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TRP Channels in Platelet Function

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Abstract Ca^{2+} entry forms an essential component of platelet activation; however, the mechanisms associated with this process are not understood. Ca^{2+} entry upon receptor activation occurs as a consequence of intracellular store depletion (referred to as store-operated Ca^{2+} entry or SOCE), a direct action of second messengers on cation entry channels or the direct occupancy of a ligand-gated $P2_{X1}$ receptor. The molecular identity of the SOCE channel has yet to be established. Transient receptor potential (TRP) proteins are candidate cation entry channels and are classified into a number of closely related subfamilies including TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin) and TRPML (mucolipins). From the TRPC family, platelets have been shown to express TRPC6 and TRPC1, and are likely to express other TRPC and other TRP members. TRPC6 is suggested to be involved with receptor-activated, diacyl-glycerol-mediated cation entry. TRPC1 has been suggested to be involved with SOCE, though many of the suggested mechanisms remain controversial. As no single TRP channel has the properties described for SOCE in platelets, it is likely that it is composed of a heteromeric association of TRP and related subunits, some of which may be present in intracellular compartments in the resting cell.

Keywords TRPC proteins · Platelets · TRPC1 · TRPC6 · Store-operated Ca^{2+} entry

1 Introduction

Platelets are anucleate cells that play an essential role in haemostasis and thrombosis. At sites of blood vessel injury, platelets adhere to the sub-endothelium and activate, releasing a number of factors that recruit and activate more platelets in addition to activating other vascular cells. These factors include ADP, serotonin and thromboxane A_2 and result in platelet–platelet aggregation and secretion of the contents of their granules, providing a surface for the coagulation reactions [via phosphatidylserine (PS) exposure] that result in the generation of thrombin, and leading to the formation of a haemostatic plug. However, inappropriate activation of platelets in diseased blood vessels, such as those occurring in unstable angina after rupture of an unstable plaque, can lead to myocardial infarction or stroke. Thus, platelets represent important targets for drug intervention. Platelets are formed from megakaryocytes by a complex process that involves the fragmentation of the mature megakaryocyte cytoplasm (Brown et al. 1997). Mature megakaryocytes form less than 0.1% of the cell population of the bone marrow, but the mature cell can be clearly distinguished by its gigantic size compared with other cells in the bone marrow. Megakaryocytes are formed from stem cells during a differentiation process that takes several weeks with one mature megakaryocyte able to produce several thousand platelets. As platelets are anucleate and have little protein synthesising activity, the expression of proteins, be it surface receptors, ion channels or intracellular signalling enzymes, is essentially dictated by their expression in mature megakaryocytes. While the ease with which megakaryocytes can be recognised allows their study at the single cell level, a study of their biochemistry is very difficult because of low numbers. This has led to the use of cell lines isolated from leukaemic patients that represent a megakaryoblastoid phenotype of which the DAMI and human erythroleukemia (HEL) cell lines are a good example. The CHRF-288 cell line is also widely used and represents a more mature megakaryocyte phenotype, as it expresses a higher content of platelet enriched proteins [such as GPIIbIIIa (CD61/CD42b) and GPIb] than the DAMI cell.

2 Ca^{2+} Signalling in Platelets and Megakaryocytes

Recent studies have confirmed an essential role for Ca^{2+} signalling during aggregate formation and thrombus growth in vitro (Nesbitt et al. 2003). Platelet activation (by collagen, thrombin, thromboxane A_2 or ADP) involves the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting in the formation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 causes the release of Ca^{2+} from in-

tracellular stores via the IP_3 receptor (IP_3R). Ca^{2+} entry mechanisms from the outside medium are poorly understood but are critical for full activation of the platelet (Nesbitt et al. 2003). A major Ca^{2+} entry pathway results as a consequence of store depletion and is referred to as store-operated Ca^{2+} entry (SOCE) (Putney et al. 2001). A Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) that represents a specific SOCE current has been demonstrated in the megakaryocytic cell line HEL (Somasundaram et al. 1997) and rat megakaryocytes (Somasundaram and Mahaut-Smith 1994). However, the link between the stores and the plasma membrane (PM) Ca^{2+} entry channel is still poorly defined. Currently favoured mechanisms include the release of a soluble factor (Randriamampita and Tsien 1993) that may involve a Ca^{2+}-independent PLA_2 ($iPLA_2$) and calmodulin (Smani et al. 2004), a coupling mechanism involving the IP_3R with the PM channel (Irvine 1990) and a model proposing incorporation of a channel or messenger molecule into the plasma membrane (Yao et al. 1999). Rearrangements of the actin cytoskeleton that may affect protein coupling have been shown to block SOCE (Patterson et al. 1999), though the mechanism remains controversial (Lockwich et al. 2001). In platelets, ATP can directly induce Ca^{2+} entry via occupancy of the ligand-gated $P2_{X1}$ receptor (MacKenzie et al. 1996). Other non-SOCE mechanisms include the action of second messengers such as IP_3, which has been reported to induce activation of Na^+ entry in rat megakaryocytes (Somasundaram and Mahaut-Smith 1995), and a DAG analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) that is able to induce Ca^{2+} or Ba^{2+} entry in platelets via a possible action on a member of the TRPC channels present in platelets (Hassock et al. 2002). Additionally, phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is the product of phosphatidylinositol 3-kinase (PI-3-K), has been reported to induce Ca^{2+} entry in rabbit platelets and T cells (Lu et al. 1998; Hsu et al. 2000). There are therefore multiple mechanisms of Ca^{2+} (and Na^+) entry and the identities of the channels involved and the molecular mechanisms gating them are not established.

3
Ca^{2+} Channels and TRP Proteins

The transient receptor potential (TRP) proteins have been proposed as candidates for SOCE and non-SOCE channels. They are thought to assemble as tetramers to form a channel. There are at least 28 TRP genes in mammalian systems (Clapham 2003), and they have been divided into closely related groups based on sequence identities—the TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPP (polycystin) and TRPML (mucolipin) groups. There is considerable information on the TRPC group, with over-expression studies suggesting most to be receptor-activated (involving PLC), non-selective cation channels. TRPC3, -6 and -7 are the most closely related members, having 75% sequence identity. Heterotetrameric association is known to occur

within the TRPC3, -6 and -7 subgroup and TRPC1 is able to complex with -4 and -5 (Hofmann et al. 2002) and may associate with TRPC3 and -7 also (Zagranichnaya et al. 2005). TRPC3, -6 and -7 are known to be activated by DAG independent of protein kinase C (PKC) and independent from store regulation (Hofmann et al. 1999). However, some studies using a variety of tissues have suggested TRPC1, -3, -4 and -5 may be gated by store depletion, though this is controversial.

4
Expression of TRPC Genes in Megakaryocytic Cells

Concerning megakaryocytes and megakaryocytic cell lines, published studies from ourselves (Berg et al. 1997) and others (den Dekker et al. 2001) have shown the expression of messenger RNA (mRNA) for TRPC1, -1A (shorter spliced form), -2, -3, -4 and -6, and for platelets, expression of TRPC1, -1A, -6 and very faintly -4 (TRPC7 was not examined). We have now carried out a further analysis of mRNA expression of TRPC channels in DAMI and CHRF-288 cells prior to studies of knock-down using RNA interference (RNAi) reagents. Excluding TRPC2, which is a pseudogene in humans, in DAMI cells we find good detection of TRPC1 and -3 mRNA using 28 cycles of polymerase chain reaction (PCR), with weak detection of TRPC4 and -6 after 40 cycles of PCR, suggesting predominant expression of TRPC1 and -3 in DAMI cells. In CHRF-288 cells, message for TRPC1 and -3 is again readily detectable at 28 cycles, with that for TRPC6 and -7 detectable at 32 cycles. For TRPC4, 40 cycles are required. In both CHRF-288 and DAMI cells we detected no TRPC5 when tested up to 40 cycles of PCR (K. S. Authi, Y. Shaifta and J.P.T. Ward, unpublished observations). Taken together these observations suggest differentiation-related changes of TRPC expression in megakaryocytes, an increased expression of TRPC6 and -7 with maturation, and potentially an increased role for the latter TRPC channels in the Ca^{2+} signalling characteristics of platelets. This suggestion was mirrored in the earlier study of den Dekker et al. (2001) who reported increased expression of TRPC4 and -6 upon differentiation of stem cells to megakaryocytes using thrombopoietin as the growth factor, with the initial stem cell having detectable levels of only TRPC1A mRNA. It is interesting that TRPC3 mRNA was not detected in the megakaryocytes but, as in our studies, was detected in the cell lines. This may reflect a property of the immortalised cell lines and remains to be established. Further differentiation of stem cells into monocytes did not lead to over-expression of TRPC3, -4 or -6, indicating a lineage-related change. Den Dekker suggested the increased expression of these TRPC channels may relate to the increased SOCE activity of mature megakaryocytes and platelets. In both our work and that of den Dekker, no detection of TRPC5 mRNA was seen in either the megakaryocytes or platelets (or in the cell lines).

5
Expression of TRPC Proteins in Platelets and Ca^{2+} Signalling

Many of the studies published to date regarding the proteins have been from our group and Sage and colleagues, and interestingly have been subject to considerable debate and controversy [see letters to *Blood* (Sage et al. 2002; Authi et al. 2002)]. A great deal of this controversy has arisen from the use of different reagents to identify the TRPC channels present. But before this, it is worth defining some of the characteristics of Ca^{2+} entry in platelets. Figure 1 illustrates important parameters of Ca^{2+} entry after platelets, labelled with Fura2, are stimulated with either a potent platelet agonist—such as thrombin or the sarco-endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin—or a cell-permeable analogue of diacylglycerol, namely OAG, using the Ca^{2+} add back protocol (Hassock et al. 2002). Essentially, store depletion by thapsigargin induces a slow release of the Ca^{2+} from stores, and when Ca^{2+} is added to the medium a rapid and strong entry ensues (Panel b). In our hands, activation of SOCE by thapsigargin is potent at inducing entry of Ca^{2+} but poor at inducing entry of Ba^{2+} when this is substituted for Ca^{2+} in the extracellular medium. This suggests that in human platelets the SOCE channel is selective for Ca^{2+} over Ba^{2+}. If the platelet agonist thrombin is used, this is potent at releasing Ca^{2+} from intracellular stores (by the formation of IP_3) but also effective at inducing the entry of Ca^{2+} and Ba^{2+} (panel c). This suggests that thrombin induces the opening of at least two plasma membrane cation channels. One of these will be the SOCE channel that is selective for Ca^{2+} and another will allow the entry of Ca^{2+} or Ba^{2+}. Figure 1 also shows that OAG induces a slow entry of Ca^{2+} as effectively as Ba^{2+} and thus activates a non-selective cation entry channel (panel a). OAG-induced cation entry occurs without an effect on Ca^{2+} release from intracellular stores (not shown) and is independent of PKC as no effect is observed by inclusion of the PKC inhibitor bisindolylmaleimide I (Bis I). As thrombin leads to the activation of PLC and the formation of DAG, it is likely that endogenous DAG is responsible for the entry of Ba^{2+} by thrombin.

The molecular identification of the channels present is not easy, and many questions regarding the role of TRP channels remain unanswered. In our studies we have used antibodies raised in our laboratory and those of collaborators to identify expression in platelets. In each case the antibody preparations were shown to recognise the corresponding over-expressed TRPC protein and were then tested with platelet membranes. Our studies have shown that there is a low level of TRPC1 and a higher level of TRPC6. TRPC6 was easily detectable using both an in-house-generated antibody and one obtained from Alomone Labs (both to the same epitope sequence); when determined using highly purified fractions of plasma and intracellular membranes prepared by free flow electrophoresis, TRPC6 was found exclusively in the plasma membrane fraction consistent with a role involving cation entry. TRPC1, however, was found predominantly in the intracellular membrane from resting platelets, us-

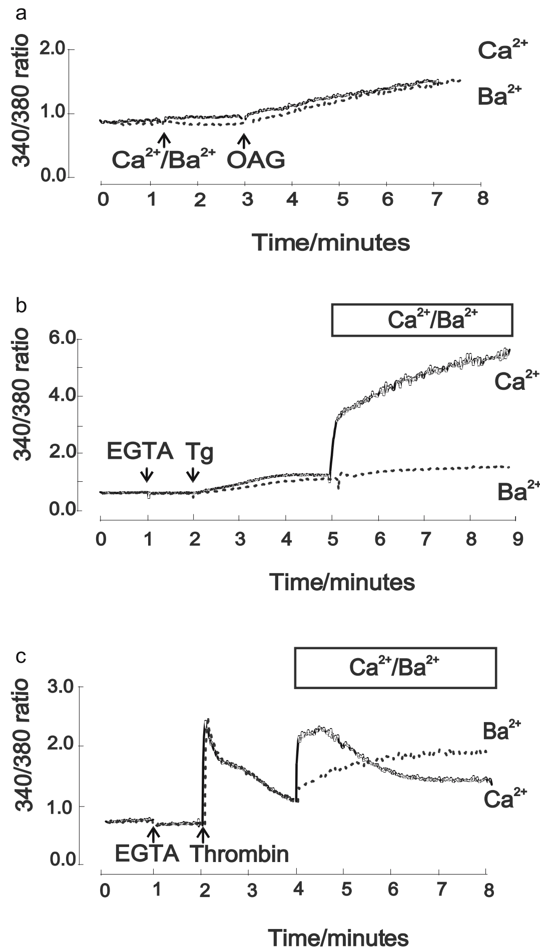

Fig. 1 a–c Characteristics of cation influx in human platelets. Fura2-loaded platelets were incubated at 37°C (additions of the agonists or analogue are marked with *arrows*); the ratio (340/380 nm) of fluorescence increase was measured using extracellular 1 mM Ca^{2+} (*continuous lines*) or Ba^{2+} (*dashed lines*). **a** Responses to 60 μM *OAG*; **b** responses to 3 μM thapsigargin (*Tg*), EGTA was added at 100 μM; **c** responses to 1 U/ml *thrombin*. Other details see text. Taken from Hassock et al. (2002)

ing an antibody raised to the first ankyrin domain of TRPC1 (Ank) and also an anti-*Xenopus* TRP1 antibody that recognises hTRPC1 (Hassock et al. 2002). Using antibodies that were shown to be specific to TRPC3 and one shown to be specific for TRPC4 and -5, we were unable to confirm expression of TRPC3, and TRPC4 or -5 in platelet membranes. This suggests that expression of these isoforms was below the level of detection for these antibodies or that they were not significantly expressed. The absence of TRPC3 detection agrees with

the absence of detection of TRPC3 mRNA from platelets and from cultured megakaryocytes (den Dekker et al. 2001), though TRPC3 mRNA is detected in the cell lines DAMI and CHRF-288. Additionally, the absence of TRPC5 also confirms the lack of detection of this isoform at the mRNA level in platelets, megakaryocytes and cell lines. With anti-TRPC4 antibodies we have observed staining of a 250-kDa protein, but the identity of this band is unknown (S.R. Hassock and K.S. Authi, unpublished observations). Studies to determine expression of TRPC7 are under way, and our preliminary studies suggest using an antibody raised to a C-terminal epitope of TRPC7 that recognises mTRPC7 overexpressed in QBI-293 cells that there is expression in platelet membranes (S.R. Hassock and K.S. Authi, unpublished observations).

A number of studies from Sage and colleagues have also reported the presence of TRPC1 in platelets using an antibody obtained from Alomone Labs (Rosado and Sage 2000a, 2001; Rosado et al. 2002), which they suggest is involved with SOCE and couples with the IP_3R type II upon store depletion. Very recently they have reported the expression of TRPC3, -4 and -5 to almost similar extents as to TRPC6 and -1, again using antibodies obtained from Alomone Labs (Brownlow and Sage 2005). This reported presence of TRPC3, -4 and -5 may reflect levels that may be undetectable by antibodies used in our laboratory, in which case their roles require elucidation. However, a number of the commercially available antibodies have been subject to considerable criticism; thus, results obtained with these require caution and also reconfirmation with better-characterised antibodies. This is particularly striking with the TRPC1 and -4 polyclonal antibodies from Alomone Labs, and many groups have used these extensively. The manufacturer's Web site (up to December 2005) indicates that the antibody to TRPC1 is raised to a cytoplasmic epitope (residues 557–571) and Western blotting with rat brain membranes reveals protein staining at greater than 250 kDa and approximately 120 kDa. Unfortunately, neither weight is the equivalent of TRPC1. Human TRPC1 migrates at approximately 80 kDa, and so does the endogenous protein from rat brain (Goel et al. 2002). Strikingly, Ong et al. (2002) have shown that the Alomone TRPC1 antibody preparation does not recognise over-expressed hTRPC1 but did recognise proteins of 120 kDa (as stated by the manufacturer) in fractions from mouse liver and brain that remain unidentified. Therefore it remains possible that the 90- to 100-kDa protein recognised in endogenous tissues with this anti-TRPC1 antibody may not be TRPC1, and its identity needs to be confirmed by protein sequencing. Similar criticism has also surfaced regarding the anti-TRPC4 antibody used in the Brownlow and Sage study to show expression of TRPC4 in platelet membranes. Flockerzi et al. (2005) have recently shown that this anti-TRPC4 antibody recognises the appropriate size band in brain microsomes from wild-type mice expressing TRPC4, but it also recognises the same band in brain microsomes prepared from $TRPC4^{-/-}$ mice, where the protein does not exist. It may be that batch difference may explain the discrepancies between laboratories, but use of these reagents alone will continue to generate confus-

ing messages that do not help our understanding of the proteins that regulate Ca^{2+} signalling. Using these antibodies, Brownlow and Sage (2005) show from immunoprecipitation experiments that "TRPC1" associated with "TRPC4" and TRPC5, and that TRPC3 associated with TRPC6 in human platelets. The study lacks any demonstration that the antibody preparations recognise the appropriate positive controls. They reported near equal expression of all these isoforms, whereas mRNA analysis suggests very unequal expression, and in our studies TRPC5, -4 and -3 proteins were not detected with the antibodies used. Verification of this data is therefore needed using better-characterised alternative antibodies.

6
Proposed Roles for TRPC1

Our finding of TRPC1 in intracellular membranes initially surprised us, but there are currently numerous examples of TRPC channels found in membrane systems distributed in the cytoplasm. When over-expressed alone, Strubing et al. (2001) found that TRPC1 did not carry a significant current and Hofmann et al. (2002) showed that it was widely distributed in the cytoplasm. Where expression is successful its cation permeability is similar for Ca^{2+}, Na^{+}, Cs^{+} or Ba^{2+} (Sinkins et al. 1998), and when co-expressed with TRPC5 again it has non-selective cation permeability (Strubing et al. 2001). Thus, by itself or in combination with TRPC5, TRPC1 does not explain the Ca^{2+} selectivity seen in platelet SOCE. However, there is evidence that in a number of cells, including salivary glands and endothelial cells, TRPC1 may be involved with store-mediated Ca^{2+} entry. Its presence in intracellular stores does not rule out its involvement in SOCE, particularly if a population of heteromeric channel complexes exists that is ready to be inserted into the plasma membrane upon signal generation. In our ongoing studies, over-expression of hTRPC1-GFP (green fluorescent protein tagged) into DAMI or CHRF-288 cells reveals expression throughout the cytoplasm and not just localised at the plasma membrane. In avian B cells, Mori et al. (2002) reported that knock-out of TRP1 (avian TRPC1) not only inhibited SOCE but also reduced agonist-induced Ca^{2+} release from stores and IP_3-mediated Ca^{2+} release from the endoplasmic reticulum. Such effects would not be seen if all of the TRP1 was present in the plasma membrane and imply, in line with our finding, that at least a part of the TRPC1 is present in intracellular stores and can interact with the IP_3R. Thus, activation of platelets by surface receptor agonists or store depletion may lead to an insertion of the channel into the plasma membrane.

In other cell systems, TRPC1 has been shown to be capable of binding to a large number of proteins; these include the IP_3R, homer, calmodulin, caveolin, FKBP25, Gq11, PLC, PKC and RhoA, plus others. We have shown that platelets express all three types of IP_3R, with the expression of the type I and

type II much greater than the type III receptor (El Daher et al. 2000). The Sage group have reported using human platelets and the Alomone anti-TRPC1 antibody that "TRPC1" couples to the type II IP_3R when stores are depleted with either thapsigargin or thrombin treatment; the TRPC1 antibody when applied extracellularly inhibited Ca^{2+} entry or Mn^{2+} entry by approximately 70% (Rosado and Sage 2000a, 2001; Rosado et al. 2002). This suggests that "TRPC1" could account for the majority of Ca^{2+} entry. They suggest a model where the type II IP_3R at the intracellular stores specifically couples with "TRPC1" at the plasma membrane when stores are depleted and cite this as evidence for a secretion-like coupling mechanism previously described by Patterson et al. (1999). "TRPC1" is proposed to be only in the plasma membrane, as platelet activation by thapsigargin did not lead to any further increase of antibody binding to intact platelets (Rosado et al. 2002) or an increase of surface biotin labelling of this protein with either thapsigargin or thrombin treatment (Brownlow and Sage 2005). Coupling and SOCE was decreased if platelets were depleted of cholesterol with methyl-β-cyclodextrin (Brownlow et al. 2004), suggesting that "TRPC1" may exist in lipid rafts, as shown in other cells, and was not affected by PKC inhibition (Brownlow and Sage 2003). Further they reported that jasplakinolide, which is known to induce cortical actin assembly and thereby erect a physical barrier, inhibited Ca^{2+} entry and disrupted the coupling of "TRPC1" with the IP_3R (Rosado et al. 2002). Surprisingly, xestospongin, an agent that inhibits IP_3R function, also inhibited the coupling of the IP_3R to "TRPC1", even if store depletion was carried out by thapsigargin (Rosado and Sage 2001). However, and in contrast, coupling induced by thapsigargin was not affected in the presence of 2-aminoethoxydiphenyl borate, which has also been shown to inhibit IP_3R and cation entry channels, suggesting that not all inhibitors of IP_3R affected this interaction (Diver et al. 2001).

Studies carried out with TRPC1 on platelets in our laboratory differ significantly from those of the Sage group. Incubation of Fura2-loaded human platelets with the Alomone TRPC1 antibody at 10 µg/ml for 10 min did not have any significant effect on Ca^{2+} entry induced by either thapsigargin (200 nM) or 0.25 U/ml thrombin (S.R. Hassock and K.S. Authi, unpublished observations). Again we cannot rule out differences in batches that may lead to loss of antibody activity, but an alternative explanation is that TRPC1 may be present predominantly inside the cell. Thus, the protein band seen in the Sage group studies needs further characterisation. In studies thus far, with our antibodies we have not observed a store depletion-dependent coupling of TRPC1 with the IP_3R type II but have seen a constitutive coupling of the two proteins (S.R. Hassock and K.S. Authi, unpublished observations). Studies by Kunzelmann-Marche et al. on HEL cells (which have a similar phenotype to DAMI cells) with the anti-TRPC1 antibody suggest that the antibody had a small significant inhibition of thapsigargin-induced Ca^{2+} entry but also A23187-mediated Ca^{2+} entry, where in the latter case these effects were linked to a similar small inhibition of PS exposure that occurs when pro-coagulant activity is expressed. But

surprisingly the antibody also significantly inhibited thapsigargin-mediated Ca^{2+} release from stores, with the authors having to use low concentrations of antibody to "avoid artefactual membrane perturbation" (Kunzelmann-Marche et al. 2002).

Studies from other systems provide an insight into the microenvironment of TRPC1. Ambudkar's group have shown that TRPC1 (using their own in-house-generated antibody) exists in the cholesterol-rich lipid raft domains in the membrane of human salivary gland cells in association with a number of other signalling proteins such as the IP_3R, caveolin 1, plasma membrane Ca^{2+} ATPase and Gαq/11 (Lockwich et al. 2000). Such an arrangement was also seen with TRPC3 in a stably transfected cell line and where rearrangement of the cytoskeleton with jasplakinolide led to an internalisation of the multimolecular complex containing the IP_3R and TRPC protein rather than a disruption of the association (Lockwich et al. 2001). Studies from Worley's group have reported that the adaptor protein homer plays an important role in the interaction of TRPC1 with IP_3R in human embryonic kidney (HEK)-293 cells and show that store depletion was associated with a disassembly of the complex in contrast to the association reported by the Sage group (Yuan et al. 2003). Homer appears to be important in the association of the IP_3R with TRPC1, and the group reported that a mutated form of homer that doesn't bind TRPC1 leads to a constitutive activation of TRPC1, suggesting that homer may hold TRPC1 in a closed state. Currently it is not known if homer is expressed in platelets. Another cytosolic protein reported to interact with and inhibit TRPC1 is I-mfa (inhibitor of myogenic family a) whose knock-down leads to an enhancement and its over-expression leads to an inhibition of TRPC1-implicated store-mediated currents in CHO-K1 cells (Ma et al. 2003). In microvessel endothelial cells a significant proportion of TRPC1 has been reported to exist in the cytosolic compartment, and its recruitment to the plasma membrane requires the activation of Rho, which increases an interaction of TRPC1 with an IP_3R and its insertion into the plasma membrane (Mehta et al. 2003). The authors reported that inhibition of Rho activation by transfected C3 transferase reduced the association and translocation of TRPC1 to the plasma membrane even if stores were depleted. In a subsequent study they reported that PKC-dependent phosphorylation of TRPC1 was also required to elicit Ca^{2+} entry via TRPC1 and this was also associated with an increase of endothelial cell permeability (Ahmmed et al. 2004). In platelets there is a suggestion that Rho and other small G proteins may play a role in SOCE, and incubation of C3 exoenzyme with platelets for 2 h is reported to cause inhibition of thapsigargin-induced Ca^{2+} entry in addition to use of pharmacological agents that inhibited methylation of these proteins (Rosado and Sage 2000b). Whether these manipulations can alter TRPC1-IP_3R coupling or translocation as is suggested in endothelial cells is not known. But PKC function is thought not to be required for SOCE in platelets, and its inhibition has been reported not to affect the "TRPC1"-IP_3R coupling (Brownlow and Sage 2003).

Phosphorylation of proteins is an effective way of regulating the activity of many proteins. TRPC1 contains consensus sequences for action by various protein kinases such as PKC, PKA (cAMP-dependent protein kinase), PKG (cGMP-), casein kinase II and tyrosine kinases. Platelet stimulatory and inhibitory agents all cause an increase in phosphorylation of a number of protein substrates. Using Western blotting techniques and [^{32}P]-labelled platelets we were unable to show an increase of phosphorylation of TRPC1 by tyrosine kinases, PKC, PKA or PKG under conditions where the activity of the kinases was demonstrated on known substrates after stimulation by appropriate agents (Hassock et al. 2002). However, TRPC1 extraction from human platelets with the Ank antibody did co-extract a number of proteins of molecular weight 250, 120 and 85 kDa that were phosphorylated by either PKA or PKG, and these phosphorylations decreased upon activation of platelets by thrombin. This suggests that the microenvironment around TRPC1 contains proteins that are substrates for PKA and PKG. This is important as, in the circulation, platelets are exposed to either prostacyclin or nitric oxide that will activate PKA and PKG, respectively. These kinases play important regulatory roles in maintaining low cytosolic Ca^{2+} levels. Thus far, however, there are no studies linking TRPC1 activity to platelet function such as shape change, aggregation, secretion or pro-coagulant expression. If TRPC1 is integrally involved with SOCE it is likely that it forms a component rather than the channel itself. Its co-association with TRPC5 or -4 opens the heteromeric channel to augmentation by 100 μM La^{3+} (Strubing et al. 2001; Schaefer et al. 2000). This is in contrast to the reported effects of inhibition of SOCE by La^{3+} and it complicates the proposal that TRPC5 and -4 are associated with TRPC1 in human platelets. The possibility that TRPC1 may exist as a complex with subunits other than TRPC members is worthy of examination. TRPC1 has been shown to bind to polycystin 2 (TRPP2), which itself has channel properties, and the combination may constitute a channel with distinct properties (Tsiokas et al. 1999). TRPP2 is predominantly associated with the endoplasmic reticulum (Koulen et al. 2002) though some is also reported in the plasma membrane (Hanaoka et al. 2000). TRPP2 has also been reported to form a distinct channel when complexed with TRPP1 (polycystin 1) (Hanaoka et al. 2000). Thus, a heteromeric complex comprising TRPC1 with three distinct subunits, some of which may be initially intracellular and translocate to the plasma membrane upon signal generation, may confer the specific properties of SOCE as seen in platelets. Though this remains to be established, it is an interesting hypothesis.

7 Expression and Role for TRPC6

TRPC6 is a non-selective cation channel but there is a fivefold preference for divalent over monovalent cations (Hofmann et al. 1999). In line with the ex-

pression of TRPC6 in the platelet plasma membrane, we have shown OAG to cause the entry of Ca^{2+} or Ba^{2+}, independent from store release or PKC activation (Hassock et al. 2002). We have also shown that thrombin stimulated entry of Ba^{2+} is inhibited by the inclusion of the PLC inhibitor U73122 and by a cell-permeable analogue of cAMP that suppresses thrombin-induced PLC activation (S. R. Hassock and K.S. Authi, unpublished observations). Thus, a link between PLC activity, DAG and increase of Ca^{2+} entry is proposed. Further inclusion of the PKC inhibitor Bis I prior to thrombin addition leads to an enhancement of Ba^{2+} entry. The slower kinetics of cation entry by OAG on platelets compared with SOCE-mediated entry suggests a role for TRPC6 during the middle and later stages of platelet activation. Thrombin (but not collagen) stimulation of platelets also results in an increase of TRPC6 translocation to the cytoskeletal fraction. This may be important, as thrombin activation leads to the focussing of a large number of signalling proteins (particularly those that are tyrosine phosphorylated) with cytoskeletal elements that allow enzymes and substrates to be in close association for rapid signalling (Fox 1996). Examples of these include actin-binding protein, α-actinin, actin, myosin, cortactin, talin, spectrin, vinculin, GPIb, GPIIbIIIa, $pp60^{c\text{-}src}$, PI-3-K, rap1b and others. TRPC6 has been reported to bind to α-actinin (Goel et al. 2005).

We have shown in platelets that agents that stimulate PKA or PKG lead to TRPC6 phosphorylation and, like TRPC1, TRPC6 also appears to be associated with proteins that are substrates of PKA (Hassock et al. 2002). This localises the channel in an environment that is regulated by cAMP concentrations. Membranes from TRPC6 over-expressing QBI-293 cells incubated with the catalytic subunit of PKA show increased incorporation of phosphate into the protein. Currently the direct consequence of this phosphorylation is not known, as in platelets OAG-stimulated Ca^{2+} or Ba^{2+} entry is unaffected. Two potential sites for PKA-mediated phosphorylation exist at $RRQT^{70}$ and $KKLS^{332}$. Both are in the N-terminal part of the protein which contains a number of ankyrin domains; whether this may alter interaction with cytoskeletal proteins remains to be determined. There are no specific inhibitors of TRPC6 and no specific function-blocking antibody available for testing yet. Analysis of the platelet activities from $TRPC6^{-/-}$ mice may yield valuable information.

There have been recent studies that implicate PIP_3 (the product of PI-3-K) as an activator of TRPC6. PI-3-kinase is heavily expressed in a number of haematopoietic cells, has been described to be important in many functions and has a major role in the activation of PLCγ during platelet stimulation by collagen. TRPC6 contains a YXXM motif that acts as a recognition site for the p85 subunit of PI-3-K. Initially Chen's laboratory showed that cell-permeable analogues of PIP_3 [such as dipalmitoyl-sn-glycerol (DiC16)-PIP_3 or dioctanoyl-sn-glycerol (DiC8)-PIP_3] activated rabbit platelets and caused entry of Ca^{2+} without release from stores (Lu et al. 1998). These studies were extended to PIP_3-activating Ca^{2+} entry in T cells and in rat basophilic leukaemia cells but

not in B cells (Hsu et al. 2000; Ching et al. 2001). This suggests that PIP_3 may represent a novel mechanism for store-independent Ca^{2+} entry in some haematopoietic cells. In a recent study using TRPC subunits stably expressed in HEK-293 cells, Chen's group have shown that PIP_3 activates a number of TRPC channels but especially TRPC6 (Tseng et al. 2004).

Because TRPC6 is expressed in platelets we have also examined this activity. As our previous studies suggested that we could use entry of Ba^{2+} to monitor SOCE-independent TRPC6 activity, we examined both activation and entry of cations in Ca^{2+} or Ba^{2+} medium. We have found that exogenously added DiC8- or DiC16-PIP_3 caused aggregation of washed human platelets in a dose-related manner with the DiC8-PI(4,5)P_2 or DiC8-PI(3,4)P_2 either poorly or not effective (S.R. Hassock and K.S. Authi, manuscript in preparation). However, DiC8-PIP_3 was not able to cause the entry of Ca^{2+}, and in only a few preparations (1 in 10) did we observe a small entry of Ba^{2+}. Thus, aggregation of platelets and induction of cation entry could not be correlated. When examined against an effector of PI-3-K, exogenous DiC8-PIP_3 was able to cause activation of Akt (or PKB) in human platelets, suggesting that it did gain entry into the cell and that PKB activation could be related to the aggregation of the cells. In agreement with this effect, an inhibitor of PKB was effective in blocking the aggregation of platelets seen with DiC8-PIP_3. We examined the ability of DiC8-PIP_3 to directly activate transiently expressed TRPC channels in QBI-293 cells. Expression of TRPC proteins was monitored by Western blotting using TRPC-specific antibodies, and the functional activity of hTRPC6, hTRPC3 and mTRPC7 was gauged by monitoring the entry of Ba^{2+} by OAG. Under these conditions we were unable to activate hTRPC6, hTRPC3 or mTRPC7 by DiC8-PIP_3 (S.R. Hassock and K.S. Authi, manuscript in preparation).

The reason for the contrast in data between these findings and those of the Chen group who used stably transfected mTRPC6 is not known, but one possibility may be that stable expression leads to alteration of the expression of PI-3-K effectors or that in our transient expression system an effector protein for PIP_3 may be reduced or missing. We suggest from our data that PIP_3 does not directly activate TRPC6 or its related channels, but we cannot rule out an action of PIP_3 on TRPC channels via another PI-3-K effector. Possible effectors such as PKC isoforms (or others) need to be tested, as these are known to affect TRPC channel activity and a number of the PKC isoforms have been reported to be activated by PIP_3 (Toker and Cantley 1997).

A recent study of TRPC6 gene knock-out in mice resulted in a two- to threefold up-regulation of TRPC3, suggesting a close association of these two members (Dietrich et al. 2005). The mice showed an elevated blood pressure and enhanced agonist-induced contractility in isolated aortic rings that is probably due to the enhanced expression of TRPC3. A number of recent studies have reported that some TRPC channels can associate with other ion-transporting proteins—such as the Na^+/K^+ATPase with TRPC6 and -5 in rat brain (Goel et al. 2005) and the Na^+/Ca^{2+} exchanger with TRPC3 in HEK-293

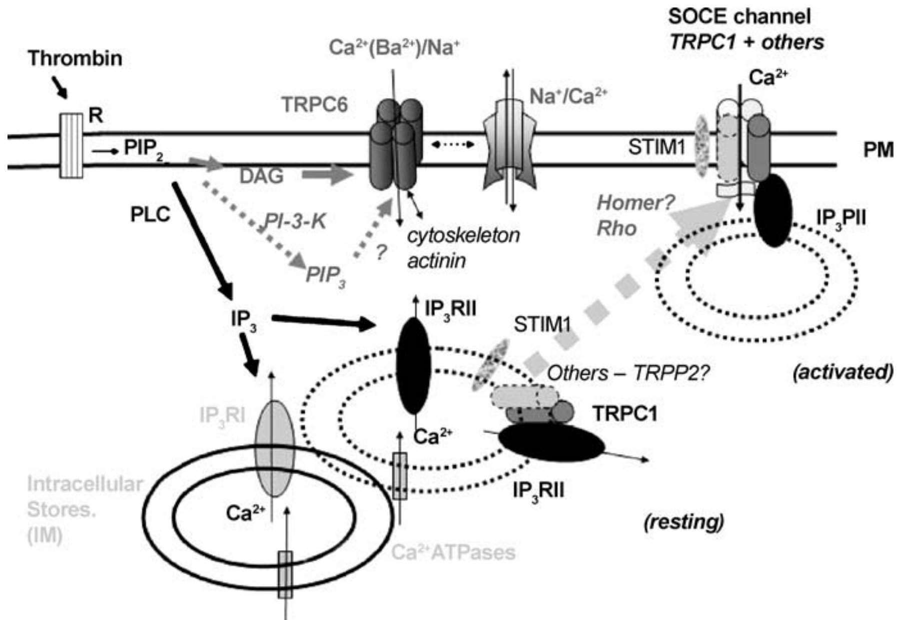

Fig. 2 Model of organisation of TRP proteins in human platelets. For full details see text. Localisation of TRPC7 and other TRPs has yet to be determined

cells (Rosker et al. 2004)—implying a role in Na^+ or K^+ movements. Platelet activation is associated with Na^+ entry (e.g. Roberts et al. 2004), and there is a well-expressed Na^+/Ca^{2+} exchanger in the plasma membrane (Rengasamy et al. 1987; El Daher et al. 2000). Thus, it is possible that TRPC6 may allow the entry of Na^+ which then activates a Na^+/Ca^{2+} exchanger (in the reverse mode) to allow Ca^{2+} entry. A simplified model of the possible organisation of TRP proteins in platelets is depicted in Fig. 2.

8 Expression of Other TRPs and Ca^{2+} Signalling Proteins

Thus far there are no reports of the expression of TRPV or TRPM members in platelets, though it is likely that a number of these subunits are expressed. In an analysis of mRNA detection for TRPM2 and TRPM7 in megakaryocytic cell lines DAMI and CHRF-288, we have observed high expression of TRPM7 in both cell lines and a restricted expression of TRPM2 to the more mature CHRF-288 cell with no detection in the DAMI cells (K.S. Authi and Y. Shaifta, unpublished observations). This is in line with the described ubiquitous expression of TRPM7, which is know to be permeable to Ca^{2+} and Mg^{2+} ions but also to heavy metal divalent cations (Clapham 2003). The channel's sug-

gested ability to sense Mg^{2+}.ATP levels may play a role in metabolic sensing. TRPM2 expression in CHRF-288 cells also suggests its expression in platelets. TRPM2 is a Ca^{2+}-permeable channel that is activated by ADP ribose and is responsive to hydrogen peroxide (H_2O_2). Platelets express CD38 which can generate ADP ribose (Torti et al. 1999), and H_2O_2 is generated as a consequence of platelet activation and has been associated with many oxidative stress-related cellular effects but also Ca^{2+} elevation, inhibition of sequestration, inhibition of extrusion and activation of $pp60^{src}$ (Rosado et al. 2004; Redondo et al. 2004). In a proteomics-based approach using three different strategies to isolate platelet membrane proteins, we have analysed the protein composition of platelet membranes using liquid chromatography linked with mass spectrometry. We have detected expression of TRPM2 and also detected stromal interaction molecule 1 (STIM1), PKD1-like 1 and TRPV3 in addition to other Ca^{2+} signalling proteins and channels (Y.A. Senis, M.G. Tomlinson, Á. García, S. Dumon, V.L. Heath, J. Herbert, S.P. Cobbold, J.C. Spalton, S. Ayman, N. Zitzman, R. Bicknell, J. Frampton, K.S. Authi, A. Martin, M.J. Wakelam, and S.P. Watson, manuscript submitted). Recently STIM1 has been shown by two groups to be the Ca^{2+} sensor, as its knock-down with siRNA inhibited Ca^{2+} entry (Roos et al. 2005; Zhang et al. 2005; Liou et al. 2005). STIM1 can be present in intracellular and plasma membranes and contains an EF hand domain near the N terminus that protrudes either into the lumen of the store or projects outwards to the extracellular medium and binds Ca^{2+}. Store depletion is thought to either induce translocation of STIM1 to the plasma membrane or its re-organisation into punctate regions around the plasma membrane that may allow the regulation of the entry channel. Again, trafficking of proteins from the intracellular compartments to the plasma membrane is stipulated.

In conclusion, platelets have been shown to express TRPC6 as a DAG-activated cation entry channel and a number of other TRP isoforms. The organisation of these subunits into the major Ca^{2+} entry channel and its possible interaction with STIM1 is yet to be established, but may bring about a better understanding of how cation entry occurs into platelets and open up new avenues for therapeutic targeting.

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References

Ahmmed GU, Mehta D, Vogel S, Holinstat M, Paria BC, Tiruppathi C, Malik AB (2004) Protein kinase Calpha phosphorylates the TRPC1 channel and regulates store-operated Ca^{2+} entry in endothelial cells. J Biol Chem 279:20941–20949

Authi KS, Hassock S, Zhu MX, Flockerzi V, Trost C (2002) TRPC channels in platelets and Ca^{2+} entry in human platelets. Blood 100:4246–4247

Berg LP, Shamsher MK, El Daher SS, Kakkar VV, Authi KS (1997) Expression of human TRPC genes in the megakaryocytic cell lines MEG01, DAMI and HEL. FEBS Lett 403:83–86

Brown AS, Erusalimsky JD, Martin JF (1997) Megakaryocytopoiesis: the megakaryocyte/platelet haemostatic axis. In: Bruchlausen FV, Walter U (eds) Antithrombotics. (Handbook of experimental pharmacology, vol. 126) Springer, Berlin Heidelberg New York, pp 3–26

Brownlow SL, Sage SO (2003) Rapid agonist-evoked coupling of type II Ins(1,4,5)P3 receptor with human transient receptor potential (hTRPC1) channels in human platelets. Biochem J 375:697–704

Brownlow SL, Sage SO (2005) Transient receptor potential protein subunit assembly and membrane distribution in human platelets. Thromb Haemost 94:839–845

Brownlow SL, Harper AG, Harper MT, Sage SO (2004) A role for hTRPC1 and lipid raft domains in store-mediated calcium entry in human platelets. Cell Calcium 35:107–113

Ching TT, Hsu AL, Johnson AJ, Chen CS (2001) Phosphoinositide 3-kinase facilitates antigen-stimulated $Ca^{(2+)}$ influx in RBL-2H3 mast cells via a phosphatidylinositol 3,4,5-trisphosphate-sensitive $Ca^{(2+)}$ entry mechanism. J Biol Chem 276:14814–14820

Clapham DE (2003) TRP channels as cellular sensors. Nature 426:517–524

den Dekker E, Molin DG, Breikers G, van Oerle R, Akkerman JW, van Eys GJ, Heemskerk JW (2001) Expression of transient receptor potential mRNA isoforms and $Ca^{(2+)}$ influx in differentiating human stem cells and platelets. Biochim Biophys Acta 1539:243–255

Dietrich A, Mederos YS, Gollasch M, Gross V, Storch U, Dubrovska G, Obst M, Yildirim E, Salanova B, Kalwa H, Essin K, Pinkenburg O, Luft FC, Gudermann T, Birnbaumer L (2005) Increased vascular smooth muscle contractility in $TRPC6^{-/-}$ mice. Mol Cell Biol 25:6980–6989

Diver JM, Sage SO, Rosado JA (2001) The inositol trisphosphate receptor antagonist 2-aminoethoxydiphenylborate (2-APB) blocks Ca^{2+} entry channels in human platelets: cautions for its use in studying Ca^{2+} influx. Cell Calcium 30:323–329

El Daher SS, Patel Y, Siddiqua A, Hassock S, Edmunds S, Maddison B, Patel G, Goulding D, Lupu F, Wojcikiewicz RJ, Authi KS (2000) Distinct localization and function of (1,4,5)IP(3) receptor subtypes and the (1,3,4,5)IP(4) receptor GAP1(IP4BP) in highly purified human platelet membranes. Blood 95:3412–3422

Flockerzi V, Jung C, Aberle T, Meissner M, Freichel M, Philipp SE, Nastainczyk W, Maurer P, Zimmermann R (2005) Specific detection and semi-quantitative analysis of TRPC4 protein expression by antibodies. Pflugers Arch 451:81–86

Fox JE (1996) Studies of proteins associated with the platelet cytoskeleton. In: Watson SP, Authi KS (eds) Platelets, a practical approach. IRL Press, Oxford, pp 217–233

Goel M, Sinkins WG, Schilling WP (2002) Selective association of TRPC channel subunits in rat brain synaptosomes. J Biol Chem 277:48303–48310

Goel M, Sinkins W, Keightley A, Kinter M, Schilling WP (2005) Proteomic analysis of TRPC5- and TRPC6-binding partners reveals interaction with the plasmalemmal $Na^{(+)}/K^{(+)}$-ATPase. Pflugers Arch 451:87–98

Hanaoka K, Qian F, Boletta A, Bhunia AK, Piontek K, Tsiokas L, Sukhatme VP, Guggino WB, Germino GG (2000) Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. Nature 408:990–994

Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS (2002) Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. Blood 100:2801–2811

Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397:259–263

Hofmann T, Schaefer M, Schultz G, Gudermann T (2002) Subunit composition of mammalian transient receptor potential channels in living cells. Proc Natl Acad Sci U S A 99:7461–7466

Hsu AL, Ching TT, Sen G, Wang DS, Bondada S, Authi KS, Chen CS (2000) Novel function of phosphoinositide 3-kinase in T cell Ca^{2+} signaling. A phosphatidylinositol 3,4,5-trisphosphate-mediated Ca^{2+} entry mechanism. J Biol Chem 275:16242–16250

Irvine RF (1990) 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates—a possible mechanism. FEBS Lett 263:5–9

Koulen P, Cai Y, Geng L, Maeda Y, Nishimura S, Witzgall R, Ehrlich BE, Somlo S (2002) Polycystin-2 is an intracellular calcium release channel. Nat Cell Biol 4:191–197

Kunzelmann-Marche C, Freyssinet JM, Martinez MC (2002) Loss of plasma membrane phospholipid asymmetry requires raft integrity. Role of transient receptor potential channels and ERK pathway. J Biol Chem 277:19876–19881

Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE Jr, Meyer T (2005) STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. Curr Biol 15:1235–1241

Lockwich T, Singh BB, Liu X, Ambudkar IS (2001) Stabilization of cortical actin induces internalization of transient receptor potential 3 (Trp3)-associated caveolar Ca^{2+} signaling complex and loss of Ca^{2+} influx without disruption of Trp3-inositol trisphosphate receptor association. J Biol Chem 276:42401–42408

Lockwich TP, Liu X, Singh BB, Jadlowiec J, Weiland S, Ambudkar IS (2000) Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. J Biol Chem 275:11934–11942

Lu PJ, Hsu AL, Wang DS, Chen CS (1998) Phosphatidylinositol 3,4,5-trisphosphate triggers platelet aggregation by activating Ca^{2+} influx. Biochemistry 37:9776–9783

Ma R, Rundle D, Jacks J, Koch M, Downs T, Tsiokas L (2003) Inhibitor of myogenic family, a novel suppressor of store-operated currents through an interaction with TRPC1. J Biol Chem 278:52763–52772

MacKenzie AB, Mahaut-Smith MP, Sage SO (1996) Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. J Biol Chem 271:2879–2881

Mehta D, Ahmmed GU, Paria BC, Holinstat M, Voyno-Yasenetskaya T, Tiruppathi C, Minshall RD, Malik AB (2003) RhoA interaction with inositol 1,4,5-trisphosphate receptor and transient receptor potential channel-1 regulates Ca^{2+} entry. Role in signaling increased endothelial permeability. J Biol Chem 278:33492–33500

Mori Y, Wakamori M, Miyakawa T, Hermosura M, Hara Y, Nishida M, Hirose K, Mizushima A, Kurosaki M, Mori E, Gotoh K, Okada T, Fleig A, Penner R, Iino M, Kurosaki T (2002) Transient receptor potential 1 regulates capacitative $Ca^{(2+)}$ entry and $Ca^{(2+)}$ release from endoplasmic reticulum in B lymphocytes. J Exp Med 195:673–681

Nesbitt WS, Giuliano S, Kulkarni S, Dopheide SM, Harper IS, Jackson SP (2003) Intercellular calcium communication regulates platelet aggregation and thrombus growth. J Cell Biol 160:1151–1161

Ong HL, Chen J, Chataway T, Brereton H, Zhang L, Downs T, Tsiokas L, Barritt G (2002) Specific detection of the endogenous transient receptor potential (TRP)-1 protein in liver and airway smooth muscle cells using immunoprecipitation and Western-blot analysis. Biochem J 364:641–648

Patterson RL, van Rossum DB, Gill DL (1999) Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. Cell 98:487–499

Putney JW Jr, Broad LM, Braun FJ, Lievremont JP, Bird GS (2001) Mechanisms of capacitative calcium entry. J Cell Sci 114:2223–2229

Randriamampita C, Tsien RY (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature 364:809–814

Redondo PC, Salido GM, Pariente JA, Rosado JA (2004) Dual effect of hydrogen peroxide on store-mediated calcium entry in human platelets. Biochem Pharmacol 67:1065–1076

Rengasamy A, Soura S, Feinberg H (1987) Platelet Ca^{2+} homeostasis: Na^{+}–Ca^{2+} exchange in plasma membrane vesicles. Thromb Haemost 57:337–340

Roberts DE, McNicol A, Bose R (2004) Mechanism of collagen activation in human platelets. J Biol Chem 279:19421–19430

Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445

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

Rosado JA, Sage SO (2000b) Farnesylcysteine analogues inhibit store-regulated Ca^{2+} entry in human platelets: evidence for involvement of small GTP-binding proteins and actin cytoskeleton. Biochem J 347:183–192

Rosado JA, Sage SO (2001) Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. Biochem J 356:191–198

Rosado JA, Brownlow SL, Sage SO (2002) Endogenously expressed Trp1 is involved in store-mediated Ca^{2+} entry by conformational coupling in human platelets. J Biol Chem 277:42157–42163

Rosado JA, Redondo PC, Salido GM, Gomez-Arteta E, Sage SO, Pariente JA (2004) Hydrogen peroxide generation induces pp60src activation in human platelets: evidence for the involvement of this pathway in store-mediated calcium entry. J Biol Chem 279:1665–1675

Rosker C, Graziani A, Lukas M, Eder P, Zhu MX, Romanin C, Groschner K (2004) $Ca^{(2+)}$ signaling by TRPC3 involves $Na^{(+)}$ entry and local coupling to the $Na^{(+)}$/$Ca^{(2+)}$ exchanger. J Biol Chem 279:13696–13704

Sage SO, Brownlow SL, Rosado JA (2002) TRP channels and calcium entry in human platelets. Blood 100:4245–4246

Schaefer M, Plant TD, Obukhov AG, Hofmann T, Gudermann T, Schultz G (2000) Receptor-mediated regulation of the nonselective cation channels TRPC4 and TRPC5. J Biol Chem 275:17517–17526

Sinkins WG, Estacion M, Schilling WP (1998) Functional expression of TrpC1: a human homologue of the Drosophila Trp channel. Biochem J 331:331–339

Smani T, Zakharov SI, Csutora P, Leno E, Trepakova ES, Bolotina VM (2004) A novel mechanism for the store-operated calcium influx pathway. Nat Cell Biol 6:113–120

Somasundaram B, Mahaut-Smith MP (1994) Three cation influx currents activated by purinergic receptor stimulation in rat megakaryocytes. J Physiol 480:225–231

Somasundaram B, Mahaut-Smith MP (1995) A novel monovalent cation channel activated by inositol trisphosphate in the plasma membrane of rat megakaryocytes. J Biol Chem 270:16638–16644

Somasundaram B, Mason MJ, Mahaut-Smith MP (1997) Thrombin-dependent calcium signalling in single human erythroleukaemia cells. J Physiol 501:485–495

Strubing C, Krapivinsky G, Krapivinsky L, Clapham DE (2001) TRPC1 and TRPC5 form a novel cation channel in mammalian brain. Neuron 29:645–655

Toker A, Cantley LC (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 387:673–676

Torti M, Bertoni A, Canobbio I, Sinigaglia F, Balduini C (1999) Hydrolysis of NADP+ by platelet CD38 in the absence of synthesis and degradation of cyclic ADP-ribose 2′-phosphate. FEBS Lett 455:359–363

Tseng PH, Lin HP, Hu H, Wang C, Zhu MX, Chen CS (2004) The canonical transient receptor potential 6 channel as a putative phosphatidylinositol 3,4,5-trisphosphate-sensitive calcium entry system. Biochemistry 43:11701–11708

Tsiokas L, Arnould T, Zhu CW, Kim E, Walz G, Sukhatme VP (1999) Specific association of the gene product of PKD2 with the TRPC1 channel. Proc Natl Acad Sci USA 96:3934–3939

Yao Y, Ferrer-Montiel AV, Montal M, Tsien RY (1999) Activation of store-operated Ca^{2+} current in Xenopus oocytes requires SNAP-25 but not a diffusible messenger. Cell 98:475–485

Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S, Worley PF (2003) Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. Cell 114:777–789

Zagranichnaya TK, Wu X, Villereal ML (2005) Endogenous TRPC1, TRPC3, and TRPC7 proteins combine to form native store-operated channels in HEK-293 cells. J Biol Chem 280:29559–29569

Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD (2005) STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature 437:902–905