Two Pathways for Store-mediated Calcium Entry Differentially Dependent on the Actin Cytoskeleton in Human Platelets

Juan A. Rosado‡§, José J. López‡, Alan G. S. Harper¶, Matthew T. Harper¶**, Pedro C. Redondo‡ ‡‡, José A. Pariente‡, Stewart O. Sage‡, and Ginés M. Salido‡‡

From the ‡Department of Physiology, University of Extremadura, Caceres, Spain and the ¶Department of Physiology, University of Cambridge, Cambridge CB2 3EG, United Kingdom

A major pathway for stimulated Ca2+ entry in non-excitable cells is activated following depletion of intracellular Ca2+ stores. Secretion-like coupling between elements in the plasma membrane (PM) and Ca2+ stores has been proposed as the most likely mechanism to activate this store-mediated Ca2+ entry (SMCE) in several cell types. Here we identify two mechanisms for SMCE in human platelets activated by depletion of two independent Ca2+ pools, which are differentially modulated by the actin cytoskeleton. Ca2+ entry induced by depletion of a 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ)-sensitive pool is increased by disassembly of the actin cytoskeleton and that induced by a TBHQ-insensitive pool is reduced. Stabilization of the actin cytoskeleton prevented Ca2+ entry by both mechanisms. We propose that the membrane-associated actin network prevents constitutive Ca2+ entry via both pathways. Reorganization of the actin cytoskeleton permits the activation of Ca2+ entry via both mechanisms, but only SMCE activated by the TBHQ-insensitive pool requires new actin polymerization, which may support membrane trafficking toward the PM.

SMCE1 is triggered by depletion of the intracellular Ca2+ stores (1), although the mechanism underlying this process is not fully understood. Several hypotheses have been proposed to account for the communication between the intracellular Ca2+ stores and the PM, which can be grouped into two main categories: the conformational or secretion-like coupling hypotheses, which propose physical coupling between elements in the Ca2+ stores and the PM, and diffusible messenger hypotheses, which propose the release of a small molecule from the Ca2+ stores that opens, directly or indirectly, Ca2+ channels in the PM (2).

Recently, the secretion-like coupling model has received support from studies showing that activation of SMCE shares properties with the activation of secretion (3, 4). In several non-excitable cells, including platelets, actin filament reorganization plays a key role in the activation of SMCE, perhaps by facilitating translocation of Ca2+ stores to the PM to enable coupling (3–7). In human platelets, where we have demonstrated a secretion-like coupling mechanism, Ca2+ entry is proposed to be based on reversible trafficking of portions of the Ca2+ stores toward the PM to facilitate de novo coupling between the type II inositol 1,4,5-trisphosphate (IP3) receptor in the store membrane and naturally expressed human canonical transient receptor potential 1 (hTRPC1) in the PM (8–10). In this process the actin cytoskeleton acts as a negative modulator of the interaction between the Ca2+ store and PM but is also required for activation of SMCE, since cytoskeletal disruption impairs Ca2+ entry (4).

Platelets express at least two isoforms of the sarco-endoplasmic reticulum Ca2+ ATPase (SERCA), with molecular masses of 100 and 97 kDa (11). The 100-kDa isoform, which is inhibited by low concentrations of TG, has been identified as SERCA 2b. The 97-kDa isoform is much less sensitive to TG (11) and, unlike the 100-kDa isoform, is inhibited by 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ) (12). This has been identified as SERCA 3 (13). Pharmacological studies suggest that these two SERCA isoforms are distributed separately in discrete sub-pool populations of the IP3-sensitive Ca2+ stores (14). TBHQ or high concentrations of TG release Ca2+ from one pool (TBHQ-sensitive store with low affinity for TG), while low (about 10 nM) concentrations of TG release Ca2+ from a different compartment (TBHQ-insensitive store with high affinity for TG) (14). Immunolocalization studies indicate that the SERCAs 2b and 3 have different distributions in platelets (15), although these have not been clearly resolved.

Here we show that SMCE in platelets is the result of the combined effects of depletion of two different Ca2+ stores and that these two pathways are differentially modulated by the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Materials—fura-2 acetoxyethyl ester and jasplakinolide were from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), apyrase from calf liver, and bovine serum albumin were from Sigma (Madrid, Spain). Ionomycin (Iono), cytochalasin D (CytD), and latrunculin A (Lat A) were from Calbiochem (Nottingham, UK). Farnesylthioic acid (FT), farnesyltransferase inhibitor (FTI), and farnesyltransferase inhibitors (FTIs) were from Alexis (Nottingham, UK). All other reagents were of analytical grade.

Platelet Preparation—fura-2-loaded platelets were prepared as described previously (4). Briefly, blood was obtained from healthy drug-free volunteers and mixed with ½ volume of acid/citrate dextrose anti-
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couplant containing (in 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) were added. Platelet-rich plasma was incubated at 37 °C with 2 μM acetyoxymethyl ester for 5 min. Cells were then collected by centrifugation at 350 × g for 20 min and resuspended in Hepes-buffered saline, pH 7.45, containing (in mM): 145 NaCl, 10 Hepes, 1 d-glucose, 5 KCl, 1 MgSO$_4$, and supplemented with 0.1% bovine serum albumin and 40 μg/ml apyrase.

Measurement of Intracellular Free Calcium Concentration ([Ca$^{2+}$]).—Fluorescence was recorded from 2-ml aliquots of magnetically stirred platelet suspension (2 × 10$^8$ cells/ml) at 37 °C using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) or a Cairn Research Spectrofluorimeter (Cairn Research Ltd., Faversham, U.K.) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in [Ca$^{2+}$], were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (16).

Ca$^{2+}$ entry was estimated using the integral of the rise in [Ca$^{2+}$], for 2.5 min after addition of CaCl$_2$ (4). Ca$^{2+}$ release was estimated using the integral of the rise in [Ca$^{2+}$], for 9 min, for TBHQ, or 2.5 min TG or TG + Iono, after Ca$^{2+}$ or agonist addition. Both Ca$^{2+}$ entry and release are expressed as nm, as described previously (17, 18). When platelets were preincubated with inhibitors, Ca$^{2+}$ entry was corrected by subtraction of the Ca$^{2+}$, elevation due to leakage of the indicator.

To investigate SMCE induced by depletion of TBHQ-sensitive and -insensitive stores individually we followed the procedure depicted in Fig. 2. This protocol consists of depleting the TBHQ-sensitive store to investigate the subsequently induced Ca$^{2+}$, entry, followed by depletion of the TBHQ-insensitive store using TG + Iono (Fig. 2, A and E) to monitor Ca$^{2+}$ influx, in this case due to depletion of both stores. Complete depletion of the TBHQ-insensitive store by TG alone required a long exposure to this agent, which might lead to deterioration of the cells; therefore we used TG (100 nM) combined with a low concentration of Iono (20 nM) to induce rapid store depletion. Ca$^{2+}$ entry activated by the TBHQ-insensitive pool was estimated by subtraction of the increase in [Ca$^{2+}$], after the addition of Ca$^{2+}$ to the medium in platelets not stimulated with TG + Iono (Fig. 2, A and D).

Statistical Analysis.—Analysis of statistical significance was performed using Student’s t test. p < 0.05 was considered to be significant for a difference.

RESULTS

We have previously shown that treatment of platelets with TG in a Ca$^{2+}$-free medium results in a sustained increase in [Ca$^{2+}$], due to Ca$^{2+}$ release from intracellular stores. Subsequent addition of Ca$^{2+}$ to the extracellular medium induces a prolonged elevation in [Ca$^{2+}$], indicative of SMCE (4, 7, 8). SMCE induced by a short (3 min) treatment with TG was significantly reduced by treatment for 40 min with the actin filament disrupter CytD (10 μM), which abolishes actin polymerization in platelets (18).

As shown in Fig. 1A, SMCE induced by longer exposures to TG (200 nM) is less sensitive to the disassembly of the actin cytoskeleton, and this modification even increases SMCE activated by a 30-min treatment with TG. We also compared the effect of CytD on SMCE induced by treatment with TG (100 nM) alone or together with a low concentration of Iono (20 nM) to accelerate depletion of the Ca$^{2+}$ stores. Treatment of platelets with CytD did not significantly modify Ca$^{2+}$ release induced by TG alone or in combination with Iono, suggesting that Ca$^{2+}$ accumulation is not affected by disruption of the actin cytoskeleton (Fig. 1B; n = 6). In CytD-treated platelets TG-induced SMCE was significantly reduced compared with controls (Fig. 1C; p < 0.05; n = 6), whereas SMCE induced by TG + Iono was increased by treatment with CytD (Fig. 1C; p < 0.05; n = 6).

There are at least two possible explanations for these observations. Either different Ca$^{2+}$ entry pathways, which are differentially modulated by the actin cytoskeleton, are activated according to the extent of Ca$^{2+}$ store depletion, or depletion of different intracellular Ca$^{2+}$ stores gives rise to the activation of two Ca$^{2+}$ entry pathways. In the latter case, a short treatment with TG would be expected to affect mainly the Ca$^{2+}$ stores with high TG affinity, while a prolonged exposure to TG might deplete Ca$^{2+}$ pools with both high and low affinity for TG.

To further investigate this phenomenon we investigated the effects of sequential application of two different SERCA inhibitors, TBHQ and TG. Treatment of platelets with TBHQ induced a concentration-dependent Ca$^{2+}$ release from a TBHQ-sensitive component of the intracellular Ca$^{2+}$ stores, reaching a maximal effect at concentrations of 20 μM (data not shown). Ca$^{2+}$ entry was also maximal after treatment with 20 μM TBHQ (not shown); therefore we used 20 μM TBHQ throughout this study.

Treatment of platelets in a Ca$^{2+}$-free medium (300 μM EGTA was added) with TBHQ (20 μM; Fig. 2) induced a sustained increase in [Ca$^{2+}$]; subsequent addition of CaCl$_2$ (500 μM) 9 min later induced a larger, prolonged increase in [Ca$^{2+}$], indicative of Ca$^{2+}$ entry (Fig. 2). The extracellular Ca$^{2+}$ was chelated 3 min later by addition of EGTA (1 mM), and cells were then treated with TG (100 nM) + Iono (20 nM; Fig. 2, B and E) for a further 5 min or with the vehicle (Me$_3$SO; Fig. 2, A and D). Treatment with TG + Iono induced a transient increase in [Ca$^{2+}$], due to Ca$^{2+}$ release from a TBHQ-insensitive component of the Ca$^{2+}$ stores. The amount of Ca$^{2+}$ released by TG + Iono was 5-fold greater than that released by TBHQ, suggesting that the TBHQ-releasable component of the pool is smaller in size than the TBHQ-sensitive component. The subsequent addition of CaCl$_2$ (1.3 mM) resulted in a rise in [Ca$^{2+}$], 6-fold higher than that observed when the TBHQ-sensitive compo-
TBHQ, TG, and Iono was reduced (Fig. 2, contrast, SMCE induced following the combined actions of \( F \)–\( F \) potentiated by disruption of the actin cytoskeleton (Fig. 2, potentiation was only observed after more extensive store depletion of one or both stores, as estimated using an established EGTA/Ca\( ^{2+} \) buffer equation (19). This is supported by the similar Ca\( ^{2+} \) entries observed on each [Ca\( ^{2+} \)], elevation in Fig. 2, A and D, in the absence of TG + Iono. Treatment of human platelets with CytD (10 \( \mu \)M for 40 min) or Lat A (3 \( \mu \)M for 1 h), two unrelated actin polymerization inhibitors, increased TBHQ-induced SMCE by 33\% in CytD- and Lat A-treated cells, respectively (Fig. 2, A–F; \( p < 0.05; n = 6–11 \)). This difference cannot be attributed to a different extracellular Ca\( ^{2+} \) concentration ([Ca\( ^{2+} \)], since a similar [Ca\( ^{2+} \)], was present at the time when SMCE was initiated after depletion of one or both stores, as estimated using an established EGTA/Ca\( ^{2+} \) buffer equation (19). This is supported by the similar Ca\( ^{2+} \) entries observed on each [Ca\( ^{2+} \)], elevation in Fig. 2, A and D, in the absence of TG + Iono. Treatment of human platelets with CytD (10 \( \mu \)M for 40 min) or Lat A (3 \( \mu \)M for 1 h), two unrelated actin polymerization inhibitors, increased TBHQ-induced SMCE by 33\% in CytD- and Lat A-treated cells, respectively (Fig. 2, A–F; \( p < 0.05; n = 6 \)). In contrast, SMCE induced following the combined actions of TBHQ, TG, and Iono was reduced (Fig. 2, B, C, E, and F). SMCE induced by the TBHQ-insensitive component of the stores alone (see “Experimental Procedures”) was reduced by 40\% and 70\% \( \pm 2\% \) in CytD- and Lat A-treated cells, respectively. These results suggest against differential depletion of a single intracellular Ca\( ^{2+} \) store being responsible for the activation of two pathways for SMCE, which are regulated differently by the actin cytoskeleton. SMCE activated following a small degree of Ca\( ^{2+} \) store depletion by treatment with TBHQ was potentiated by disruption of the actin cytoskeleton (Fig. 2, A–F), whereas cytoskeletal disruption inhibited SMCE activated following brief treatments with TG (Fig. 1, A and C); potentiation was only observed after more extensive store depletion due to longer exposures to TG (Fig. 1A) or the combined actions of Iono and TG (Fig. 1C). Rather, these results support the hypothesis that two mechanisms for SMCE that are differentially regulated by the actin cytoskeleton are activated following depletion of two discrete Ca\( ^{2+} \) stores, one with a low sensitivity to TG but sensitive to TBHQ and another that is insensitive to TBHQ but highly sensitive to TG. Thus these two Ca\( ^{2+} \) entry pathways appear to be activated by the depletion of the two intracellular Ca\( ^{2+} \) stores identified by Cavallini et al. (14).

We further explored this issue by testing the involvement of the Ras superfamily of proteins in the two pathways for SMCE. Ras family proteins are required for the activation of SMCE in platelets (18) and other cells (20). In platelets this mechanism involves the reorganization of the actin cytoskeleton. To investigate the involvement of Ras family proteins we used a combination of FTA and AGGC, inhibitors of methylation, and thus activation of prenylated and geranylglyceranlated Ras family proteins (18, 21). As observed previously with the actin cytoskeleton disruptors, treatment of platelets for 30 min with 40 \( \mu \)M FTA combined with 30 \( \mu \)M AGGC increased TBHQ-induced SMCE by 350\% \( \pm 72\% \) (Fig. 3; \( p < 0.05; n = 8 \)). In contrast, total SMCE induced by depletion of both stores was clearly reduced. SMCE induced by the TBHQ-insensitive store alone was reduced by 52\% \( \pm 6\% \) (Fig. 3). As expected, since Ras family proteins participate in the maintenance and the dynamics of the cytoskeletal structure, the effect of these proteins on both...
Ca\textsuperscript{2+} entry models is similar to that observed following disruption of the actin cytoskeleton.

In the "secretion-like coupling" hypothesis, the cortical actin network acts as a clamp that blocks the interaction between the Ca\textsuperscript{2+} stores and PM; therefore disorganization of the actin cytoskeleton would be expected to facilitate Ca\textsuperscript{2+} entry. Consistent with this, we have observed an increase in the amount of Ca\textsuperscript{2+} entry when this event was initiated by depletion of the TBHQ-sensitive stores. In contrast, when the TBHQ-insensitive pool was emptied, actin-depolymerizing agents reduced Ca\textsuperscript{2+} entry, suggesting that the actin cytoskeleton also plays a positive role in the activation of this mechanism.

To investigate whether the membrane-associated cytoskeleton acts as a physical barrier that prevents Ca\textsuperscript{2+} entry, we used jasplakinolide (JP), a cell-permeant peptide that induces polymerization and redistribution of actin filaments into a thick cortical layer subjacent to the PM (3, 22). Interestingly, treatment of platelets for 30 min with 10 \mu M JP significantly reduced SMCE induced by depletion of TBHQ-sensitive and -insensitive stores by 50\% (Fig. 4; \( p < 0.05; n = 5 \)). These observations suggest that the cortical actin cytoskeleton exerts a negative regulatory role on both SMCE pathways, perhaps preventing the coupling between IP\textsubscript{3} receptors in the Ca\textsuperscript{2+} stores and TRP channels in the PM as previously reported (8, 9).

**DISCUSSION**

The existence of two Ca\textsuperscript{2+} stores and their differentially regulated Ca\textsuperscript{2+} entry pathways in human platelets requires a reinterpretation of the previously described model for SMCE in these cells. We believe that the secretion-like coupling model explains Ca\textsuperscript{2+} entry induced by the TBHQ-insensitive store, since it shows high affinity for TG and must be the store affected by short treatments with TG. According to this model, and consistent with the results presented here, the actin cytoskeleton plays a dual role in the activation of SMCE by this pathway: a positive role as a support for the transport of portions of the Ca\textsuperscript{2+} store to the proximity of the PM to allow coupling to occur, which is impaired by cytoskeletal disruptors.
This new two-store model for SMCE explains the different effects of CytD on platelets treated for different periods of time with TG or with TG ± Iono. After short treatments with TG alone, SMCE is activated by the TBHQ-insensitive store and therefore is reduced by CytD. When Iono was added together with TG, or after long exposures to TG, both stores were emptied and a combination of effects was observed, and the CytD-induced inhibition of SMCE initiated by the TBHQ-insensitive store is compensated for by the enhanced SMCE activated by the TBHQ-sensitive pool.

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