Platelet Signalling: Calcium

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Abstract

A rise in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) is central to platelet activation. Agonists stimulate a rise in $[Ca^{2+}]_{cyt}$ through a combination of Ca^{2+} release from intracellular stores located in the dense tubular system (DTS) and acidic organelles as well as Ca^{2+} entry across the plasma membrane via several channel types. $[Ca^{2+}]_{cyt}$ may be reduced by Ca^{2+} sequestration into the intracellular stores by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and via a H^+-dependent mechanism, whilst Ca^{2+} may be removed across the plasma membrane by plasma membrane Ca^{2+}-ATPases (PMCAs) and by Na^+/Ca^{2+} exchangers (NCXs). Ca^{2+} signals are shaped by differential employment of these basic Ca^{2+} entry and removal processes and by Ca^{2+} buffers present in the platelet cytosol and other cellular compartments. In turn, Ca^{2+} signals can be transduced into a number of platelet responses by an array of effector proteins which may be activated in some cases by Ca^{2+} signals confined to specific cellular microdomains.

Introduction

Human platelets maintain a low resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) estimated to be around 50–100 nM. To maintain the resting $[Ca^{2+}]_{cyt}$ against leakage of Ca^{2+} from intracellular stores or across the plasma membrane, or to restore resting $[Ca^{2+}]_{cyt}$ after the generation of Ca^{2+} signals, several Ca^{2+} removal mechanisms are used. Ca^{2+} is sequestered into organelles by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and by a vacuolar H^+-ATPase (vH^+-ATPase), probably coupled to a H^+/Ca^{2+} exchanger. Ca^{2+} is removed across the plasma membrane either by primary active transport via plasma membrane Ca^{2+}-ATPases (PMCAs) and by Na^+/Ca^{2+} exchangers (Fig. 1).

Platelet activation is associated with a rise in $[Ca^{2+}]_{cyt}$, which may reach values of several μM and be associated with oscillations or repetitive spikes in elevated $[Ca^{2+}]_{cyt}$ in individual cells. Stored Ca^{2+} may be released from the dense tubular system and acidic organelles by the second messengers inositol 1,4,5-trisphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively, and the entry of Ca^{2+} across the plasma membrane may be activated in response to store depletion (store-operated Ca^{2+} entry; SOCE), in response to the second messenger diacylglycerol (DAG) or by direct activation of an ionotropic receptor in the case of ATP acting at the P2X1 receptor. The calcium signals generated by different platelet agonists are shaped not just by the calcium signalling elements recruited but also by the Ca^{2+} buffers in the platelet cytosol.

Many of the basic aspects of platelet calcium signalling have been appreciated for a decade or more. However, many studies have relied on measuring $[Ca^{2+}]_{cyt}$ alone. Such studies are limited by their ability to only monitor the net effect

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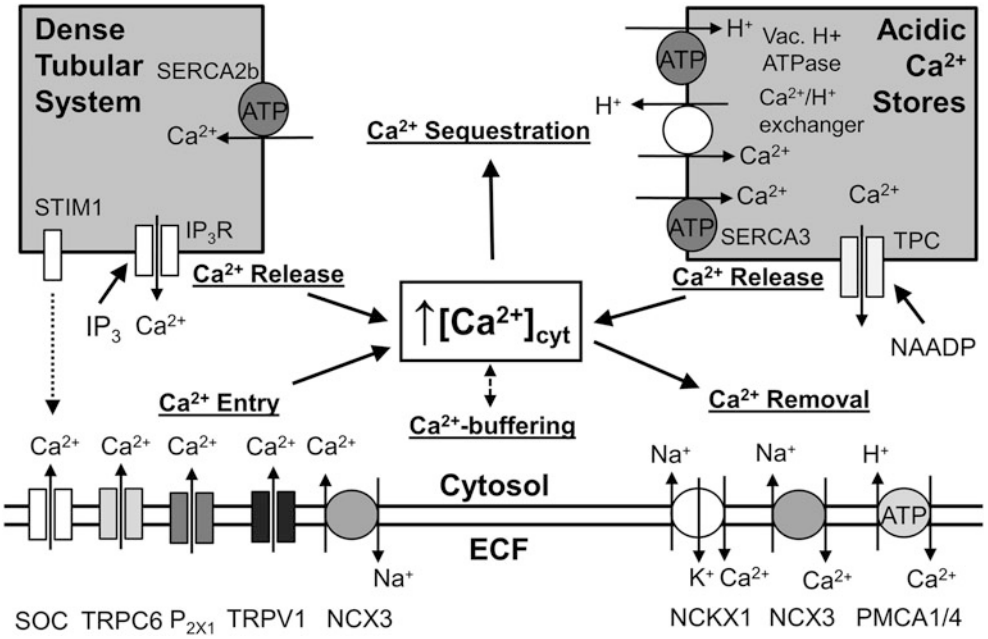

Fig. 1 Summary of the mechanisms that generate and shape platelet Ca^{2+} signals. Ca^{2+} enters the platelet cytosol by entry across the plasma membrane via several channels and possibly by reverse-mode Na^+/Ca^{2+} exchange, as well as by release from intracellular stores and acidic organelles. The Ca^{2+} signal is shaped by various cytosolic Ca^{2+} buffers. Ca^{2+} is removed across the plasma membrane by plasma membrane Ca^{2+} ATPases and forward mode Na^+/Ca^{2+} exchange, and Ca^{2+} is sequestered back into the DTS by SERCA2b and into acidic organelles by SERCA3 and Ca^{2+}/H^+ exchange powered by a V-type H^+-ATPase

of experimental manipulations on the combined actions of the component processes involved in controlling $[Ca^{2+}]_{cyt}$ (Ca^{2+} buffering, sequestration, release, entry and removal) and lack the ability to resolve the source of the Ca^{2+} or the transporters or channels affected. More recent work in human platelets has highlighted the many pitfalls of drawing conclusions as to the molecular pathways involved in eliciting Ca^{2+} signals based solely on measurements of $[Ca^{2+}]_{cyt}$ rather than methods to measure agonist-evoked changes in extracellular and intracellular store Ca^{2+} concentrations ($[Ca^{2+}]_{ext}$ and $[Ca^{2+}]_{st}$, respectively) as well as the platelet pericellular Ca^{2+} concentration ($[Ca^{2+}]_{peri}$), which can be utilised alongside the measurement of agonist-evoked changes in $[Ca^{2+}]_{cyt}$ to improve the interpretation of results compared with simple cytosolic Ca^{2+} measurements alone. Here we summarise current information on platelet calcium signalling, paying particular attention to more recent findings.

Processes That Increase Platelet Cytosolic Calcium Concentration

Release of Stored Ca^{2+}

Most platelet agonists evoke the release of Ca^{2+} from intracellular stores. Two stores are involved, the dense tubular system (DTS; or endoplasmic reticulum (ER)) and acidic stores found in lysosomes and dense granules.

The DTS represents the largest releasable Ca^{2+} store in platelets. DTS Ca^{2+} is released following the formation of inositol 1,4,5-trisphosphate (IP_3). Many agonists, including ADP, platelet activating factor (PAF), thrombin and thromboxane A_2 increase platelet IP_3 levels (Rink and Sage 1990). These agonists activate metabotropic receptors coupled to phospholipase C (PLC) β isoforms 1–3 via a heterotrimeric GTP-binding protein, Gq (Heemskerk and Sage 1994; Boyanova et al. 2012). Additionally, collagen and the low affinity antibody Fc receptor, FcγRII, activate PLC-γ2 via tyrosine phosphorylation (Daniel et al. 1994; Blake et al. 1994). Some studies have implicated PLC-γ_1 in thrombin-evoked responses, although recent data suggest this PLC isoform is undetectable in the platelet proteome (Boyanova et al. 2012). IP_3 releases stored Ca^{2+} in human platelets (O'Rourke et al. 1985) and platelets express all three isoforms of the IP_3-receptor (IP_3RI-III), with the Type I and II receptors being expressed in internal membranes and therefore apparently responsible for IP_3-evoked Ca^{2+} release (Quinton and Dean 1996; El-Daher et al. 2000).

The releasable acidic Ca^{2+} store in platelets is smaller than that in the DTS. The acidic store is released by thrombin acting via protease activated receptor-4 (PAR-4) and glycoprotein (GP) Ib-IX-V, but ADP and arginine vasopressin are apparently without effect on this store (Rosado 2011). The second messenger responsible for releasing Ca^{2+} from acidic stores is nicotinic acid adenine dinucleotide phosphate (NAADP) and this messenger has been shown to release stored Ca^{2+} in permeabilised platelets (López et al. 2006). The collagen receptor GPVI has been reported to elevate NAADP in human platelets and to release Ca^{2+} from the acidic store (Coxon et al. 2012). The identity of the NAADP receptor is a matter of ongoing debate. Much attention has focussed on two-pore channels (TPCs) although conflicting evidence exists (Morgan et al. 2015). Recent work on

platelet dense granules isolated from a megakaryocytic cell line suggests that TPC2 is present in the dense granule membrane and can mediate Ca^{2+} release from this acidic organelle (Ambrosio et al. 2015). Although the agonist-releasable Ca^{2+} pool in the acidic stores is relatively small, it may play an important role in platelet Ca^{2+} signalling by acting to sensitise IP_3Rs and so promote Ca^{2+} release from the DTS, as proposed in the trigger hypothesis demonstrated in other cell types (Galione 2015).

A third known Ca^{2+}-releasing second messenger, cyclic ADP-ribose (cADPR), has been reported to be formed in platelets in response to thrombin but this messenger is without Ca^{2+}-releasing effect in human platelets (Ohlmann et al. 1998).

Ca^{2+} Entry Across the Plasma Membrane

Plasma membrane Ca^{2+} entry channels may be receptor-, second messenger- or store-operated (Sage 1997). In addition, Ca^{2+} may enter cells by Na^+/Ca^{2+} exchangers operating in reverse mode.

Receptor-Operated Channels The only receptor-operated channel identified in human platelets is P2X1 (MacKenzie et al. 1996). This ionotropic receptor is stimulated by ATP and is non-selective, allowing Na^+ as well as Ca^{2+} across the plasma membrane (Sage et al. 1991). Earlier work suggested that this receptor was stimulated by ADP, but it was later shown that the stimulus was the contaminating ATP present in commercial ADP preparations (Mahaut-Smith et al. 2000). The presence of this receptor-operated channel was first suggested on the basis of elevations in $[Ca^{2+}]_{cyt}$ that commenced without discernible latency upon stimulation (Sage and Rink 1987) and was later confirmed in the first patch-clamp recordings from stimulated platelets (Mahaut-Smith et al. 1990). Although P2X1 receptors desensitise rapidly at high agonist concentrations they may be active for tens of seconds at lower levels of stimulation allowing P2X1 receptor stimulation to sustain Ca^{2+} signals evoked by other platelet agonists that stimulate ATP secretion from dense granules (Mahaut-Smith et al. 2011). Ca^{2+} and Na^+ entry via P2X1 receptors also results in membrane depolarisation which may enhance signalling through Gq-coupled receptors such as P2Y1 (Mahaut-Smith et al. 2011). The availability of selective agonists such as α-β-methylene ATP has demonstrated that the rises in $[Ca^{2+}]_{cyt}$ evoked by P2X1 stimulation can result in functional responses including shape change and low levels of $\alpha_{IIb}\beta_3$ activation leading to reversible aggregation, whilst transgenic mouse models indicate a role for P2X1 receptors in amplifying thrombus development (Mahaut-Smith et al. 2011). Although the P2X1 receptor is selective for ATP, it was suggested that a splice variant of this receptor, P2X1del, might mediate P2X1-like responses to ADP (Greco et al. 2001). However, electrophysiological studies indicate that P2X1del does not form functional ion channels and furthermore P2X1del protein is reported to be below the level of detection in human platelets (Vial et al. 2003).

Second Messenger-Operated Channels Second messenger-operated channels (SMOCs) are activated indirectly following the stimulation of metabotropic receptors. The leading candidates for SMOCs in human platelets are channels formed by one or more members of the transient receptor potential canonical sub-family (TRPCs). Human platelets are varyingly reported to express TRPCs 1, 3, 4, 5, 6 and 7 (Hassock et al. 2002; Brownlow and Sage 2005; Liu et al. 2008; Boyanova et al. 2012), with possible associations between TRPCs 1, 4 and 5 and between TRPCs 3 and 6 (Brownlow and Sage 2005). There is conflicting evidence regarding the gating mechanisms of the various TRPC isoforms (Hardie 2007) and at least with TRPC3 the gating mechanism may depend on expression level (Vasquez et al. 2003). TRPCs 3, 6 and 7 are generally regarded as activated by diacylglycerol (DAG; Hardie 2007). In human platelets TRPCs 3 and 6 appear to be responsible for Ca^{2+} entry downstream of DAG formation by PLCs (Hassock et al. 2002; Harper et al. 2013). TRPC6 is reported to be absent from the plasma membrane of resting human platelets and to be inserted upon stimulation by the DAG analogue, OAG, or by thrombin (Harper et al. 2013). Since TRPCs form non-selective cation channels, they gate a substantial Na^+ entry which may result in secondary Ca^{2+} entry by Na^+/Ca^{2+} exchangers (NCXs) operating in reverse mode (Harper et al. 2013).

Another potential SMOC in human platelets may be formed by transient receptor potential vanilloid-1 (TRPV1). TRPV1 has been reported to be expressed in human (Harper et al. 2009; Savini et al. 2010) but not murine platelets (Sage et al. 2014). The TRPV1 agonist, capsaicin, elevates $[Ca^{2+}]_{cyt}$ in human platelets, a response that is inhibited by the TRPV1 antagonists 5′-iodoresiniferatoxin or AMG9810 (Harper et al. 2009). These antagonists reduce rises in $[Ca^{2+}]_{cyt}$ evoked by ADP and thrombin, suggesting a contribution from TRPV1 activation, however the endogenous activator of TRPV1 in human platelets remains to be identified (Harper et al. 2009). Potential candidates include several endovanilloids (Harper et al. 2009), including 12-HPETE, which is produced upon platelet activation (Coffey et al. 2004).

Store-Operated Calcium Entry The phenomenon of store-operated Ca^{2+} entry (SOCE), where depletion of intracellular Ca^{2+} stores leads to the activation of plasma membrane channels permeable to Ca^{2+}, was first described by Putney in 1986. A kinetic study of ADP-evoked rises in $[Ca^{2+}]_{cyt}$ suggested SOCE operated in human platelets since divalent

cation entry was activated temporally coincident with store release (Sage et al. 1990). The existence of SOCE in platelets was confirmed by the use of SERCA inhibitors to deplete intracellular Ca^{2+} stores in the absence of agonist stimulation (Sargeant et al. 1992). In platelets as in other cells the quest to identify the store-operated channel (SOC) proved lengthy. There is now consensus that a highly Ca^{2+}-selective channel (under physiological conditions) is formed by Orai1 and that the Ca^{2+} sensor in the membrane of the endoplasmic reticulum responsible for its activation is STIM1 (Feske et al. 2006; Roos et al. 2005). STIM1–Orai1 coupling has been demonstrated in human platelets (Jardin et al. 2008). Although an essential role for Orai1 in SOCE and thrombus formation has been demonstrated in murine platelets in knock-out studies (Braun et al. 2009), humans with Orai1 mutations affecting channel function do not suffer from major platelet functional defects (Feske 2010).

TRPC1 has also been suggested to play a role in SOCE in human platelets (Rosado et al. 2002), although this does not appear to be the case in murine platelets (Varga-Szabo et al. 2008). There is good evidence to support a role for TRPC1 in SOCE in many other cell types (Cheng et al. 2013). As indicated earlier, TRPCs form non-selective cation channels that are permeable to Na^+ as well as Ca^{2+}. In human platelets, Ca^{2+} store depletion following SERCA inhibition elevates the cytosolic Na^+ concentration, an event blocked by an anti-TRPC1 antibody directed to the pore-forming region of the protein (Harper and Sage 2007). The same antibody reduces the rise in $[Ca^{2+}]_{cyt}$ when Ca^{2+} is added to platelet suspensions following store depletion by SERCA inhibition (Rosado et al. 2002). Although a role for TRPC1 in SOCE in human platelets remains controversial, the presence of this protein in the human platelet proteome allows for this possibility (Boyanova et al. 2012). Although it was suggested that TRPC1 might be activated by conformational coupling to the type II IP_3R, it now appears such coupling is not essential for activation (Harper and Sage 2007). TRPC1 is reported to couple to STIM1 upon Ca^{2+} store depletion in human platelets (Jardin et al. 2008), and this likely serves to activate the channel as reported in other cell types (Cheng et al. 2013).

Processes That Decrease Platelet Cytosolic Calcium Concentration

Ca^{2+} Removal Across the Plasma Membrane

Ca^{2+} is removed across the platelet plasma membrane either by primary active transport via a plasma membrane Ca^{2+}-ATPase (PMCA) or by secondary active transport via Na^+/Ca^{2+} exchangers (NCXs). PMCAs are high affinity Ca^{2+} transporters but have relatively low turnover rates, whilst NCXs have a lower Ca^{2+} affinity but higher turnover rates than PMCAs (Blaustein and Lederer 1999). Consequently, PMCAs are important in the maintenance of resting $[Ca^{2+}]_{cyt}$ whilst Na^+/Ca^{2+} exchangers are more important in the restoration of $[Ca^{2+}]_{cyt}$ after it has been elevated during Ca^{2+} signalling.

Of the four isoforms on PMCA, only PMCA1b and PMCA4b have been detected in human platelets (Martin et al. 2000). PMCA1b expression in human platelets is very low (Pászty et al. 1998), suggesting PMCA4b is the dominant isoform responsible for active Ca^{2+} extrusion (Dean 2010). In platelets the PMCA is subject to several regulatory influences (Dean 2010). The pump is stimulated by Ca^{2+}/calmodulin (Zabe and Dean 2001) and by protein kinase A-dependent phosphorylation (Dean et al. 1997), whilst tyrosine phosphorylation inhibits PMCA activity (Dean et al. 1997), an action that potentiates agonist-evoked rises in $[Ca^{2+}]_{cyt}$ and so platelet functional responses (Bozulic et al. 2007). The PMCA is also modulated by calpain-dependent proteolytic cleavage, with work in vitro and in erythrocytes showing removal of a C-terminal autoinhibitory domain increases activity, whereas further cleavage results in inactivation (Dean 2010). Similar cleavage patterns in human platelets suggest modulation of platelet PMCA by calpain (Brown and Dean 2007).

There are two types of plasma membrane Na^+/Ca^{2+} exchangers: those that exchange Na^+ for Ca^{2+} (NCXs) and those that exchange Na^+ for Ca^{2+} and K^+ (NCKXs) (Lytton 2007). Early functional studies suggested that human platelets possessed an NCKX (Kimura et al. 1993). Messenger RNA for three isoforms of NCKX are reported to be detectible in human platelets (Bugert et al. 2003), however only one study has reported detection of low levels of NCKX1 by Western blotting (Roberts et al. 2012), and others have concluded that NCKX isoforms are absent from the platelet proteome (Boyanova et al. 2012). Low levels of the cardiac NCX isoform, NCX1, have been reported in some Western blot studies (Roberts et al. 2012), whilst others have failed to detect this isoform in human platelets (Harper et al. 2010). A proteomic screen of platelet plasma membrane proteins (Lewandrowski et al. 2009) as well as Western blotting studies (Harper et al. 2010; Roberts et al. 2012) all suggest that NCX3 is the predominant NCX isoform in human platelets. NCX3, like the better studied NCX1, is believed to exchange three Na^+ ions for one Ca^{2+} ion (Lytton 2007). Some early studies concluded that NCXs played little role in influencing $[Ca^{2+}]_{cyt}$ in resting or stimulated platelets (Sage and Rink 1987; Rosado and Sage 2000), however more recent work has provided evidence that forward mode Na^+/Ca^{2+} exchange exports Ca^{2+} across the plasma membrane in resting and thrombin-stimulated human platelets (Roberts et al. 2012). The roles of NCXs in shaping platelet Ca^{2+} signals are complex, with

evidence indicating that these exchangers can promote a rise in $[Ca^{2+}]_{cyt}$ in stimulated platelets. It has been suggested that in collagen-stimulated platelets NCXs transiently reverse to mediate Ca^{2+} entry (Roberts et al. 2012), whilst in thrombin-stimulated platelets there is evidence that NCXs operating in forward mode promote Ca^{2+} recycling via the open canalicular system, supporting the Ca^{2+} signal directly and by promoting the secretion of autocoids from dense granules (Sage et al. 2013) (see section "Signal Magnitude").

Ca^{2+} Sequestration by Intracellular Organelles

Ca^{2+} is sequestered into the main releasable platelet Ca^{2+} store in the DTS by SERCA2b (Enouf et al. 1992), whilst Ca^{2+} is sequestered into the acidic Ca^{2+} stores that are probably located in the dense granules and lysosomes by SERCA3 (Wuytack et al. 1994). SERCA3 shows a much lower sensitivity to thapsigargin than SERCA2b, but is inhibited by 2,5-di-(*t*-butyl)-1,4-hydroquinone (TBHQ) (Cavallini et al. 1995). The acidic Ca^{2+} store membranes also contain a vacuolar H^+-ATPase (vH^+-ATPase), which may be coupled to a H^+/Ca^{2+} exchanger to facilitate Ca^{2+} loading (Sage et al. 2011). Evidence for such a SERCA-independent mechanism of Ca^{2+} sequestration comes from experiments in which the intracellular store $[Ca^{2+}]$ ($[Ca^{2+}]_{st}$) was monitored using Fluo-5 N and $[Ca^{2+}]_{cyt}$ was monitored using Fura-2 (Sage et al. 2011). SERCA2b and SERCA3 were inhibited using high concentrations of thapsigargin (1 μM) and TBHQ (20 μM) and non-acidic Ca^{2+} stores were depleted using the Ca^{2+} ionophore, ionomycin. Since ionomycin is a $Ca^{2+}/H+$ ionophore (Fasolato and Pozzan 1989; Fasolato et al. 1991), it is unable to deplete acidic Ca^{2+} stores due to the absence of a favourable electrochemical gradient for exchange. With SERCAs inhibited, ionomycin caused a rise in $[Ca^{2+}]_{cyt}$ and a fall in $[Ca^{2+}]_{st}$, changes which then gradually reversed indicating Ca^{2+} sequestration in the absence of SERCA activity (Sage et al. 2011). Addition of the pronophore, nigericin, after SERCA inhibitors and ionomycin, caused a further decrease in $[Ca^{2+}]_{st}$ and rise in $[Ca^{2+}]_{cyt}$ which did not reverse over several minutes, indicating that SERCA-independent sequestration was into acidic stores. The nature of the novel SERCA-independent Ca^{2+} sequestration mechanism remains to be identified, but a Ca^{2+}/H^+ exchanger coupled to vH^+-ATPase activity has been suggested on the basis of similarities between platelet dense granules and the acidosomes found in trypanosomes (Ruiz et al. 2004), where such a system operates (Vercesi et al. 1994). Another possibility is that a secretory pathway Ca^{2+}-ATPase may be involved, as found in the dense core vesicles of neuroendocrine cells (Mitchell et al. 2001). A further possibility is that the mitochondrial Ca^{2+} uniporter plays a role (Kirichok et al. 2004). However, the use of antimycin A and oligomycin to inhibit mitochondrial Ca^{2+} uptake does not affect changes in $[Ca^{2+}]_{cyt}$ in platelets treated with thapsigargin and ionomycin (Rosado and Sage 2000).

The SERCA-dependent and pH-dependent Ca^{2+} sequestration mechanisms found in human platelets appear to have different effects in shaping agonist-evoked Ca^{2+} signals (Sage et al. 2011). SERCA inhibition increases the peak of the initial agonist-evoked rise in $[Ca^{2+}]_{cyt}$ and fall in $[Ca^{2+}]_{st}$, whilst blocking pH-dependent Ca^{2+} sequestration using nigericin is without effect on early agonist-evoked changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{st}$, but enhances the later plateau in $[Ca^{2+}]_{cyt}$. Hence pH-dependent Ca^{2+} sequestration into acidic stores appears to commence with some delay after the initial stimulus.

Ca^{2+} Buffering in the Platelet Cytosol

In addition to the Ca^{2+} transport mechanisms detailed earlier, the free $[Ca^{2+}]_{cyt}$ is also influenced by the binding of Ca^{2+} to cytosolic proteins. The intrinsic ability of cellular proteins to buffer agonist-evoked Ca^{2+} signals is often overlooked by many investigators despite the ability of these events to shape the spatiotemporal dynamics of Ca^{2+} signals (Schwaller 2010; Sabatini et al. 2001). However, Ca^{2+} buffering is likely to be essential to the platelet's ability to utilise Ca^{2+} as a second messenger, as influx of Ca^{2+} through a single channel has the potential to deliver a toxic dose of Ca^{2+} to the cell within a split second in the absence of any other systems to restrain Ca^{2+} accumulation (Sage et al. 2013). Estimates of the Ca^{2+}-binding capacity of the platelet cytosol indicate that it is particularly high, with increases in free Ca^{2+} accounting for only around 0.01–0.1 % of all the Ca^{2+} ions entering this cellular compartment (Sage et al. 2013; Valant et al. 1992). These results suggest that Ca^{2+} buffering is likely to play a significant role in modulating platelet Ca^{2+} signal dynamics and the downstream functional responses. Despite this, there has been no study into which molecular targets may underlie this heavy cytosolic Ca^{2+} buffering, although a proteomic study of human platelets has identified a number of candidate Ca^{2+}-binding proteins including calmodulin, S100 proteins, annexins and tubulin (Burkhart et al. 2012) that may be responsible.

Whilst our understanding of the molecular machinery that creates agonist-evoked Ca^{2+} signals in platelets is indebted to the use of fluorescent Ca^{2+} indicators, it is also important to appreciate that the introduction of these compounds artificially increases the Ca^{2+} buffering capacity of the cells. This in turn can markedly alter the spatiotemporal characteristics of any agonist-evoked Ca^{2+} signal (Sabatini et al. 2001). A recent study demonstrated that loading platelets with the commonly used, high-affinity cytosolic Ca^{2+} indicator, Fura-2, could essentially double the Ca^{2+}-binding capacity of the platelet cytosol when compared to

the lower affinity Ca^{2+} indicators, Fura-4F and Fura-FF (Sage et al. 2013). This effect on the buffering capacity of the platelet cytosol could be observed to directly buffer Ca^{2+} signals as well as indirectly interfering with Ca^{2+}-release from intracellular stores (Sage et al. 2013), demonstrating the potential for Ca^{2+} indicators to disrupt normal Ca^{2+} signalling processes. In addition, the introduction of fluorescent Ca^{2+} indicators has also been demonstrated to modulate platelet functional responses to aggregating stimuli (Hatayama et al. 1985; Lanza et al. 1987). Thus, whilst Ca^{2+} indicators may allow us to understand the basic molecular machinery that is responsible for the creation of Ca^{2+} signals, the enhanced Ca^{2+} buffering elicited by their introduction may interfere with the platelet's ability to modulate or respond to this Ca^{2+} signal by interfering with the signal's interaction with its downstream effector molecules. Thus, caution must be taken into account when interpreting the functional responses of platelets loaded with these indicators.

Modulation of Platelet Ca^{2+} Signals

Ca^{2+} signalling systems in cells are strongly non-linear, because in addition to their ability to be activated by agonists, the activity of individual Ca^{2+}-transporting proteins can also be modulated in a time-dependent manner by Ca^{2+}-dependent signalling pathways. In platelets the most studied example of Ca^{2+}-dependent modulation of Ca^{2+} signalling comes from the known role of protein kinase C (PKC) to reduce agonist-evoked rises in $[Ca^{2+}]_{cyt}$ (Lever et al. 2015). However, despite PKC being a well-known modulator of platelet Ca^{2+} signalling, the mechanisms by which it does so have been uncertain, with suggested potential mechanisms including actions on IP_3 production (Rittenhouse and Sasson 1985; Zavoico et al. 1985; King and Rittenhouse 1989; Connolly et al. 1986) to effects on PMCAs and SERCAs (Cavallini and Alexandre 1994; Pollock et al. 1987; Tao et al. 1992). This difficulty in identifying the mechanisms of such modulations relates to the difficulty in studying the effect of a signalling system which has many potential targets when measuring $[Ca^{2+}]_{cyt}$ alone. More recent analysis of the effects of PKC on agonist-evoked Ca^{2+} fluxes into and out of the platelet cytosol has demonstrated a role for Ca^{2+}-dependent conventional PKC isoforms in accelerating Ca^{2+} removal from the cytosol both via an effect on SERCA activity, as well as by indirectly affecting NCX-mediated Ca^{2+} removal via increased Na^+/K^+-ATPase activity (Lever et al. 2015). This ability of PKC to limit agonist-evoked Ca^{2+} signals has been previously shown to play a key role in reducing the development of a procoagulant phenotype in collagen- and thrombin-stimulated platelets (Strehl et al. 2007), thus demonstrating that Ca^{2+}-dependent feedback pathways may play a role in tuning platelet functional responses to physiological stimuli.

In addition to the ability of Ca^{2+} to modulate the generation of its own signal, other physiologically relevant signalling pathways are also able to engage in cross talk with the Ca^{2+} signalling system. These include the ability of endothelial-derived inhibitors such as prostacyclin and nitric oxide to trigger rises in cAMP and cGMP in the platelet which are able to inhibit platelet Ca^{2+} signalling through effects on IP_3-mediated Ca^{2+} release, and possibly via effects on Ca^{2+} entry (for review see Smolenski 2012). In addition to other chemical signals, it has also been shown that shear stress can play a role in upregulating platelet Ca^{2+} signals, both in isolation of other chemical stimuli (Nesbitt et al. 2009) as well as in platelets translocating over surfaces coated with adhesive ligands (Goncalves et al. 2005; Mazzucato et al. 2002).

Controlling Stimulus–Response Coupling of Ca^{2+} Signals in Human Platelets

Calcium Effectors

The central role of Ca^{2+} in mediating platelet activation is through the ability of this second messenger system to translate extracellular signals into functional responses in these cells. The Ca^{2+} signalling system is therefore responsible for triggering and coordinating a range of different processes required for thrombus formation. To mediate this change in platelet activity, the cells require Ca^{2+}-sensitive effector proteins that can translate Ca^{2+} signals into appropriate intracellular responses. Platelets are known to possess a number of such effector proteins: Calmodulin, scinderin, gelsolin and calpain are involved in reorganisation of the actin cytoskeleton and platelet shape change (Paul et al. 1999; Witke et al. 1995; Rodríguez Del Castillo et al. 1992; Croce et al. 1999); CalDagGEFI and CIB1 are involved in integrin $\alpha_{IIb}\beta_3$ activation (Naik et al. 2009; Stefanini et al. 2009); PKC, Munc13-4, SNARE proteins and calpain are involved in granule secretion (Strehl et al. 2007; Golebiewska and Poole 2014; Croce et al. 1999); $cPLA_2$ underlies thromboxane production (Murthy et al. 1995) and TMEM16F is responsible for phosphatidylserine exposure (Mattheij et al. 2015).

The presence of a variety of Ca^{2+}-sensitive signal transduction pathways in one cell requires mechanisms to ensure coordinated activation of these pathways at the correct time and place to produce the desired functional response to any particular extracellular signals. If all the different Ca^{2+}-sensitive processes were fully activated in every platelet then a self-propagating cycle of platelet activation, incorporation into a forming thrombus, secretion of autocrine stimulants and recruitment of additional platelets would lead to uncontrolled thrombus growth, which could eventually occlude the vessel leading to deleterious consequences for the tissue downstream. However, thrombus formation in vivo can be

seen to be a self-limiting process, which does not lead to occlusion of the damaged vessel (Furie and Furie 2008). This suggests that there are a range of regulatory mechanisms that tightly control the ability of Ca^{2+} signals to activate their effector systems. Understanding the systems that prevent excessive activity may provide a valuable asset in devising treatments to prevent excessive blood clotting from occurring (Brass et al. 2011). Recent work has demonstrated that there is heterogeneity in platelet responses during thrombus formation (Munnix et al. 2007; Stalker et al. 2013; London et al. 2006), thus indicating that Ca^{2+}-stimulated processes are able to be differentially activated within the developing platelet aggregate. Whilst this can be explained through spatial control of the physical and chemical stimuli which individual platelets are exposed to when encountering the thrombus (Stalker et al. 2013; Nesbitt et al. 2009; Smolenski 2012), there is still a requirement for the platelet to translate these extracellular signals into a Ca^{2+} signal which can itself be translated into the selective activation of the various Ca^{2+} regulated processes found in these cells. How can individual platelets translate the Ca^{2+} signals they generate to elicit distinguishable phenotypes?

The versatility of Ca^{2+} signalling in cells comes from the ability of cells to selectively decode different agonist-evoked rises in $[Ca^{2+}]_{cyt}$ into specific cellular responses leading to responses over a remarkable range of different timescales (Berridge et al. 2003). The simplest Ca^{2+} signal available in this cellular language is a single Ca^{2+} transient, which can be seen to possess three distinct parameters that can be decrypted by the cell: the signal magnitude, its duration and its subcellular localisation. Previous work in other cells has demonstrated that Ca^{2+}-sensitive effector proteins with different Ca^{2+}-binding affinities and kinetics are able to selectively decode each of these parameters (Dolmetsch et al. 1997).

Signal Magnitude

In platelets, early work identified a role for gradually increasing thresholds of $[Ca^{2+}]_{cyt}$ for the activation of platelet shape change (400–500 nM), serotonin secretion (800 nM) and platelet aggregation (2 μM) (Rink et al. 1982; Hallam et al. 1985). These data establish the potential for the differential Ca^{2+} affinity of effector proteins in mediating these responses in platelets, yet the use of Ca^{2+} ionophores to artificially create Ca^{2+} signals of different amplitude does lead to questions over the physiological relevance of these early studies. Both studies demonstrated that stimulation with physiological agonists such as thrombin and platelet-activating factor could trigger similar responses below the $[Ca^{2+}]_{cyt}$ thresholds defined with ionophores, suggesting that additional regulatory mechanisms were also triggered by agonist stimulation. In addition to other known signalling pathways that may sensitise responses to elevated $[Ca^{2+}]_{cyt}$ (e.g. Paul et al. 1999), it may be that Ca^{2+} microdomains elicited in the vicinity of Ca^{2+}-permeable ion channels play a role in facilitating stimulation of Ca^{2+}-dependent processes in platelets even when the average $[Ca^{2+}]_{cyt}$ reported by fluorescent indicators is relatively low (Parekh 2008).

Signal Duration

The duration of the agonist-evoked rises in $[Ca^{2+}]_{cyt}$ may play an important role in determining important shifts in the functional properties of platelets. Previous work examining platelet interaction with VWF under flow conditions has shown that the duration of Ca^{2+} signalling is crucial in determining the final physiological response of these cells (Nesbitt et al. 2002; Mazzucato et al. 2002). Both of these studies demonstrated that the duration of platelet arrest onto a VWF-coated surface was determined by the length of the Ca^{2+} transient. These transients were not dependent on the presence of extracellular Ca^{2+} and were therefore dependent upon Ca^{2+} release from intracellular stores. In contrast, when the Ca^{2+} signals were prolonged oscillatory responses dependent on Ca^{2+} entry, they led to integrin-dependent, irreversible adhesion and platelet aggregation. This relationship between Ca^{2+} signalling and incorporation within a platelet aggregate was also demonstrated in vivo by van Gestel et al. (2002). This group demonstrated that platelets that were observed to have a sustained increase in their $[Ca^{2+}]_{cyt}$ after binding to the surface of a growing thrombus become stably incorporated within this structure, whilst platelets whose $[Ca^{2+}]_{cyt}$ returned to baseline were found to embolise. Beyond a role in primary haemostatic reactions, prolongation of platelet Ca^{2+} signals has also been demonstrated to play a key role in regulating the blood coagulation process (Heemskerk et al. 2013). Activated platelets create a catalytic surface for the activation of the tenase and prothrombinase complexes through the expression of anionic phospholipids such as phosphatidylserine on the extracellular face of their plasma membrane. Previous work has demonstrated that a large, sustained increase in $[Ca^{2+}]_{cyt}$ is key to the ability of platelets to develop a procoagulant phenotype (Heemskerk et al. 1997; Kulkarni and Jackson 2004; Smeets et al. 1993; Jackson and Schoenwaelder, 2010).

As can be seen from the earlier discussion, prolonged Ca^{2+} signalling in platelets plays a role in determining the rate and extent of both the primary and secondary haemostatic systems that they elicit. Therefore, blocking aspects of the Ca^{2+} signalling system that could elicit a shortening of cytosolic Ca^{2+} signals may be useful for limiting the extent of unwanted platelet aggregation in thrombotic conditions.

A number of signalling pathways are important in eliciting a large, sustained increase in $[Ca^{2+}]_{cyt}$ in agonist-stimulated platelets. These include key roles for store-operated- and receptor-operated Ca^{2+} entry pathways

(Gilio et al. 2010; Harper et al. 2013), autocrine stimulation by the contents of the dense granules (Weiss and Lages 1997; Lages and Weiss 1999) and the activity of Na^+/Ca^{2+} exchangers (Harper et al. 2013; Sage et al. 2013). However, how these systems are coordinated to control the maintenance of agonist-evoked Ca^{2+} signals is less clear. Recent studies have suggested a unified mechanism for prolonging Ca^{2+} signalling via a pericellular Ca^{2+} recycling system. This work has suggested that Ca^{2+} removed from the cell by the NCX can accumulate to a high concentration within the narrow confines of the open canalicular system, such that it can then be recycled back into the cell down its concentration gradient through Ca^{2+}-permeable ion channels and possibly by reverse mode NCX activity, thus prolonging Ca^{2+} signalling directly as well as indirectly through triggering dense granule secretion (Sage et al. 2013; Walford et al. 2015). Further work will be required to define whether this system could be responsible for determining if a platelet adopts proaggregatory and procoagulant phenotypes during thrombus formation under physiological flow conditions.

Subcellular Localisation

In other cells selective activation of Ca^{2+}-sensitive signalling pathways can be achieved through tight spatial coupling of Ca^{2+}-permeable channels and Ca^{2+}-sensitive effectors in specific microdomains of the cell (Rizzuto and Pozzan 2006). Yet the size of the resting platelet is comparative in size or smaller than the spatial spread of elementary Ca^{2+} signals generated through the isolated opening of individual groups of ion channels in other cells (Niggli and Shirokova 2007; Sage et al. 2013). This would seem to suggest that Ca^{2+} signals should always spread throughout the entire platelets. However, Ca^{2+} microdomains may be masked in the tiny platelet volume by the ability of high-affinity, highly diffusible fluorescent Ca^{2+} indicators to act as highly mobile shuttles for rapid transport of Ca^{2+} through the cell (Sala and Hernandez-Cruz 1990). Similar distortions have previously been reported in the spines of Purkinje neurons (Schmidt et al. 2007). In spite of this, previous single cell imaging studies have reported the presence of either localised cytosolic Ca^{2+} microdomains or Ca^{2+} gradients across human platelets (Ariyoshi and Salzman 1996; Tsunoda et al. 1988). In addition, Nesbitt et al. (2009) reported that a localised Ca^{2+} signal was generated that allowed membrane tether formation in adherent platelets, without notable activation of shape change or aggregatory mechanisms. These data suggest that platelets can significantly restrict Ca^{2+} diffusion from the source of entry into the cytosol, and thus use the subcellular localisation of Ca^{2+} signals to selectively activate some Ca^{2+} effectors.

To create localised rises of Ca^{2+} in specific areas would require platelets to localise Ca^{2+}-transporting processes to specific subregions of the cell. This possibility is suggested by a number of previous studies which have shown a key role for the platelet cytoskeleton (Ariyoshi and Salzman 1996, Walford et al. 2015) and lipid raft domains (Brownlow et al. 2004) in facilitating normal agonist-evoked Ca^{2+} signals. Single cell imaging studies have also found evidence for specific subregions of platelets being the sites of Ca^{2+} mobilisation (Heemskerk et al. 2002) as well as Ca^{2+} removal from the platelet cytosol (Sage et al. 2013), suggesting that Ca^{2+}-transporting proteins are inhomogenously located in these cells. In addition to locating Ca^{2+} channels and exchangers in specific regions, platelets would also require cellular mechanisms to restrict the spatial spread of Ca^{2+} through the cytosol to account for the Ca^{2+} microdomains and gradients described earlier. Recent work has suggested that the membrane complex, formed by close association of the open canalicular system with the dense tubular system, may create a suitable cellular architecture to generate such localised Ca^{2+} signals in platelets (Walford et al. 2015). The close association of both the open canalicular system and the dense tubular system at the membrane complex creates a nanojunction, which in other cell types has been shown to play a role in creating a region of the cytosol in which Ca^{2+} concentration can be controlled in isolation from the rest of the cell (van Breemen et al. 2013; White 1972). The possibility that the membrane complex may control platelet Ca^{2+} signalling has been suggested by the identification of a family with a bleeding disorder characterised by the lack of a membrane complex and a defect in thrombin-evoked Ca^{2+} signalling (Parker et al. 1993).

Coincidence Detection

Although individual platelet function may be affected principally by the Ca^{2+} signal within its cytosol, it has also become apparent that the outcome of that signal is also dependent on whether other platelets encountered on the surface of the thrombus are also simultaneously active. Previous work has demonstrated that thrombus growth is dependent on the presence of a coincident Ca^{2+} signal in a neighbouring cell (Nesbitt et al. 2003). Although intracellular Ca^{2+} signalling may facilitate thrombus growth, recent work has also suggested that Ca^{2+} signalling can also upregulate nitric oxide production in platelets, which may work to prevent thrombus growth (Cozzi et al. 2015). Thus, further research will be necessary to understand how these distinct pathways interact to regulate the Ca^{2+}-dependent growth of a thrombus.

Whilst we now have a reasonable understanding of the molecular machinery involved in mediating agonist-evoked rises in $[Ca^{2+}]_{cyt}$, there is still much to learn about how the channels and transporters concerned work to alter the

spatiotemporal patterns of Ca^{2+} signals, how these Ca^{2+} signals regulate downstream Ca^{2+}-sensitive processes and how these systems work alongside other signalling pathways to help regulate both the growth of a thrombus and the patterning of individual platelet responses within it.

Take Home Messages

- A rise in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) is central to platelet activation.
- Agonists stimulate a rise in $[Ca^{2+}]_{cyt}$ by releasing Ca^{2+} from intracellular stores and stimulating Ca^{2+} entry across the plasma membrane.
- Stored Ca^{2+} may be released from the dense tubular system (DTS) and acidic organelles by the second messengers inositol 1,4,5-trisphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively.
- Ca^{2+} entry across the plasma membrane may be activated in response to store depletion (store-operated Ca^{2+} entry; SOCE), in response to the second messenger diacylglycerol (DAG) or by activation of an ionotropic receptor (ATP acting at the P2X1 receptor).
- Platelet activation may increase $[Ca^{2+}]_{cyt}$ from 50 to 100 nM to several μM and result in oscillations or repetitive spikes in elevated $[Ca^{2+}]_{cyt}$ in individual cells.
- Imaging studies have revealed platelet Ca^{2+} signals show subcellular microdomains and the platelet membrane complex may form a specialised nanojunction for Ca^{2+} released by the DTS to be removed into the open canalicular system for subsequent recycling back to the cytosol.
- Platelets Ca^{2+} signals are transduced into function responses by many effector proteins including calmodulin, scinderin, gelsolin, calpain, CalDagGEFI, CIB1, PKC, Munc13-4, SNARE proteins, $cPLA_2$ and TMEM16F.
- Ca^{2+} is removed from the platelet cytosol by sequestration into organelles and removal across the plasma membrane.
- Ca^{2+} is sequestered by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and by a vacuolar H^+-ATPase (vH^+-ATPase), probably coupled to a H^+/Ca^{2+} exchanger.
- Ca^{2+} is removed across the plasma membrane either by primary active transport via plasma membrane Ca^{2+}-ATPases (PMCAs), and by Na^+/Ca^{2+} exchangers.

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