Protein kinase C activates non-capacitative calcium entry in human platelets

Juan A. Rosado and Stewart O. Sage

Department of Physiology, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK

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1. In many non-excitable cells Ca^{2+} influx is mainly controlled by the filling state of the intracellular Ca^{2+} stores. It has been suggested that this store-mediated or capacitative Ca^{2+} entry is brought about by a physical and reversible coupling of the endoplasmic reticulum with the plasma membrane. Here we provide evidence for an additional, non-capacitative Ca^{2+} entry mechanism in human platelets.

2. Changes in cytosolic Ca^{2+} and Sr^{2+} were measured in human platelets loaded with the fluorescent indicator fura-2.

3. Depletion of the internal Ca^{2+} stores with thapsigargin plus a low concentration of ionomycin stimulated store-mediated cation entry, as demonstrated upon Ca^{2+} or Sr^{2+} addition. Subsequent treatment with thrombin stimulated further divalent cation entry in a concentration-dependent manner.

4. Direct activation of protein kinase C (PKC) by phorbol-12-myristate-13-acetate or 1-oleoyl-2-acetyl-*sn*-glycerol also stimulated divalent cation entry, without evoking the release of Ca^{2+} from intracellular stores. Cation entry evoked by thrombin or activators of PKC was abolished by the PKC inhibitor Ro-31-8220.

5. Unlike store-mediated Ca^{2+} entry, jasplakinolide, which reorganises actin filaments into a tight cortical layer adjacent to the plasma membrane, did not inhibit divalent cation influx evoked by thrombin when applied after Ca^{2+} store depletion, or by activators of PKC. Thrombin also activated Ca^{2+} entry in platelets in which the release from intracellular stores and store-mediated Ca^{2+} entry were blocked by xestospongin C. These results indicate that the non-capacitative divalent cation entry pathway is regulated independently of store-mediated entry and does not require coupling of the endoplasmic reticulum and the plasma membrane.

6. These results support the existence of a mechanism for receptor-evoked Ca^{2+} entry in human platelets that is independent of Ca^{2+} store depletion. This Ca^{2+} entry mechanism may be activated by occupation of G-protein-coupled receptors, which activate PKC, or by direct activation of PKC, thus generating non-capacitative Ca^{2+} entry alongside that evoked following the release of Ca^{2+} from the intracellular stores.

The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) modulates a large number of cellular processes ranging from short term responses such as contraction and secretion to longer term modulation of cell growth (Monteith & Roufogalis, 1995). Stimulation of non-excitable cells by various agonists results in a rise in $[Ca^{2+}]_i$, which consists of two components: release of Ca^{2+} from the intracellular stores and the entry of Ca^{2+} across the plasma membrane (Rink & Sage, 1990; Parekh & Penner, 1997).

The mechanisms involved in the generation of Ca^{2+} signals in platelets have much in common with those operating in other non-excitable cells. Thus platelets are a good model for the study of Ca^{2+} signalling in normal human non-excitable cells. In these cells, store-mediated Ca^{2+} entry (SMCE), a mechanism regulated by the degree of filling of the intracellular Ca^{2+} stores (Putney, 1990; Sage, 1997), is a major pathway for Ca^{2+} influx (Sage *et al.* 1990). The mechanism of activation of SMCE is not fully understood and current hypotheses fall into two main categories: those suggesting a diffusible messenger that gates plasma membrane channels and those suggesting a conformational coupling between elements in the endoplasmic reticulum (possibly the InsP_3 receptors) and the plasma membrane (Berridge, 1995). Recently, the physical coupling model has received support

from studies which show that the activation of SMCE shares properties with those of the secretory mechanism (Patterson *et al.* 1999; Putney, 1999; Yao *et al.* 1999; Rosado *et al.* 2000). These studies suggest that SMCE is mediated by a physical and reversible coupling of the endoplasmic reticulum and the plasma membrane and that the actin cytoskeleton might play a key regulatory role in the activation and maintenance of SMCE (Rosado *et al.* 2000).

In platelets there is in addition evidence for the existence of a purinoceptor-activated receptor-operated channel (Sage & Rink, 1986; Mackenzie *et al.* 1996). Adenine nucleotides have been shown to be able to evoke Ca^{2+} entry in human platelets with a short latency via a P_{2X1} purinoceptor (Sage & Rink, 1986; Mackenzie *et al.* 1996). This purinoceptor-operated non-selective cation channel can be blocked by desensitisation of the $P_{2\,X1}$ receptors by addition of the selective $P_{2\,X1}$ receptor agonist α,β-methylene ATP (Mackenzie *et al.* 1996).

Here we provide evidence for a novel mechanism for receptor-evoked divalent cation entry in human platelets, which is independent of the filling state of the intracellular Ca^{2+} stores and different from the purinoceptor-activated receptor-operated cation entry. In this mechanism Ca^{2+} entry occurs through a divalent cation permeable channel that is activated via protein kinase C (PKC).

METHODS

Platelet preparation

Experiments were carried out on human blood platelets obtained from healthy drug-free volunteers who gave written informed consent, with local ethical committee approval and in accordance with the Declaration of Helsinki. Fura-2-loaded platelets were prepared as described previously (Rosado & Sage, 2000*b*). Briefly, blood was mixed with a one-sixth volume of acid/citrate dextrose anticoagulant containing (mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 *g* and aspirin (100 μM) and apyrase (40 μg ml^{-1}) were added. The platelet-rich plasma was incubated at 37 °C with 2 μM fura-2 AM for 45 min. Cells were then collected by centrifugation at 350 *g* for 20 min and resuspended in Hepes-buffered saline (HBS) containing (mM): 145 NaCl, 10 Hepes, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7·45, and supplemented with 0·1 % (w/v) bovine serum albumin (BSA) and 40 μg ml^{-1} apyrase.

Measurement of $[Ca^{2+}]_i$

Fluorescence was recorded from 1·5 ml aliquots of a magnetically stirred platelet suspension (10^8 cells ml^{-1}) at 37 °C using a Cairn Research Spectrophotometer (Cairn Research Ltd) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340 nm/380 nm fluorescence ratio and calibrated in terms of $[Ca^{2+}]_i$ according to the method of Grynkiewicz *et al.* (1985).

Determination of Sr^{2+} entry

In a number of experiments, Sr^{2+} was used to monitor divalent cation entry. This was done to avoid complications arising from stimulation of the platelet plasma membrane Ca^{2+}-ATPase by PKC (Rink & Sage, 1987), since Sr^{2+} is transported with lower affinity than Ca^{2+} by this Ca^{2+}-ATPase (Graf *et al.* 1982). Sr^{2+} entry was measured in Ca^{2+}-free HBS containing EGTA (100 μM) to minimise the effects of contaminating Ca^{2+}. Cytosolic Sr^{2+} was monitored using the fura-2 340 nm/380 nm fluorescence ratio. Store depletion-evoked Sr^{2+} entry was calculated using the integral of the rise in the 340 nm/380 nm fluorescence ratio for 2·5 min after addition of $SrCl_2$. Thrombin-evoked Sr^{2+} influx was measured as the integral of the rise in the 340 nm/380 nm fluorescence ratio above basal levels for 1 min after addition of thrombin in the presence of external Sr^{2+}. When platelets were preincubated with various compounds, Sr^{2+} entry was corrected by subtraction of the change in the 340 nm/380 nm fluorescence ratio (due to leakage of the indicator) that occurred when Sr^{2+} was added to vehicle-treated (non-depleted) controls.

Confocal microscopy

Samples of platelet suspension (200 μl) were transferred to 200 μl ice-cold 3 % (w/v) formaldehyde in PBS (mM: 137 NaCl, 2·7 KCl, 5·62 Na_2HPO_4, 1·09 NaH_2PO_4 and 1·47 KH_2PO_4, pH 7·2) for 10 min. Fixed platelets were permeabilised by incubation for 10 min with 0·025 % (v/v) Nonidet P-40 detergent dissolved in PBS. The platelets were then incubated for 30 min with fluorescein isothiocyanate (FITC)-labelled phalloidin (1 μM) in PBS supplemented with 0·5 % (w/v) BSA. Cells were collected by centrifugation in an MSE Micro-Centaur centrifuge (MSE Scientific Instruments) for 60 s at 3000 *g* and resuspended in PBS. The platelets were visualised using a Leica TCS4D confocal microscope.

Materials

Fura-2 acetoxymethyl ester (fura-2 AM) was from Texas Fluorescence (Austin, TX, USA). Apyrase (grade VII), aspirin, BSA, paraformaldehyde, Nonidet P-40, FITC-labelled phalloidin, thrombin and thapsigargin (TG) were from Sigma (Poole, Dorset, UK). Ionomycin (IONO), xestospongin C (Xest C), phorbol-12-myristate-13-acetate (PMA), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and Ro-31-8220 were from Calbiochem (Nottingham, UK). Jasplakinolide (JP) was from Molecular Probes (Leiden, The Netherlands). All other reagents were of analytical grade.

Statistical analysis

Analysis of statistical significance was performed using Student's *t* test. For multiple comparisons, one-way analysis of variance combined with Dunnett's test was used.

RESULTS

Thrombin-evoked receptor-operated divalent cation entry

Addition of TG (1 μM) and a low concentration of IONO (50 nM) to platelets in a Ca^{2+}-free medium caused a transient increase in $[Ca^{2+}]_i$ as the intracellular Ca^{2+} stores were depleted (Fig. 1*A*). A combination of TG and IONO was used since platelets are reported to possess two Ca^{2+} stores with high and low Ca^{2+} leakage rates (Doni *et al.* 1994; Cavallini *et al.* 1995) and both agents are required to ensure extensive Ca^{2+} store depletion. Subsequent stimulation with a maximally effective concentration of thrombin (10 U ml^{-1}; Sage & Rink, 1987) was unable to further increase $[Ca^{2+}]_i$, indicating that the agonist-releasable Ca^{2+} stores were fully depleted (Fig. 1*A*). We have previously shown that depletion of intracellular Ca^{2+} stores in platelets induces divalent cation entry (Sargeant *et al.* 1992; Jenner *et*

al. 1994), hence addition of Sr^{2+} (300 μM) to the external medium after depletion of the Ca^{2+} stores resulted in a rapid Sr^{2+} entry (Fig. 1*B*) which did not occur in non-depleted cells (Control). Addition of the physiological platelet agonist thrombin (1 or 10 U ml^{-1}) to platelets in which Sr^{2+} entry had already been stimulated by complete depletion of the intracellular Ca^{2+} stores caused a further, concentration-dependent increase in Sr^{2+} entry (Fig. 1*C*). The peak elevations in the 340 nm/380 nm fluorescence ratio above the level attained following store-mediated Sr^{2+} entry were 0·44 ± 0·05 and 0·31 ± 0·04 arbitrary units after treatment with 10 or 1 U ml^{-1} thrombin, respectively ($n = 7$). The addition of thrombin (10 U ml^{-1}) after store-mediated Ca^{2+} entry had been initiated by the addition of 300 μM Ca^{2+} to store-depleted platelets resulted in further Ca^{2+} entry (Fig. 1*D*). The additional thrombin-evoked divalent cation entry was not due to thrombin-evoked hyperpolarisation of the cells, since similar responses were elicited when the membrane potential was clamped close to the K^+ reversal potential using valinomycin (5 μM; Mahaut-Smith *et al.* 1990; data not shown). Since addition of thrombin to TG- and IONO-treated platelets did not evoke further release of Ca^{2+} from the stores (Fig. 1*A*), the thrombin-evoked entry of Sr^{2+} or Ca^{2+} observed in addition to that evoked by store depletion suggests the existence of a novel and Ca^{2+} store-independent divalent cation entry pathway, stimulated following receptor occupation.

Previous studies have reported a non-capacitative purinoceptor-activated cation entry pathway in human platelets (Mackenzie *et al.* 1996). To investigate whether this pathway could be responsible for the cation entry mechanism described above, through thrombin-evoked secretion of adenine nucleotides, we desensitised the P_{2X1} receptor by adding α,β-methylene ATP, a non-hydrolysable ATP analogue. In agreement with previous observations (Mackenzie *et al.* 1996; Vial *et al.* 1997), the selective P_{2X1} agonist α,β-methylene ATP (10 μM) induced a transient rise in $[Ca^{2+}]_i$ in the presence of external calcium ($n = 4$; Fig. 2*A*). As reported previously (Vial *et al.* 1997), addition of α,β-methylene ATP desensitised the P_{2X1} purinoceptor as indicated by the lack of effect of a subsequent addition of 10 μM α,β-methylene ATP. The addition of α,β-methylene ATP to platelets in the absence of external Ca^{2+} (with 100 μM EGTA added) was, as expected (MacKenzie *et al.* 1996), without effect on $[Ca^{2+}]_i$ (Fig. 2*B*). The subsequent addition of 1 mM $CaCl_2$ was also without effect, indicating that P_{2X1} receptor stimulation in the absence of external

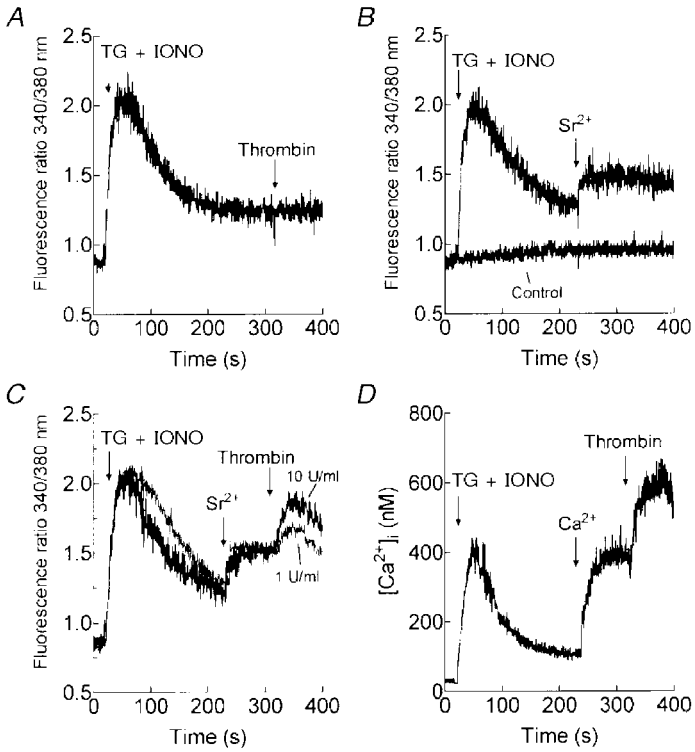

Figure 1. Capacitative and non-capacitative cation entry pathways in human platelets

A, fura-2-loaded human platelets were stimulated with TG (1 μM) and IONO (50 nM) in Ca^{2+}-free HBS (100 μM EGTA added) and 5 min later thrombin (10 U ml^{-1}) was added ($n = 5$). *B*, cells were stimulated with TG and IONO or the vehicles alone (Control) in Ca^{2+}-free HBS (100 μM EGTA added) and 3·5 min later $SrCl_2$ (300 μM) was added ($n = 7$). *C*, cells were stimulated with TG and IONO in Ca^{2+}-free HBS (100 μM EGTA added); 3·5 min later $SrCl_2$ (300 μM) was added to initiate Sr^{2+} entry followed by thrombin (1 or 10 U ml^{-1}; $n = 7$). *D*, cells were stimulated with TG and IONO in Ca^{2+}-free HBS (100 μM EGTA added); 3·5 min later $CaCl_2$ (300 μM) was added to initiate Ca^{2+} entry followed by thrombin (10 U ml^{-1}; $n = 6$).

Ca^{2+} still results in desensitisation of the receptor, a conclusion supported by the fact that a further addition of α,β-methylene ATP also did not affect $[Ca^{2+}]_i$ (Fig. 2*B*). As shown in Fig. 2*C* and *D*, prior desensitisation of the $P_{2\,xi}$ receptors by addition of 10 μM α,β-methylene ATP failed to inhibit thrombin-evoked Sr^{2+} entry in store-depleted cells (0·53 ± 0·04 arbitrary 340 nm/380 nm fluorescence units in α,β-methylene ATP-treated platelets *vs.* 0·50 ± 0·02 units in control; $n = 4$), indicating that this pathway is different from the purinoceptor-activated receptor-operated cation entry pathway. We subsequently refer to the novel entry pathway as a non-capacitative process to differentiate it from the purinoceptor-activated receptor-operated divalent cation entry pathway.

Protein kinase C activates non-capacitative cation entry

It is well established that thrombin activates phospholipase C in human platelets, resulting in the generation of diacylglycerol (DAG) which in turn activates PKC (Haslam *et al.* 1990; Sargeant *et al.* 1993). To investigate the possible role of PKC in the activation of non-capacitative cation entry, we assessed the effects of activators of PKC, PMA and the DAG analogue OAG. Treatment of platelets with PMA (1 μM) elicited a transient entry of both Ca^{2+} and Sr^{2+} without releasing Ca^{2+} from the internal stores (Fig. 3*A* and *B*). The initial peak elevations in $[Ca^{2+}]_i$ or 340 nm/380 nm fluorescence ratio above the basal level after treatment with PMA (1 μM) were 149 ± 41 nM and 0·42 ± 0·05 arbitrary units, respectively ($n = 6$). Moreover, addition of PMA (1 μM) to platelets where Sr^{2+} entry had already been stimulated by treatment with TG (200 nM) resulted in further Sr^{2+} entry (Fig. 3*C* and *D*) of similar magnitude to that stimulated by thrombin (Fig. 1*C*). The peak elevation in the 340 nm/380 nm fluorescence ratio above the level attained following store-mediated Sr^{2+} entry after treatment with PMA was 0·46 ± 0·07 arbitrary units ($n = 6$). Similar results were obtained with 100 μM OAG (not shown). Treatment of platelets with OAG (100 μM) elicited a transient elevation in cytosolic Ca^{2+} or Sr^{2+}, reaching a peak elevation in $[Ca^{2+}]_i$ or 340 nm/380 nm fluorescence ratio above the basal level of 137 ± 34 nM and

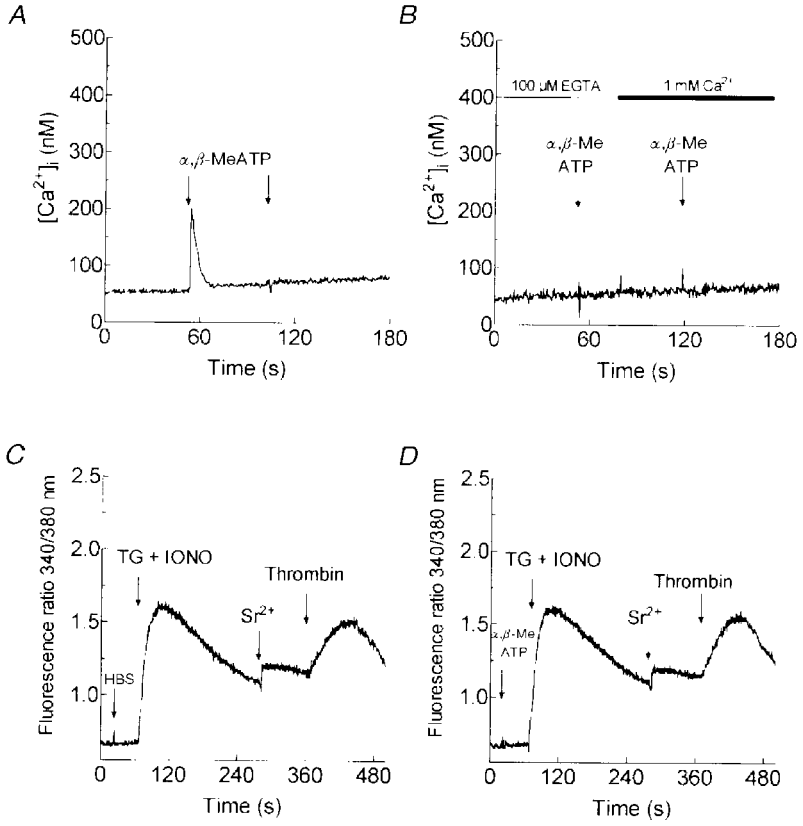

Figure 2. Effect of P_{2x1} receptor desensitisation on thrombin-evoked non-capacitative Sr^{2+} entry in human platelets

Fura-2-loaded human platelets were resuspended in HBS containing 1 mM $CaCl_2$ (*A*) or in Ca^{2+}-free HBS (100 μM EGTA added; *B*) and then α,β-methylene ATP (α,β-MeATP; 10 μM) was added. A second addition of 10 μM α,β-methylene ATP in the presence of 1 mM external Ca^{2+} demonstrates desensitisation of the $P_{2\,xi}$ receptor ($n = 4$; *A*). Addition of 1 mM $CaCl_2$ after 10 μM α,β-methylene ATP and a second addition of 10 μM α,β-methylene ATP were without effect, indicating that desensitisation occurs independently of Ca^{2+} entry ($n = 4$; *B*). *C* and *D*, platelets were treated in Ca^{2+}-free HBS (100 μM EGTA added) with α,β-methylene ATP (10 μM; *D*) or the vehicle (HBS; *C*) prior to stimulation with TG (1 μM) and IONO (50 nM); 3·5 min later $SrCl_2$ (300 μM) was added followed by thrombin (10 U ml^{-1}; $n = 4$).

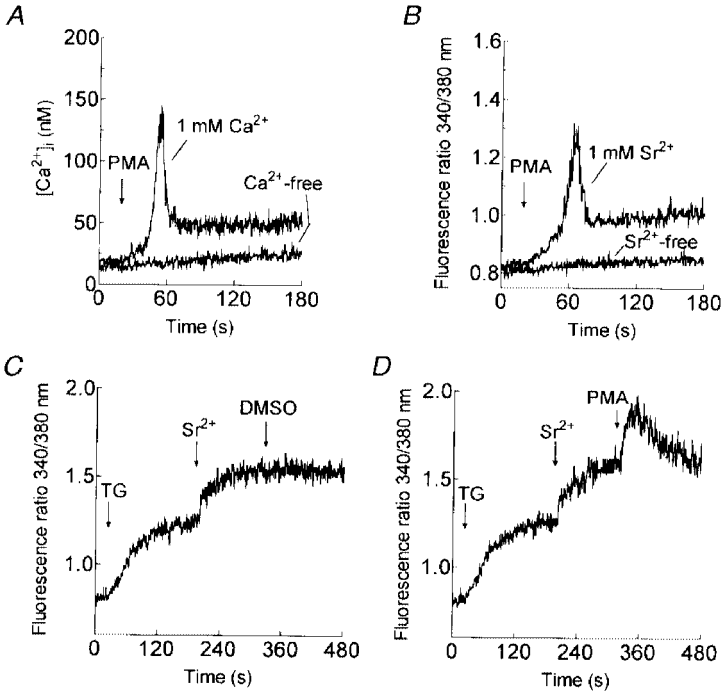

Figure 3. Effect of PMA on Ca^{2+} and Sr^{2+} entry in human platelets

A and *B*, platelets were treated with 1 μM PMA in HBS containing 1 mM Ca^{2+} (*A*) or 1 mM Sr^{2+} (*B*; $n = 6$). The same experiments were performed in the absence of external Ca^{2+} (*A*) or Sr^{2+} (*B*; $n = 6$). *C* and *D*, cells were treated with TG (200 nM) in Ca^{2+}-free HBS (100 μM EGTA added) and 3 min later $SrCl_2$ (final concentration, 300 μM) was added followed by PMA (1 μM, *D*) or the vehicle (DMSO) alone (*C*; $n = 6$).

$0{\cdot}38 \pm 0{\cdot}04$ arbitrary units, respectively ($n = 6$). In addition, in platelets in which Sr^{2+} entry had been activated by Ca^{2+} store depletion, OAG (100 μM) evoked additional Sr^{2+} entry ($0{\cdot}05 \pm 0{\cdot}05$ 340 nm/380 nm fluorescence arbitrary units above the level attained by store-mediated Sr^{2+} entry; $n = 6$). These results indicate that the activation of PKC stimulates non-capacitative cation entry in human platelets.

Time-dependent effect of PKC on cation entry

Previous studies in *Xenopus* oocytes have shown that PKC activation initially potentiates Ca^{2+} entry while more

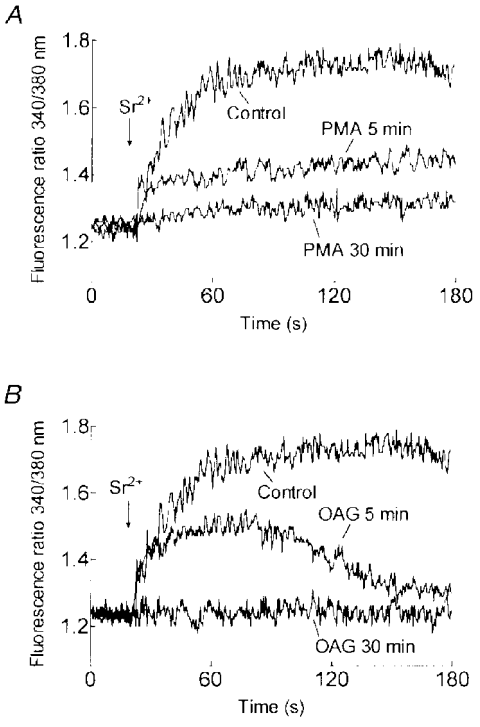

Figure 4. Time-dependent effect of PKC activation on cation entry

A, platelets were preincubated at 37 °C for 5 or 30 min in the absence (Control) or presence of PMA (1 μM). Cells were then treated with TG (200 nM) and 3 min later $SrCl_2$ (final concentration, 300 μM) was added ($n = 6$). *B*, platelets were preincubated at 37 °C for 5 or 30 min in the absence (Control) or presence of OAG (100 μM). Cells were then treated with TG (200 nM) and 3 min later $SrCl_2$ (final concentration, 300 μM) was added ($n = 6$).

prolonged activation abolishes this process (Petersen & Berridge, 1994). We therefore investigated the time-dependent effect of PKC activation on store-mediated cation entry in human platelets. Pretreatment of platelets at 37 °C with PMA (1 μM) for 5 min significantly inhibited TG-induced Sr^{2+} entry by $39{\cdot}4 \pm 7{\cdot}7\%$ ($P < 0{\cdot}01$; $n = 6$; Fig. 4*A*). Preincubation of platelets with PMA for 30 min abolished Sr^{2+} entry ($P < 0{\cdot}001$; $n = 6$; Fig. 4*A*). Similarly, pretreatment of platelets with OAG (100 μM) for 5 min reduced TG-evoked Sr^{2+} entry by $54{\cdot}0 \pm 12{\cdot}5\%$ ($P < 0{\cdot}01$; $n = 6$) while pretreatment for 30 min completely inhibited Sr^{2+} entry ($P < 0{\cdot}001$; $n = 6$; Fig. 4*B*). Treatment of platelets with PMA or OAG had no effect on the TG-evoked release of Ca^{2+} from the intracellular stores, indicating that Ca^{2+} storage was unaffected by PKC activation (data not shown).

Desensitisation of PKC by incubation with PMA (1 μM) for 30 min almost abolished non-capacitative divalent cation entry stimulated by a maximal concentration of thrombin (10 U ml^{-1}; Fig. 5). Sr^{2+} entry evoked by 10 U ml^{-1} thrombin was inhibited by $91{\cdot}5 \pm 3{\cdot}8\%$ ($P < 0{\cdot}001$; $n = 6$; Fig. 5). Consistent with the results presented above, prolonged stimulation of PKC with PMA for 30 min also inhibited TG plus IONO-induced Sr^{2+} entry without affecting Ca^{2+} release from the intracellular stores (Fig. 5).

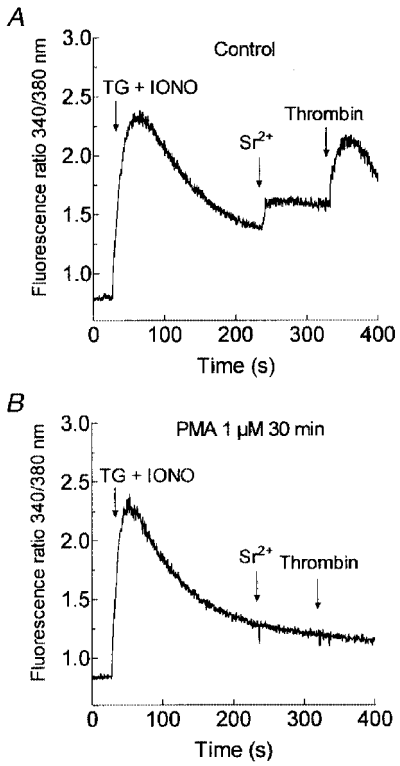

Figure 5. Desensitisation of PKC inhibits non-capacitative Sr^{2+} entry in human platelets

Cells were pretreated for 30 min at 37 °C with 1 μM PMA (*B*) or the vehicle (DMSO) alone (Control; *A*). The platelets were then stimulated with TG (1 μM) and IONO (50 nM) in Ca^{2+}-free HBS (100 μM EGTA added) and 3·5 min later $SrCl_2$ (300 μM) was added followed by thrombin (10 U ml^{-1}; $n = 6$).

These findings indicate that although activation of PKC initially leads to stimulation of non-capacitative cation entry, continued activation or desensitisation of this protein kinase inhibits both store-mediated and non-capacitative divalent cation entry.

Effect of Ro-31-8220 on non-capacitative cation entry

In further support of a role for PKC in the thrombin-evoked non-capacitative cation entry, we observed that treatment of platelets for 5 min with the PKC inhibitor Ro-31-8220 (3 μM), which abolished PMA-evoked Sr^{2+} entry ($P < 0{\cdot}001$; $n = 6$; Fig. 6*A*), significantly inhibited thrombin-evoked non-capacitative Sr^{2+} entry by $89 \pm 6\%$ ($P < 0{\cdot}001$; $n = 6$; Fig. 6*B*). Treatment of human platelets with Ro-31-8220 did not alter the elevation in $[Ca^{2+}]_i$ evoked by TG and IONO indicating that storage of Ca^{2+} in the intracellular stores was unaffected by this compound. This is in agreement with the lack of effect of PKC activation on Ca^{2+} storage described above. In addition, treatment of platelets with Ro-31-8220 had no effect on store depletion-evoked Sr^{2+} entry (Fig. 6*B*), indicating that this compound is not a non-selective cation channel blocker.

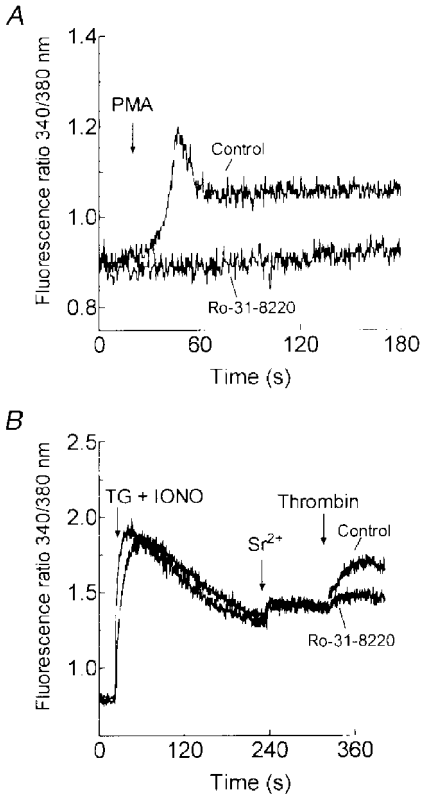

Figure 6. Effect of PKC inhibition on non-capacitative Sr^{2+} entry in human platelets

A, cells were pretreated for 5 min at 37 °C with 3 μM Ro-31-8220 or the vehicle (DMSO) alone (Control). The platelets were then treated with 1 μM PMA in HBS containing 1 mM Sr^{2+} ($n = 6$). *B*, cells were pretreated for 5 min at 37 °C with 3 μM Ro-31-8220 or the vehicle (Control). The platelets were then stimulated with TG (1 μM) and IONO (50 nM) in Ca^{2+}-free HBS (100 μM EGTA added) and 3·5 min later $SrCl_2$ (300 μM) was added followed by thrombin (10 U ml^{-1}; $n = 6$).

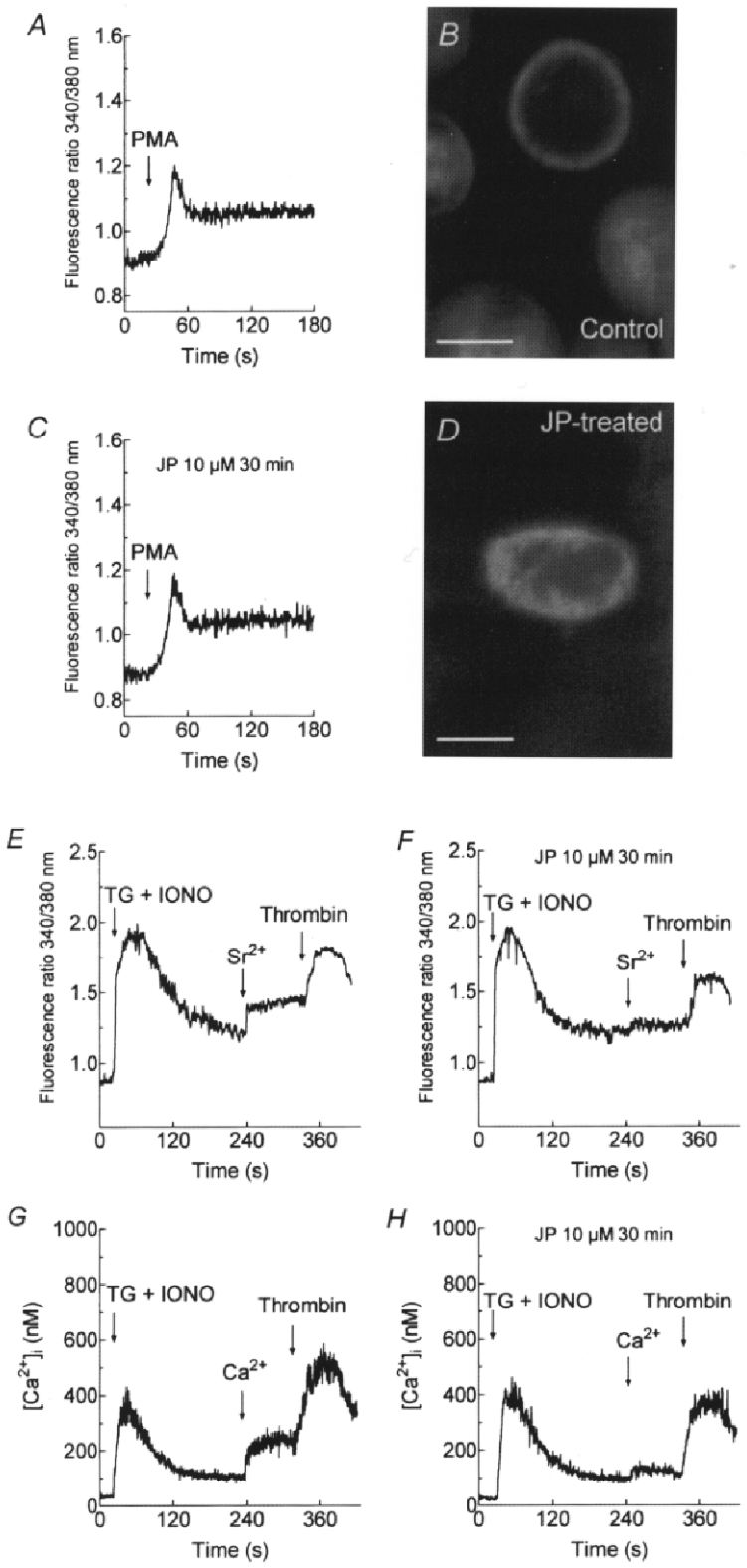

Figure 7. Effect of jasplakinolide on non-capacitative cation entry in human platelets

A–D, platelets were pretreated for 30 min at 37 °C with 10 μM JP (*C* and *D*; $n = 6$) or the vehicle (ethanol; *A* and *B*; $n = 6$). Cells were stimulated with PMA (1 μM) in HBS containing 1 mM Sr^{2+}. *B* and *D* show, respectively, confocal images of F-actin labelled with FITC-conjugated phalloidin in control or JP-treated human platelets. Scale bars represent 1 μm. *E–H*, cells were pretreated at 37 °C with 10 μM JP (*F* and *H*) or the vehicle (ethanol; *E* and *G*) for 30 min. The platelets were then treated with TG (1 μM) and IONO (50 nM) in Ca^{2+}-free HBS (100 μM EGTA added) and 3 min later $SrCl_2$ (final concentration, 300 μM; *E* and *F*) or $CaCl_2$ (final concentration 300 μM; *G* and *H*) was added followed by thrombin (10 U ml^{-1}; $n = 7$).

Effect of jasplakinolide on divalent cation entry

It has recently been proposed that SMCE is mediated by a reversible trafficking and coupling of the endoplasmic reticulum with the plasma membrane in several cell types (Patterson *et al.* 1999; Yao *et al.* 1999), including human platelets (Rosado *et al.* 2000). In this model, cortical actin filaments play an inhibitory role, as demonstrated by treatment with JP, a cell-permeant peptide that induces polymerisation and stabilisation of actin filaments (Cooper, 1987). JP has been shown to block SMCE, without altering receptor-evoked Ca^{2+} release, by formation of a cortical actin barrier at the plasma membrane, so preventing close association between the plasma membrane and the endoplasmic reticulum and thus providing evidence that association between the endoplasmic reticulum and the plasma membrane is required for the activation of SMCE (Patterson *et al.* 1999; Rosado *et al.* 2000; Rosado & Sage, 2000*a*,*c*). We have previously shown that treatment of human platelets with 10 μM JP for 30 min induces full actin polymerisation (Rosado *et al.* 2000). This action correlates with the formation of a dense cortical layer of actin filaments (Fig. 7*D*), which contrasts with the more restricted cortical actin distribution in resting platelets (Fig. 7*B*). If non-capacitative cation entry was directly stimulated by receptor occupation via PKC activation, one would expect that this pathway might be insensitive to JP treatment. In contrast, activation of the store-mediated (capacitative) cation entry, which requires coupling between the endoplasmic reticulum and the plasma membrane, is, as might be expected, blocked by JP (Rosado *et al.* 2000). JP treatment did not alter either thrombin or PMA-evoked activation of non-capacitative Sr^{2+} entry (Fig. 7*A*, *C*, *E* and *F*; $n = 6$–7) or thrombin-evoked non-capacitative Ca^{2+} entry (Fig. 7*G* and *H*; $n = 7$). In contrast, in agreement with our previous studies (Rosado *et al.* 2000), treatment of human platelets with JP essentially abolished store depletion-induced Sr^{2+} and Ca^{2+} entry but had no effect on the release of Ca^{2+} from the intracellular stores (Fig. 7*F* and *H*; $n = 7$). These findings suggest that the novel non-capacitative cation entry in human platelets is stimulated by direct activation of plasma membrane divalent cation permeable channels and does not involve interaction between the plasma membrane and another organelle. In addition, these data indicate that the non-capacitative cation entry and store-mediated Ca^{2+} entry pathways are independently regulated, since the non-capacitative pathway operates normally under conditions in which SMCE is blocked.

Effect of xestospongin C on divalent cation entry

Further evidence for the independent regulation of the store-mediated and non-capacitative divalent cation entry pathways was obtained using the inhibitor of inositol 1,4,5-

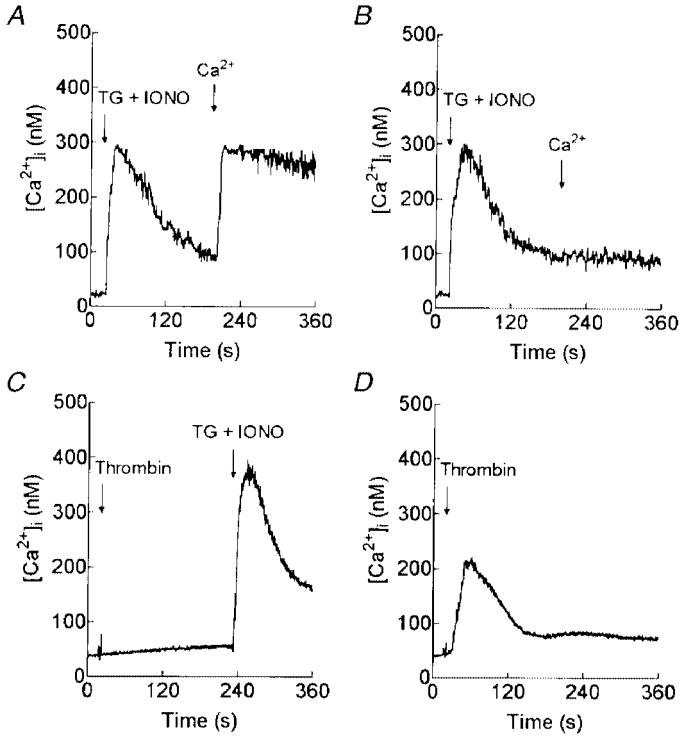

Figure 8. Thrombin-induced non-capacitative Ca^{2+} entry is independent of store depletion

Fura-2-loaded human platelets were pretreated for 30 min at 37 °C with 20 μM Xest C (*B*–*D*) or the vehicle (DMSO) alone (*A*). In *A* and *B*, cells were then stimulated with TG (1 μM) and IONO (50 nM) in Ca^{2+}-free HBS (100 μM EGTA added) and 3 min later $CaCl_2$ (final concentration, 200 μM) was added to initiate Ca^{2+} entry ($n = 4$). *C*, cells were stimulated with 10 U ml^{-1} thrombin in Ca^{2+}-free HBS (100 μM EGTA added) and 3·5 min later TG (1 μM) and IONO (50 nM) were added ($n = 5$). *D*, cells were stimulated with 10 U ml^{-1} thrombin in the presence of 1 mM external Ca^{2+} ($n = 6$).

trisphosphate receptor ($InsP_3R$) function, Xest C (Gafni *et al.* 1997). We have previously shown that Xest C treatment, which blocks thrombin-evoked release of Ca^{2+} from intracellular stores, also blocks SMCE in TG-treated platelets (Rosado & Sage, 2000*c*). Treatment with 20 μM Xest C for 30 min completely inhibited SMCE in platelets treated with TG and IONO to maximally deplete the agonist-releasable Ca^{2+} stores (Fig. 8*A* and *B*; $n = 4$). Addition of a maximally effective concentration of thrombin (10 U ml^{-1}) to Xest C-treated platelets in the absence of external Ca^{2+} (100 μM EGTA added) was without effect on $[Ca^{2+}]_i$, indicating complete blockade of thrombin-evoked release of Ca^{2+} from intracellular stores (Fig. 8*C*). TG plus IONO were able to increase $[Ca^{2+}]_i$ in Xest C-treated platelets, confirming that the intracellular Ca^{2+} stores were intact (Fig. 8*C*). However, addition of thrombin (10 U ml^{-1}) to Xest C-treated cells in the presence of 1 mM external Ca^{2+} still evoked a rise in $[Ca^{2+}]_i$ ($n = 6$; Fig. 8*D*), even though both thrombin-evoked release of Ca^{2+} from intracellular stores (Fig. 8*C*) and SMCE (Fig. 8*B*) were blocked.

DISCUSSION

The activation of Ca^{2+} entry following receptor occupation is a signalling process of great physiological relevance. Receptor-operated Ca^{2+} entry might occur by several different mechanisms (Putney, 1990; Sage, 1992). These include a receptor-operated channel formed by the subunits of the receptor protein, a channel closely linked with the receptor via a GTP-binding protein, a second messenger-operated channel gated by a diffusible molecule generated by receptor occupation and a store-mediated (or capacitative) mechanism, where Ca^{2+} entry is controlled by the filling state of the intracellular Ca^{2+} stores (Putney, 1990). In many non-excitable cells the latter pathway is a major mechanism for Ca^{2+} entry. Here we describe the existence of a new receptor-operated non-capacitative divalent cation entry pathway in human platelets, which are a good model for the study of Ca^{2+} signalling in normal human non-excitable cells.

This non-capacitative cation entry pathway can be activated by physiological agonists, such as thrombin, which activate PKC. Our data using activators and inhibitors of PKC indicate that the activity of this enzyme is required for the non-capacitative divalent cation entry pathway to operate. Our findings suggest that activation of PKC initially leads to stimulation of a non-capacitative divalent cation entry pathway that would add to the well-established store-mediated cation entry in these cells (Sargeant *et al.* 1992). However, in agreement with previous studies (Petersen & Berridge, 1994), continued activation of PKC inhibited both store-mediated and non-capacitative divalent cation entry in platelets. This inhibition may prevent a dangerously high $[Ca^{2+}]_i$ being reached during prolonged cell stimulation.

Our results showing the stimulation of Ca^{2+} entry by the PKC activators PMA and OAG contrast with an earlier study in which OAG and the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) were reported not to affect $[Ca^{2+}]_i$ in quin-2-loaded human platelets (Rink *et al.* 1983). The failure to detect changes in $[Ca^{2+}]_i$ in response to PKC activators in this earlier study may reflect the low concentrations used (15–20 nM), the much higher cytosolic Ca^{2+} buffering power after the introduction of approximately 1 mM quin-2, or both of these factors.

A number of receptor-operated non-capacitative Ca^{2+} entry pathways have been reported including those activated by ADP and ATP in platelets (Mackenzie *et al.* 1996), vasopressin in rat A7r5 smooth muscle cells (Byron & Taylor, 1995) and hepatocytes (Kass *et al.* 1994), and carbachol in PC12 cells (Clementi *et al.* 1992) and exocrine avian nasal gland cells (Shuttleworth & Thompson, 1996). In addition, several intracellular messengers have been identified as regulators of non-capacitative divalent cation entry pathways, including DAG (Helliwell & Large, 1997), PKC (Oike *et al.* 1993) and arachidonic acid (Van Delden *et al.* 1993; Broad *et al.* 1999; Shuttleworth & Thompson, 1999). Our results indicate that thrombin-evoked non-capacitative divalent cation entry in platelets is mediated by activation of PKC, since direct activation of PKC using PMA or the DAG analogue OAG stimulated the pathway and the PKC inhibitor Ro-31-8220 blocked the thrombin-evoked entry. In addition, we have previously reported that arachidonic acid does not mediate rises in $[Ca^{2+}]_i$ in human platelets in the presence of the cyclo-oxygenase inhibitors aspirin (used throughout the present study) or indomethacin (Vindlacheruvu *et al.* 1991), indicating that a hydrolysis product of DAG is unlikely to be responsible for the effect. Taken together, these results indicate that PKC mediates the regulation of receptor-operated non-capacitative divalent cation entry in human platelets.

The novel divalent cation entry pathway described here is regulated independently of the filling state of the intracellular Ca^{2+} stores and thus is different from the well-established SMCE mechanism, since the PKC inhibitor Ro-31-8220 reduced non-capacitative cation entry without modifying store-mediated entry and, in addition, the non-capacitative pathway could operate under conditions in which SMCE was completely inhibited (in JP- or Xest C-treated platelets). Further evidence comes from the finding that thrombin evoked non-capacitative cation entry alongside store-mediated cation entry when the Ca^{2+} stores were completely depleted, so that thrombin could not induce further Ca^{2+} release. In addition we found that PKC activators could stimulate the non-capacitative pathway without releasing Ca^{2+} from the intracellular stores.

The non-capacitative cation entry pathway is independent of the purinoceptor-activated receptor-operated Ca^{2+} entry pathway previously reported to be mediated by P_{2X1} receptors in platelets (Mackenzie *et al.* 1996). Firstly, prior desensitisation of the P_{2X1} receptors by addition of 10 μM α,β-methylene ATP (Vial *et al.* 1997), a non-hydrolysable ATP analogue, had no effect on the non-capacitative cation

entry subsequently evoked by thrombin. Secondly, the magnitude of divalent cation entry evoked by thrombin was comparable with that of store depletion-evoked entry and substantially larger than the reported cation entry evoked by $P_{2\,XI}$ receptor stimulation (Mackenzie *et al.* 1996). Thirdly, the occupation of purinoceptors by secreted ADP would be expected to evoke the release of Ca^{2+} from internal stores as well as the activation of divalent cation entry through the $P_{2\,XI}$ receptor, which was not the case when platelets were stimulated with PMA.

The results presented here are consistent with those reported recently in T3-65 clonal human embryonic kidney (HEK293) cells stably transfected to express the human homologue of the *Drosophila* transient receptor potential channel, TRP3 (Ma *et al.* 2000). In these cells, an increase in DAG levels brought about by application of DAG lipase inhibitors, or treatment with DAG analogues, stimulated TRP3-mediated Sr^{2+} entry. Although speculative, there is a potential relationship between the results presented here and the presence of TRP channels in human platelets (D. Molin, E. den Dekker, G. Breikers, R. van Oerle, J. W. Akkerman & J. W. A. Heemskerk, unpublished observations; Rosado & Sage, 2000*c*). These channels may be activated following occupation of G-protein-coupled receptors by physiological agonists which activate phospholipase C and therefore PKC, thereby generating non-capacitative Ca^{2+} entry alongside that initiated following the release of Ca^{2+} from the intracellular stores.

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Corresponding author

S. O. Sage: Department of Physiology, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK.

Email: sos10@cam.ac.uk