacturer’s protocol (Qiagen, Limburg, Netherlands), and quantified by nanodrop. The hPanx1-C primer set was used to amplify the hPanx1 sequence (table 2.4) plus restriction sites for Hind III (AAGCTT) and XhoI (CTCGAG) at the N- and C-termini, respectively. A single product of expected size was seen for both annealing temperatures (56, 58°C; Fig. 2.3C); these bands were excised and cDNA extracted and quantified as described above.

Table 2.4 Primer sequences for cloning the human pannexin-1 channel.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence 5'-3'</th>
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<tbody>
<tr>
<td>tot-hPanx1-F</td>
<td>CCGGCCGCTGAACCTGAGGTGAAG</td>
</tr>
<tr>
<td>tot-hPanx1-R</td>
<td>CTCCGGCTCTCGACAGGGCTAC</td>
</tr>
<tr>
<td>hPanx1-C-Fw</td>
<td>TAATAAAAGCTTCCGCGCCGTGA</td>
</tr>
<tr>
<td>hPanx1-C-Rv</td>
<td>TAATAAAACTCGAGCAGAAAGAAATCCAGA</td>
</tr>
</tbody>
</table>

Both cDNA and pcDNA3 vector were digested by Hind III and XhoI restriction enzymes, according to the manufacturer’s instructions and recommended conditions (https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder). Digests were separated on a 0.8% agarose gel and bands corresponding to the vector (5400bp) and hPanx1 sequence (1451bp) were extracted (Fig. 2.3D). Digested vector and hPanx1 sequence were ligated (5:1) by T4 DNA ligase (16h, 4°C; NEB) and transformed into competent DH5α E. coli cells (Invitrogen). Briefly, DH5α cells (50µl) were incubated on ice with 5µl of either ligated vector, unligated vector or pUC control vector for 30 min. Tubes were heat shocked at 37°C for 30-40s and replaced on ice for 2 min. Cells were diluted with SOC media (500µl; Invitrogen), placed in a shaking incubator (37°C, 50 min) and poured onto LB agar plus ampicillin (100 µg ml⁻¹); plates were incubated overnight at 37°C. Next, plates were inspected and six single colonies (G-L) were sub-cultured in LB broth plus ampicillin (100 µg ml⁻¹) in a shaking incubator (overnight, 37°C). Glycerol stocks of these cultures were made by adding 250µl culture medium to 750µl sterile glycerol and were stored at -80°C. Vectors were extracted using miniprep kits (Qiagen), according to the manufacturer’s guidelines. An aliquot of each vector was digested by Hind III
and Xho I and separated on a 0.8% agarose gel (Fig. 2.3E); of the six colonies, only G gave bands that corresponded to the vector (5400bp) and hPanx1 sequence (1366bp). Construct G was sequenced by the protein and nucleic acid chemistry laboratory (PNACL; University of Leicester) and was identical to the hPanx1 sequence.

The hPanx1 vector was then transfected into HEK-293 cells as described in Chapter 2.3.2, to generate a stable hPanx1-HEK-293 cell line. Expression of the hPanx1 construct was assessed by Western blot using antibodies raised against either Panx1 or the FLAG tag (Fig. 2.3F). These data show more intense staining for Panx1 in hPanx1- than native-HEK-293 cells. Furthermore, only hPanx1-HEK-293 cells were positive for FLAG indicating successful transfection of the hPanx1 construct. A stable cell line was established by extreme dilution of hPanx1-HEK-293 cells into a 96 well plate. This dilution gave 17 wells with a single cell, of these six clones were derived and cryopreserved in liquid nitrogen. Clone 3 was selected for further studies based on the level of hPanx1 protein expression observed by Western blot.

2.10 Statistical analysis

Platelet aggregation, ATP release and [Ca$^{2+}$]i measurements were normalised as a percentage of a paired control response, whilst whole cell currents are expressed as current density (pA pF$^{-1}$) to account for variation in cell size. Data are presented as the mean ± SEM and significance was determined using either Student’s paired t test or ANOVA with Bonferroni post-testing within GraphPad Prism 6 as appropriate. The level of significance is indicated as not significant ($P > 0.05$; ns), or significant at $P < 0.05$ (**), $P < 0.01$ (***).
Figure 2.3 Generation of a stable hPanx1-His-FLAG HEK-293 cell line.
Expression vector and construct map for the hPanx1 clone (A). Amplification of the full length Panx1 sequence from leukocyte cDNA (B). PCR products using hPanx1-C primer set (C). Restriction digests of the pcDNA3 vector and hPanx1 cDNA sequence by HindIII and XhoI (D). Minipreps from 6 colonies (G-L) were digested by HindIII and XhoI and screened by agarose gel electrophoresis (E). Western blot detection of Panx1 (left) and FLAG-tag (right) expression by native HEK-293 cells and those transfected with the hPanx1 construct (F). Note bands were extracted prior to capturing images of agarose gels to reduce risk of mutations.
Chapter 3

3.0 Functional Pannexin-1 channels are expressed on the surface of human platelets

3.1 Background

The connexin family of mammalian gap junctions were first identified in 1967 as intercellular connections between cardiac myocytes and hepatocytes (Revel and Karnovsky, 1967). By 2004, twenty one human connexin proteins had been identified (Sohl and Willecke, 2004), which are annotated using the Cx(n) nomenclature, whereby Cx denotes connexin and (n) is the predicted molecular weight. Cx proteins have four TMDs with intracellular amino- and carboxyl-termini and hemichannels are formed by six Cx subunits (Hervé, 2004, Hervé, 2005). Conserved cysteine residues on their extracellular loops facilitate docking of apposite hemichannels, resulting in gap junction formation between adjacent cells (Foote et al., 1998). Multiple lines of evidence suggest that Cx’s function as hemichannels and gap junctions. Atomic force microscopy and electrophysiological recordings have shown that Cx hemichannels are closed at physiological levels of [Ca\(^{2+}\)]\(_o\) (1-4 mM), however they do open in the nominal absence of [Ca\(^{2+}\)]\(_o\) (Ebihara and Steiner, 1993, Pfahnl and Dahl, 1999, Thimm et al., 2005, Allen et al., 2011). To date, four Cx family members have been identified in platelets; Cx32, Cx37, Cx40, Cx43 (Vaiyapuri et al., 2012, Vaiyapuri et al., 2013, Angelillo-Scherrer et al., 2011). These proteins have been shown to have roles in both haemostasis and thrombosis, as assessed using pharmacological tools and knock-out mouse strategies.

Cx proteins were identified as the mammalian equivalent of the invertebrate gap junction family, the innexins (Phelan and Starich, 2001). A third family of three proteins with 20% sequence homology to the innexins was identified in 2000; these proteins were named Pannexin-1, 2 and 3 (Panx1/2/3), and are expressed by both vertebrate and invertebrate organisms (Panchin et al., 2000). Despite a lack of sequence homology, both pannexin and connexin proteins have strikingly similar membrane topology; both families have four TMDs with two extracellular loops with intracellular amino and carboxyl termini (Scemes et al., 2009). However, Panx proteins have only two extracellular cysteine residues
and are extensively glycosylated on their second extracellular loop at Asn254 (Boassa et al., 2007, Boassa et al., 2008, Scemes et al., 2009).

Expression profiling of the pannexin family identified ubiquitous expression of Panx1, whilst Panx2 expression was restricted to the brain and CNS and Panx3 was only detected in bone and skin (Bruzzone et al., 2003). Panx1 subunits assemble into hexamers before being glycosylated and trafficked to the plasma membrane (Boassa et al., 2007, Boassa et al., 2008). Glycosylation prevents gap junction formation between apposite Panx1 channels; instead they function as plasma membrane channels that are capable of opening at physiological levels of $[\text{Ca}^{2+}]_o$ (1-4 mM) (Sosinsky et al., 2011). Electrophysiological studies have shown that Panx1 channels are anion-selective with permeability up to $\approx1$ kDa (Bao et al., 2004, Ma et al., 2012). Panx1 channels open in response to a variety of stimuli, including oxygen-glucose deprivation (OGD), caspase cleavage, an increase in $[\text{Ca}^{2+}]_i$ and mechanical stimulation (Thompson et al., 2006, Sandilos et al., 2012, Locovei et al., 2006b, Bao et al., 2004).

To date, Panx1 channel activity has been associated with multiple pathological conditions, including ulcerative colitis (Gulbransen et al., 2012), HIV infection (Séror et al., 2011) and ischaemic stroke (Bargiotas et al., 2011, Xiong et al., 2014). Panx1 channels may also contribute to inflammatory conditions, such as atherosclerosis (Velasquez and Eugenin, 2014). Thus, it is important to establish the role of these proteins in platelet function.

### 3.1.1 Aims

Within this laboratory a modified protocol for the purification and extraction of human platelet mRNA has been developed (Amisten, 2012, Wright et al., 2013). Analysis of platelet mRNA by qPCR identified transcripts for $\text{PANX1}$, but not $\text{PANX2}$ or $\text{PANX3}$ (Taylor et al. 2014). Thus, this chapter focuses on the expression and localisation of Panx1 channels in platelets, MKs and related cell lines. In addition, the mechanism(s) by which these channels are activated in human platelets will be explored.
Figure 3.1 Pannexin-1 is expressed at the periphery of human platelets and megakaryocytes. A) Immunocytochemistry of native and hPanx1-His-FLAG HEK-293 cells, human platelets and primary rat and mouse MKs. Inset images show that there was no staining in the secondary antibody only controls. B) Profile plots of the fluorescence intensity for the respective cross-section (A, dashed line). Scale bars represent 10 µm and data are representative of a minimum of three independent experiments.
3.2 Results

3.2.1 Pannexin-1 channels are expressed by human platelets and rodent megakaryocytes

Panx1 protein expression has previously been reported in HEK-293 cells using an anti-Panx1 antibody raised against its C-terminus (Ma et al., 2009). ICC using this antibody demonstrated strong fluorescence for Panx1 at the periphery of both native HEK-293 cells and a stable cell line overexpressing human Panx1 tagged with His and FLAG domains (hPanx1-HEK-293; see methods, Chapter 2.9, for cloning strategy and creation of this cell line). A similar profile was seen for human platelets and both mouse and rat primary MKs (Fig. 3.1A). The inset images (Fig. 3.1A) show that there was no fluorescence detected in the secondary antibody only controls for HEK-293 cells and platelets, whilst very weak staining was observed for both mouse and rat MK samples. This non-specific binding may be due to species cross reactivity of the secondary antibody. Line plot analyses (Fig. 3.1B) indicated that the most intense staining is observed at the periphery of these cells, with some fluorescence observed within the cytosol of native and hPanx1-HEK-293 cells; a similar pattern was observed for mouse and rat MK samples. This suggests that Panx1 is predominantly located on the surface membrane, with a small proportion of protein residing intracellularly. The staining within the cytoplasm of the mouse and rat MKs may also result from the presence of Panx1 on the DMS, which acts as a membrane reserve for the generation of platelets (Radley and Haller, 1982). Western blotting was performed to assess Panx1 expression by native and hPanx1 HEK-293 cells and platelets. Full length Panx1 (48 kDa) was observed in native HEK-293 cells and platelets and a 50 kDa variant in the hPanx1 HEK-293 cells (Fig. 3.2A), as predicted due to the inclusion of a 2 kDa His-FLAG tag within the construct (Fig. 2.3). hPanx1 HEK-293 cell lysates were diluted twenty fold owing to their greatly enhanced level of Panx1 expression. Intense staining for Panx1 was observed by ICC in human erythroleukaemic (HEL) cells (Fig. 3.1A), which was supported by Western blot detection of Panx1 both in HEL cells and a related megakaryoblastic, Meg-01, cell line (Fig. 3.2B). Panx1 channels in HEL cells are explored further by patch clamp in Chapter 5. To further investigate the localisation of platelet Panx1, membrane
Figure 3.2 Pannexin-1 protein is present on the surface of platelets and is fully glycosylated. A) Western blot detection of Panx1 protein in native HEK-293, hPanx1-His-FLAG HEK-293 cells and platelets; relative protein loading was assessed by GAPDH (* hPanx1-His-FLAG HEK-293 lysates were 20-fold more dilute [0.5 µg per lane] than for platelets and native HEK-293 cells [10 µg per lane]). B) Western blot detection of Panx1 protein in the HEL and Meg-01 cell lines. C) Platelet membrane biotinylation shows Panx1 expression in the surface enriched fraction, as assessed by Panx1:ERK 1/2 density. D) Treatment of native and hPanx1-His-FLAG HEK-293 cells and platelets with PNGaseF shows a decrease in molecular weight of approximately 11 kDa. Data are representative of a minimum of three independent experiments.
Figure 3.3 Pannexin-1 contributes to Ca\(^{2+}\) response evoked by multiple platelet agonists. [Ca\(^{2+}\)]\(_i\) responses were measured from Fura-2 loaded washed platelet suspensions in the presence of 2 mM [Ca\(^{2+}\)]\(_o\). Platelets were stimulated by 0.5 µg ml\(^{-1}\) collagen (A), 0.03 U ml\(^{-1}\) thrombin (B) or 500 nM U46619 (C) in the presence of either 100 µM Prb (left panel) or 10 µM Cbx (right panel). (D) Average peak [Ca\(^{2+}\)]\(_i\) increases were normalised to the paired vehicle control (saline or 0.2% DMSO). Statistical significance was calculated using a Student’s paired t-test. Representative traces are from paired-control runs from individual donors and representative of at least five independent experiments.
biotinylation experiments were performed. Contamination from cytosolic proteins in the surface fraction was assessed by probing membranes for the cytosolic protein, ERK1/2. Panx1 band intensity was normalised to ERK1/2 such that an increase in Panx1:ERK1/2 ratio indicated an enrichment of Panx1 at the surface. Densitometry using this method demonstrated a 4.3-fold enhancement in Panx1:ERK1/2 ratio for biotinylated proteins (\( P < 0.05 \), n=3, Fig. 3.2C). With the exception of platelets, additional bands between 37-50kDa were observed for all lysates (Fig. 3.2A-B). Panx1 has previously been shown to be glycosylated on its second extracellular loop (Asn254), giving rise to the fully glycosylated (48 kDa) and unglycosylated (≈37 kDa) species (Boassa et al., 2007, Boassa et al., 2008, Penuela et al., 2009). Consistent with these previous studies, the Panx1 band in platelets, native HEK-293 cells (48 kDa) and hPanx1-HEK-293 cells (50 kDa), was reduced to ≈37 kDa and ≈39 kDa, respectively, after treatment with the glycosidase, PNGaseF (Fig. 3.2D). This finding, combined with the ICC and biotinylation data, suggests that Panx1 is predominantly located on the surface membrane of platelets.

3.2.2 Evidence for a role of pannexin-1 channels in the Ca\(^{2+}\) responses to physiological agonists

Locovei and co-workers (Locovei et al., 2006b) demonstrated that Panx1 can be activated by increased [Ca\(^{2+}\)], resulting in ATP release and amplification of Ca\(^{2+}\) responses in oocytes co-expressing both Panx1 and P2Y1 receptors. I therefore examined the contribution by Panx1 to Ca\(^{2+}\) influx evoked by low concentrations of the major platelet agonists; collagen (0.5 µg ml\(^{-1}\)), thrombin (0.03 U ml\(^{-1}\)) and the TXA\(_2\) analogue U46619 (500 nM), which all require secondary activation of P2X1 receptors to amplify the Ca\(^{2+}\) response (Fung et al., 2007). The collagen-evoked Ca\(^{2+}\) response was reduced to 67.3 ± 4.9% (\( P < 0.001 \), n=8,) and 69.1 ± 2.1% (\( P < 0.001 \), n=5) of control responses by Prb and Cbx, respectively (Fig. 3.3A). These two structurally unrelated and widely used Panx1 blockers (Silverman et al., 2008, Ma et al., 2012) were applied at concentrations (100 µM Prb and 10 µM Cbx) that do not affect connexin channels reported in platelets (Vaiyapuri et al., 2012, Niles and Smith, 1982, Ma et al., 2009, Vaiyapuri et al., 2013, D'Hondt et al., 2009). Inhibition of Panx1
Figure 3.4 Pannexin-1 does not contribute to release of Ca\(^{2+}\) from the dense tubular system. [Ca\(^{2+}\)]\(_i\) responses were measured from Fura-2 loaded washed platelet suspensions in the nominal absence of [Ca\(^{2+}\)]. Platelets were stimulated with 0.5 µg ml\(^{-1}\) collagen (A), 0.03 U ml\(^{-1}\) thrombin (B) or 500 nM U46619 (C) in the presence of either 100 µM Prb (left panel) or 10 µM Cbx (right panel). (D) Average peak [Ca\(^{2+}\)]\(_i\) increases were normalised to the paired vehicle control response (saline or 0.2% DMSO). Statistical significance was calculated using a Student’s paired t-test. Data are representative of five independent experiments.
caused a similar reduction of thrombin-evoked Ca$^{2+}$ influx, to 68.2 ± 4.5% ($P < 0.01$, n=6) and 77.5 ± 2.8% ($P < 0.001$, n=5) of control by Prb and Cbx, respectively (Fig. 3.3B). Finally, U46619 Ca$^{2+}$ responses were also reduced to 79.3 ± 3.0% of control ($P < 0.001$, n=6) by Prb and 74.7 ± 2.8% of control ($P < 0.001$, n=5) by Cbx (Fig. 3.3C). For each agonist there was no difference between the reductions caused by the two inhibitors ($P > 0.05$, Fig. 3.3D).

Given that Panx1 channels are anion-selective (Ma et al., 2012), their contribution to the Ca$^{2+}$ responses above must be through modulation of a Ca$^{2+}$ entry pathway. Thus, Panx1 could influence platelet Ca$^{2+}$ responses by modulation of either Ca$^{2+}$ release from the DTS or a plasma membrane Ca$^{2+}$ entry pathway. There was no effect of Prb or Cbx on agonist-evoked Ca$^{2+}$ store release, measured in the absence of external Ca$^{2+}$ ($P > 0.05$, Fig. 3.4), suggesting that Panx1 acts at the plasma membrane, consistent with its predominant location (Figs. 3.1-2). SOCE and P2X1 receptors represent the main plasma membrane Ca$^{2+}$ entry pathways at the low agonist concentrations used in this study (Fung et al., 2007, Harper et al., 2013). Neither Prb nor Cbx had any effect on SOCE activated by store depletion with the SERCA inhibitor thapsigargin (TG; $P > 0.05$, Fig. 3.5). These findings suggest a role for Panx1 in Ca$^{2+}$ influx across the plasma membrane, which is most likely via P2X1 receptors. This possibility is explored further in Chapter 4.

The concentration-response relationship between the thrombin-evoked (0.03 U ml$^{-1}$) Ca$^{2+}$ response and Panx1 inhibitors, Prb and Cbx, was investigated. Prb was applied at concentrations between 0.001-1 mM (Fig. 3.6A), whilst Cbx, a more potent Panx1 inhibitor, was tested across a lower concentration range; 0.1-200 µM (Fig. 3.6B). The IC$_{50}$ values for Prb and Cbx were 455 ± 146µM (Hill slope = -0.55) and 96.3 ± 35µM (Hill slope = -0.63), respectively (Fig. 3.6C).

### 3.2.3 Pannexin-1 channels are opened by thrombin

Open Panx1 channels have permeability to anions ≤1 kDa in size (Bao et al., 2004), including some high molecular weight, cell-impermeant dyes. Thus, efflux of such dyes has been used as a surrogate marker of Panx1 channel opening in response to various stimuli (Thompson et al., 2006, Bargiotas et al.,
Figure 3.5 Pannexin-1 does not contribute to store-operated Ca\(^{2+}\) entry. [Ca\(^{2+}\)]\(_i\) responses were measured from Fura-2 loaded washed platelet suspensions. Platelet Ca\(^{2+}\) stores were depleted by incubation with 1 µM thapsigargin (TG) for five minutes in the nominal absence of [Ca\(^{2+}\)]\(_o\). Incubation with either 100 µM Prb (A) or 10 µM Cbx (B) did not significantly affect the time taken to reach 500 nM [Ca\(^{2+}\)]\(_i\) (C) nor the rate of Ca\(^{2+}\) influx (D) after adding 2 mM CaCl\(_2\) (2Ca\(^{2+}\)). Statistical significance was calculated using a Student’s paired t-test. Data are representative of five independent experiments.
Figure 3.6 Concentration response relationships for the pannexin-1 inhibitors probenecid and carbenoxolone. 

$[\text{Ca}^{2+}]_i$ responses were measured from Fura-2 loaded washed platelet suspensions in the presence of 2 mM $[\text{Ca}^{2+}]_o$. Platelets were treated with either Prb (1 µM to 1 mM), Cbx (100 nM to 200 µM) or vehicle control (0.2% DMSO). Representative $\text{Ca}^{2+}$ responses in the presence of high and low concentrations of Prb (A) and Cbx (B) are shown. The concentration-response relationship was plotted as a percentage of paired control response (C) and gave IC$_{50}$ values of 455 ± 145 µM and 96.3 ± 35 µM for Prb (circles) and Cbx (squares), respectively. Summary data are derived from a minimum of five independent experiments.
To investigate thrombin-evoked (0.03 U ml⁻¹) Panx1 channel opening, efflux of the fluorophore calcein, a 0.62 kDa anion, from the platelet cytosol was assessed by flow cytometry (Fig. 3.7A). Calcein was loaded into the cytoplasm by incubation with its membrane-permeant acetoxymethylester derivative (Chapter 2.7) and thrombin-evoked dye efflux was measured from washed platelets at 60s time points by flow cytometry. Platelets were selected based on their forward and side scatter properties so that ≥90% of events were counted in both stimulated and unstimulated conditions. Platelet activation by thrombin induces shape change and degranulation, ultimately leading to aggregation (Aoki et al., 1998, Wang et al., 2014). In agreement with these reports, platelets were reduced in size (forward scatter) and granularity (side scatter) 300s after stimulation by thrombin compared to unstimulated cells (Fig. 3.7A).

Unstimulated samples were collected and interrogated by flow cytometry at 60s intervals to ascertain whether dye efflux occurs in unstimulated cells. Gate F1 was set as the baseline level of fluorescence for calcein-loaded, unstimulated cells and events in this gate have not extruded the dye. Thus, dye efflux is reported as a percentage reduction of cells that fall within gate F1. There was no dye efflux observed for unstimulated cells across the time course (100.0 ± 0.0 vs. 107.7 ± 6.7% at t=0s and t=300s, respectively; \( P > 0.05 \), n=5, Fig. 3.7C). Stimulation by thrombin caused the fluorescence histogram to shift to the left at t=300s (Fig. 3.7B), which represents an efflux of dye and was quantified as a 31.4 ± 10.4% (\( P < 0.001 \), n=5) loss of events from gate F1 (Fig. 3.7C). Addition of Panx1 inhibitors, Prb and Cbx, rescued thrombin-evoked calcein dye efflux. Indeed, there was an insignificant 2.1 ± 1.7% and 1.1 ± 1.2% increase of cells within gate F1 for Thr + Prb and Thr + Cbx treated samples, respectively (\( P < 0.001 \), n=5, Fig. 3.7C). These data provide further evidence for the expression of functional Panx1 channels on the surface of human platelets.

### 3.2.4 No evidence for activation of Pannexin-1 channels by reduced oxygen tension or caspase cleavage

Several mechanisms have been proposed for the activation of Panx1 channels including: mechanical stimulation (Bao et al., 2004), oxygen-glucose deprivation (OGD) (Thompson et al., 2006), caspase cleavage (Sandilos et al., 2012) and a
Figure 3.7 Thrombin opens pannexin-1 channels on the platelet surface. Flow cytometry was used to assess dye efflux from calcein-loaded washed platelet suspensions at rest and after stimulation by thrombin. (A) Scatter plot analysis of forward and side scatter properties of 10,000 events; the gated area (red coloured) represents typical properties of platelets and include >90% of all events in resting (left) and thrombin- (0.03 U ml⁻¹) stimulated (right) conditions. (B) Histograms of calcein fluorescence at t=0s (black) and t=300s (red) under unstimulated conditions (Unstim), following exposure to thrombin alone (Thr), or after thrombin in the presence of either 100 µM Prb (Thr + Prb) or 10 µM Cbx (Thr + Cbx). (C) The percentage reduction in the proportion of cells within F1 was calculated at each 60s time point and is expressed relative to 0s. Inhibitor-free runs for unstimulated and thrombin-stimulated conditions contained 0.2% DMSO as the vehicle control. Statistical significance was calculated using an ANOVA. Data are representative of five independent experiments.
rise in $[Ca^{2+}]_i$ (Locovei et al., 2006b). Data presented above demonstrates that Panx1 channels open in response to physiological agonists and modulate subsequent $Ca^{2+}$ influx; here, Panx1 activation by low oxygen tension and caspase cleavage is explored.

Sodium dithionite (dithionite) has been shown to reduce the oxygen tension of a solution to 0 kPa for up to fifteen minutes when applied at a concentration of 2.5 mM (Johansen et al., 2011). Thus, activation of Panx1 by dithionite was assessed using the calcein dye efflux protocol described above. Unstimulated (Unstim) platelets were sampled at 1 min intervals between t=0 to 5 min and then at 5 min intervals up to t=30 min. No difference was observed between dye efflux in Unstim and dithionite-treated (Dith; 2.5 mM) platelets; 10.4 ± 11.2% vs. 2.3 ± 11.2% ($P > 0.05$, n=3, Fig. 3.8). In the presence of Prb (Dith + Prb) and Cbx (Dith + Cbx), dithionite-induced dye efflux at t=30 min was not significantly different from Unstim control; 19.8 ± 14.9% and 27.5 ± 15.9%, respectively ($P > 0.05$, n=4 [Prb] n=3 [Cbx], Fig. 3.8C).

Caspase activity can be studied using a variety of methods, including ELISA, pharmacological inhibitors and Western blotting. Experiments within this chapter have demonstrated that Panx1 channels contribute to platelet activation in response to threshold agonist concentrations (Figs. 3.3-7). However, caspase activation requires significantly higher agonist concentrations (Shcherbina and Remold-O'Donnell, 1999). To this end, platelet aggregation and ATP release was assessed in response to stimulation by either 10 µg ml$^{-1}$ collagen plus 1 U ml$^{-1}$ Thr (Col + Thr) or 1 µM ionomycin. The pan-caspase inhibitor z-VAD-fmk (80 µM) (Chekeni et al., 2010) was used to assess contributions by caspases to platelet aggregation and ATP release. Peak aggregation responses to ionomycin or Col + Thr were unaffected by z-VAD-fmk; 105.9 ± 6.9% ($P > 0.05$, n=3) and 95.3 ± 2.0% ($P > 0.05$, n=6) of paired control, respectively (Fig. 3.9A). Furthermore, aggregation responses were not reduced by co-application of z-VAD-fmk and Prb (n=3 [ionomycin], n=6 [Col + Thr], $P > 0.05$, Fig. 3.9A). Panx1 channels are permeable to ATP (Bao et al., 2004), therefore, ionomycin- and Col + Thr-evoked ATP release was assessed in the presence of z-VAD-fmk alone and in combination with Prb. Ionomycin-evoked ATP release was 89.6 ± 15.0% ($P > 0.05$, n=3) and 100.4 ± 24.8% ($P > 0.05$, n=3) of control responses

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Figure 3.8 Platelet pannexin-1 channels do not open in response to low oxygen tension. Flow cytometry was used to assess dye efflux from calcein-loaded washed platelet suspensions at rest and after exposure to the oxygen scavenging compound dithionite. (A) Washed platelets loaded with 0.5 µM calcein were selected using forward and side scatter properties to include >90% of events in resting (left) and dithionite treated (2.5 mM, right) conditions. (B) Histograms of calcein fluorescence at t=0 min (black) and t=30 min (red) for either unstimulated platelets (Unstim.), dithionite treated (Dith), dithionite plus 100 µM Prb (Dith + Prb) or dithionite plus 10 µM Cbx (Dith + Cbx). (C) The proportion of cells within F1 was calculated at each interval and is expressed relative to t=0 min. Inhibitor-free runs for unstimulated and dithionite-stimulated conditions contained 0.2% DMSO as the vehicle control. Statistical significance was calculated using an ANOVA. Data are representative of five independent experiments.
in the presence of z-VAD-fmk and z-VAD-fmk plus Prb, respectively (Fig. 3.4B). Col + Thr-evoked ATP release was modestly reduced to 87.5 ± 5.9% and 88.7 ± 3.1% of paired control responses by z-VAD-fmk alone and in combination with Prb, respectively (\( P > 0.05 \), \( n=6 \), Fig. 3.9B).

Induction of Panx1 currents by caspase-3 mediated cleavage of its C-terminus has been demonstrated through patch clamp studies (Sandilos et al., 2012). C-terminal cleavage of platelet Panx1 was investigated by Western blotting, using a C-terminal antibody; in this assay cleavage of Panx1 by caspases should result in either a reduction of molecular weight or loss of immunoreactivity. Platelets were stimulated by 10 µM ionomycin ± z-VAD-fmk, 1 µM ionomycin or threshold concentrations of the major platelet agonists; collagen, thrombin and U46619 (across a 15 min time course). There was no obvious change in molecular weight of Panx1 in response to either agonist (Fig. 3.9C). In addition, densitometry performed on these blots did not show a change in immunoreactivity between t=0 and t=15 min (\( P > 0.05 \), \( n=3 \), \( P > 0.05 \), Fig. 3.9D). These data indicate that platelet Panx1 channels are not activated by caspase cleavage.
Platelet pannexin-1 channels are not activated by caspase cleavage of its carboxyl terminus. Peak platelet aggregation (A) and ATP release (B) in response to stimulation by ionomycin (1 µM) or Col + Thr (10 µg ml⁻¹ collagen plus 1 U ml⁻¹ thrombin; black). Peak responses in the presence of z-VAD-fmk (80 µM; grey) or z-VAD-fmk plus Prb (100 µM; white) are expressed as a percentage of the paired control response (1% DMSO; black bars). C,D) Western blot analysis (C) of Panx1 expression by platelets after stimulation by ionomycin (10 and 1 µM), collagen (0.5 µg ml⁻¹), thrombin (0.03 U ml⁻¹) or U46619 (500 nM) across a 15 min time course. Average band intensities (D) from Western blots were quantified at t=0 and t=15 min by densitometry and are expressed as percentage change from t=0 to t=15 min. Statistical significance was calculated using an ANOVA. Aggregation and ATP release data are representative of 3 (ionomycin) and 6 (Col + Thr) independent experiments. Western blot data are representative of 3 experiments.
3.3 Discussion

Work conducted within this chapter set out to assess the presence of Panx1 channels in platelets, MKs and related cell lines. In addition, the mechanism by which these channels are activated was investigated. These data have provided the first evidence that platelets express functional Panx1 channels.

Purified human platelet mRNA contains transcripts for PANX1, but not other pannexin family members, at a comparable level to another ion channel (P2X1) (Taylor et al., 2014). Importantly, Panx1 protein was detected in platelets using both ICC and Western blot (Figs. 3.1-2). Staining for platelet Panx1 suggested a predominantly plasma membrane localisation (Fig. 3.1), a conclusion which was supported by biotinylation experiments (Fig. 3.2C). In contrast to platelets, ICC of HEK-293 cells (native and hPanx1) revealed both plasma membrane and cytoplasmic staining for Panx1 (Fig. 3.1). This likely represents recently synthesised Panx1 present within the cytosol of these cells. Panx1 channels undergo glycosylation at Asn254, which is a pre-requisite for insertion into the plasma membrane as unglycosylated channels are retained within the cytosol (Boassa et al., 2007, Boassa et al., 2008, Penuela et al., 2009). A single band for Panx1 from platelets was detected by Western blot, whilst HEK-293 cells had two additional bands of lower molecular weight. Treatment with the glycosidase PNGaseF demonstrated that platelet Panx1 exists exclusively in its glycosylated form, whilst both glycosylated and unglycosylated species were detected in HEK-293 cells (native and hPanx1; Fig. 3.2D). These findings are consistent with cell types that are terminally differentiated (platelets) and mitotically active (HEK-293). Given the long half-life of panx1 channels (=54-h), de novo synthesis is not essential during the typical platelet lifespan of 7-10 days (Gehi et al., 2011). Panx1 channels were also detected from both primary mouse and rat MK’s and related myeloid cell lines (HEL and Meg-01; Figs. 3.1-2). The MK DMS acts as a membrane reserve for thrombopoiesis (Chapter 1.2) (Radley and Haller, 1982); thus, proteins expressed along the DMS, including Panx1, will be present at the plasma membrane of resultant platelets. Western blot studies to further investigate MK Panx1 expression were not performed due to their limited abundance (<1% of bone marrow cells) and documented
differences in ion channel transcripts between primary and cultured MK’s (Tolhurst, 2006).

To date there have been only two studies that report Panx1 gap junction activity (Bruzzone et al., 2003, Sahu et al., 2014). In these studies Panx1 gap junction activity was recorded from cells that have greatly enhanced heterologous Panx1 expression, i.e. ≈34-fold increase (Sahu et al., 2014). Such overexpression of Panx1 likely exhausts the glycosylation machinery, allowing unglycosylated channels to be trafficked to the membrane (MacVicar and Thompson, 2010). This hypothesis is supported by recordings of Panx1 gap junctions from paired oocytes, when n-linked glycans were removed by application of PNGaseF (Boassa et al., 2008). Thus, it is accepted that glycosylation abrogates the formation of gap junctions and that Panx1 hexamers function as bona fide channels (Sosinsky et al., 2011). Given that pannexin and connexin hemichannels have distinct biophysical and pharmacological properties, it is likely that they perform distinct roles within the platelet.

Panx1 channels from a variety of cell types and tissues have been shown to open in response to OGD, caspase cleavage of the C-terminus, mechanosensation and a rise of [Ca^{2+}]; (Thompson et al., 2006, Sandilos et al., 2012, Bao et al., 2004, Locovei et al., 2006b). Neuronal Panx1 channels open in response to OGD; furthermore both Panx1 and Panx2 double knockout mice have improved outcomes in a stroke model (middle cerebral artery occlusion; MCAO) (Thompson et al., 2006, Bargiotas et al., 2011, Bargiotas et al., 2012). Treatment of wild-type mice with Prb before or after MCAO-induced stroke also resulted in smaller infarct volumes and improved functional outcomes (Xiong et al., 2014). Thus, it was surprising that reduction of oxygen tension by dithionite failed to open platelet Panx1 channels; indeed, dye efflux was modestly enhanced at t=30 min in the presence of Panx1 inhibitors (Fig. 3.8). This effect was not significant, which is likely due to the low n number reported. The percentage dye efflux in these experiments was smaller than that observed for thrombin-stimulated platelets (Fig. 3.9). Studies of dithionite-induced calcein efflux from HEK-293 cells (native and hPanx1), using a real-time imaging assay, were also unsuccessful (data not shown). Therefore, it is likely that these conditions are not optimal to activate Panx1 channels in the manner described
previously (Thompson et al., 2006). Alternatively, other co-factors and signalling pathways required for OGD-induced activation of neuronal Panx1 channels may be absent from platelets. Nevertheless, the data reported here suggest that platelet Panx1 channels do not open in response to reduced oxygen tension alone.

Caspases are required for apoptosis (Cohen, 1997) but their role in platelet activation is controversial (Shcherbina and Remold-O'Donnell, 1999, Vogler et al., 2011). Activation of caspases 3, 5 and 8 has been reported in response to platelet stimulation by both physiological agonists and Ca\(^{2+}\) ionophores (Mutlu et al., 2012, Shcherbina and Remold-O'Donnell, 1999). Caspase 3 and 7 cleavage sites have been identified within the C-terminus of Panx1 (Chekeni et al., 2010, Silverman et al., 2009); cleavage of Panx1 by caspase 3 induces currents in Jurkat T-cells (Sandilos et al., 2012). However, in the present study there was no contribution by caspases to Col + Thr- or ionomycin-evoked platelet ATP release (Fig. 3.9B). Consistent with previous findings, aggregation responses (Fig. 3.9A) to these agonists was independent of caspase activation (Shcherbina and Remold-O'Donnell, 1999). ATP and aggregation responses were equally unaffected by co-application of Prb with caspase inhibitors (Fig. 3.9B). Finally, assessment of Panx1 cleavage by caspase 3 using Western blot failed to show a reduction of molecular weight or immunoreactivity of Panx1 in response to several agonists (Fig. 3.9C-D). These findings indicate that caspase 3 cleavage does not represent a mechanism for activation of platelet Panx1. In addition, it is unlikely that platelet caspases become activated in response to the low agonist concentrations applied in the remainder of this study; conditions under which Panx1 exerts its greatest contribution to platelet functional responses.

If neither low oxygen tension nor caspase activation induces platelet Panx1 channel opening (Figs. 3.8-9), a rise of [Ca\(^{2+}\)]\(_i\) represents a possible mechanism for the activation of Panx1 channels, as described in a Xenopus oocyte expression system (Locovei et al., 2006b). In that study, P2Y receptor-evoked, IP\(_3\)-dependent Ca\(^{2+}\) release from intracellular stores (Burnstock and Knight, 2004) was proposed as a stimulus to open Panx1 channels, which release nucleotides to stimulate P2Y1 receptors on neighbouring cells. Assessment of
Ca\textsuperscript{2+} release from the DTS demonstrated a relatively small contribution of total Ca\textsuperscript{2+}-influx by collagen, compared to thrombin and U46619 (Figs. 3.3 and 3.4). In agreement with previous [Ca\textsuperscript{2+}i] measurements, experiments in this thesis (Fig. 4.4) show that ≈70% of the Ca\textsuperscript{2+} response to 0.5 µg ml\textsuperscript{-1} collagen is mediated via plasma membrane P2X1 receptors (Fung et al., 2005). Panx1 inhibitors reduced collagen-, thrombin- and U46619- evoked Ca\textsuperscript{2+} responses by ≈ 30% in the presence of external Ca\textsuperscript{2+} (Fig. 3.3), whereas these inhibitors had no effect in the absence of extracellular Ca\textsuperscript{2+} (Fig. 3.4). Given that patch clamp studies reported Panx1 channels to be anion-selective (Ma et al., 2012), they are unlikely to directly generate the Prb/Cbx-sensitive Ca\textsuperscript{2+}-influx. Furthermore, SOCE was unaffected by Panx1 inhibitors (Fig. 3.5). Thus, modulation of Ca\textsuperscript{2+} entry by Panx1 is via a plasma membrane pathway; leaving TRPC6 channels and P2X1 receptors as potential candidates. Under these conditions, TRPC6 channels do not significantly contribute to Ca\textsuperscript{2+}-influx (Harper et al., 2013), thus, ATP-gated P2X1 receptors remain the prime candidate for Panx1-modulated Ca\textsuperscript{2+}-influx; this possibility is explored further in Chapter 4.

Panx1 channels are likely activated by a common early signalling event downstream given the equivalent effect of the Panx1 inhibitors on Ca\textsuperscript{2+} responses to collagen, thrombin and TxA\textsubscript{2} receptors. Generation of IP\textsubscript{3} through PLC-β and PLC-γ mediated hydrolysis of PIP\textsubscript{2} occurs directly downstream of ITAM receptor (GPVI, collagen) and G\textsubscript{q}-coupled GPCR (PAR1, PAR4 [thrombin], TP [TxA\textsubscript{2}]) activation. The resultant Ca\textsuperscript{2+} release from the DTS (Varga-Szabo et al., 2009) and rise in [Ca\textsuperscript{2+}] may activate Panx1 channels, as described previously (Locovei et al., 2006b). Indeed, thrombin stimulation resulted in channel opening and efflux of calcein dye (Fig. 3.7), an anionic dye that is permeable through Panx1 channels (Thompson et al., 2006, Bargiotas et al., 2011). Panx1 was also shown to contribute to \textit{in vitro} thrombus development in a platelet adhesion assay; both thrombus height and volume were reduced by Panx1 inhibitors without affecting surface coverage (Taylor et al., 2014). Platelet adhesion under these conditions has been shown to induce Ca\textsuperscript{2+}-oscillations (Goncalves et al., 2003, Goncalves et al., 2005, Nesbitt et al., 2009), which may serve to activate Panx1 channels. Subsequent release of
ATP (and/or ADP) through Panx1 channels could promote thrombus development via paracrine stimulation of platelets.

The role of Panx1 in this study relies upon the use of two inhibitors that are not totally selective for Panx1 channels. However, these drugs were applied at concentrations shown to block Panx1 channels by patch clamp studies and do not affect other known targets; namely, Cx hemichannels (Cbx) or anion transporters (Prb) (Silverman et al., 2008, D'Hondt et al., 2009, Lipman et al., 1990, Pritchard et al., 1999). Furthermore, atomic force microscopy data demonstrated that Cx hemichannels are closed under the conditions of these experiments (2 mM [Ca$^{2+}$]o) (Ebihara and Steiner, 1993, Pfahnl and Dahl, 1999, Gomez-Hernandez et al., 2003, Allen et al., 2011, Thimm et al., 2005). Thus, Cx family channels are unlikely to contribute to either the Ca$^{2+}$ movements observed in Figs. 3.3 and 3.5 or calcein dye efflux (Fig. 3.7). Furthermore, both Prb and Cbx abrogated thrombin-evoked calcein dye efflux (Fig. 3.7), in agreement with studies of Panx1 function (Thompson et al., 2006, Bargiotas et al., 2011). Concentration:response curves gave IC$_{50}$ values of 455 ± 146 µM and 96.3 ± 35 µM for Prb and Cbx, respectively (Fig. 3.8). There is a documented variation in the IC$_{50}$ values of these compounds at Panx1 channels between cell types; however these values from platelets fall within the expected range (D'Hondt et al., 2009). Given the complexity of platelet activation within the circulation, it will be important to conduct future studies of the contribution of platelet Panx1 to haemostasis and thrombosis using in vivo studies within a murine model. However, since Panx1 has been reported in other blood cell types (Woehrle et al., 2010, Sridharan et al., 2010), it will be crucial to develop a mouse line in which altered expression of this channel is specifically targeted to the platelet and MK lineage.

Data presented here show that platelets express membrane-localised, Panx1 channels. These ATP-permeable channels modulate Ca$^{2+}$ entry across the plasma membrane in response to several agonists; which is most likely via stimulation of P2X1 receptors. This hypothesis is explored further in Chapter 4.
Chapter 4

4.0 Pannexin-1 amplifies platelet activation through stimulation of P2X1 receptors

4.1 Background

Multiple studies have identified Panx1 channels as a *bona fide* route for non-lytic and non-vesicular release of cytosolic ATP to the extracellular space (Woehrle et al., 2010, Gulbransen et al., 2012, Locovei et al., 2006a). ATP release by Panx1 has been shown to contribute to the activation of ATP-gated P2X1, 4 and 7 receptors. To date, Panx1 channels have been implicated in the activation of T cells (Woehrle et al., 2010), erythrocytes (Locovei et al., 2006b) and neurons (Gulbransen et al., 2012). P2X7 receptors are distinct from other P2X family members in that they are activated by millimolar concentrations of ATP (North, 2002) (compared to nanomolar potency of this nucleotide at P2X1 receptors); prolonged stimulation of P2X7 receptors leads to formation of a large pore (Surprenant et al., 1996). Although some reports have proposed that this large pore is comprised of Panx1 channels (Pelegrin and Surprenant, 2006), this concept is controversial. Overall, data from a combination of electrophysiological and measurements of dye efflux in cell lines lacking Panx1 channels suggest that Panx1 is likely redundant in the transition of P2X7 to this large pore conformation (Alberto et al., 2013).

The P2X1 receptor is the only P2X receptor subtype that has been shown to be functional in platelets (Sun et al., 1998, Wang et al., 2003) and accounts for up to 80% of the Ca$^{2+}$ response to threshold concentrations of collagen (Fung et al., 2005). P2X1 receptors represent the fastest route whereby an extracellular agonist can stimulate Ca$^{2+}$ entry in platelets (Mahaut-Smith, 2012). *In vitro* and *in vivo* studies, including use of pharmacological tools and P2X1$^{-/-}$ mice (Hechler et al., 2003), have identified an important role for this ion channel in thrombus formation, particularly under high shear conditions and thus in the arterial circulation. The exact mechanism(s) whereby this ATP-gated cation channel is activated following stimulation by collagen or other major platelet agonists is incompletely understood, however, evidence suggests a predominantly autocrine mechanism of activation by released ATP (Fung et al.,
Chapter 3 provides evidence for functional Panx1 channels on the surface of human platelets, which may therefore contribute to agonist-evoked ATP release and secondary P2X1 receptor activation.

4.1.1 Aims

Work within this chapter set out to investigate the contribution by Panx1 to ATP release and whether this is linked to subsequent P2X1 receptor activation. The relationship between Panx1 and P2X1 is explored under conditions where P2X1 receptors make a significant contribution to platelet activation; 0.5 µg ml\(^{-1}\) collagen in the presence of 0.32 U ml\(^{-1}\) apyrase. Furthermore, the spatial interaction between P2X1 receptors and Panx1 channels is investigated by co-immunoprecipitation studies.
Figure 4.1 Pannexin-1 contributes to ATP release from the platelet cytosol in response to stimulation by 0.5 µg ml\(^{-1}\) collagen. Representative collagen-evoked (0.5 µg ml\(^{-1}\)) platelet ATP release (A) and aggregation (B) traces in the presence of either 100 µM Prb (upper panel), 10 µM Cbx (middle panel) or after P2X1 receptor-desensitisation with 0.6 µM α,βmeATP (lower panel). C,D) Average responses from four donors for peak ATP release (C) and peak aggregation (D) as a percentage of paired vehicle control response. Statistical significance was calculated using an ANOVA. Data are representative of four independent experiments.
4.2 Results

4.2.1 Pannexin-1 plays a key role in platelet aggregation and ATP release stimulated by threshold concentrations of collagen

To determine the contribution by Panx1 to platelet activation, simultaneous ATP release and aggregation responses to collagen were assessed. Platelets stimulated by 0.5 µg ml\(^{-1}\) collagen undergo shape change followed by a moderate aggregation response (Fig. 4.1A). ATP release follows a more rapid time course with a peak response observed within 30s of agonist application compared with ≈70s for aggregation (Figs. 4.1 and 4.7B). The contribution by Panx1 to platelet aggregation and ATP release was assessed by pre-incubation of platelets with either 100 µM Prb (Fig. 4.1A) or 10 µM Cbx (Fig. 4.1B). In the presence of Prb, collagen-evoked ATP release and aggregation were reduced to 18.2 ± 5.7% and 16.2 ± 3.3% (\(P < 0.001\), n=4) of control responses, respectively (Fig. 4.1C-D). Equally, ATP release and aggregation responses were reduced to 14.7 ± 8.6% and 12.6 ± 2.4% (\(P < 0.001\), n=4) of control responses in the presence of Cbx. There was no difference between the inhibition of aggregation and ATP release in the presence of either Prb or Cbx (Fig. 4.1C-D). Contributions by P2X1 receptors were assessed before and after desensitisation by application of 0.6 µM α,β-meATP in nominally Ca\(^{2+}\)-free saline 60s prior to the addition of 2 mM CaCl\(_2\) and the agonist (Fung et al., 2007). Desensitisation virtually abolished Ca\(^{2+}\) influx through P2X1 receptors, in response to a maximal concentration of α,β-meATP (10 µM; peak increase of 30.3 ± 3.9 nM compared to the control responses of 215.7 ± 44.3 nM; \(P < 0.01\), n=5, Fig. 4.2), in agreement with previous reports (Rolf et al., 2001, Fung et al., 2007). Co-application of Panx1 inhibitors to α,β-meATP-desensitised platelets gave an equivalent decrease of 0.5 µg ml\(^{-1}\) collagen-evoked aggregation (Fig. 4.1C; 17.1 ± 8.8% [Prb + α,β-meATP], 15.7 ± 7.0% [Cbx + α,β-meATP], of control responses; \(P < 0.001\), n=4) and ATP release (Fig. 4.1D; 9.7 ± 6.6% [Prb + α,β-meATP], 6.0 ± 5.0% [Cbx + α,β-meATP], of control responses; \(P < 0.001\), n=4).

Studies have shown that P2X1 receptors are dispensable for platelet aggregation to collagen concentrations above 2.5 µg ml\(^{-1}\) (Hechler et al., 2003, Toth-Zsamboki et al., 2003). Thus, the effect of Panx1 inhibition on aggregation
was also assessed following stimulation by higher collagen concentrations, where α,β-meATP pre-addition caused smaller or no significant effects; 80.8 ± 4.3% (P < 0.05, n=4) and 92.5 ± 5.6% (P > 0.05, n=4), of control aggregation responses to 5 and 10 µg ml\(^{-1}\) collagen, respectively (Fig. 4.3). Likewise, collagen-evoked ATP release was only moderately reduced by desensitisation of P2X1 receptors, with peak responses of 81.1 ± 3.1% (P < 0.05, n=4) at 5 µg ml\(^{-1}\) and no effect at 10 µg ml\(^{-1}\) (104.1 ± 3.9%, P > 0.05, n=4). Panx1 inhibition had no significant effect on ATP release or aggregation in response to stimulation with 10 µg ml\(^{-1}\) (P > 0.05, n=4, Fig. 4.3B-C), and an intermediate effect was observed at 5 µg ml\(^{-1}\) collagen (Fig. 4.3A,C). These data indicate that contributions by Panx1 to aggregation and ATP release are greatest in response to threshold concentrations of collagen; conditions that have been shown to require P2X1 receptor-mediated Ca\(^{2+}\) influx in order to amplify platelet activation (Fung et al., 2005).

Figure 4.2 Desensitisation of P2X1 receptors abolishes α,β-meATP-evoked Ca\(^{2+}\) influx. [Ca\(^{2+}\)]\(_i\) measurements from Fura-2 loaded washed platelet suspensions. A) Representative Ca\(^{2+}\) responses to stimulation by 10 µM α,β-meATP for control (black) and 0.6 µM α,β-meATP desensitised (grey) platelets. B) Average peak platelet Ca\(^{2+}\) responses (nM) to 10 µM α,β-meATP under control (vehicle) and P2X1 receptor-desensitised (pre-addition of 0.6 µM α,β-meATP) conditions. Statistical significance was calculated using a Student’s paired t-test. Data are representative of five independent experiments.
Figure 4.3 Pannexin-1 is dispensable for platelet aggregation to higher concentrations of collagen. Representative collagen-evoked platelet ATP release (left) and aggregation (right) responses to 5 µg ml$^{-1}$ (A) or 10 µg ml$^{-1}$ (B) collagen. Overlaid traces show responses from control (black), 10 µM Cbx (blue) or 0.6 µM α,β-meATP (grey) treated platelets. C) Average responses from four independent donors are shown as a percentage of paired control response. Statistical significance was calculated using an ANOVA.
Figure 4.4 Pannexin-1 contributes to P2X1-dependent Ca\textsuperscript{2+} influx. A) Collagen-evoked (0.5 µg ml-1) responses before (black trace) and after (grey trace) desensitisation of P2X1 receptors with 0.6 µM α,β-meATP. No further significant decrease was observed upon addition of 100 µM Prb (red trace) or 10 µM Cbx (blue trace) to P2X1 receptor-desensitised platelets. B) Average Ca\textsuperscript{2+} responses expressed as a percentage of paired vehicle control; Cbx or Prb addition to P2X1 receptor desensitised platelets did not significantly reduce the peak Ca\textsuperscript{2+} response compared with desensitisation with α,β-meATP alone. For reference the peak collagen-evoked Ca\textsuperscript{2+} responses in the presence of Prb (red stripes) or Cbx (blue stripes) alone have been taken from Fig. 3.5. Data are means of five independent experiments. Statistical significance was calculated using an ANOVA.
4.2.2 Pannexin-1-dependent Ca^{2+}-influx is mediated through activation of P2X1 receptors

Experiments in Chapter 3 show that collagen-evoked (0.5 µg ml\(^{-1}\)) Ca\(^{2+}\) responses are reduced by ≈30-35% by Panx1 blockers (Fig. 3.5, and striped bars of 4.4B). Furthermore, it was demonstrated that this occurs through a plasma membrane Ca\(^{2+}\)-influx pathway rather than modulation of Ca\(^{2+}\) release from the DTS (Figs. 3.3-5). Under the conditions of these experiments this is most likely to be through P2X1 channels, given the lack of contribution by TRPC6 at low collagen concentrations (Harper et al., 2013). Pre-desensitisation of P2X1 receptors, by treatment with α,β-meATP, reduced the peak Ca\(^{2+}\)-response to 33.2 ± 3.1% \((P < 0.001, n=5, \text{Fig. 4.4})\) of the control response, as described previously (Fung et al., 2005). No further significant decrease was observed following co-addition of Prb with α,β-meATP (23.3 ± 3.3%, \(P = 0.13, n=5\)) or Cbx with α,β-meATP (21.3 ± 3.5%; \(P = 0.08, n=5, \text{Fig. 4.4}\)).

To determine whether Panx1 inhibitors directly affect P2X1 receptor activity, α,β-meATP-evoked (10 µM) Ca\(^{2+}\) responses were assessed in the presence and absence of these antagonists. P2X1 receptors are the sole target for α,β-meATP (non-hydrolysable ATP analogue) on platelets (Rolf et al., 2001), therefore, the effect of various drugs on P2X1 receptor activity can be directly measured using this assay. Application of 10 µM α,β-meATP induced a rapid and transient Ca\(^{2+}\) influx through P2X1 receptors (Fig. 4.5) under control conditions. Peak \([\text{Ca}^{2+}]_i\) responses were unaffected by pre-incubation with either Prb or Cbx; 107.7 ± 9.8% and 101.3 ± 4.0% \((P > 0.05, n=5, \text{Fig. 4.5})\) of paired control responses, respectively. These data suggest a mechanism whereby Panx1 activity leads to P2X1 receptor activation, possibly via efficient coupling of ATP release and stimulation of P2X1 receptors.

4.2.3 Pannexin-1 co-localises with P2X1

Previous studies within this laboratory have shown that activation of P2X1 receptors by collagen and other major platelet agonists occurs via a predominantly autocrine mechanism (Fung et al., 2012). In addition, it has been proposed that ATP release, through dense granule release or ATP-permeable channels, may be targeted to membrane regions close to P2X1 receptors, to
Figure 4.5 Pannexin-1 inhibitors do not directly inhibit P2X1 receptor-mediated Ca\(^{2+}\) influx. 

\([\text{Ca}^{2+}]_i\) measurements from Fura-2 loaded washed platelet suspensions in the presence of 2 mM \([\text{Ca}^{2+}]_o\). A,B) Platelet P2X1 receptors were maximally stimulated by 10 µM \(\alpha,\beta\)-meATP under control (0.2% DMSO; black trace) conditions and in the presence of 100 µM Prb (A) or 10 µM Cbx (B). C) There was no significant difference in the peak Ca\(^{2+}\) response between vehicle control and inhibitor treated groups. Statistical significance was calculated using a Student's paired t-test. Data shown are representative of five independent experiments.
facilitate efficient activation of these channels (Mahaut-Smith et al., 2011, Fung et al., 2012). Contributions by Panx1 to aggregation and calcium influx were dependent on functional P2X1 receptors (Figs. 4.1, 3-4); co-operation between these proteins may be enhanced by either physical coupling or co-localisation. Co-immunoprecipitation studies revealed staining for Panx1 when P2X1 receptors were immunoprecipitated from resting platelet lysates. In the reciprocal experiment, weak staining for P2X1 was observed when Panx1 was immunoprecipitated (Fig. 4.6A). Stronger staining was observed in the P2X1 receptor co-immunoprecipitation experiment (Fig. 4.6A upper panel), which may be due to the location of the P2X1 receptor and Panx1 antibody epitopes. This interaction was not a result of antibody cross-reactivity as there was no staining by Panx1 (raised in goat) or P2X1 (raised in rabbit) antibodies in the rabbit and goat IgG control experiments, respectively (Fig. 4.6B). Densitometry shows that the signal for Panx1 was stronger when P2X1 was immunoprecipitated than vice versa, 0.373 ± 0.027 vs 0.152 ± 0.068 (P < 0.001, Fig. 4.6C). A possible explanation of these data is that the stoichiometry between Panx1 and P2X1 is 2:1, which is consistent with the quaternary structure of these proteins that form hexamers and trimers, respectively.
Figure 4.6 Pannexin-1 has a weak physical interaction with P2X1 in resting platelets. Panx1 and P2X1 receptors were co-immunoprecipitated from platelet lysates and detected by Western blot (A). hPanx1-His-FLAG and hP2X1R expressing HEK-293 cells were included to control for Panx1 and P2X1R antibody binding. Protein loading was assessed by re-probing for β-actin. Low level Panx1 staining was observed in the P2X1R IP and weak staining for P2X1R was observed in the Panx1 IP. Non-specific interactions between the agarose beads and goat (Gt, Panx1) or rabbit (Rb, P2X1) IgG were tested and show no cross reactivity in the co-immunoprecipitation lanes (B). Quantification of the co-immunoprecipitation experiments show that the Panx1 density in the P2X1 receptor pull down is approximately double that of the P2X1 receptor density in the Panx1 pull down (C). Data shown are representative of at least three independent experiments. Statistical significance was calculated using a Student’s paired t-test.
4.3 Discussion

Experiments performed in this chapter have identified a role for Panx1 in collagen-evoked ATP release and subsequent aggregation, which is dependent on functional P2X1 receptors. Furthermore, the contribution by Panx1 to Ca\(^{2+}\)-influx is likely via stimulation of P2X1 receptors, which were shown to be colocalised by co-immunoprecipitation studies. Taken together, these findings suggest that Panx1 constitutes a possibly localised ATP release site for the efficient activation of platelet P2X1 receptors; in turn, amplifying responses to threshold agonist concentrations.

Data presented in Chapter 3 identified that Panx1, an anion-selective channel (Ma et al., 2012), modulates Ca\(^{2+}\)-influx across the plasma membrane in response to multiple agonists (Figs. 3.5-7); however, the identity of the Ca\(^{2+}\) entry pathway was not determined. Elevations of [Ca\(^{2+}\)]\(_i\) by Ca\(^{2+}\) release from the DTS (Fig. 3.4), SOCE (Fig. 3.5) and direct stimulation of P2X1 receptors (Fig. 4.5) were equally unaffected by Panx1 inhibitors. In addition, at the low agonist concentrations used in this study, TRPC6 does not significantly contribute to Ca\(^{2+}\) entry (Harper et al., 2013). Therefore, given that Prb and Cbx reduce collagen-evoked ATP release, it is likely that Prb and Cbx inhibit Ca\(^{2+}\) increases via reduction of secondary P2X1 receptor activation. This hypothesis was tested by comparing peak Ca\(^{2+}\) responses from platelets stimulated by 0.5 µg ml\(^{-1}\) collagen in the presence of Panx1 inhibitors after α,β-meATP desensitisation of P2X1 receptors. Under these conditions Panx1 inhibitors did not further reduce the peak Ca\(^{2+}\) response as compared to P2X1 receptor desensitisation alone (Fig. 4.4). The greater reduction of the 0.5 µg ml\(^{-1}\) collagen-evoked Ca\(^{2+}\) response by P2X1 receptor desensitisation (≈70%) than Panx1 inhibition (≈30%; Fig. 4.4) suggests that ATP release involves other pathways (e.g. dense granule secretion) at this low agonist concentration (Toth-Zsamboki et al., 2003). Interestingly, ATP release through Panx1 has also been shown to activate P2X1 receptors in T cells (Woehrle et al., 2010).

It is well established that platelets secrete ATP and ADP from dense granules in a Ca\(^{2+}\)- (Varga-Szabo et al., 2009) and PKC- (Hashimoto et al., 1994) dependent manner, resulting in important amplification of platelet responses through P2X and P2Y receptors. Indeed, platelets from Unc13d\(^{-/-}\) mice (a
mouse model that lacks the vesicle priming factor Munc 13-4) do not undergo dense granule secretion in response to several agonists (Ren et al., 2010). In these experiments a small amount of ATP release still occurs, suggesting that there is an alternative, dense granule-independent pathway for ATP release. Given that Panx1 is known to serve as an ATP release pathway (MacVicar and Thompson, 2010), this channel represents a novel pathway for ATP release from the platelet cytosol. Panx1 inhibitors reduced ATP release following stimulation by a threshold concentration of collagen (Fig. 4.1). There are reported differences between ATP measurements made from bulk solutions and those that use plasma membrane bound luciferin:luciferase (Beigi et al., 1999). In this study, surface-attached luciferin:luciferase was able to detect ATP concentrations of up to 15-20 µM; whereas bulk solution measurements only detected a peak value of several hundred nanomolar of ATP. This likely reflects the spatial averaging of ATP released into bulk solution compared to direct recordings at the plasma membrane. Furthermore, ATP released into the solution will be degraded by endogenous (e.g. CD39) and exogenous (i.e. apyrase) ecotonucleotidases. Contributions by Panx1 to ATP release in this study are therefore likely underestimated using this approach (Figs. 4.1 and 4.3). P2X1 receptor desensitisation also reduced peak ATP release to the same extent as Panx1 inhibition (Fig. 4.1), consistent with the observation that P2X1 knockout mice have reduced dense granule secretion (Hechler et al., 2003).

In agreement with previous studies, P2X1 receptors were shown to be required for aggregation to 0.5, but not 5 or 10 µg ml⁻¹, collagen (Fig. 4.1-2); this requirement of P2X1 receptors is directly related to Ca²⁺-influx (Fung et al., 2005). When platelets were treated with Panx1 inhibitors the 0.5 µg ml⁻¹ collagen-evoked aggregation response was equally blunted and in a non-additive manner, suggesting a degree of co-operation between Panx1 channels and P2X1 receptors. The redundancy of P2X1 receptors at higher collagen concentrations (Fig. 4.3) can be explained by alternative signalling pathways and routes for Ca²⁺-influx at play under these conditions. Panx1 inhibitors also had a reduced effect under these conditions (Fig. 4.3); further supporting the hypothesis that P2X1 receptors and Panx1 channels function in a cooperative manner. Upon closer inspection, the ATP traces show a biphasic response to
0.5 µg ml\(^{-1}\) collagen (Fig. 4.1) compared to a singular rate in response to 5 and 10 µg ml\(^{-1}\) collagen. Overlaying the aggregation and ATP release traces (Fig. 4.7) shows that this second phase of ATP release coincides with reaching a threshold level of platelet aggregation. Thus, this second phase of ATP release is likely in response to outside-in signalling through integrin \(\alpha_{IIb}\beta_3\) (Shattil et al., 1998). A PKC phosphorylation consensus sequence has been identified in the C-terminus of Panx1, which may influence channel gating (Barbe et al., 2006). Therefore, attempts to delineate the relative contribution of Panx1 and dense granule secretion to ATP release by use of a PKC inhibitor (GF109203X) were unsuccessful. In these experiments 20 µM GF109203X completely abolished the ATP release in response to 0.5 µg ml\(^{-1}\) collagen (data not shown), which could be explained by blockade of dense granule secretion, Panx1 channels or both. This relationship could be further investigated by use of the \(^{[3]}\text{H}\)-5-hydroxytryptamine assay (Crosby and Poole, 2004) and possibly flow cytometry to assess dense granule secretion in the presence of P2X1, Panx1 and PKC inhibitors. However, given the reliance on ATP-gated P2X1 receptors to observe full platelet activation to 0.5 µg ml\(^{-1}\) collagen it will be difficult to fully dissociate the role of each pathway.

Data presented here suggests that there is a functional co-operation between P2X1 and Panx1, which facilitates the efficient activation of P2X1 receptors. A predominantly autocrine route for P2X1 activation has been suggested by assessment of Ca\(^{2+}\) and ATP responses in serially diluted platelet suspensions (Fung et al., 2012, Fung et al., 2007). Two hypotheses for efficient autocrine activation were proposed by these studies: firstly, dense granule secretion and P2X1 receptors could be co-targeted to discrete regions of the plasma membrane; secondly, an unidentified, ATP permeable protein is present close to P2X1 receptors. The former hypothesis remains plausible but would be difficult to test, as this requires measurements of ATP release at plasma membrane microdomains containing P2X1 receptors. The second hypothesis was tested by co-immunoprecipitation experiments (Fig. 4.6); these experiments identified a weak, but consistent, physical interaction between P2X1 and Panx1 proteins. Co-localisation of P2X1 receptors and Panx1 channels has been described previously in T-cells (Woehrle et al., 2010).
Blockade of Panx1 channels virtually abolished both aggregation and ATP release, yet Ca\(^{2+}\) responses were only reduced by \(\approx 30-35\%\). This apparent discrepancy is likely explained by considering the temporal profile of the Ca\(^{2+}\) aggregation and ATP release responses. These three responses following stimulation by 0.5\(\mu\)g ml\(^{-1}\) collagen under control and Panx1 inhibited (Prb) conditions are overlaid in Fig. 4.7A (data have been normalised such that control is 100\% and Prb is proportionate to peak responses reported earlier). Collagen stimulation promotes the formation of IP\(_3\) via PLC\(\gamma\) (Varga-Szabo et al., 2009), which leads to Ca\(^{2+}\) release from the DTS. This initial rise of [Ca\(^{2+}\)]\(_i\) may be sufficient to activate Panx1 channels (Fig. 4.7C), as proposed in Chapter 3 for platelets and in oocytes heterologously expressing the channel (Locovei et al., 2006b). Open Panx1 channels release ATP from the cytosol (Fig. 4.1), which activates localised P2X1 receptors (Figs. 4.6). Ca\(^{2+}\)-influx is subsequently amplified by the recruitment of P2X1 receptors (Fig. 4.4); this further rise of [Ca\(^{2+}\)]\(_i\) serves to promote dense granule release and platelet shape change. Dense granules contain the nucleotides ATP and ADP in a ratio of approximately 2:1 (Weiss et al., 1979), thus measurements of ATP using surface attached luciferin: luciferase predict that local extracellular ADP will also reach a level of 7-10 \(\mu\)M following platelet secretion (Beigi et al., 1999). Release of ATP and ADP is sufficient to further promote platelet activation by autocrine and paracrine stimulation of P2X1 (Fung et al., 2005) and P2Y1/12 receptors (Léon et al., 1997), ultimately leading to aggregation (described in further detail in Chapter 1; Figs. 4.1, 4.7C). The temporal profile of Fig. 4.7 shows that platelet aggregation is dependent on nucleotide release, which in turn is dependent on a sustained rise of [Ca\(^{2+}\)]\(_i\). Thus, the reduction in platelet aggregation in the presence of Panx1 inhibitors (Fig. 4.1) is likely due to reduced P2X1 receptor stimulation as a result of reduced ATP release by Panx1 channels (Fig. 4.7Aii). Panx1 may also contribute to platelet P2Y receptor activation either by direct passage of ADP or by degradation of
Figure 4.7 A model for Pannexin-1 dependent amplification of platelet activation based on the time course of Ca^{2+}, ATP release and aggregation responses. Normalised collagen-evoked Ca2+ (blue), ATP release (red) and aggregation (black) traces overlaid from control (A i) and 100 µM Prb (A ii) treated platelets. (B) Mean time to peak Ca2+, ATP release and aggregation response to 0.5 µg ml^{-1} collagen for control, Prb, Cbx and α,βmeATP-desensitised platelets. (C) Cartoon of the proposed pathway for Panx1-dependent amplification of collagen-evoked platelet activation. Statistical significance was calculated using an ANOVA.
released ATP to ADP by ectonucleotidase activity. ADP release through Panx1 channels has not been reported but this is likely due to the technical limitations relating to the measurement of ADP. Attempts to investigate the contribution of Panx1 to ADP-dependent platelet activation were unsuccessful (data not shown) since there was very little aggregation and ATP release observed when Panx1 maximally contributes to aggregation but P2X1 receptors are desensitised (threshold agonist concentrations and 0.02 U ml⁻¹ apyrase) It was therefore not possible to determine the contribution by Panx1-dependent ADP release and P2Y activation in these experiments. If Panx1 does contribute to the release of ADP, this could represent another mechanism for amplification of platelet activation at low agonist concentrations.

In summary, the data presented in this chapter supports a role for Panx1 channels in the amplification of platelet activation, in response to threshold concentrations of collagen. Furthermore, there is evidence to suggest that this amplification is achieved through stimulation of P2X1 receptors following release of ATP through colocalised Panx1 channels. Given that Panx1 also contributes to thrombus development, without affecting surface coverage, under arteriolar shear rates (Taylor et al., 2014), these channels may represent a novel target for future antiplatelet therapies; this possibility is discussed in Chapter 7.
Chapter 5

5.0 Electrophysiological characterisation of Ca\(^{2+}\)-activated ion channels expressed by human erythroleukaemia cells.

5.1 Background

Work conducted in the latter half of this thesis examines the biophysical properties of Ca\(^{2+}\)-activated ion channels expressed by HEL cells (Chapter 5) and primary MKs (Chapter 6). MKs express the vast majority of channels and receptors found in platelets and as such represent a *bona fide* model of platelet signalling (Tolhurst et al., 2005). HEL cells were established from a patient with Hodgkin’s lymphoma and show characteristics of both erythroid and non-erythroid cells (Martin and Papayannopoulou, 1982). These cells express megakaryocytic (GPIb and GPIIb/IIIa) and erythroid (α and γ globin chains) markers (Tabilio et al., 1984) and have been used to model human MK and platelet signalling, particularly the activity of ion channels (Kapural et al., 1995, Somasundaram et al., 1997, Stoneking et al., 2013).

5.1.1 Ca\(^{2+}\)-activated K\(^{+}\) channels

Ca\(^{+}\) activated K\(^{+}\) channels are discussed in Chapter 1.7 and can be split into three subfamilies based on their conductance: the large-conductance channel (K\(_{\text{Ca}}\)1.1); small-conductance channels (K\(_{\text{Ca}}\)2.1, K\(_{\text{Ca}}\)2.2 and K\(_{\text{Ca}}\)2.3) and the intermediate-conductance channel (K\(_{\text{Ca}}\)3.1) (Vergara et al., 1998). Patch clamp recordings of human platelets and HEL cells identified K\(_{\text{Ca}}\)3.1-like conductances from these cells (Mahaut-Smith, 1995, Lu et al., 1999, Stoneking et al., 2013). The Kd for Ca\(^{2+}\) at these channels is \(\approx\)310 nM and they are maximally activated by 1 µM [Ca\(^{2+}\)] (Mahaut-Smith and Schlichter, 1989, Grissmer et al., 1993). The Dami cell line, which was derived from a patient with megakaryocytic leukemia (Greenberg et al., 1988), has also been shown to express functional K\(_{\text{Ca}}\)3.1-like and Ca\(^{2+}\)-activated Cl\(^{-}\) channels, which are activated by thrombin stimulation (Sullivan et al., 1996, Sullivan et al., 1998). In considering these recordings it is important to note that genotyping and karyotyping of the Dami cell line revealed cross-contamination by HEL cells, which occurred prior to entering these cells into the American Type Culture Collection (MacLeod et al., 1997). To date, K\(_{\text{Ca}}\)3.1 channels are the only Ca\(^{2+}\)-activated channels reported in HEL cells.
However, cross-contamination of the Dami cell line by HEL cells raises the possibility that the Ca$^{2+}$-activated Cl$^{-}$ conductance described above may also be present in HEL cells.

5.1.2 TMEM16F channels

An overview of the TMEM16 family of proteins is given in Chapter 1.7. Of the ten family members, TMEM16F has been identified in platelets and MKs (Yang et al., 2012). TMEM16F proteins form Ca$^{2+}$-activated Cl$^{-}$ channels that are predicted to have eight TMDs with intracellular amino and carboxyl termini (Schreiber et al., 2010, Yu et al., 2012). Activation of these channels requires sustained (>5 min) elevation of [Ca$^{2+}$] to 100 µM (Grubb et al., 2013). TMEM16F currents, recorded from several human and mouse cell types, are outwardly rectifying and principally permeable to Cl$^{-}$ (Martins et al., 2011, Szteyn et al., 2012, Grubb et al., 2013). It was therefore surprising that patch clamp recordings from primary mouse MK’s reported cation-selective TMEM16F channels (Yang et al., 2012).

5.1.3. Pannexin-1

Data presented in Chapters 3 and 4 of this thesis investigated platelet Panx1 channels. These channels modulate platelet Ca$^{2+}$ influx in response to multiple agonists (Fig. 3.3); most likely via stimulation of P2X1 receptors (Fig. 4.7). In these studies, platelet Panx1 channels were proposed to be activated by a rise of [Ca$^{2+}$], in response to agonist stimulation, as reported previously in a HEK-293 expression system (Locovei et al., 2006b). Panx1 expression studies (ICC and Western blotting) also identified these channels in myeloid cell lines (CHRF, HEL and Meg-01) and primary mouse and rat MK’s (Figs. 3.1-2).

5.1.4 Aims

Work conducted within this chapter set-out to identify Ca$^{2+}$-activated ion channels expressed by HEL cells. In particular, the biophysical properties of Ca$^{2+}$-activated anion channels are investigated. This includes members of the TMEM16 family and also Panx1 channels, which were suggested to be present in HEL cells using Western blotting and ICC experiments in Chapter 3.
Figure 5.1 Ca$^{2+}$-activated ion channels stimulated by ionomycin. Whole cell patch clamp recordings of HEL cells in a variety of K$^+$ gradients in response to depolarising voltage ramps (from -60mV to potentials in the range -120 to +120mV). A-D,F) Baseline currents were recorded in 1 mM CaCl$\text{\textsubscript{2}}$-containing bath solution (green trace), prior to addition of 1 µM ionomycin (red trace). Cells were subsequently perfused with 0.5 mM EGTA-buffered bath solution (blue trace). In all cases K$^+$ was exchanged by equimolar substitution of Na$^+$. Where appropriate, $P_{\text{Na}}/P_{\text{K}}$ values were calculated from the Goldman-Hodgkin-Katz equation and are indicated in the figure. Theoretical $E_{\text{rev}}$ values for a K$^+$-selective channel were calculated for paradigms A-D and plotted against the $\log_{10} \left[ \frac{[K^+]_o}{[K^+]_i} \right]$ (Black squares); experimental $E_{\text{rev}}$ values are plotted also (red diamonds). F) Ionomycin-evoked currents in K$^+$-free recording solutions. Data are normalised for current density (pA pF$^{-1}$) and are representative of five independent experiments.
5.2 Results

5.2.1 Assessment of Ca\(^{2+}\)-activated ion channels in HEL cells using the Ca\(^{2+}\) ionophore ionomycin

Functional K\(_{Ca}\)3.1 channels have been recorded from the plasma membrane of HEL cells by whole cell (WC) patch clamp (Lu et al., 1999, Stoneking and Mason, 2013). Similar channels have also been reported in Dami cells, which may be contaminated with the HEL cell line (Sullivan et al., 1996, MacLeod et al., 1997). K\(_{Ca}\)3.1 channels are inactive at basal [Ca\(^{2+}\)]\(_i\) and can be opened by raising [Ca\(^{2+}\)]\(_i\) to ≥300 nM for example using Ca\(^{2+}\) ionophores (Mahaut-Smith and Schlichter, 1989). HEL cells were WC patch clamped in bath solutions supplemented with 1 mM CaCl\(_2\) (Fig. 5.1 details the ionic conditions for each experimental paradigm). Initially, depolarising voltage ramps from -120 to +120mV (0.36 V s\(^{-1}\)) were applied at 15s intervals to obtain instantaneous I-V relationships. Ca\(^{2+}\)-activated currents were recorded in response to ionomycin (1 µM)-evoked elevation of [Ca\(^{2+}\)]\(_i\) under a range of K\(^+\) gradients, as indicated in figure 5.1. In a thirty-fold K\(^+\) gradient (5 mM [K\(^+\)]\(_o\) vs 150 mM [K\(^+\)]\(_i\)) ionomycin induced a large current that reversed at -84.0 ± 2.0mV (Fig. 5.1A, n=5). These currents were Ca\(^{2+}\)-dependent as they were gradually eliminated upon perfusion with EGTA-buffered (0.5 mM) bath solution (Fig. 5.1A blue trace), which leads to a slow reversal of the ionomycin-evoked [Ca\(^{2+}\)]\(_i\) increase. To assess the K\(^+\)-selectivity of these currents, the same protocol was applied with different [K\(^+\)]\(_o\) and [K\(^+\)]\(_i\), using equimolar Na\(^+\) substitution (Fig. 5.1B-D). When [K\(^+\)]\(_o\) was set at 5, 28 and 150 mM, ionomycin-evoked currents were inwardly rectifying and E\(_{rev}\) shifted from -84.0 ± 2.0mV to -40.3 ± 0.6mV and 0.0 ± 0.0mV, respectively (Fig. 5.1B-C, n=5). Furthermore, when [K\(^+\)]\(_i\) was reduced to 28 mM Ca\(^{2+}\)-activated currents recorded from 150 mM KCl bath solution reversed at +36.0 ± 2.0mV (Fig. 5.1D, n=5). Theoretical and experimental E\(_{rev}\) values were not significantly different and could be fitted by a line with gradient \(y = 58x\) and \(y = 54.54x\), respectively (\(P > 0.05\), n=5, Fig. 5.1E). The relative permeability of Na\(^+\) over K\(^+\) was calculated as ≤ 0.052 for each K\(^+\) gradient (equation 2.3). Taken together, these data are consistent with the expression of a highly K\(^+\)-selective, K\(_{Ca}\)3.1-like channel by HEL cells. Interestingly, outwardly rectifying Ca\(^{2+}\)-activated currents persisted when all K\(^+\) was substituted by Na\(^+\) (Fig.
Figure 5.2 Evidence for TMEM16F expression by human platelets and cell lines. A) Immunoblot detection of TMEM16F protein from whole cell lysates of native HEK-293, HEL and Meg-01 cell lines and primary human platelets. A strong band in the region of 75 to 100 kDa was observed for all lysates using a TMEM16F antibody raised against an intracellular epitope (predicted molecular weight 106 kDa). B) ICC using the same antibody shows strong fluorescence at the periphery of native HEK-293, HEL cells and platelets. HEL and platelet samples also show some cytosolic staining. No fluorescence was detected for secondary antibody only controls for each cell type tested. Scale bars represent 5 µm and data are representative of three independent experiments.
5.1F). It is unlikely that these remaining currents represent Na\(^+\) permeability through \(K_{Ca}\)3.1 given the low \(P_{Na}/P_K\) values obtained following Na\(^+\) substitution for K\(^+\), even at low [K\(^+\)]\(_i\) or [K\(^+\)]\(_o\). The remaining currents in K\(^+\)-free salines reversed close to 0mV and thus could represent a Na\(^+\) or Cl\(^-\)-permeable channel or indeed a totally non-selective channel. It is possible that TMEM16F is partly responsible for the currents observed in symmetrical NaCl salines, however it is unclear how effective ionomycin is in achieving the high, sustained increase of [Ca\(^{2+}\)] required to activate this conductance.

### 5.2.2 TMEM16F is expressed by both HEL cells and human platelets

TMEM16F channels are ubiquitously expressed (Schreiber et al., 2010) and have recently been studied in MKs (Yang et al., 2012) and platelets isolated from a Scott syndrome patient (van Kruchten et al., 2013). These channels are Ca\(^{2+}\)-dependent and outwardly rectifying in pseudophysiological solutions, which is comparable to the currents observed in Fig. 5.1F. Western blot detection of TMEM16F from mouse dendritic cells has been shown using a polyclonal antibody raised against an intracellular epitope (Szteyn et al., 2012). Thus, this antibody was used to assess TMEM16F expression by human platelets, native HEK-293 cells and myeloid cell lines (HEL and Meg-01) by Western blot. A strong band was detected for all cell types between 75 and 100 kDa (Fig. 5.2A), corresponding to the predicted molecular weight of TMEM16F (≈106 kDa). An additional lower band was observed for native HEK-293, HEL and Meg-01 cell lines but not platelet lysates. A similar finding was described in Chapter 3 for Panx1 channels whereby both unglycosylated and glycosylated species were detected from cell lines, whereas platelets only expressed the fully glycosylated species (Fig. 3.2). Six putative N-linked glycosylation sites have been identified within the TMEM16F sequence, however, it is not yet known whether these proteins are glycosylated (Suzuki et al., 2013). Related TMEM16A channels have been shown to be glycosylated on their final extracellular loop at N854; a site that is conserved between all TMEM16 family members (Fallah et al., 2011, Hartzell et al., 2009). Treatment with the glycosidase PNGaseF did not result in a decrease of the apparent molecular weight of TMEM16F (data not shown). Thus, it is likely that this assay will require development to assess the glycosylation status of TMEM16F proteins.
Figure 5.3 HEL cells express functional TMEM16F-like channels. Whole cell patch recordings from HEL cells. A) Pipette and bath solutions contained 150 mM NaCl and voltage ramps were applied to cells when the [Ca^{2+}]_i was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM Ca^{2+} pipette solutions (F). Voltage steps (Gii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA, 1 and 100 µM [Ca^{2+}]_i experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero-current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density and are representative of five independent experiments.
TMEM16F channel expression was investigated further using ICC. These experiments revealed strong fluorescence at the periphery of native HEK-293 and HEL cells, whilst platelets had both peripheral and diffuse cytosolic staining (Fig. 5.2B). These data are indicative of TMEM16F channel expression along the plasma membrane of these cells. Importantly, no fluorescence was observed in the secondary antibody control for each cell type (Fig. 5.2B). Comparable Western blot and ICC results were obtained using a second TMEM16F antibody raised against an extracellular epitope (tables 2.1-2; data not shown).

5.2.3 Currents in HEL cells during dialysis with salines set at different Ca\textsuperscript{2+} concentrations

The data in Fig. 5.1F suggest that HEL cells express more than one Ca\textsuperscript{2+}-activated channel that persists in K\textsuperscript+-free recording solutions. TMEM16F Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels are a candidate for this conductance. K\textsubscript{Ca}3.1 currents are maximally activated by 1 µM [Ca\textsuperscript{2+}]\textsubscript{i} (Mahaut-Smith and Schlichter, 1989, Grissmer et al., 1993), whereas TMEM16F channels require considerably greater levels of [Ca\textsuperscript{2+}]\textsubscript{i} for activation, with a reported EC\textsubscript{50} ≈100 µM (Grubb et al., 2013). Thus, Ca\textsuperscript{2+}-activated currents were further assessed by dialysis with pipette salines containing a mixture of EGTA and CaCl\textsubscript{2} in which the Ca\textsuperscript{2+} was set at three different levels: very low (no added Ca\textsuperscript{2+}, estimated free Ca\textsuperscript{2+} of ≈5nM), 1 µM or 100 µM (see Chapter 2 for further details). Given that activation of TMEM16F currents requires prolonged exposure (>5 min) to a high [Ca\textsuperscript{2+}]\textsubscript{i} environment, voltage ramps were applied for up to ten minutes after transition to the WC configuration. Currents over this time course were initially recorded with symmetrical NaCl (K\textsuperscript+-free) recording solutions (Fig. 5.3A). Voltage ramps applied to cells dialysed with EGTA activated small outwardly rectifying currents, which were stable across the ten minute recording period (+120mV; 3.9 ± 1.1 pA pF\textsuperscript{-1} at t=0 vs 2.1 ± 0.3 pA pF\textsuperscript{-1} at t=10, Fig. 5.3B, P > 0.05, n=5). In the presence of 1 and 100 µM [Ca\textsuperscript{2+}]\textsubscript{i}, outward currents at t=0 min were significantly larger than in the Ca\textsuperscript{2+}-free condition, with peak amplitudes at +120mV of 10.1 ± 1.5 and 13.3 ± 2.0 pA pF\textsuperscript{-1}, respectively (Fig. 5.3B, P < 0.001, n=5). With 1 and 100 µM Ca\textsuperscript{2+} in the pipette, outward currents declined with time; at t=5 min they were 4.4 ± 1.3 and 6.5 ± 1.2 pA pF\textsuperscript{-1}, respectively (P <
0.05, n=5, Fig. 5.3). This reduction likely represents the dialysis of intracellular K+ and subsequent loss of the K\textsubscript{Ca}3.1-like currents. The loss of K+ currents was calculated for the 100 µM Ca\textsuperscript{2+} condition and occurred after ≈3 min (171.4 ± 15.7 s) of dialysis with the pipette solution (Fig. 5.7B left panel). There were no further changes observed to these currents within the time course monitored. TMEM16F currents are known to be completely blocked by 20 µM CaCC\textsubscript{inh}-A01 (A01) (De La Fuente et al., 2008, van Kruchten et al., 2013). Thus, this compound was used to assess contributions by TMEM16F to the currents recorded in Fig. 5.3B. Currents recorded with the Ca\textsuperscript{2+}-free (1mM EGTA) pipette saline were unaffected by A01 across the t=10 min time course (P > 0.05, n=5, Fig. 5.3B-D). However, 1 µM [Ca\textsuperscript{2+}]-evoked currents were reduced from 10.1 ± 1.5 to 4.8 ± 1.3 pA pF\textsuperscript{-1} by A01 at t=0 (P < 0.05, n=5). The cause of this reduction is unclear; however, this effect was lost at later time points (Fig. 5.3E). 100 µM [Ca\textsuperscript{2+}]-evoked currents were blocked by A01 at each time point shown: t=0 (13.3 ± 1.5 vs 2.1 ± 0.5 pA pF\textsuperscript{-1}, P < 0.001, n=5), t=5 (6.5 ± 1.7 vs 1.7 ± 0.4 pA pF\textsuperscript{-1}, P < 0.05, n=5); t=10 (6.7 ± 1.8 vs 1.8 ± 0.4 pA pF\textsuperscript{-1}, P < 0.05, n=5, Fig. 5.3D).

At t=10+ min voltage steps (Fig. 5.3G) were applied to the cells in the presence of vehicle control (0.04% DMSO, Fig. 5.3H) or A01 (Fig. 5.3I). With no added Ca\textsuperscript{2+} (EGTA) in the pipette, small A01-resistant currents with mild outward rectification were recorded (Fig. 5.3H-J left panels). Elevation of [Ca\textsuperscript{2+}]: to 1 and 100 µM gave currents with intermediate and strong outward rectification, respectively (Fig. 5.3K,L). Application of A01 reduced 100 µM [Ca\textsuperscript{2+}]-evoked currents at +120mV from 14.1 ± 2.3 pA pF\textsuperscript{-1} to 2.0 ± 0.4 pA pF\textsuperscript{-1}, representing an 85.8% decrease (P < 0.001, n=5, Fig. 5.3H,I,L right panel). Currents in the presence of 1 µM Ca\textsuperscript{2+} were unaffected by application of A01 (P > 0.05, n=5, Fig. 5.3K). With 100 µM Ca\textsuperscript{2+} in the pipette currents reversed at -1.7 ± 0.3 and -3.2 ± 0.8mV, under vehicle control and A01-treated conditions, respectively (P > 0.05, n=5, Fig. 5.7D left panel). In these experiments both E\textsubscript{Na} and E\textsubscript{Cl} are 0mV, thus, these data may represent either a Na\textsuperscript{+} or Cl\textsuperscript{-} conductance.

5.2.4 Currents induced by 1 µM intracellular Ca\textsuperscript{2+} are not dependent on pannexin-1

Figs. 3.1 and 3.2 demonstrate that Panx1 is expressed by HEL cells and
**Figure 5.4** Ca²⁺-activated currents are not dependent on pannexin-1. Whole cell patch clamp recordings from HEL cells. A) Pipette and bath solutions contained 150 mM NaCl and voltage ramps were applied to cells when [Ca²⁺]ᵢ was set to 0 (B) or 1 µM (C). Summary data are shown for voltage ramps across a ten minute time course in the presence of either vehicle control (0.04% DMSO; black trace), Prb (100 µM; red trace) or Cbx (10 µM; blue trace). Peak inward (-120mV) and outward (+120mV) currents are shown for control (black), Prb- (red) and Cbx-treated (blue) cells, in the presence of either EGTA (D) or 1 µM [Ca²⁺]ᵢ (E). Fii) Voltage steps were applied to cells at t=10+ min. Representative traces are shown for 1mM EGTA (G) and 1 µM [Ca²⁺]ᵢ experiments (H) performed in the presence of either control (squares), Cbx (traingles) or Prb (diamonds); arrowheads indicate zero-current level. I-J) summary I-V relationships for in response to voltage steps in the presence of EGTA or 1 µM [Ca²⁺]. Statistical significance was calculated using an ANOVA. Data are normalised for current density and are representative of five (control and Prb) and four (Cbx) independent experiments.
primary mouse and rat MKs. Panx1 channels have been shown to open in response to physiological elevations of $[\text{Ca}^{2+}]_i$ (Locovei et al., 2006b) and may therefore contribute to the currents recorded in response to 1 µM $[\text{Ca}^{2+}]_i$ (Fig. 5.3). This hypothesis was tested by use of the Panx1 inhibitors Cbx (10 µM) and Prb (100 µM). Currents in the presence of these inhibitors were compared to those obtained in the presence of vehicle control for Fig. 5.3B. In response to voltage ramps, Prb reduced currents recorded with EGTA and 1 µM Ca$^{2+}$ pipette solutions to $1.6 \pm 0.2$ and $2.8 \pm 0.3$ pA pF$^{-1}$, respectively ($P < 0.001$, n=5); however this effect was lost at later time points (Fig. 5.4B-E).

Interestingly, Cbx had no effect on the currents recorded at each time point ($P > 0.05$, n=5, Fig. 5.4B-E). Moreover, neither Cbx nor Prb affected currents recorded in the presence of EGTA or 1 µM Ca$^{2+}$ in response to voltage steps at t=10+ min ($P > 0.05$, n=4 [Cbx], n=5 [Prb], Fig. 5.4G-J). These findings suggest that Panx1 channels may contribute to currents at t=0 min but are redundant at later time points.

### 5.2.5 Currents activated by 100 µM intracellular Ca$^{2+}$ in HEL cells are permeable to anions

Experiments conducted with equimolar NaCl solutions revealed a Ca$^{2+}$-activated current that was outwardly rectifying and A01-sensitive (Fig. 5.3), which is consistent with the biophysical and pharmacological properties of TMEM16F channels (Grubb et al., 2013). Currents carried by TMEM16F ion channels are anion-selective (Pedemonte and Galietta, 2014), although a recent study reported that these channels are cation-selective in mouse MKs (Yang et al., 2012). Thus, the ionic selectivity of the Ca$^{2+}$-activated, A01-sensitive currents in HEL cells was assessed by reducing $[\text{Cl}^-]_o$ to 5 mM by equimolar gluconate substitution. Under these conditions $E_\text{Na} = 0$ mV and $E_\text{Cl} = +85.7$ mV. Voltage ramps applied to cells at t=0 min yielded outwardly rectifying currents for all three pipette solutions (Fig. 5.5B). These currents reversed close to -60mV and gradually declined in amplitude over ≈2.5 minutes (147.0 ± 14.5s; Figs. 5.5B and 5.7B centre panel), which is consistent with the loss of $K_{Ca3.1}$-like currents described in Fig. 5.3. For cells dialysed with 100 µM Ca$^{2+}$, a linear current that reversed direction close to +40mV was activated ≈7 min (417.0 ± 18.6s) after transition to the WC configuration (Figs. 5.5B left panel and 5.7C).
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Figure 5.5 TMEM16F currents are anion-selective. Whole cell patch clamp recordings from HEL cells. Ai) Pipette solutions contained 150 mM NaCl, whilst [Cl\textsubscript{o}] was reduced to 5 mM by equimolar substitution of Na-gluconate. Voltage ramps (Aiii) were applied to cells when the [Ca\textsuperscript{2+}]\textsubscript{i} was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCC\textsubscript{inh}-A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM [Ca\textsuperscript{2+}]\textsubscript{i} (F). Voltage steps (Gii) were applied to the cells at t=10+ min. Representative traces are shown for 1mM EGTA, 1 and 100 µM [Ca\textsuperscript{2+}] experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero-current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density. Control data are representative of four (EGTA) and five (1 and 100 µM) cells, whilst A01 data are representative of three, five and four cells for EGTA, 1 and 100 µM Ca\textsuperscript{2+} pipette solutions, respectively.
centre panel). For cells treated with A01, 100 µM [Ca^{2+}]-induced currents were abrogated across the entire voltage range. This effect was most pronounced at -120mV where inward currents were reduced from 5.2 ± 0.8 to 0.6 ± 0.2 pA pF⁻¹, representing an 88.5% decrease \((P < 0.001, \text{n}=5 \text{ [control]} \text{ and } \text{n}=4 \text{ [A01]}\), Fig. 5.5B,C,F right panels). These data support the hypothesis that the currents recorded in the presence of 100 µM Ca^{2+} are carried by a TMEM16F-like conductance.

To further investigate Ca^{2+}-activated currents, 1s duration voltage steps (-60mV holding to -120 up to +120mV) were applied to each cell at t=10+ min (Fig. 5.5G). In response to this protocol, small A01-resistant currents with a linear I-V relationship were recorded with EGTA and 1 µM Ca^{2+} pipette solutions \((P > 0.05, \text{n}=3 \text{ [EGTA]}, \text{n}=5 \text{ [1 µM Ca^{2+}]}\), Fig. 5.5H-K left and centre panels).

Elevation of [Ca^{2+}] to 100 µM led to the activation of a linear conductance that reversed at +37.2 ± 2.1mV \((\text{n}=5, \text{Figs. 5.5H,L right panels and 5.7D centre panel})\). These currents were virtually abolished at each test potential upon application of A01, in agreement with data collected in response to voltage ramps. For example, inward currents at -120mV were reduced by 86.9% from 6.1 ± 0.9 to 0.8 ± 0.3 pA pF⁻¹, whilst outward currents at +120mV decreased by 65.3% from 4.9 ± 1.5 to 1.7 ± 0.6 pA pF⁻¹ \((P < 0.01, \text{n}=5 \text{ [control]} \text{ and } \text{n}=4 \text{ [A01]}\), Fig. 5.5H,I,L right panels). Furthermore, A01 shifted \(E_{\text{rev}}\) for 100 µM [Ca^{2+}]-induced currents by -38.7mV to -1.5 ± 0.6mV \((P < 0.001, \text{n}=4, \text{Figs. 5.5L and 5.7D centre panel})\).

\(E_{\text{rev}}\) for currents induced by 100 µM [Ca^{2+}] was +37.2 ± 2.1mV in NaGluconate bath saline and -1.7 ± 0.3mV in symmetrical NaCl salines. This shift is smaller than the +85.7mV predicted for a Cl⁻-selective conductance. This difference may be explained by either an underlying NSCC or a significant permeability to the substitute anion, gluconate⁻. This smaller than predicted shift in \(E_{\text{rev}}\) has been reported previously for TMEM16F conductances recorded from B lymphocytes (Kmit et al., 2013). In addition, studies of reconstituted TMEM16 proteins concluded that the channel pore is sufficiently large enough to permeate both gluconate⁻ and NMDG⁺ (Malvezzi et al., 2013). Overall, however the above data are indicative of a Ca^{2+}-activated Cl⁻-permeable conductance within HEL cells.
Figure 5.6 TMEM16F currents persist in the absence of external Na⁺. Whole cell patch clamp recordings of HEL cells. Ai) pipette solutions contained 150 mM NaCl, whilst the bath solution contained 5 mM Cl⁻, 145 mM gluconate⁻ and 150 mM NMDG⁺. Voltage ramps (Aii) were applied to cells when the [Ca²⁺]i was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCCₐₙ₉₋ₐ₀₁ (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM [Ca²⁺]: (F). Voltage steps (Gii) were applied to the cells at t=10+ min. Representative traces are shown for 1mM EGTA, 1 and 100 µM [Ca²⁺] experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero-current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Data are normalised for current density. Statistical significance was calculated using an ANOVA. Data are normalised for current density and are representative of four independent experiments for each condition.
To assess whether currents activated by 100 µM Ca$^{2+}$ are influenced by an underlying NSCC conductance, all [Na$^+$]$_o$ was substituted by equimolar NMDG$^+$, whilst maintaining [Cl$^-$]$_o$ at 5mM. Given that lowering of [Cl$^-$]$_o$ did not shift $E_{\text{rev}}$ for currents recorded with 1 µM [Ca$^{2+}$], these currents were not investigated further. In response to voltage ramps, currents recorded from cells dialysed with EGTA alone were small and A01-resistant; comparable to data obtained in parallel experiments performed in symmetrical NaCl recording solutions (Fig 5.3B-D). Furthermore, currents recorded in response to voltage steps applied to these cells at t=10+ min were unaffected by application of A01. For comparison, peak currents at +120mV were 1.1 ± 0.5 and 2.9 ± 0.8 pA pF$^{-1}$ under control and A01-treated conditions, respectively ($P > 0.05$, n=4, Fig. 5.6G-I left panels). Dialysis of cells with 100 µM Ca$^{2+}$ induced large currents with weak inward rectification. These currents initiated ≈6.5 min (401.3 ± 31.5s) after transition to the WC configuration and reversed direction close to +40mV (Figs. 5.6B and 5.7C right panels). Incubation of cells with A01 substantially reduced 100 µM [Ca$^{2+}$]-induced currents across the voltage range. The greatest effect was observed at t=10 min where inward currents at -120mV were reduced by 92.8% from 8.3 ± 2.1 to 0.6 ± 0.5 pA pF$^{-1}$ ($P < 0.01$, n=4, Fig. 5.6B,C,E right panels). In response to voltage steps, 100 µM [Ca$^{2+}$]-induced currents displayed strong inward rectification and reversed at +40.4 ± 3.9mV (Figs. 5.6G,J and 5.7D right panels). Incubation with A01 abrogated these currents at each test potential. For example inward currents at -120mV were reduced by 80.6% from 9.3 ± 1.8 to 0.9 ± 0.5 pA pF$^{-1}$ ($P < 0.001$, n=4), whereas outward currents at +120mV were decreased by 76.1% from 4.6 ± 0.9 to 1.1 ± 0.5 pA pF$^{-1}$ ($P < 0.01$, n=4, Fig. 5.6G,H,J right panels). In the presence of A01 100 µM [Ca$^{2+}$]-induced currents reversed at -4.8 ± 0.6mV ($P < 0.001$, n=4, Figs. 5.6H,J and 5.7D right panels).

Given that $E_{\text{rev}}$ was unchanged in NMDG-gluconate (+40.4 ± 3.9 mV) vs NaGluconate (+37.2 ± 2.1 mV) bath solutions ($P > 0.05$, n=5 [Na-gluconate] n=4 [NMDG-gluconate], Fig. 5.7D), these data suggest that the smaller than predicted shift of $E_{\text{rev}}$ for a Cl$^-$-selective conductance is not due to an underlying NSCC. Thus, these data indicate that HEL cells express an anion-permeable channel that is highly Ca$^{2+}$-dependent and A01-sensitive.
Figure 5.7 Biophysical properties of 100 µM Ca\(^{2+}\)-activated currents. A) I-V relationships for 100 µM Ca\(^{2+}\)-evoked currents (EGTA-subtracted) in response to voltage steps. Data shown are from experiments where the bath solution contained, in mM: 150 NaCl (left), 150 Na\(^+\), 5 Cl\(^-\) (centre) and 150 NMDG, 5 Cl\(^-\) (right) under control (circles) and A01-inhibited (squares) conditions. The time taken for initial K\(^+\) currents to dialyse (B) and TMEM16F currents to activate (C) were calculated from voltage ramp data under control and A01-treated conditions. D) The point where EGTA and 100 µM Ca\(^{2+}\) currents in response to voltage steps intersect under control and A01-treated conditions was taken as the reversal potential for Ca\(^{2+}\)-activated currents. Statistical significance was assessed using an ANOVA (A) or student’s paired t-Test (B-D).
In summary, $K_{Ca3.1}$-like currents had similar profiles in each bath solution (NaCl, Na-gluconate and NMDG-gluconate) and the time course for $K^+$ current dialysis from HEL cells was unaffected by application of A01 (Fig. 5.7B). Initiation of the 100 µM $[Ca^{2+}]_i$-induced conductance was calculated from the voltage ramp data as the point where either outward (150 mM $[Cl^-]_o$) or inward (5 mM $[Cl^-]_o$) currents were first observed. These data demonstrate that 6-7 min exposure to 100 µM $[Ca^{2+}]_i$ is required to activate this conductance (Fig. 5.7C); which is consistent with a previous study of TMEM16F currents recorded from HEK-293 cells (Grubb et al., 2013). Moreover, activation of this conductance in low $[Cl^-]_o$ bath solutions shifted $E_{rev}$ by ≈+40mV, suggesting that it is predominantly carried by $Cl^-$ (Fig. 5.7D). In the presence of A01 the 100 µM $[Ca^{2+}]_i$-induced conductance failed to activate and currents reversed close to 0mV (Fig. 5.7C,D). Taken together these data are consistent with the activation of an anion-permeable TMEM16F-like conductance in HEL cells.
5.3 Discussion

Data presented within this chapter have focussed on the biophysical properties of Ca\(^{2+}\)-activated ion channels expressed by HEL cells. This cell line, which expresses both erythroid and megakaryocytic markers, was derived from the bone marrow of a leukaemia patient (Martin and Papayannopoulou, 1982, Tabilio et al., 1984). These cells have been used previously for patch clamp studies of ion channels expressed by platelets and MKs (Kapural et al., 1995, Somasundaram et al., 1997, Mahaut-Smith, 2012, Stoneking et al., 2013). Experiments in this Chapter have identified a highly Ca\(^{2+}\)-dependent, A01-sensitive Ca\(^{2+}\)-activated ion channel with biophysical and pharmacological properties consistent with those described for TMEM16F channels (Grubb et al., 2013). Taken together with Western blot and ICC data, these recordings represent the first report of functional TMEM16F channels in HEL cells.

5.3.1 \(\text{K}_{\text{Ca3.1}}\)

HEL cells proved a useful tool for the evaluation of Ca\(^{2+}\)-activated currents in human cells of myeloid origin given previous evidence for \(\text{K}_{\text{Ca3.1}}\)-like channels in this cell line (Lu et al., 1999, Stoneking and Mason, 2013). Stimulation of HEL cells by ionomycin yielded a highly K\(^+\)-selective, Ca\(^{2+}\)-activated current with biophysical properties typical of \(\text{K}_{\text{Ca3.1}}\) channels (Fig. 5.1). Currents in a thirty fold K\(^+\) gradient gave \(P_{\text{Na}}/P_{\text{K}} = 0.002\), in agreement with a previous report of lymphocyte \(\text{K}_{\text{Ca3.1}}\) currents (Grissmer et al., 1993). Furthermore, ionomycin-evoked currents reversed at potentials close to those predicted for a K\(^+\)-selective conductance across a range of K\(^+\) gradients (Fig. 5.1E). \(\text{K}_{\text{Ca3.1}}\) currents could also be induced by setting [Ca\(^{2+}\)]\(_i\) at 1 or 100 µM. With 150 mM NaCl in the pipette \(\text{K}_{\text{Ca3.1}}\) currents current amplitudes were comparable in the presence of both 1 and 100 µM [Ca\(^{2+}\)]\(_i\): (Fig. 5.3), in agreement with previous reports that these currents are maximally activated by 1 µM [Ca\(^{2+}\)]\(_i\): (Mahaut-Smith and Schlichter, 1989, Grissmer et al., 1993). These currents were lost within 3-4 min (Fig. 5.7B), which likely represents dialysis of intracellular K\(^+\). Furthermore, \(\text{K}_{\text{Ca3.1}}\)-like currents were not affected by the TMEM16F inhibitor A01 (Figs. 5.3 and 5.5-6). In some cases, currents typical of \(\text{K}_{\text{Ca3.1}}\) were observed when using EGTA-buffered pipette solutions (Fig. 5.5). Activation of \(\text{K}_{\text{Ca3.1}}\) channels in these experiments is most likely via spontaneous activation
of Ca\(^{2+}\) release and/or SOCE by the patch clamp recording process, leading to a rise of [Ca\(^{2+}\)]; before complete dialysis of the cell with EGTA.

5.3.2 Pannexin-1

ICC and Western blot experiments in Chapter 3 demonstrated that HEL cells express Panx1 channels (Figs. 3.1 and 3.2). These channels have been shown to open in response to a rise of [Ca\(^{2+}\)] (Locovei et al., 2006b) and may therefore contribute to the 1 µM [Ca\(^{2+}\)]-activated currents observed in Fig. 5.3. However, currents in symmetrical NaCl with 1 µM [Ca\(^{2+}\)] at t=0 min were only modestly reduced by incubation with Prb and were resistant to Cbx (Fig. 5.4); suggesting that there was only a small or insignificant contribution by Panx1 to these currents. Furthermore, there was no shift of reversal potential for 1 µM [Ca\(^{2+}\)]-activated currents when [Cl\(^-\)]\(_o\) was lowered; indicating that these currents are cation- rather than anion-selective (Fig. 5.4). Panx1 currents have been recorded from a variety of cell types using both patch clamp (mammalian cells) and two electrode voltage clamp (Xenopus laevis oocytes). However, a large variation exists between the experimental paradigms used to record Panx1 currents. For example, recordings of Panx1 currents from oocytes have utilised voltage step protocols that range in duration from 2s (Bruzzone et al., 2005) to 70s (Suadicani et al., 2012). However, studies of Panx1 currents from mammalian cells have used voltage ramps (Zhan et al., 2012), voltage steps (Poornima et al., 2011) or a combination of the two (Bunse et al., 2011). Furthermore, the rate of voltage ramps ranges from 0.015 (Zhan et al., 2012) to 0.8 V s\(^{-1}\) (Bunse et al., 2011). In light of this variation, a standardised protocol for recording Panx1 currents from neuronal cells has been suggested (Grundken et al., 2011). This protocol suggests that preconditioning voltage ramps are required to record stable Panx1 currents. Thus, it is possible that the protocols employed in the present chapter are not optimal for recording Panx1 currents.

5.3.3 TMEM16F

Western blot and ICC data show that HEL cells express TMEM16F channels at their periphery (Fig. 5.2), which is supported by recordings of membrane currents with TMEM16F-like properties: Ca\(^{2+}\)-dependent, delayed activation and
outwardly rectifying (Figs. 5.1 and 5.7; for comparison see (Szteyn et al., 2012, Grubb et al., 2013)). The Ca\(^{2+}\)-dependence of these TMEM16F-like currents was assessed using either ionomycin or Ca\(^{2+}\)-containing pipette solutions. Consistent with a previous report of TMEM16F currents from mouse dendritic cells (Szteyn et al., 2012), ionomycin-evoked currents in symmetrical NaCl solutions were inactivated by removal of extracellular Ca\(^{2+}\) (Fig. 5.1F). WC recordings have shown that TMEM16F channels are activated by elevation of [Ca\(^{2+}\)] with an EC\(_{50}\) = 105.6 ± 1.2 µM (Grubb et al., 2013). In agreement with this report, currents typical of TMEM16F were observed when [Ca\(^{2+}\)] was raised to 100 µM, but not 1 µM (Fig. 5.3). Furthermore, 100 µM Ca\(^{2+}\)-activated currents were abrogated by A01, a TMEM16F inhibitor (Fig. 5.7). This compound was identified during a screen for inhibitors of the endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channel and was shown to block TMEM16F channels with an IC\(_{50}\) = 10 µM (De La Fuente et al., 2008). Finally, in agreement with the reported time-dependent activation of TMEM16F channels (Grubb et al., 2013), TMEM16F-like currents initiated ≈6 min after the onset of dialysis with the 100 µM Ca\(^{2+}\) pipette solution (Fig. 5.7). Taken together, these data provide evidence for the expression of functional TMEM16F channels at the plasma membrane of HEL cells, which are activated by sustained elevation of [Ca\(^{2+}\)] to 100 µM.

It was shown recently that TMEM16F channels form small conductance NSCCs in primary mouse MKs (Yang et al., 2012). This report is controversial as studies of TMEM16F channels from lymphocytes, dendritic cells and HEK-293 cells have all shown that these channels are anion-selective (Szteyn et al., 2012, Grubb et al., 2013, Kmit et al., 2013). Thus, ion substitution experiments were performed to determine whether HEL TMEM16F currents are anion- or cation-selective. Substitution of [Cl\(^{-}\)]\(_{o}\) by gluconate\(^{-}\) shifted the reversal potential of 100 µM [Ca\(^{2+}\)]-induced currents to ≈+40mV, indicating that these currents are predominantly permeable to anions (Figs. 5.5 and 5.7). This shift was smaller than predicted for a Cl\(^{-}\) selective current (+85.7mV), which could be explained either by activation of another channel(s) or by significant permeability to the substituted anion. A recent study of reconstituted TMEM16 channels reported that the ion pore is 4-6.5 Å in diameter, which is sufficiently large enough to permeate both gluconate\(^{-}\) and NMDG\(^{+}\) (Malvezzi et al., 2013). Thus, these large
ions may influence $E_{\text{rev}}$ in these experiments (Fig. 5.7). In addition, a study of TMEM16F currents from human lymphocytes reported a similar shift of $E_{\text{rev}}$ to $\approx +40\text{mV}$ in response to lowering $[\text{Cl}^-]_o$ (Kmit et al., 2013). Contributions by an underlying NSCC to the 100 µM $[\text{Ca}^{2+}]_i$-induced currents were tested by substitution of extracellular Na$^+$ by NMDG$^+$; in these experiments there was no further shift of $E_{\text{rev}}$ (Figs. 5.6 and 5.7). Thus, these data demonstrate that HEL cells express anion-permeable TMEM16F-like channels that have low permeability to cations.

Data presented in this chapter demonstrate that HEL cells express functional $\text{Ca}^{2+}$-activated $K^+$ and $\text{Cl}^-$ channels consistent with the properties of $K_{\text{Ca}3.1}$ and TMEM16F channels; which can be activated either by ionomycin or clamping $[\text{Ca}^{2+}]_i$ at 1 or 100 µM. These represent the first recordings of anion-permeable TMEM16F-like currents from HEL cells. The HEL cell line, derived from a leukaemia patient, expresses both erythroid and megakaryocytic markers, but it is important to note that ion channel expression can be markedly altered in myeloid cell lines (Kapural et al., 1995). Consequently, the aim of Chapter 6 is to investigate the biophysical properties of TMEM16F channels in primary mouse and rat MKs utilising the approaches developed in the present chapter.
Chapter 6

6.0 Electrophysiological characterisation of Ca\(^{2+}\)-activated ion channels from primary mouse and rat megakaryocytes

6.1 Background

Through a combination of electrophysiological and biochemical techniques, experiments in Chapter 5 provided the first evidence that HEL cells express functional TMEM16F channels. This protein is clearly important for platelet function as both homozygous and compound heterozygous mutations of the TMEM16F gene result in the bleeding diathesis Scott syndrome (Suzuki et al., 2010, Castoldi et al., 2011). This disease is extremely rare with three patients reported worldwide and is characterised by defective Ca\(^{2+}\)-dependent phospholipid scrambling (Kunzelmann et al., 2014). Furthermore, patch clamp recordings demonstrated that B lymphocytes, derived from a Scott syndrome patient, lack a Ca\(^{2+}\)-activated Cl\(^{-}\) conductance, which was identified as TMEM16F (Kmit et al., 2013). Recently, further evidence that Cl\(^{-}\) channels contribute to the procoagulant response of human platelets has been provided using channel blockers and altering [Cl\(^{-}\)]\(_o\) (Harper and Poole, 2013).

Hyperpolarisation of platelets and Dami cells upon stimulation by thrombin requires functional Cl\(^{-}\) channels (Sullivan et al., 1998, Harper and Poole, 2013). In the latter study, experiments were restricted to the use of potentiometric dyes and inhibitors. Thus, the identity and biophysical properties of the Cl\(^{-}\) channels could not be determined. The gold standard technique for the study and characterisation of ion channels is patch clamp electrophysiology. Within our laboratory, Ca\(^{2+}\)-activated Cl\(^{-}\) channel currents have been recorded from human platelets (Mahaut-Smith, 1990, MacKenzie and Mahaut-Smith, 1996). These channels were shown to activate in response to raising [Ca\(^{2+}\)] between 0.3-10 \(\mu\)M. Although the molecular identity of the channels in these studies remains unclear, Panx1 channels and TMEM16 family members are potential candidates (Chapter 1.7). Given the small and fragile nature of platelets, patch clamp recordings from these cells are technically challenging. However, platelet progenitor cells, MKs, have been shown to express many of the same channels and receptors as platelets (Tolhurst, 2006, Mahaut-Smith, 2012). Thus, these
cells are commonly used as a model to study the electrophysiological properties of platelet ion channels.

TMEM16F channels have a ubiquitous expression pattern (Schreiber et al., 2010) and have been studied in a variety of tissues by use of TMEM16F-deficient mice and pharmacological inhibitors (Pedemonte and Galietta, 2014). Patch clamp recordings of TMEM16F currents from human and mouse cells have shown that these form Ca\(^{2+}\)-activated Cl\(^-\) channels (Szteyn et al., 2012, Grubb et al., 2013). Surprisingly, excised inside-out patch clamp recordings of mouse MKs reported the expression of cation-selective TMEM16F channels from these cells (Yang et al., 2012). It was proposed that this difference was due to mutations within the pore region of TMEM16F channels formed by mouse MKs (Yang et al., 2012). However, this study remains controversial and to date there have not been any further attempts to record Ca\(^{2+}\)-activated TMEM16F currents from MKs.

### 6.1.1 Aims

Work presented in this chapter focusses on identifying the biophysical properties and ion selectivity of Ca\(^{2+}\)-activated ion channels expressed by mouse and rat MKs; using the patch clamp protocols developed in Chapter 5 that characterised TMEM16F currents in HEL cells. The expression and localisation of TMEM16F channels within primary MKs is also assessed by ICC.
Figure 6.1 TMEM16F channels are expressed by both mouse and rat megakaryocytes. TMEM16F expression in primary rat (A) and mouse (B) MKs was assessed by ICC using a TMEM16F antibody, raised against an intracellular epitope. Cells were permeabilised with 0.1% triton X-100. The fluorescence intensity (FI) along the dashed line is shown for both rat (C) and mouse (D) MKs. These show that TMEM16F staining is most intense at the periphery of these cells, with more diffuse staining within the cytosol. In parallel, there was no fluorescence detected from cells incubated with only the secondary antibody. Scale bars represent 10 µm and data are representative of three independent experiments.
6.2 Results

6.2.1 Megakaryocytes express TMEM16F ion channels

Western blot and ICC experiments in Chapter 5 revealed expression of TMEM16F channels at the periphery of human platelets (Fig. 5.2). In the present chapter, ICC studies were performed to assess TMEM16F expression by primary mouse and rat MKs, using the same intracellular epitope antibody employed in Chapter 5. MKs were fixed and permeabilised and could be distinguished from other bone marrow cells due to their large diameter (Fig. 6.1). Strong fluorescence was observed at the periphery of MKs isolated from both mouse and rat bone marrow (Fig. 6.1A,B). Line plots of fluorescence intensity (FI) across these cells show that staining is predominantly along their periphery, however, fluorescence was also detected within the cytoplasmic volume (Fig. 6.1C,D). The latter may be due intracellular localisation of the target protein or its presence on the DMS, which runs through this region (Mahaut-Smith et al., 2003). Importantly, there was no fluorescence detected within the secondary antibody only controls (Fig. 6.1).

6.2.2 Ca\(^{2+}\)-activated currents from primary rat megakaryocytes

The majority of MK patch clamp recordings have been performed using cells isolated from rat (Uneyama et al., 1993, Romero and Sullivan, 1997, Thomas et al., 2001, Mahaut-Smith et al., 2003) compared to mouse (Kawa, 1996, Carter et al., 2006); which is, in part, due to the higher yield of MKs from rat vs mouse bone marrow (Mahaut-Smith, 2004). Thus, rat MKs were used to assess currents in the nominal absence of intracellular Ca\(^{2+}\) (≈5 nM; 1 mM EGTA) and Ca\(^{2+}\)-activated currents in the presence of low (1 µM) and high (100 µM) [Ca\(^{2+}\)]. MKs are known to express Kv1.3 channels (McCloskey et al., 2010), which are selective for K\(^+\) over other physiologically relevant ions and activated by depolarisation of V\(_m\) ≥-60mV (Maruyama, 1987, Mahaut-Smith et al., 1990a, McCloskey et al., 2010). Thus, NaCl-based recording solutions were used to eliminate K\(^+\) currents. Initially, voltage ramps between -120 and +120mV (0.36 V s\(^{-1}\)) were used to obtain instantaneous I-V relationships (Fig. 6.2A) and thus monitor the time course of voltage-activated and Ca\(^{2+}\)-dependent currents during the first 10 minutes of WC recording. This represents a similar time
Figure 6.2 Rat megakaryocytes express functional TMEM16F-like channels. Whole cell patch clamp recordings from rat MKs. A) Pipette and bath solutions contained 150 mM NaCl and voltage ramps were applied to cells when the $[Ca^{2+}]_i$ was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM $[Ca^{2+}]_i$ (F). Voltage steps (Gii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA, 1 and 100 µM $[Ca^{2+}]_i$ experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density and are representative of five independent experiments.
course used in HEK-293 cells to assess TMEM16F currents, which require sustained elevation (>5 min) of [Ca^{2+}] for activation (Grubb et al., 2013). Voltage ramps applied immediately after conversion to the WC configuration (t=0 min) gave currents typical of Kv1.3 channels (strong voltage-dependent activation within the range -40mV to +120mV; Fig. 6.2B). No significant difference was observed between the currents at the different [Ca^{2+}]. The lack of clear outward K_{Ca}3.1 currents (see Chapter 5) is likely due to the much larger amplitude of Kv1.3 currents compared to K_{Ca} currents in the MK. For all three pipette solutions, Kv1.3-like currents dialysed within ≈3 min, which possibly reflects the loss of intracellular K^+ from these cells (compare I-V relationships in Fig. 6.2B, left and centre panels).

With 100 µM Ca^{2+} in the pipette solution, outwardly rectifying currents, which reversed direction close to 0mV, developed ≈7 min (418.0 ± 17.1s) after transition to the WC configuration (Figs. 6.2B right panel and 6.5C). At t=10 min these currents were significantly larger than those recorded with EGTA and 1 µM Ca^{2+} in the pipette solution (average current densities at +120mV of 13.4 ± 3.2 vs 3.3 ± 0.7 and 5.2 ± 1.9 pA pF^{-1}, respectively for 100µM Ca^{2+}, EGTA and 1µM Ca^{2+} (P < 0.001, n=5, Fig. 6.2B). Currents for both Ca^{2+}-free and 1 µM Ca^{2+} pipette solutions were unaffected by application of the TMEM16F inhibitor A01 (P > 0.05, n=5, Fig. 6.2C-E). In contrast, this compound reduced 100 µM [Ca^{2+}]-induced currents to levels similar to those recorded with low [Ca^{2+}]; (Fig. 6.2B,C,F, right panels). For example at +120mV, A01 reduced currents by 84.3% from 13.4 ± 3.2 to 2.1 ± 0.5 pA pF^{-1} (P < 0.001, n=5, Fig. 6.2C,F).

Voltage steps from a holding potential of -80mV to potentials in the range -120 to +120mV were applied to each MK at t=10+ min, to further assess Ca^{2+}-activated currents (Fig. 6.2G). With EGTA in the pipette solution, small, A01-resistant, currents that reversed close to 0mV were recorded (Fig. 6.2H-J, left panels). Outward currents at +120mV were 4.0 ± 0.8 and 3.1 ± 0.8 pA pF^{-1} under control (0.04% DMSO) and A01-treated conditions, respectively (P > 0.05, n=4, Fig. 6.2H-J left panels). Elevation of [Ca^{2+}] to 1 µM gave small outwardly rectifying currents that reversed at ≈0mV with peak amplitude of 6.3 ± 1.4 pA pF^{-1} (Fig. 6.2 H,K centre panels). These currents decreased by 55.6% to 2.8 ± 0.6 pA pF^{-1} in the presence of A01 (P < 0.001, n=5, Fig. 6.2I,K centre.
panels). Dialysis of MKs with 100 µM Ca\textsuperscript{2+} stimulated a conductance that displayed clear outward rectification at positive potentials. Application of A01 virtually abolished this conductance; at +120mV the current was reduced by 94.7% from 47.3 ± 10.1 to 2.5 ± 0.7 pA pF\textsuperscript{-1} (P < 0.001, n=5, Fig. 6.2H,I,L). The biophysical and pharmacological properties of this Ca\textsuperscript{2+}-activated conductance are similar to those reported for TMEM16F channels in HEK 293 cells (Grubb et al., 2013). In the rat MK, currents activated by 1 and 100 µM Ca\textsuperscript{2+} reversed at ≈0mV (Figs. 6.2 and 6.5D). Given that these recordings were made with symmetrical NaCl solutions, the Ca\textsuperscript{2+}-activated currents may result from Na\textsuperscript{+} and/or Cl\textsuperscript{-} movement.

6.2.2.1 Ca\textsuperscript{2+}-activated currents in the primary rat megakaryocyte are anion-permeable

To investigate the ion selectivity of Ca\textsuperscript{2+}-activated currents from rat MKs, ion substitution experiments were performed. [Cl\textsuperscript{-}]\textsubscript{o} was reduced to 5 mM by equimolar substitution of gluconate\textsuperscript{-} (Fig. 6.3A). As observed for symmetrical NaCl salines, currents typical of Kv1.3 were activated in response to voltage ramps applied immediately after transition to the WC configuration (t=0; Fig. 6.3B). No significant effect of altering [Ca\textsuperscript{2+}]\textsubscript{i} was observed on the initial currents recorded in this set of experiments (P > 0.05, n=5, Fig. 6.3B; the large variation in currents within these experiments may result from different cell volumes and thus variation in the time course of dialysis of both Ca\textsuperscript{2+} into the cytoplasm and exchange of Na\textsuperscript{+} for K\textsuperscript{+}). The Kv1.3-like currents dialysed within ≈3 min; consistent with the time course observed under normal Cl\textsuperscript{-} conditions (Fig. 6.5B left and centre panels). With ≈5 nM (EGTA) or 1 µM Ca\textsuperscript{2+} in the pipette, linear and A01-resistant currents were observed between t=5 to t=10 min (Fig. 6.2B-E). Following ≈6.5 min (393.0 ± 8.8s) of dialysis with the 100 µM Ca\textsuperscript{2+} pipette saline, a conductance developed that displayed larger inward currents than those observed in symmetrical NaCl solutions (Figs. 6.3B right panel and 6.5C centre panel). At t=10 min inward currents at -120mV were 8.3 ± 3.5 pA pF\textsuperscript{-1} (at -120mV) and reversed direction at +35.2 ± 0.9mV (n=5, Fig. 6.3B right panel). Application of A01 substantially decreased 100 µM [Ca\textsuperscript{2+}]-evoked currents across the voltage range and the remaining currents reversed at ≈0mV; for example at -120mV the inward current was reduced by 91.6% from 8.0 ± 3.6 to
Figure 6.3 TMEM16F-like currents from rat megakaryocytes are anion-permeable. Whole cell patch clamp recordings from rat MKs. A) Pipette solutions contained 150 mM NaCl, whilst [Cl]o was reduced to 5 mM by equimolar substitution of Na-gluconate. Voltage ramps (Aii) were applied to cells when the \([\text{Ca}^{2+}]_i\) was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCC\textsubscript{inh}-A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM \([\text{Ca}^{2+}]_i\) (F). Voltage steps (Gii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA, 1 and 100 µM \([\text{Ca}^{2+}]_i\) experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density. Control data are representative of five independent experiments, whilst experiments in the presence of A01 are representative of three, five and four cells for EGTA, 1 and 100 µM \text{Ca}^{2+}pipette solutions, respectively.
0.7 ± 0.2 pA pF⁻¹ \( (P < 0.05, n=5 \text{ [control]} \text{ and } n=4 \text{ [A01]; Fig. 6.3B,C,F}) \). These data suggest that 100 µM Ca²⁺-activated currents are anion-permeable.

Voltage steps from -80mV to potentials in the range -120 to +120mV were applied to each MK at \( t=10^+ \text{ min} \) (Fig. 6.3G). In response to this protocol, small currents that reversed close to 0mV were recorded with EGTA in the pipette. Given the small nature of these currents \( (3.5 ± 0.4 \text{ pA pF}^{-1} \text{ at +120mV}) \) and the A01-resistance observed across the voltage ramp time course; the A01-sensitivity of these currents in response to voltage steps was not tested (Fig. 6.3E,H,J left panels). Voltage steps applied to MKs dialysed with 1 µM Ca²⁺ yielded small outwardly rectifying currents (Fig. 6.3 H,K). Elevation of [Ca²⁺]ᵢ to 100 µM activated a larger outwardly rectifying conductance. This rectification was less pronounced than in symmetrical NaCl; at +120mV, the outward current was 61.3% smaller in the presence of 5 vs 150 mM [Cl⁻]. \( (P < 0.05, n=5, \text{ Figs. 6.2L and 6.3L}) \). 1 and 100 µM [Ca²⁺]-activated currents reversed at +31.5 ± 1.6 and +33.5 ± 2.7mV, respectively (Figs. 6.3K,L and 6.5D). This represents a shift of +40.6 ± 4.0mV and +36.5 ± 2.9mV, relative to symmetrical NaCl, which is indicative of Ca²⁺-activated anion-permeable conductances within these cells.

With 1 µM Ca²⁺ in the pipette, A01 did not affect the currents recorded across the voltage range and \( E_{rev} \) remained at \( ≈+30 \text{mV} \) \( (P > 0.05, \text{ n=5 [control]} \text{ and } n=4 \text{ [A01], Fig. 6.3H,I,K}) \). However, in MKs dialysed with 100 µM Ca²⁺, A01 abrogated currents at each test potential; outward currents at +120mV were reduced from 18.3 ± 5.4 to 2.8 ± 1.0 pA pF⁻¹ \( (P < 0.001, n=5 \text{ [control]} \text{ and } n=4 \text{ [A01], Figs. 6.3H,I,L right panels}) \). Furthermore, A01 shifted \( E_{rev} \) for 100 µM [Ca²⁺]-induced currents to -2.2 ± 0.8mV, consistent with the block of an anion-permeable conductance \( (P < 0.001, n=5 \text{ [control]} \text{ and } n=4 \text{ [A01], Fig. 6.5D}) \).

Taken together, these data suggest that rat MKs express more than one Ca²⁺-activated anion channel with distinct biophysical and pharmacological properties. 100 µM [Ca²⁺]-activated TMEM16F-like currents required prolonged exposure to Ca²⁺ and were outwardly rectifying and A01-sensitive, whereas currents recorded with 1 µM [Ca²⁺] were smaller in amplitude, A01-resistant and did not display any significant time-dependent activation (Fig. 6.3). Panx1 channels are a candidate for these A01-resistant currents since this conductance has been suggested to be Ca²⁺-activated (Locovei et al., 2006b)
and ICC experiments demonstrate their expression on the peripheral plasma membrane of MKs (Fig. 3.1). However, given the difficulties associated with recording Panx1 currents (discussed in Chapter 5), these small 1 µM Ca\(^{2+}\)-activated currents were not explored further.

In the above low [Cl\(^{-}\)]o experiments, E_{Na}= 0mV and E_{Cl}= +85.7mV. Under these conditions, 100 µM [Ca\(^{2+}\)]-activated currents reversed at +33.5 ± 2.7mV, compared to -3.3 ± 0.5mV in symmetrical NaCl. This smaller than predicted shift of E_{rev} may be explained by a significant permeability to the substituted anion (gluconate\(^{-}\)), as described previously for a number of anion channels including TMEM16F channels recorded from B lymphocytes (Kmit et al., 2013). Furthermore, it has been shown that the ion pore of TMEM16 family members is large enough to permeate gluconate\(^{-}\) and NMDG\(^{+}\) (Malvezzi et al., 2013). An alternative explanation is that cations are permeable either through these TMEM16F-like channels or a separate A01-sensitive channel in the rat MK.

To test whether 100 µM [Ca\(^{2+}\)]-induced currents are influenced by a NSCC, all extracellular Na\(^{+}\) was substituted by equimolar NMDG\(^{+}\) (Fig. 6.4A). Currents typical of KV1.3 were activated immediately after conversion to the WC configuration (t=0 min, Fig. 6.4B left panel). These currents dialysed within ≈3 min for both EGTA and 100 µM Ca\(^{2+}\) pipette solutions, which likely reflects the time course for dialysis of intracellular K\(^{+}\) (Fig. 6.5B right panel). Following the loss of KV1.3 currents, currents recorded with EGTA in the pipette were linear and A01-resistant (Fig. 6.4B,D). Elevating [Ca\(^{2+}\)] to 100 µM yielded large currents that were activated on average ≈6 min (352.5 ± 19.8s) after the transition to the WC configuration, although for some cells this current was first observed around t=5 min (Figs. 6.4B,E and 6.5C). This conductance reversed at ≈+40mV (Fig. 6.4B right panel), consistent with observations in Na-gluconate bath solutions and thus suggesting that it is carried predominantly by anions (Fig. 6.3). For cells treated with A01 currents at t=10 min were substantially reduced across the voltage range. The greatest effect of A01 was observed at -120mV and +120mV where amplitudes were reduced from 8.2 ± 1.1 to 1.2 ± 0.6 pA pF\(^{-1}\) and from 11.3 ± 2.1 to 2.7 ± 0.4 pA pF\(^{-1}\), respectively (P < 0.001, n=4 [control] and n=5 [A01], Fig. 6.4B,C,E right panels).
A

Rat MK

Bath:
150 NMDG
145 Gluconate
5 Cl

B

100 μM Ca²⁺
EGTA

C

20 μM CaCC_{inh}-A01

D

EGTA

100 μM Ca²⁺

E
Figure 6.4 TMEM16F-like currents in the rat megakaryocyte persist in the absence of external Na\(^+\). Whole cell patch clamp recordings from rat MKs. A) Pipette solutions contained 150 mM NaCl, whilst the bath solution contained, in mM; 5 Cl\(^-\), 145 gluconate\(^-\) and 150 NMDG\(^+\). Voltage ramps (Aii) were applied to cells when the [Ca\(^{2+}\)]\(_i\) was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCC\(_{inh}\)-A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM [Ca\(^{2+}\)]\(_i\) (F). Voltage steps (Gii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA, 1 and 100 µM [Ca\(^{2+}\)]\(_i\) experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density. Control data are representative of four independent experiments, whilst experiments in the presence of A01 are representative of three and five cells for EGTA and 100 µM.
Ca\textsuperscript{2+} pipette solutions, respectively.

At t=10+ min, voltage steps from -80mV to potentials in the range of -120mV to +120mV were applied to each MK (Fig. 6.4F). With EGTA in the pipette, small A01-resistant currents that reversed close to 0mV were recorded in response to this protocol (\(P > 0.05\), n=4 [control] and n=3 [A01]; Fig. 6.4G-I left panels). At +120mV mean current densities were 2.6 ± 0.9 pA pF\textsuperscript{-1} and were comparable to currents recorded in NaCl (4.0 ± 0.8 pA pF\textsuperscript{-1}) and Na-gluconate (3.5 ± 0.4 pA pF\textsuperscript{-1}) bath solutions (Figs. 6.2J, 6.3J and 6.4I). Elevating [Ca\textsuperscript{2+}]\textsubscript{i} to 100 µM induced a large conductance with a linear I-V relationship (Figs. 6.4G,J right panels). Application of A01 significantly reduced these currents across the voltage range; for example currents at -120mV and +120mV were reduced from 10.0 ± 0.8 to 1.2 ± 0.3 pA pF\textsuperscript{-1} and 8.1 ± 1.9 to 3.5 ± 0.6 pA pF\textsuperscript{-1}, respectively (\(P < 0.001\), n=4 [control] and n=5 [A01], Fig. 6.4G,H,J right panels). In addition, A01 shifted \(E_{\text{rev}}\) for 100 µM [Ca\textsuperscript{2+}]\textsubscript{i}-activated currents from +41.9 ± 3.4 to -4.8 ± 0.8mV (Figs. 6.4G,H,J and 6.5D right panels). Thus, these data suggest that the 100 µM [Ca\textsuperscript{2+}]\textsubscript{i}-induced conductance is predominantly carried by anions.

Taken together, experiments performed in rat MKs provide evidence that these cells express a Ca\textsuperscript{2+}-dependent, anion-permeable conductance, consistent with the properties of TMEM16F (Grubb et al., 2013). When [Cl\textsubscript{o}] was reduced to 5 mM, without altering [Na\textsuperscript{+} \textsubscript{o}], 100 µM Ca\textsuperscript{2+} currents reversed at +33.5 ± 2.7mV (Fig. 6.5D). This shift was smaller than predicted for a Cl\textsuperscript{-}-selective conductance (+85.7mV). This may be explained by significant permeability to the substitute anion (gluconate\textsuperscript{−}) or by an underlying cation conductance. The latter hypothesis was tested by substitution of external Na\textsuperscript{+} by NMDG\textsuperscript{+}, which caused a further shift of \(E_{\text{rev}}\) by +8.4 ± 4.3mV (\(P < 0.05\), n=5 [Na-gluconate], n=4 [NMDG-gluconate], Fig. 6.5D centre and right panels). Thus, these data suggest that the 100 µM [Ca\textsuperscript{2+}]\textsubscript{i}-induced conductance is predominantly carried by anions but also show some permeability to cations.
Figure 6.5 Biophysical properties of rat TMEM16F-like currents. A) I-V relationships for 100 µM Ca\(^{2+}\)-evoked currents (EGTA-subtracted) in response to voltage steps. Data shown are from experiments where the bath solution contained, in mM; 150 NaCl (left), 150 Na\(^{+}\), 5 Cl\(^{-}\) (centre) and 150 NMDG, 5 Cl\(^{-}\) (right) under control (circles) and A01-inhibited (squares) conditions. The time taken for initial K\(^{+}\) currents to dialyse (B) and TMEM16F currents to activate (C) were calculated from voltage ramp data under control and A01-inhibited conditions. D) The point where EGTA and 100 µM Ca\(^{2+}\) currents in response to voltage steps intersect under control and A01-treated conditions was taken as the reversal potential for Ca\(^{2+}\)-activated currents. Statistical significance was assessed using an ANOVA (A) or student’s paired t-Test (B-D).
6.2.3 Ca\textsuperscript{2+}-activated currents recorded from primary mouse megakaryocytes

Thus far, data presented in this chapter have identified the Ca\textsuperscript{2+}-activated ion channels expressed by rat MKs. These cells express a highly Ca\textsuperscript{2+}-dependent, anion-permeable and A01-sensitive TMEM16F-like channel (Grubb et al., 2013). Mouse MKs are known to express TMEM16F channels, but patch clamp recordings have shown that they are cation-selective in these cells (Yang et al., 2012). However, this report remains controversial. All MK recordings by Yang and colleagues (Yang et al., 2012) were conducted using excised inside-out patch clamp recordings and to date is the only study to have examined TMEM16F currents in primary MKs. It is also the only study to have applied the inside-out patch clamp configuration to these cells. Thus, the following experiments have been designed to evaluate the biophysical properties of TMEM16F channels expressed by mouse MKs using WC patch clamp, which has been successfully used to record other conductances in this cell type (eg. Kv1.3 and P2X1 (Kawa, 1996, Vial et al., 2002, McCloskey et al., 2010)).

Mouse MKs express voltage-gated Kv1.3 channels (McCloskey et al., 2010) but not K\textsubscript{Ca}3.1 channels (Martinez-Pinna, McCloskey, Mahaut-Smith, unpublished observations). Thus, present experiments focussed on currents activated by 100 µM [Ca\textsuperscript{2+}]\texttextit{i}. In NaCl-based recording solutions (Fig. 6.6A), Kv1.3-like currents were observed immediately after transition to the WC configuration (t=0 min; Fig. 6.6B left panel). These currents were comparable in the presence of ≈5 nM (EGTA) and 100 µM Ca\textsuperscript{2+} pipette solutions and were unaffected by application of A01 (P > 0.05, n=3 [EGTA] and n=4 [100 µM Ca\textsuperscript{2+}], Fig. 6.6B left panel). Furthermore, Kv1.3-like currents dialysed quickly and could no longer be detected ≈2 min into the voltage ramp protocol (Fig. 6.9B left panel); reflecting the time course for dialysis of intracellular K\textsuperscript{+} from these cells.

To assess the temporal profile of Ca\textsuperscript{2+}-activated currents, voltage ramps were applied to each MK ten minutes. When the pipette contained EGTA, small A01-resistant currents with mild outward rectification were recorded. These currents were stable and outward currents at t=5 and t=10 min were 2.0 ± 0.7 and 2.5 ± 0.4 pA pF\textsuperscript{-1}, respectively (+120mV; Fig. 6.6B,D centre and right panels). These data are consistent with observations from rat MKs under the same conditions.
Figure 6.6 Mouse megakaryocytes express functional TMEM16F-like channels. Whole cell patch clamp recordings from mouse MKs. A) Pipette and bath solutions contained 150 mM NaCl and voltage ramps were applied to cells when the $[Ca^{2+}]_{i}$ was set to either 0 (EGTA, blue trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCC$_{inh}$-A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D) and 100 µM $[Ca^{2+}]_{i}$ (E). Voltage steps (Fii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA and 100 µM $[Ca^{2+}]_{i}$ experiments conducted in the presence of either control (G) or A01 (H); arrowheads indicate zero current level. I-J) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density. EGTA experiments are representative of three (control) and four (A01) cells, whilst 100 µM $Ca^{2+}$ data are representative of four (control) and five (A01) cells taken from independent experiments.
Elevating $[Ca^{2+}]_i$ to 100 µM yielded a larger current that initiated ≈6 min (367.5 ± 18.9s) after transition to the WC configuration (Figs. 6.6B and 6.9C). In cells treated with A01, currents were significantly reduced across the voltage range; at t=10 min current amplitudes at -120mV and +120mV were reduced from 3.5 ± 0.4 to 0.1 ± 0.9 and from 6.1 ± 0.6 to 2.0 ± 0.6 pA pF$^{-1}$, respectively ($P < 0.01$, n=4 [control] and n=3 [A01], Fig. 6.6B,C,E). These data are consistent with the presence of functional TMEM16F-like channels in intact mouse MKs.

Ca$^{2+}$-activated currents were further assessed in response to voltage steps from a holding potential of -80mV to potentials in the range -120 to +120mV (t=10+ min; Fig. 6.6F). With EGTA in the pipette solution, small A01-resistant outward currents that reversed close to 0mV were recorded (Fig. 6.6I). Current densities at +120mV were 2.4 ± 0.4 and 3.1 ± 1.3 pA pF$^{-1}$ in the presence of vehicle control and A01, respectively ($P > 0.05$, n=3, Fig. 6.6G-I left panels). Elevating $[Ca^{2+}]_i$ to 100 µM led to activation of a large conductance with a linear I-V relationship that reversed at -2.7 ± 0.9mV (Figs. 6.6G,J right panel and 6.9D left panel). In the presence of A01 these currents were substantially reduced at each test potential; this effect was most pronounced at +120mV, where currents were reduced by 81.7% from 11.5 ± 3.8 to 2.1 ± 0.8 pA pF$^{-1}$ ($P < 0.01$, n=4 [control] n=3 [A01], Fig. 6.6 G,H,J). In symmetrical NaCl recording solutions, 100 µM $[Ca^{2+}]_i$-induced currents at +120mV were smaller in mouse (11.5 ± 3.8 pA pF$^{-1}$) vs rat (47.3 ± 10.1 pA pF$^{-1}$) MKs ($P < 0.05$, n=4 [mouse], n=5 [rat], Figs. 6.2L and 6.6J). This may reflect differences in the density of TMEM16F-like channels expressed by MKs isolated from each species. In these experiments both cation- and anion-selective conductances are predicted to reverse at 0mV. Given that Ca$^{2+}$-activated currents recorded from mouse MKs reversed at -2.7 ± 0.9mV (Fig. 6.9D left panel), further experiments are required to determine whether they represent a Na$^+$ or Cl$^-$ conductance.

6.2.3.1 TMEM16F-like currents from mouse megakaryocytes are cation-selective

To further investigate the ionic selectivity of 100 µM $[Ca^{2+}]_i$-induced currents, $[Cl^-]_o$ was reduced to 5 mM by equimolar substitution of gluconate$^-$ (Fig. 6.7A). In these experiments, large KV1.3-like currents were observed immediately after
**Ai** Mouse MK

Bath:
- 5 NaCl
- 145 Na Gluconate

**ii**

- 120mV
- 0.38 \( V_{th} \)
- 666ms

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**B**

- **Control**
  - 20pm CaCC\textsubscript{inh-A01}

- **EGTA**

- **100\mu M Ca\textsuperscript{2+}**

---

**D**

- **EGTA**

**E**

- **100\mu M Ca\textsuperscript{2+}**
Figure 6.7 TMEM16F-like currents from mouse megakaryocytes are cation-selective. Whole cell patch clamp recordings from mouse MKs. A) Pipette solutions contained 150 mM NaCl, whilst [Cl\textsubscript{o}] was reduced to 5 mM by equimolar substitution of Na-gluconate. Voltage ramps (Aii) were applied to cells when the [Ca\textsuperscript{2+}]\textsubscript{i} was set to either 0 (EGTA, blue trace) or 100 µM (black trace). Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCC\textsubscript{inh}-A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM [Ca\textsuperscript{2+}] (F). Voltage steps (Gii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA, 1 and 100 µM [Ca\textsuperscript{2+}] experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Statistical significance was calculated using an ANOVA. Data are normalised for current density. Control data are representative of four (EGTA) and five (1 and 100 µM Ca\textsuperscript{2+}) independent experiments, whilst experiments in the presence of A01 are representative of three, five and four cells for EGTA, 1 and 100 µM Ca\textsuperscript{2+} pipette solutions, respectively.
transition to the WC configuration (t=0 min; Fig. 6.7B left panel). Consistent with the recordings from symmetrical NaCl solutions (Fig. 6.6B left panel), these currents dialysed within 2-3 min and were unaffected by elevating [Ca\textsuperscript{2+}] to 100 µM (Figs. 6.7B left panel and 6.9B centre panel). For MKs dialysed with EGTA, small A01-resistant currents were observed for the remainder of the ten minute recording period; peak currents (+120mV) at t=10 min were 1.8 ± 0.5 and 0.8 ± 0.4 pA pF\(^{-1}\) under vehicle control and A01-treated conditions, respectively (P > 0.05, n=3 [control] and n=4 [A01] Fig. 6.7B-D right panels). With 100 µM Ca\textsuperscript{2+} in the pipette, larger currents with linear I-V relationships developed ≈6.5 min (378.8 ± 12.8s) after transition to the WC configuration (Figs. 6.7B right panel and 6.9C centre panel). Incubation with A01 reduced currents across the voltage range, although this effect did not reach significance in these studies. For example, outward currents at +120mV were reduced from 5.3 ± 2.6 to 1.9 ± 0.6 pA pF\(^{-1}\) (P > 0.05, n=4 [control] and n=5 [A01], Fig. 6.7C,E right panels). Interestingly, 100 µM [Ca\textsuperscript{2+}]-induced currents reversed at 0.7 ± 1.8mV (n=4); indicative of a conductance predominantly permeable to Na\textsuperscript{+} under these conditions of these experiments (Fig. 6.7B right panel).

Upon completion of the voltage ramps (t=10+ min) voltage steps from a holding potential of -80mV to potentials in the range -120 to +120mV were applied to each MK (Fig. 6.7F). For cells dialysed with EGTA, this protocol yielded small and A01-resistant currents that had a linear I-V relationship and reversed close to 0mV (Fig. 6.7G,I left panels). When [Ca\textsuperscript{2+}] was set at 100 µM, large and rapidly activating currents with a linear I-V relationship were recorded (Fig. 6.7G,J). These currents reversed at -3.7 ± 1.3mV, further suggesting that they represent a Na\textsuperscript{+}-permeable conductance (Fig. 6.9D). In support of the hypothesis that 100 µM [Ca\textsuperscript{2+}]-induced currents are carried by a TMEM16F-like channel, A01 reduced these currents across the voltage range. This effect was most pronounced at highly negative (-120mV) and positive (+120mV) potentials, where A01 reduced currents from -8.9 ± 3.4 to -2.0 ± 0.64 pA pF\(^{-1}\) (77.5% decrease) and 11.0 ± 3.4 to 4.8 ± 1.0 pA pF\(^{-1}\) (56.3% decrease), respectively (P < 0.05, n=4 [control] n=5 [A01], Fig. 6.7G,H,J). Under these conditions E\textsubscript{Na}\textsuperscript{=} 0mV and E\textsubscript{Cl}\textsuperscript{=} +85.7mV. Thus, these data indicate that 100 µM [Ca\textsuperscript{2+}]-induced currents are most likely carried by Na\textsuperscript{+} rather than Cl\textsuperscript{−}. This finding is in
A) **Mouse MK**

**Bath:**
- 150 NaCl
- 150 NMDG
- 145 Gluconate
- 5 Cl

---

B) **Control**

C) **20μM CaCC_{inh-A01}**

D) **EGTA**

E) **100μM Ca^{2+}**
Figure 6.8 Inward currents through TMEM16F have reduced amplitude in the absence of external Na⁺. Whole cell patch clamp recordings from mouse MKs. A) Pipette solutions contained 150 mM NaCl, whilst the bath solution contained, in mM; 5 Cl⁻, 145 gluconate⁻ and 150 NMDG⁺. Voltage ramps (Aii) were applied to cells when the [Ca²⁺]i was set to either 0 (EGTA, blue trace), 1 (red trace) or 100 (black trace) µM. Summary data are shown for voltage ramps across a ten minute time course in the presence of either control (B; 0.04% DMSO) or CaCC inh-A01 (C; 20 µM). Peak inward (-120mV) and outward (+120mV) current densities under control (black) and A01-treated (red) conditions are shown for 1 mM EGTA (D), 1 (E) and 100 µM [Ca²⁺]; (F). Voltage steps (Gii) were applied to the cells at t=10+ minutes. Representative traces are shown for 1mM EGTA, 1 and 100 µM [Ca²⁺] experiments conducted in the presence of either control (H) or A01 (I); arrowheads indicate zero current level. J-L) Summary I-V relationships in response to voltage steps under control (squares) and A01 treated (triangles) conditions. Data are normalised for current density (pA pF⁻¹). Statistical significance was calculated using an ANOVA. Control data are representative of three (EGTA) and four (100 µM Ca²⁺) cells whilst A01 data are representative of four cells taken from independent experiments for each condition.
agreement with excised inside-out patch clamp recordings of TMEM16F channels from mouse MKs (Yang et al., 2012).

Data presented above in Fig. 6.7 indicates that Ca\(^{2+}\)-activated currents from mouse MKs are cation- rather than anion-selective. To further investigate the cation permeability of this conductance, extracellular Na\(^+\) was substituted by equimolar NMDG\(^+\) (Fig. 6.8A). As predicted for a highly K\(^+\)-selective conductance, removal of extracellular Na\(^+\) had little effect on Kv1.3-like currents, which dialysed within \(\approx\)1.5 min (Figs. 6.8B left panel and 6.9B right panel).

When MKs were dialysed with 100 µM Ca\(^{2+}\), small, outwardly rectifying currents that reversed close to 0mV initiated \(\approx\)5 min (307.5 \(\pm\) 14.4s) after transition to the WC configuration (Figs. 6.8B,C,E centre and right panels and 6.9C right panel). Peak outward currents were 3.4 \(\pm\) 1.7 and 2.1 \(\pm\) 0.9 pA pF\(^{-1}\) under control and A01-treated conditions, respectively (+120mV; \(P > 0.05\), \(n=5\) [control] and \(n=4\) [A01], Fig. 6.8B,C,E).

Next, currents were recorded in response to voltage steps from a holding potential of -80mV to potentials in the range -120 to +120mV (t=10+ min; Fig. 6.8F). Consistent with data presented in Figs. 6.6 and 6.7, voltage steps applied to cells dialysed with EGTA gave small, A01-resistant currents (\(P > 0.05\), \(n=3\) [control] \(n=4\) [A01], Fig. 6.8G-I). Elevation of [Ca\(^{2+}\)] to 100 µM gave currents with clear outward rectification that reversed at -8.0 \(\pm\) 1.6 mV (\(n=4\), Figs. 6.8G,J and 6.9D right panels). In cells treated with A01 currents were reduced across the voltage range with the most pronounced effect observed when \(V_m\) was depolarised beyond +60mV (Fig. 6.8J). For example, currents at +120mV were reduced from 10.5 \(\pm\) 1.4 to 2.9 \(\pm\) 1.1 pA pF\(^{-1}\), representing a 72.3% reduction (\(P < 0.001\), \(n=4\), Fig. 6.8G,H,J). Furthermore, peak inward currents were 5.0 \(\pm\) 3.9 pA pF\(^{-1}\) smaller in NMDG-gluconate compared to Na-gluconate bath solution (-120mV; \(P > 0.05\), \(n=4\), Figs. 6.7 and 6.8). This reduction of inward current provides further evidence that 100 µM [Ca\(^{2+}\)]-induced currents in the mouse MK are cation-permeable. Significant permeability to NMDG\(^+\) may explain \(E_{\text{rev}}\) shifts to only -8mV following replacement of Na\(^+\) with this large cation. Another possibility is a high Ca\(^{2+}\) permeability since \(E_{\text{Ca}}\) is at +29mV. However, experiments conducted in symmetrical NaCl recording solutions with this Ca\(^{2+}\) gradient reversed close to 0mV (Fig. 6.9D), suggesting that the Ca\(^{2+}\)
Figure 6.9 Biophysical properties of mouse TMEM16F currents. A) I-V relationships for 100 µM Ca\(^{2+}\)-evoked currents (EGTA-subtracted) in response to voltage steps. Data shown are from experiments where the bath solution contained, in mM; 150 NaCl (left), 150 Na\(^{+}\), 5 Cl\(^{-}\) (centre) and 150 NMDG, 5 Cl\(^{-}\) (right) under control (circles) and A01-inhibited (squares) conditions. The time taken for initial K\(^{+}\) currents to dialyse (B) and TMEM16F currents to activate (C) were calculated from voltage ramp data under control and A01-inhibited conditions. D) The point where EGTA and 100 µM Ca\(^{2+}\) currents in response to voltage steps intersect under control and A01-treated conditions was taken as the reversal potential for Ca\(^{2+}\)-activated currents. Statistical significance was assessed using an ANOVA (A) or student’s paired t-Test (B-D).
permeability of these cells is relatively low. Given the fragile nature of cells in low Ca\textsuperscript{2+} solutions, Ca\textsuperscript{2+} substitution experiments were not performed to further investigate Ca\textsuperscript{2+} permeability.

In summary, TMEM16F-like currents recorded from HEL cells and rat MKs in gluconate bath solutions were shown to be anion-selective (table 6.1 and Figs. 5.7 and 6.5). However, parallel recordings from mouse MKs reversed close to 0mV, which is indicative of a cation-selective conductance (table 6.1 and Fig. 6.9). Taken together, these data provide further evidence for the expression of a Ca\textsuperscript{2+}-activated and cation-selective TMEM16F-like conductance by mouse MKs.

**Table 6.1 Reversal potentials of currents induced by elevating intracellular Ca\textsuperscript{2+} to 100 µM in HEL cells and rat and mouse MKs**

<table>
<thead>
<tr>
<th>Pipette saline (mM)</th>
<th>Bath Saline (mM)</th>
<th>HEL</th>
<th>Treatment</th>
<th>A01</th>
<th>Rat MK</th>
<th>DMSO</th>
<th>A01</th>
<th>Mouse MK</th>
<th>DMSO</th>
<th>A01</th>
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<tr>
<td>150 NaCl</td>
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<td>150 NaCl</td>
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<tr>
<td>150 Na\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>150 NaCl</td>
<td>HEL</td>
<td>-1.7 ± 0.3</td>
<td>+37.2 ± 2.1</td>
<td>+40.4 ± 3.9</td>
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<tr>
<td>150 Na\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>150 NaCl</td>
<td>A01</td>
<td>-3.2 ± 0.8</td>
<td>-1.5 ± 0.6</td>
<td>-4.8 ± 0.6</td>
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<tr>
<td>150 Na\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>150 NMDG\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>HEL</td>
<td>-3.3 ± 0.5</td>
<td>+33.5 ± 2.7</td>
<td>+41.9 ± 3.4</td>
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<tr>
<td>150 Na\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>150 NMDG\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>A01</td>
<td>-2.5 ± 0.5</td>
<td>-2.2 ± 0.8</td>
<td>-4.9 ± 0.8</td>
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<tr>
<td>150 Na\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>150 NMDG\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>Mouse MK</td>
<td>-2.7 ± 0.9</td>
<td>-3.7 ± 1.3</td>
<td>-8.0 ± 1.6</td>
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<tr>
<td>150 Na\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>150 NMDG\textsuperscript{+} 145 Gluconate\textsuperscript{-} 5 Cl\textsuperscript{-}</td>
<td>A01</td>
<td>-2.7 ± 1.2</td>
<td>-3.5 ± 0.7</td>
<td>-6.8 ± 0.8</td>
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6.3 Discussion

Experiments performed within this chapter have investigated the expression and biophysical properties of Ca$^{2+}$-activated ion channels from freshly isolated mouse and rat MKs. The mature MK has previously been shown to serve as an authentic model for studies of platelet ion channels (Shattil and Leavitt, 2001, Carter et al., 2006, Mahaut-Smith, 2012). ICC experiments show that these cells express TMEM16F channels at their periphery (Fig. 6.1). Furthermore, under conditions designed to abolish K$^{+}$ currents, 100 µM [Ca$^{2+}$]-induced currents from rat MKs were outwardly rectifying, A01-sensitive and required prolonged exposure to Ca$^{2+}$ for activation; these are all properties consistent with TMEM16F channels recorded in other cell types (Szteyn et al., 2012, Grubb et al., 2013). In mouse MKs, current densities were smaller, the I-V relationship was less outwardly rectifying and most importantly the conductance was predominantly permeable to cations rather than anions.

6.3.1 K$^{+}$ currents of primary megakaryocytes

K$^{+}$ channels expressed by rat MKs have not been molecularly characterised; however the voltage-gated (Romero and Sullivan, 1997) and Ca$^{2+}$-activated K$^{+}$ channels (Uneyama et al., 1993) of these cells display properties consistent with Kv1.3 channels reported in mouse MKs (McCloskey et al., 2010) and K$_{Ca}$3.1 channels recorded from human platelets and related cell lines (Mahaut-Smith, 1995, Sullivan et al., 1998). Studies using knockout mice demonstrated that Kv1.3 is the exclusive voltage-gated K$^{+}$ channel expressed by MKs (McCloskey et al., 2010). In addition, our laboratory has shown that mouse MKs do not express functional Ca$^{2+}$-activated K$^{+}$ channels (Martinez-Pinna, McCloskey and Mahaut-Smith, unpublished observations). Thus, rat MKs express Kv1.3 and K$_{Ca}$3.1 channels, whilst mouse MKs express Kv1.3 but not K$_{Ca}$3.1 channels. Kv1.3 channels are responsible for the largest amplitude conductance of resting MKs (McCloskey et al., 2010). K$^{+}$ current dialysis occurred over a similar time course in both mouse and rat MKs for each of the recording solutions (Figs. 6.5 and 6.9) These data likely reflect the fact that Kv1.3 is the major K$^{+}$ conductance in both species (Maruyama, 1987, Mahaut-Smith et al., 1990a).
6.3.2 Expression and localisation of TMEM16F channels by primary megakaryocytes

ICC and Western blot studies from Chapter 5 indicated that human platelets express TMEM16F channels at their periphery (Fig. 5.2). ICC experiments performed in the present chapter revealed staining for TMEM16F at the peripheral plasma membrane of rat and mouse MKs. In addition, TMEM16F staining was observed within the cytoplasmic volume of rat MKs, which may be indicative of channel expression along the DMS that is known to be present within this space (Mahaut-Smith et al., 2003). Given that the DMS acts as a membrane reserve for platelet production (Radley and Haller, 1982), TMEM16F channel expression in this region may correlate with expression of these channels by platelets. This finding is supported by previous reports of platelet ion channels being expressed by these progenitor cells (Tolhurst et al., 2005, Carter et al., 2006). Ideally, biotinylation and Western blotting studies should be performed to further investigate the localisation of MK TMEM16F channels. However, MKs comprise <1% of cells within the bone marrow, making it difficult to obtain a suitably pure population of cells for such studies. Instead, patch clamp recordings of functional TMEM16F-like channels (Figs. 6.5 and 6.8) provide evidence that a proportion of these channels are functional and present at the plasma membrane. The biophysical properties of 100 µM Ca$^{2+}$ currents recorded from mouse and rat MKs, closely match those described for TMEM16F channels: Ca$^{2+}$-dependent, outwardly rectifying, time-dependent activation (Szteyn et al., 2012, Grubb et al., 2013). Moreover, these currents could be completely blocked by incubation with the TMEM16F channel blocker A01 (De La Fuente et al., 2008) (Figs. 6.5 and 6.9).

6.3.3 Differential ion selectivity of TMEM16F channels formed by mouse and rat megakaryocytes

TMEM16F channels formed by human (e.g. HEK-293 cells) and mouse (e.g. dendritic cells) cells have been shown to be anion-selective and thus are principally permeable to Cl$^{-}$ in physiological salines (Szteyn et al., 2012, Grubb et al., 2013, Pedemonte and Galietta, 2014). To date, there have not been any studies of rat TMEM16F channels; however, they share 71% and 76% sequence homology with human and mouse orthologs, respectively (Fig.
Thus, it is likely that these channels perform similar roles. Indeed, rat MK TMEM16F-like channels had comparable biophysical properties to those reported for HEL cells in Chapter 5 (Figs. 5.7 and 6.5). When Cl⁻ in the bath solution was substituted by gluconate⁻, TMEM16F-like currents from rat MKs reversed at ≈+40 mV, which was smaller than that predicted for a Cl⁻-selective conductance (Fig. 6.5D and table 6.1). This can be explained by either the activation of other channels under these conditions or by a significant permeability by this conductance to cations or gluconate⁻. A study of reconstituted TMEM16 proteins demonstrated that these channels have large pores (4-6.5 Å), which allow conductance of gluconate⁻ and NMDG⁺ (Malvezzi et al., 2013). Furthermore, smaller than predicted shifts of E_{rev} have been reported in patch clamp studies of platelet Ca²⁺-activated Cl⁻ channels and B lymphocyte TMEM16F channels (Mahaut-Smith, 1990, MacKenzie and Mahaut-Smith, 1996, Kmit et al., 2013). Thus, these data likely reflect gluconate⁻ conductance by these channels. Given that TMEM16F channels in both HEL cells and primary rat MKs were shown to be predominantly permeable to anions, it was surprising to find that TMEM16F currents from mouse MKs were cation-selective (Fig. 6.9). Cation selectivity of TMEM16F channels in mouse MKs has been described previously (Yang et al., 2012). However, it is not yet understood why TMEM16F channels from these cells have distinct properties to those recorded from other mouse cell types (Szteyn et al., 2012). Possible theories regarding the differential ion selectivity between TMEM16F orthologs are discussed below.

### 6.3.4 Relationship between human, mouse and rat TMEM16F channels

Cl⁻ substitution experiments in Chapters 5 and 6 demonstrated that both human and rat TMEM16F currents are anion-permeable, whilst TMEM16F currents from mouse MKs have a preference for cations (Figs. 5.7, 6.5 and 6.9). However, mouse TMEM16F currents are equally sensitive to A01 and have comparable biophysical properties to their human and rat orthologs (Figs. 5.4, 6.2 and 6.6). It has been proposed that altered ionic selectivity of TMEM16F currents from mouse MKs is due to a mutation within the pore region (Yang et al., 2012). In that study, the authors increased the anion permeability of TMEM16F channels by introducing a single point mutation within TM5; Q559K
Figure 6.10 Comparison between human mouse and rat TMEM16F amino acid sequences. A) Membrane topology of TMEM16F monomers (based on structural studies of TMEM16A channels (Yu et al., 2012)); these channels have eight TMDs with a re-entrant P loop between TM6 and TM7. Highlighted orange is the Ca$^{2+}$-binding domain E-Y-M-E-M for TMEM16A, which corresponds to E-Y-L-E-M in the TMEM16F sequence. B) The primary amino acid sequence is shown for mouse TMEM16A (mANO1), human TMEM16F (hANO6), mouse TMEM16F (mANO6) and rat TMEM16F (rANO6) proteins between TM4 and TM7, which includes the pore region (dashed box), is shown.
(Fig. 6.10B). Thus, this apparent discrepancy may be explained by a splice variant or mutations within the mouse \textit{ANO6} gene, which is restricted to the MK lineage.

To explore this possibility, the primary amino acid sequences of human (hANO6), mouse (mANO6) and rat (rANO6) TMEM16F proteins were aligned (Fig. 6.10B). The greatest similarity was observed between hANO6 and mANO6, 90%, whilst hANO6 and rANO6 showed 71% identity and mANO6 was 76% similar to rANO6 (Fig. 6.10B). To date, there have not been any studies of TMEM16F channel topology. However, \textit{in silico} predictions and studies of related family members, TMEM16A and TMEM16G, suggest that TMEM16F channels have eight transmembrane domains, intracellular amino and carboxyl termini and a re-entrant pore loop (Das et al., 2008, Hartzell et al., 2009). More detailed studies of TMEM16A (ANO1) channels have been performed, leading to the discovery of the Ca\textsuperscript{2+} binding domain and pore region (Fig. 6.10A) (Yu et al., 2012). Thus, the TMEM16A sequence, which has 34\% homology with mANO6, was been used as a template to predict the location of the TMDs, Ca\textsuperscript{2+} binding domain and pore region within the h/m/rANO6 sequences (Fig. 6.10B). These alignments show a high degree of homology within the pore region (TM5-TM7; Fig. 6.10B). The Ca\textsuperscript{2+} binding domain (E-Y-M-E-M in mANO1 and E-Y-L-E-M in ANO6) is conserved within all four sequences and Ca\textsuperscript{2+} binding at this site likely influences channel gating (Chapter 1.7). In addition, charged residues present within the pore region were 100\% conserved between TMEM16F orthologs (Fig. 6.10). This was unsurprising, given that Ca\textsuperscript{2+}-activated anion channel activity has been demonstrated previously for each ortholog (Fig. 6.5) (Martins et al., 2011, Szteyn et al., 2012). Thus, differential ion selectivity of TMEM16F channels in HEL cell and rat and mouse MKs is most likely explained by a splice variant of the mouse ortholog, which is restricted to the MK lineage. To date, there has only been one sequence reported for mANO6; transcript ID ENSMUST00000071874. However, this hypothesis could be tested by sequencing of the pore regions of TMEM16F channels formed by HEL cells and mouse and rat MKs (discussed in Chapter 7.3).
6.3.5 Conclusions

Data presented in this chapter indicate that rat MK’s express both TMEM16F anion-permeable channels and an additional Ca\(^{2+}\)-activated anion channel(s) (Fig. 6.3). The latter was observed when \([\text{Ca}^{2+}]_i\) was raised to 1 µM and was resistant to the TMEM16F inhibitor A01. Analysis of purified platelet cDNA by qPCR has shown that platelets contain transcripts for multiple Ca\(^{2+}\)-activated ion channels, which include Panx1, TMEM16A, TMEM16B (Wright et al., 2013). These channels are discussed in greater detail in Chapter 1.7. Furthermore, patch clamp studies of human platelets have identified Ca\(^{2+}\)-dependent Cl\(^-\) channels that are activated by elevations of \([\text{Ca}^{2+}]_i\) between 0.3-10 µM (Mahaut-Smith, 1990, MacKenzie and Mahaut-Smith, 1996). The molecular identity of these channels remains unknown and further studies are required to understand the complement of Ca\(^{2+}\)-activated ion channels expressed by MKs and platelets.

In conclusion, the data presented in Chapters 5 and 6 represent the first WC recordings of TMEM16F currents from both HEL cells and primary MKs. These experiments have shown that these channels are highly Ca\(^{2+}\)-dependent and A01-sensitive. These studies were designed to resolve the controversy regarding the ionic selectivity of human and mouse TMEM16F channels. To this end, data presented here agree with previous reports that human and mouse MK TMEM16F channels are anion- and cation-selective, respectively. However, the consequences of differential ionic conductance by these channels on the procoagulant activity of these cells will require further investigation.
7.0 General discussion

7.1 Recapitulation of findings

Studies conducted within this thesis have assessed the expression and function of Ca$^{2+}$-activated ion channels within platelets and MKs, with particular emphasis on Panx1 and TMEM16F. Chapters 3 and 4 demonstrated that Panx1 channels amplify platelet activation in response to threshold concentrations of platelet agonists. Furthermore, platelet adhesion assays identified a role for Panx1 in thrombus development (Taylor et al., 2014). Previous studies have shown that TMEM16F channels contribute to Ca$^{2+}$-dependent phospholipid scrambling activity, which is required for thrombin generation and formation of a stable clot (Chapter 1.1.2) (Suzuki et al., 2010, Yang et al., 2012). Patch clamp recordings demonstrated that TMEM16F channels from a human myeloid cell line and rat MKs are anion-selective, whilst mouse MK currents are cation-selective.

Data presented in Chapter 3 demonstrated that Panx1 channels modulate Ca$^{2+}$ entry via a plasma membrane pathway, which is independent of SOCE (Figs. 3.3-5). It was later shown that this Ca$^{2+}$ influx was generated by co-localised P2X1 receptors, most likely activated by autocrine stimulation via ATP release through Panx1 channels (Chapter 4). Interestingly, in vitro thrombus formation experiments demonstrated that Panx1 channels contribute to thrombus development but are redundant for adhesion to a collagen coated surface (Taylor et al., 2014). These findings led to the development of a temporal threshold model of Panx1-dependent amplification of platelet activation (Fig. 4.7). Within this model, it was proposed that Panx1 channels are activated in response to agonist-evoked elevation of [Ca$^{2+}$] as described previously within an oocyte expression system (Locovei et al., 2006b). Open Panx1 channels release ATP (Figs. 4.1 and 4.3) (Bao et al., 2004), which can stimulate P2X1 receptors that are either co-localised with Panx1 (Fig. 4.6) or expressed on surrounding platelets, in an autocrine and paracrine manner, respectively (Fig. 4.7). This provides a mechanism whereby these channels both amplify platelet activation and facilitates platelet recruitment into a developing thrombus. In
addition, antibody techniques demonstrated that Panx1 channels are expressed by human myeloid cells lines (CHRF, HEL and Meg-01) and primary MKs derived from mouse and rat marrow (Figs. 3.1-2). These data suggest that Panx1 channels may play a role in MK development and platelet production (Chapter 1.2). However, patch clamp recordings from HEL cells failed to identify Ca\(^{2+}\)-activated Panx1 channels (Fig. 5.4), which may be due to the parameters of the protocols applied within these studies (discussed in Chapter 5).

In the latter half of this thesis (Chapters 5 and 6), WC patch clamp was used to evaluate Ca\(^{2+}\)-activated TMEM16F channels. ICC studies revealed expression of these channels at the surface of HEL cells, human platelets and mouse and rat MKs (Figs. 5.2 and 6.1). Activation of TMEM16F channels was achieved by elevating [Ca\(^{2+}\)]\(_{i}\) to 100 µM using EGTA-buffered pipette solutions supplemented with CaCl\(_2\) (Chapter 2.1.2). In agreement with previous studies (Szteyn et al., 2012, Grubb et al., 2013), TMEM16F currents recorded in symmetrical NaCl solutions required prolonged (>5 min) exposure to [Ca\(^{2+}\)]\(_{i}\), were outwardly rectifying and A01-sensitive (Figs. 5.7, 6.5 and 6.9).

Subsequent ion substitution experiments revealed anion-selective TMEM16F channels expressed by HEL and rat MKs, whilst currents recorded from mouse MKs were cation-selective. Studies of TMEM16F-deficient mice reported a loss of a Ca\(^{2+}\)-activated cation conductance from excised MK membrane patches (Yang et al., 2012). This is controversial as previous reports of TMEM16F currents from mouse dendritic cells (Szteyn et al., 2012) and human cells (Grubb et al., 2013, Kmit et al., 2013) indicate that these channels are anion-selective. This interspecies difference of TMEM16F channels could be explained by mutations within the pore region that alter ion selectivity. However, analysis of the primary amino acid sequences in Chapter 6 demonstrated that there was >96% homology between the pore region of human, mouse and rat TMEM16F orthologs (Fig. 6.10B). Possible strategies to elucidate the underlying cause of differential ion selectivity are discussed below (section 7.4).

### 7.2 Translational perspective

Current interventions for the management of arterial thrombosis are highlighted in Chapter 1.5. These strategies include receptor antagonists (clopidogrel; P2Y12), enzyme inhibitors (aspirin; COX-1/2) and vitamin K antagonists
(warfarin) (Jackson and Schoenwaelder, 2003). At present, there are no antiplatelet therapies in clinical use that directly target ion channels.

Data presented in Chapters 3 and 4 support the hypothesis that Panx1 channels represent a novel target for antiplatelet therapies. Reported Panx1 channelopathies include HIV infiltration (Séror et al., 2011), ulcerative colitis (Gulbransen et al., 2012), epileptiform activity (Thompson et al., 2008) and other inflammatory conditions (Velásquez and Eugenin, 2014). Interestingly, the Panx1 channel inhibitors used in this study, Prb and Cbx, are licenced for the treatment of gouty arthritis (Cunningham et al., 1981) and gastrointestinal ulcers (Doll et al., 1965), respectively. Moreover, pharmacokinetic studies indicate that the plasma concentration of patients taking oral Prb is ≈78 µM (Cunningham et al., 1981). Also worth noting are reports that Prb improves the therapeutic response of indomethacin (Cunningham et al., 1981) and prolongs the effects of heparin (Sanchez, 1975). These studies also demonstrated that the therapeutic benefits of Prb were not due to changes in the plasma half-life of these drugs. Circulating plasma concentrations of Prb reported by Cunningham and colleagues (Cunningham et al., 1981) are comparable to those employed in our in vitro thrombus formation assay, which demonstrated a reduction of thrombus volume and height without affecting surface coverage (Taylor et al., 2014). Thus, clinical use of Prb may represent a valuable tool for the management of thrombosis, with minimal impact on haemostatic function. Several advantages exist for the use of Cbx and Prb; for example both of these drugs are approved by the FDA with a wealth of safety data available. Additionally, as these drugs are out of patent, they are relatively cheap.

Therapeutic interventions involving TMEM16F channels are more challenging to envisage. In reality, Scott syndrome is a rare bleeding disorder with three patients worldwide (Zwaal et al., 2004). This disease is caused by missense mutations of the TMEM16F gene (Suzuki et al., 2010, Kmit et al., 2013). At present this condition is managed primarily through blood or platelet transfusions, as required (Nurden et al., 2012). In the longer term it may be possible to develop gene therapies to introduce wild type TMEM16F genes into HSCs. Given the pronounced antithrombotic phenotype of TMEM16F-deficient
mice (Yang et al., 2012), it is also possible that down-regulating TMEM16F activity may be represent an antithrombotic therapy.

7.3 Current gaps in understanding

Research into the function of Panx1 and TMEM16F channels is still in its infancy with less than 300 and 50 original articles published in the 10-15 years following the discovery of each channel, respectively (Panchin et al., 2000, Katoh, 2004). Studies investigating the expression and function of these channels in a range of tissues and species have identified various channelopathies, including Scott syndrome and ischaemic stroke. However, the mechanism(s) by which these channels are regulated and contribute to platelet signalling events is incompletely understood.

Within this thesis I have proposed that Panx1 channels are activated by a rise of [Ca^{2+}], in response to stimulation by several platelet agonists (Chapters 3 and 4). However, the precise Ca^{2+}-dependence and mechanism by which Panx1 channels are gated by Ca^{2+} is unknown (Chapter 1.7.2). Ca^{2+}-dependent activation of Panx1 channels was briefly assessed in HEL cells by elevating [Ca^{2+}] to 1 µM (Fig. 5.4). In these experiments a Prb-sensitive current was observed immediately following transition to the WC configuration (Fig. 5.4C). However, this effect was lost at later time points and could not be reproduced by application of Cbx (Fig. 5.4C-E). These data are likely explained by differences between the protocols used for the study of Panx1 and TMEM16F channels, as discussed in Chapter 5.3. Another explanation may be that Panx1 channels have a discrete range of Ca^{2+}-dependence and become inactivated at higher [Ca^{2+}]. Experiments in Chapter 3 demonstrated Panx1 channel opening by thrombin-evoked release of intracellularly loaded calcein dye (Fig. 3.7).

Thus, this method could be applied to assess Ca^{2+}-dependent activation of Panx1 channels using platelets loaded with a Ca^{2+} chelator, i.e. BAPTA.

In silico analysis of the Panx1 sequence has predicted phosphorylation sites for PKA, PKC and Src family kinases (SFKs) (Barbe et al., 2006, Weilinger et al., 2012). Both SFK inhibitors and interference with the SFK consensus sequence of Panx1 have been shown to inhibit anoxia-induced channel activation (Weilinger et al., 2012). At present there are no reports of PKA or PKC
phosphorylation-dependent modulation of Panx1 channel opening. Activation of PKC isoforms regulate multiple processes in the platelet including dense granule release and aggregation (Harper and Poole, 2010). Data presented in Figs. 4.1 and 4.3 demonstrate that Panx1 channels contribute to the release of cytosolic ATP from platelets in response to stimulation by collagen. However, attempts to delineate the relative contribution of Panx1 and PKC-dependent ATP release using a pan-PKC inhibitor (GF109203X) were unsuccessful (data not shown). Finally, elevation of cAMP and activation of PKA represents a potent endogenous inhibitory mechanism within platelets (Chapter 1.4) (Schwarz et al., 2001). Interestingly, related Panx3 channels have been shown to be regulated by cAMP and PKA activity in chondrocytes and osteoprogenitor cells (Iwamoto et al., 2010, Ishikawa et al., 2014). Clearly, further investigation is required to understand the relevance of PKA, PKC and SFK activity in relation to Panx1 channels. These questions could be addressed by use of an overexpression system (i.e. hPanx1-HEK-293 cell line) to evaluate phosphorylated residues within Panx1 by mass spectrometry or Western blotting.

TMEM16F proteins function both as Ca\(^{2+}\)-activated ion channels (Hartzell et al., 2009) and phospholipid scramblases (Suzuki et al., 2010). However, there is some debate surrounding the relationship between the ion channel and phospholipid scrambling activity of these channels (Harper and Poole, 2013, Kmit et al., 2013). Within this thesis I have focussed on the biophysical properties of TMEM16F channels without investigating their contribution to phospholipid scrambling (Chapters 5 and 6). PS exposure is routinely measured using fluorescent annexin V probes by flow cytometry (Goodall and Appleby, 2004). This high throughput technique allows for the discrimination of cell populations based on size, granularity and fluorescence intensity. This technology can be easily applied to homogenous cell populations (i.e. platelet and HEL cell suspensions). However, primary MKs comprise <1% of cells within the bone marrow and can be difficult to purify in order to analyse using this method. A limitation of this method is that they only provide a snapshot of PS exposure at discrete time points, usually several hours apart. Thus, it would be advantageous to develop an assay whereby PS exposure at the surface of individual cells may be monitored in real-time. Within our laboratory, a similar
assay has been developed to monitor real-time fibrinogen binding and [Ca\textsuperscript{2+}]\textsubscript{i} measurements, from primary MKs (Bye, 2014). This protocol could be adapted to investigate the temporal relationship between [Ca\textsuperscript{2+}]\textsubscript{i} and PS exposure from primary MKs and HEL cells. In addition, these studies could be coupled with patch clamp recordings to directly assess the relationship between TMEM16F ion channel activity and PS exposure.

Data presented in Chapters 5 and 6 have demonstrated that TMEM16F currents from HEL cells and rat MKs are anion-selective (Figs. 5.7 and 6.5), whilst currents recorded from mouse MKs are cation-selective (Fig. 6.9). Though these data agree with previous reports of TMEM16F currents from mouse MKs (Yang et al., 2012), the underlying cause of this dramatic difference in ionic selectivity remains unclear. This is particularly intriguing as TMEM16F currents from other mouse tissues are anion-selective (Szteyn et al., 2012) and there are no reported splice variants of the mouse gene. Anion-selectivity could be introduced to heterologously expressed mouse TMEM16F channels with the point mutation Q559K (Fig. 6.10), which reduced $P_{Na}/P_{Cl}$ values from 6.8 to 2.2 (Yang et al., 2012). Thus, it is likely that differential ion selectivity arises from mutations within the pore region (TM5-TM7) that occur along the MK lineage. This hypothesis could be tested by sequencing the pore regions of TMEM16F channels expressed by HEL cells, mouse and rat MKs and another mouse cell type (e.g. dendritic cells). For this purpose, individual MKs could be selected using patch pipettes, as described previously (Carter et al., 2006).

### 7.4 Future directions

Work presented in this thesis has examined the role of Panx1 and TMEM16F channels in platelet and MK function by use of pharmacological tools within \textit{in vitro} assays. These inhibitors have been selected based upon their use in previous studies and were applied at concentrations known to not affect other channels. For example, used at higher concentrations, Panx1 channel inhibitors (Prb and Cbx) also block organic anion transporters and Cx proteins (for discussion see Chapter 3.3). Thus, further characterisation of Panx1 and TMEM16F channels will benefit from the use of transgenic models.
Current technologies allow for the generation of transgenic mice where protein expression can be altered either globally or within specific tissues and cells. Such strategies have been adopted in the study of Panx1 channels in models of ischaemic stroke (Bargiotas et al., 2011) and ulcerative colitis (Gulbransen et al., 2012). To date, there are no reports of a bleeding diathesis associated with Panx1-deficient mice. This is perhaps unsurprising given that Panx1-inhibited platelets adhere normally to a collagen coated surface (Taylor et al., 2014); suggesting haemostatic function is unaffected by Panx1 blockade. These data did however demonstrate that Panx1 channels are required for thrombus propagation. It would be interesting therefore to assess thrombus formation in vivo using either a laser induced injury (Falati et al., 2006) or a pulmonary embolism (Tymvios et al., 2008) model. In addition, tail bleeding assays could be used to determine the contribution by Panx1 to haemostasis. These studies would provide valuable insight into the future of Panx1 as a therapeutic target for the management of thrombosis. Given that Panx1 has been detected in erythrocytes (Bao et al., 2004) and leukocytes (Woehrle et al., 2010), it will not be possible to directly attribute outcomes from global knockout mice to platelet Panx1 channels. To this end, targeted knockdown of Panx1 in the platelet lineage, by use of Pf4-Cre mice, would be required to assess the specific role of platelets in these events (Tiedt et al., 2007). Ultimately, the therapeutic potential of Panx1 inhibition could be assessed by performing thrombus formation assays in wild type mice treated with Panx1 inhibitors. This approach was recently adopted for a study of MCAO-induced stroke (Xiong et al., 2014); whereby mice treated with Prb had reduced infarct size and improved functional outcomes.

TMEM16F knockout mice have defective phospholipid scrambling, reduced thrombin generation and increased bleeding times but are protected against arterial thrombosis (Yang et al., 2012). As discussed earlier, this study also recorded cation-selective TMEM16F channels from isolated primary MKs. However, data presented in this thesis has demonstrated that human and rat TMEM16F channels are anion-selective, in agreement with previous reports (Pedemonte and Galietta, 2014). These data suggest that a knockout rat model may be more appropriate for modelling the role of TMEM16F channels in human platelet function. Rats are considered to be a better model of human
cardiovascular physiology and drug metabolism than mice (Jacob and Kwitek, 2002). However, the relative ease with which mice can be manipulated genetically has seen these animals favoured for in vivo studies. Over the past decade however, knockout rat technologies have been developed that enable targeted knockdown of specific genes, in a manner comparable to that described for mice (Zan et al., 2003, Izsvák et al., 2010). Thus, development of a TMEM16F-deficient rat may be required to more fully evaluate the role of this channel in human platelet and MK function.

7.5 Conclusions

Work presented within this thesis has explored the expression and function of Ca\textsuperscript{2+}-activated ion channels from platelets, MKs and related cell lines. These studies have focussed in particular on the anion channels Panx1 and TMEM16F. Panx1 channels were shown to represent a novel route for ATP release from the platelet cytosol, which likely amplifies platelet activation responses through stimulation of localised P2X1 receptors. In addition, Panx1 channels may influence platelet activation by stimulation of P2Y1 and P2Y12 receptors either via direct release of ADP or by degradation of ATP to ADP by ectonucleotidase activity (Marcus et al., 1997). These findings suggest a role for Panx1 channels in aetiology of arterial thrombosis. Meanwhile, TMEM16F channels are known to be mutated in patients with the bleeding disorder (Scott syndrome), characterised by defective Ca\textsuperscript{2+}-dependent phospholipid scrambling. Here, the ionic selectivity of TMEM16F channels formed by mouse and rat MKs and a related human cell line (HEL) were investigated. These experiments demonstrated membrane expression of functional TMEM16F channels, which are activated by elevation of [Ca\textsuperscript{2+}] to 100 µM. In addition, previously unreported TMEM16F channels formed by HEL and rat MKs were shown to be anion-permeable, whilst channels expressed by mouse MKs were cation-permeable. These differences are likely the result of mutations of the TMEM16F gene that are restricted to the MK lineage. Further studies are required to understand the contribution by both of these channels to the pathogenesis of arterial thrombosis in humans. However, modulating the activity of these channels in vivo may represent novel therapeutic strategies for the management of CVD.


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