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Microtubule remodeling mediates the inhibition of store-operated calcium entry (SOCE) during mitosis in COS-7 cells

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Summary. Regulation of the intracellular calcium ion concentration ($[Ca^{2+}]_i$) is critical, because calcium signaling controls diverse and vital cellular processes such as secretion, proliferation, division, gene transcription, and apoptosis. Store-operated calcium entry (SOCE) is the main mechanism through which non-excitable cells replenish and thus maintain this delicate balance. There is limited evidence which indicates that SOCE may be inhibited during mitosis, and the mechanisms leading to the presumed inhibition has not been elucidated. In the present study, we examined and compared the $[Ca^{2+}]_i$ dynamics of COS-7 cells in mitotic and non-mitotic phases with special reference paid to SOCE. Laser scanning confocal microscopy to monitor $[Ca^{2+}]_i$ dynamics revealed that SOCE was progressively inhibited in mitosis and became virtually absent during the metaphase. We used various cytoskeletal modifying drugs and immunofluorescence to assess the contribution of microtubule and actin filaments in SOCE signaling. Nocodazole treatment caused microtubule reorganization and retraction from the cell periphery that mimicked the natural mitotic microtubule remodeling that was also accompanied by SOCE inhibition. Short exposure to paclitaxel, a microtubule-stabilizing drug, bolstered SOCE, whereas long exposure resulted in microtubule disruption and SOCE inhibition. Actin-modifying drugs did not affect SOCE. These findings indicate that mitotic microtubule remodeling plays a significant role in the inhibition of SOCE during mitosis.

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Introduction

Calcium signaling controls diverse cellular processes such as secretion, division, proliferation, gene transcription and apoptosis (Berridge *et al*., 2000; Carafoli *et al*., 2001). Under *in vivo* conditions, the signaling cascade is achieved through a wide range of agonists such as hormones, neurotransmitters and metabolites whereas in *in vitro* settings, thapsigargin, adenosine 5' triphosphate (ATP) and its derivatives, histamine, and other amines have been extensively employed (Holda *et al*., 1998; Putney, 2001; Parekh and Putney, 2005; Targos *et al*., 2005).

When the cells are stimulated, they show an increase in intracellular calcium concentration ($[Ca^{2+}]_i$); in non-excitable cells. This is typically characterized by a successive, or overlapping, first acutely increasing phase, and a second plateau and slowly declining phase. The acute phase that lasts for a couple of seconds to a few minutes is due to the release of Ca^{2+} from intracellular stores, particularly from the endoplasmic reticulum (ER),

or mitochondria. This release of stored Ca^{2+} follows the binding of the agonist to the cell surface G-protein-coupled receptors or to the receptor tyrosine kinases, a process that activates the enzyme phospholipase C, which is necessary in the cleavage of phosphatidylinostol, a membrane phospholipid. This process culminates in the formation of two important signaling molecules: inositol triphosphate (IP_3) and diacylglycerol. The former binds IP_3 receptors (IP_3R) located on the ER membranes, thereby releasing Ca^{2+} from intracellular stores into the cystosol. Thapsigargin, however, passively raises $[Ca^{2+}]_i$ by blocking the sarco-endoplasmic ATPase pump. Diacylglycerol, the other product of the cleavage, is involved in the activation of protein kinase C, which is an important enzyme family involved in phosphorylation reactions of various proteins and in the modulation of a non-store dependent Ca^{2+} entry pathway known as non-capacitative calcium entry (NCCE) (Rosado and Sage, 2000a; Venkatachalan *et al.*, 2002; Spassova *et al.*, 2004]).

The second plateau and slowly declining phase, usually a sustained one lasting in the order of minutes *in vitro* or probably a continuous process *in vivo*, ensues following the depletion of internal Ca^{2+} stores by the first phase. It involves an influx of Ca^{2+} from an extracellular milieu through structurally diverse channels that have been dubbed store-operated channels (SOCs), due to the fact that calcium entry through them depends on the state of the cation amount in intracellular stores. Originally known as capacitative calcium entry (CCE) (Putney, 1986; Putney, 1990; Berridge, 1995), this process has increasingly come to be known as store-operated calcium entry (SOCE) (Machaca *et al.*, 2000; Lockwitch et al., 2001; Targos *et al.*, 2005). Unlike excitable cells such as neurons, muscle, and glial cells which replenish their $[Ca^{2+}]_i$ through voltage-operated channels (VOCs) or through a reverse Na^+—Ca^{2+} exchanger, non-excitable cells depend on SOCE as a predominant means of calcium replenishment (Tsien *et al.*, 1995; Parekh and Penner, 1997; Rosado and Sage, 2000b; Putney, 2003; Parekh and Putney, 2005). Once considered an exclusive feature of non-excitable cells, it is now accepted that SOCE signals are also expressed in excitable cells such as neurons (Arakawa *et al.*, 2000; Emptage *et al.*, 2001), skeletal muscle cells (Kurebayashi and Ogawa, 2001; Launikonis and Rìos, 2007), and neuroendocrine cells (Ely *et al.*, 1991)— although seemingly in a secondary role (Targos *et al.*, 2005). The contribution of NCCE to cellular calcium homeostasis in both excitable and non-excitable cells has not been qualified.

Despite its apparent potential in areas such as cancer biology, little research has been dedicated to an understanding of how SOCE is regulated during mitosis. There are few studies that compare the $[Ca^{2+}]_i$ dynamics of mitotic and interphase cells. We recently observed that, unlike interphase cells, the depletion of intracellular calcium stores with ATP was not followed by SOCE in mitotic cells which indicates the inhibition of this vital $[Ca^{2+}]_i$ -regulating mechanism. A few reports have implied either a down-regulation or a complete absence of SOCE in mitotic cells presumably due to uncoupling between cell surface channels and the ER (Volpi and Berlin, 1988; Preston *et al.*, 1991; Tani *et al.*, 2007). However, no attempts have been made to substantiate these findings and possibly shed some light on the cellular mechanisms involved. It therefore remains unclear whether—and to a large extent how—SOCE is inhibited during mitosis.

For SOCE signaling to occur, several studies have argued for an obligate intact spatial proximity between the plasma membrane and the ER that is dependent on either actin or on microtubules or on both (Holda and Blatter, 1997; Venkatachalan *et al.*, 2002; Lin *et al.*, 2003; Smyth *et al.*, 2007). In lieu of that, one plausible cause of the putative inhibition of SOCE during mitosis is the extensive reorganization of the cytoskeleton that accompanies mitosis. However, there has been no empirical evidence to support this argument (Parekh and Penner, 1997). Using COS-7 as a model, the present study aimed to clarify two main issues: 1) whether SOCE is inhibited or augmented during mitosis, and 2) the role of mitotic cytoskeletal remodeling in the inhibition process. To achieve these goals, we used cytoskeletal disruption, calcium imaging, and immunocytochemical techniques to show that microtubule remodeling mediates SOCE inhibition during mitosis.

Materials and Methods

Cell line selection

Due to the need for a high spatial resolution in the analysis of the subcellular components, large and flat cells with a short cell cycle were needed. COS-7, a cell line of immortalized kidney fibroblasts from the African green monkey, was deemed ideal for our study because of the fairly large size and robust expression of the cytoskeletal elements.

Cell culture and cytoskeletal disruption

COS-7 cells from RIKEN Institute (RIKEN, Wako City), were maintained in Dulbecco's Modified Eagle Medium

Nutrient Mixture F-12 X1 (DMEM) (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Gibco-Ivitrogen) in a humidified incubator in 5% CO_2. For subsequent experiments, cells between passages 3 and 15 were grown on 30-mm diameter cover slips or 35-mm glass base dishes with 150 μm grids (Asahi Techno Glass, Iwaki). The use of grid-bottomed dishes made it possible to follow specific cells during and after $[Ca^{2+}]_i$ imaging wherever necessary. Control cells were maintained in fresh DMEM for 12–24 h and immediately used for $[Ca^{2+}]_i$ imaging or cytoskeletal staining, as described below. Cells for microtubule disruption were incubated with 5 μM nocodazole (Sigma, St. Louis, MO, USA) in DMEM for 15 min or 5 μM paclitaxel (taxol) (Sigma) for periods ranging from 30 to 90 min and then were monitored for $[Ca^{2+}]_i$ followed by cytoskeleton staining. For actin disruption, cells were similarly maintained in DMEM for 12–24 h, treated with either 25 nM calyculin-A (BIOMOL, Plymouth Meeting, PA, USA) for 20 min. or with 5 μM cytochalasin D (BIOMOL) for 1 h, and then were either monitored for $[Ca^{2+}]_i$ dynamics or used for cytoskeleton staining.

$[Ca^{2+}]_i$ imaging

Cells were washed twice with DMEM and then loaded with oxygen-infused calcium-sensitive indicator fluo-4/AM (Molecular Probes-Invitrogen, Carlsbad, CA, USA) in standard HEPES-buffered Ringer's solution (HR) at 2 μM. The standard HR contained the following: 2.5 Mm $CaCl_2$, 118 mM NaCl, 5.5 mM D-glucose, 2 mM sodium—L-glutamate, 2% v/v MEM amino acids solution X50 (Gibco-Invitrogen), 10 mM HEPES-NaOH, 1 mM NaH_2PO_4, 4.7 mM KCl, and 1.13 mM $MgCl_2$ supplemented with 0.2% bovine serum albumin (Sigma), pH-adjusted to 7.44 with NaOH. Thereafter, the cover slips were placed into modified Sykus-Moore chambers, loaded with the calcium indicator, and incubated at 37 ℃ for 30 min. Chambers were mounted on a pre-warmed microscope stage (30 ℃), and $[Ca^{2+}]_i$ was monitored using a confocal laser scanning microscope (LSM 510, Zeiss, Jena, Germany).

During the imaging, cells were continuously perfused at a rate of 1 mℓ/min with either of the following: standard HR (extracellular Ca^{2+}, $[Ca^{2+}]_o$: 2.5 mM), calcium-free HR ($[Ca^{2+}]_o$: 0 mM), 20 μM ATP (an extracellular purinergic stimulant), 1 μM thapsigargin (a SERCA inhibitor), or 1 μM ionomycin (non-specific Ca^{2+} influx-inducing agent). To minimize fluorophore photobleaching and cellular phototoxic effects, both laser output and laser power were set at a maximum of 25% and 1%, respectively, with pinhole size set at a maximum of 1.6 airy units. Images of 512 × 512 pixel picture frame size were acquired at 6 s intervals. These settings were maintained as constant as possible throughout all the experiments. Fluorescence intensity from regions of interest covering ca. 1.3 μm-thick optical slices from randomly selected individual cells was calculated as the ratio of the fluorescence (F_t) to the initial fluorescence (F_0), the normalization F_t/F_0. After live calcium imaging, wherever necessary, Hoechst 33342 (Molecular Probes-Invitrogen) staining and differential interference contrast (DIC) imaging were used to segregate cells as either mitotic or interphase.

Cytoskeletal staining and imaging

Cells for cytoskeletal staining were washed twice with phosphate buffered saline (PBS). PBS was also used in subsequent cell washings and antibody dilutions. The cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 1% Triton X-100 (Sigma) for 5 min; they were then washed and incubated with monoclonal mouse anti-β tubulin Clone TUB 2.1 (Sigma) at a dilution of 1:100 for 1 h at room temperature. The cells were then washed twice and incubated with Alexa Flour 488-labeled anti mouse IgG secondary antibody (Molecular Probes-Invitrogen) at a dilution of 1:100 for 1 h at room temperature. For F-actin staining, the cells were washed twice and incubated with 4 units/ml Alexa Fluor 594-labeled phalloidin (Molecular Probes-Invitrogen) for 30 min at room temperature. They were then washed twice and counter-stained with 2 μM Hoechst 33342 (Molecular Probes) for 10 min at room temperature. For endoplasmic reticulum staining, cells were incubated with 2.25 μM ER-Tracker Green (Molecular Probes- Invitrogen) in a standard HR for 15 min. Following this, the cells were washed twice and examined with a confocal laser-scanning microscope. Hoechst 33342, Alexa Fluor 488, and Alexa Fluor 594-phalloidin were excited with UV, blue, and green lasers respectively.

Statistical analysis

Whenever necessary, descriptive values for fluorescence normalization, F_t/F_0, are presented as mean ± standard deviation (SD) from the three independent experiments. Outlying values and damaged cells were excluded from the analyses.

Results

ATP and thapsigargin elicits SOCE in the interphase but not in mitotic cells

$[Ca^{2+}]_i$ profiles were monitored in the interphase and mitosis of COS-7 cells using Fluor-4/AM following thapsigargin and ATP stimulation. In a classical model, thapsigargin and ATP deplete the intracellular calcium stores (particularly the ER) by mobilizing the release of free ionic calcium from the stores into the cytosol. TG does so by inhibiting the sarco-endoplasmic calcium ATPase enzyme whereas ATP depletes the stores by stimulating the classical phospholipase C enzyme pathway—whose downstream inositol triphosphate (IP_3) product stimulates the IP_3 receptors (IP_3R) to release calcium from the intracellular stores. In both scenarios, this constitutes the first cytosolic calcium peak (Peak 1) that is followed by a second peak (Peak 2) constituting the store-operated calcium entry (SOCE) of free calcium ion from the extracellular milieu (Holda *et al*., 1998; Putney, 2001; Bishara *et al*., 2002; Targos *et al*., 2005).

In the present study, Peak 1 and Peak 2 could be elicited by both ATP and thapsigargin in interphase cells. ATP elicited a sharper and a more robust first peak with a mean amplitude of 5.8 ± 1.3 (SD) (n= 51 cells), a mean

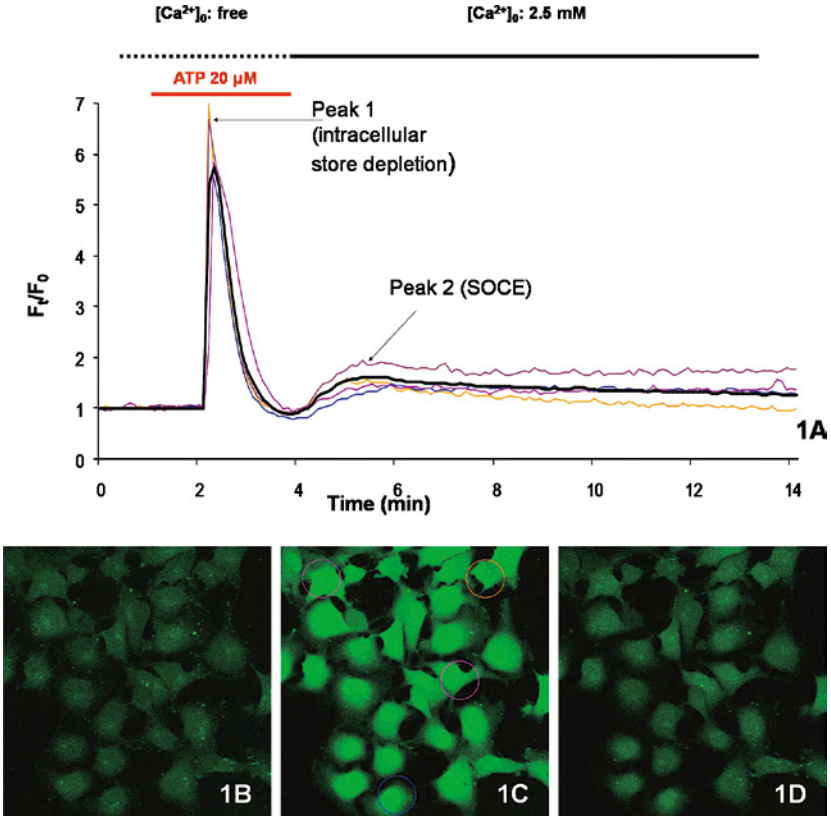


Fig. 1. A: ATP-induced intracellular calcium ion $[Ca^{2+}]_i$ dynamics in interphase cells as expressed by four randomly selected individual cells (thin traces) and mean (thick trace) normalized fluorescence (F_i/F_0). Cells were continuously perfused with 20 μM ATP in an extracellular calcium ($[Ca^{2+}]_0$) free solution which elicited the first peak in $[Ca^{2+}]_i$ (Peak 1) thereby depleting calcium from cellular internal stores. After $[Ca^{2+}]_i$ returned to basal levels, $[Ca^{2+}]_o$ was restored to 2.5 mM which stimulated the second peak (Peak 2) due to SOCE. **B–D:** Cells showing a fluorescent intensity of Fluo-4 during resting (B), Peak 1 (C), and Peak 2 (D). Four selected cells whose respective $[Ca^{2+}]_i$ dynamic traces appear in Figure 1A are highlighted with circles.

time to peak of 8.2 s ± 2.9 (SD), and a mean time to return to basal fluorescence of 58.7 s ± 11.7 (SD). The second peak, which indicates SOCE, was relatively gentle and plateau-like, reaching a mean amplitude of 1.6 ± 0.3 (SD) in an average time of 73.2 s ± 21 (SD) (Fig. 1). In contrast to ATP stimulation, thapsigargin elicited a less intense first peak with a mean amplitude of 2.4 ± 0.57 (SD) (n= 48 cells), a mean time to peak of 33 s ± 5.4 (SD) and a mean time to basal fluorescence of 72 s ± 14.1 (SD). However, the peak due to SOCE was stronger than that elicited by ATP, with a mean amplitude of 2 ± 0.28 (SD), and a mean time to peak of 108 s ± 24.0 (SD) (Fig. 2). These differences in $[Ca^{2+}]_i$ rising and falling patterns could signify the different pathways through which the two drugs elicit SOCE.

Unlike the flat evenly spread interphase cell, mitotic cells are characterized by morphological changes such as rounding off and chromatin condensation. Using these features to identify the two main cell-cycle phases, we monitored $[Ca^{2+}]_i$ in both mitotic and interphase cells in the same microscopic field. Peak 1 was comparable in sharpness and amplitude for both interphase and mitotic cells. Peak 2, however, could only be elicited in interphase cells but notably absent (or severely suppressed) in mitotic cells, which indicates that SOCE is inhibited in mitotic cells but was not in interphase

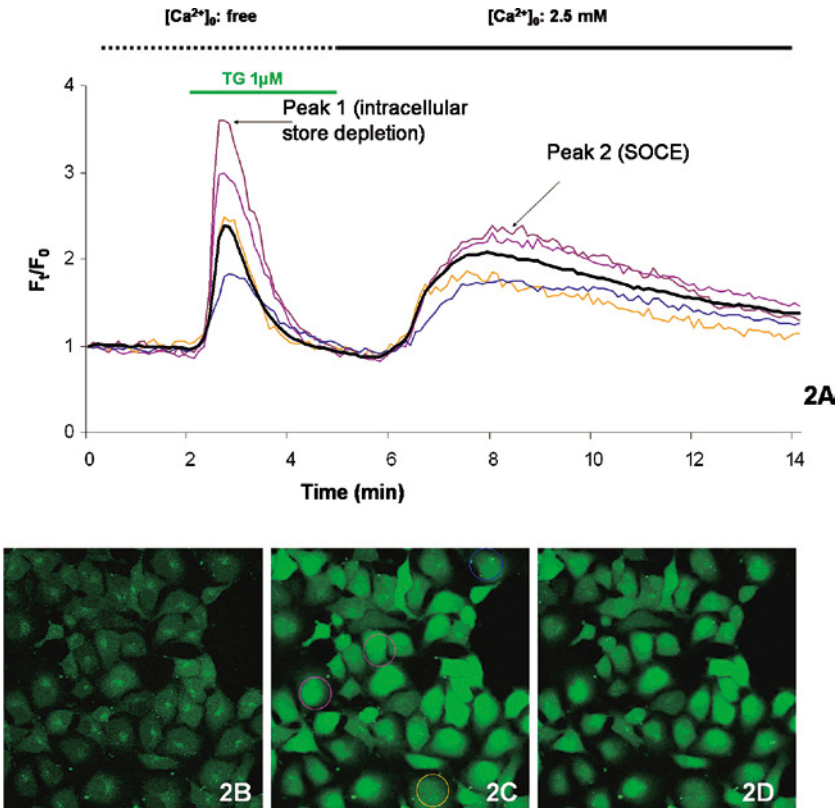


Fig. 2. A: Thapsgargin (TG)-induced intracellular calcium ion $[Ca^{2+}]_i$ dynamics in interphase cells as expressed by four randomly selected individual cell recordings (thin traces) and mean (thick trace) normalized fluorescence (F_1/F_0). Cells were continuously perfused with 1 μM thapsigargin in a $[Ca^{2+}]_0$ free solution which elicited the first peak in $[Ca^{2+}]_i$ (Peak 1), thereby depleting calcium from cellular internal stores. After $[Ca^{2+}]_i$ returned to basal levels, $[Ca^{2+}]_o$ was restored to 2.5 mM, which stimulated the second peak (Peak 2) due to SOCE. **B–D:** Cells showing fluorescent intensity of Fluo-4 during resting (**B**), Peak 1 (**C**) and Peak 2 (**D**). Four selected cells whose respective $[Ca^{2+}]_i$ dynamic traces appear in Figure 2A, are highlighted with circles.

cells. This pattern held true for ATP and thapsigargin treatments (Fig. 3, 4).

SOCE is maximally dysfunctional during the metaphase

To further comprehend the inhibition of SOCE during mitosis, we analyzed $[Ca^{2+}]_i$ dynamics in various stages of the mitotic phase from early prophase to the metaphase, during which most mitotic features are attained. Like thapsigargin, when applied in sub-micromolar concentrations, ionomycin stimulates SOCE through its ability to deplete ER calcium stores by mobilizing the release of calcium into the cytosol (Morgan and Jacob, 1994; Dedkova *et al.*, 2000). We monitored thapsigargin-evoked or ionomycin-evoked $[Ca^{2+}]_i$ in these mitotic stages (Fig. 5, 6). Interphase cells showed a normal SOCE peak as compared to those in the early prophase or those in mitosis proper. Metaphase cells, however, showed a complete absence (for thapsigargin-elicited)—or at least a severe suppression (for ionomycin-induced)—of the SOCE peak. Taken together, these data point to the possibility that SOCE is progressively suppressed through early mitosis until the metaphase stage, when it is virtually completely dysfunctional.

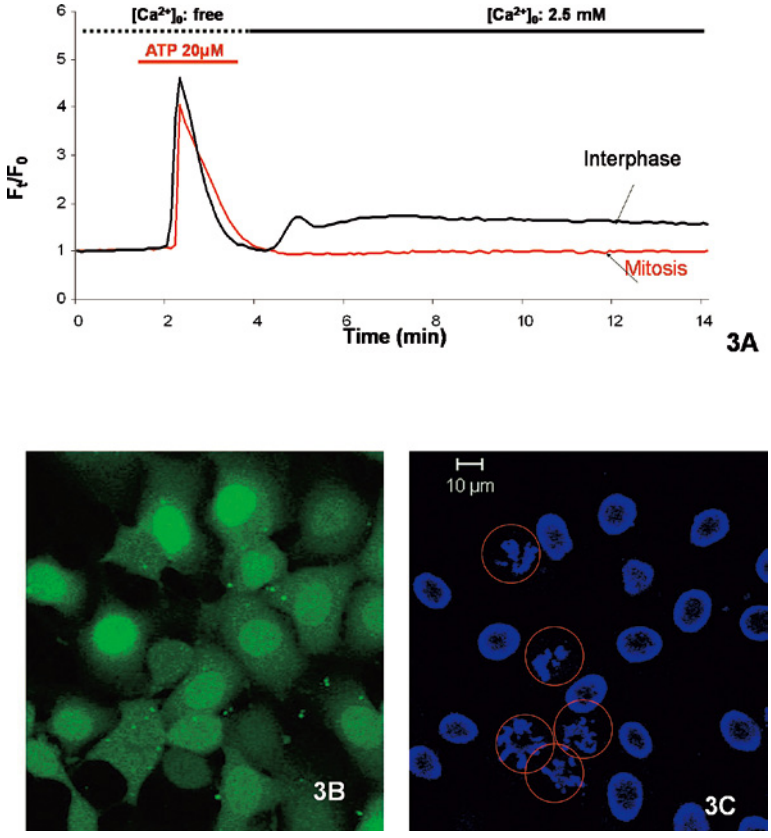


Fig. 3. A: Effect of ATP on $[Ca^{2+}]_i$ dynamics SOCE in mitotic and interphase cells. Mean normalized fluorescence (F_1/F_0) profiles in naturally occurring mitotic (n=5) and interphase (n=15) cells were compared. Mitotic and interphase cells were identified using DIC microscopy based on morphological characteristics. Cells were then monitored for ATP-elicited $[Ca^{2+}]_i$ dynamics as explained in the legend in Figure 1. Both the interphase and mitotic cells exhibited Peak 1. Unlike the interphase cells, mitotic ones did not express Peak 2 (due to SOCE). **B, C:** Interphase and mitotic cells (red circles) stained with Fluo 4/AM at Peak 1 (**B**) and Hoechst 33342 (**C**) following $[Ca^{2+}]_i$ imaging.

Microtubule disruption suppresses SOCE whereas stabilization bolsters it

Microtubules are presumed to play a significant role in the intracellular spatial localization of the ER, the main organelle involved in SOCE regulation (Smyth *et al*., 2007). In the hunt for the cause of a dysfunctional SOCE in mitotic cells, as shown above, we investigated the role of cytoskeletons in the mechanism. We used gridded glass-base dishes to locate the interphase cells, followed by microtubule disruption with 5 μM nocodazole, and then $[Ca^{2+}]_i$ was monitored. A 15-min exposure to NOC disrupted the microtubules and completely inhibited thapsigargin-invoked SOCE in about 30% of cells (Fig. 7), while producing a mitosis-like morphology in those cells with a dysfunctional SOCE. Analogous to thapsigargin results, ATP also failed to elicit SOCE in nocodazole-induced mitosis-like cells (data not shown), which indicates that SOCE is inhibited in microtubule-disrupted cells irrespective of the $[Ca^{2+}]_i$ depletion pathway.

Microtubules are important structural elements involved in organelle trafficking and positioning, and are known to regulate the position of the ER with respect to the plasma membrane (Terasaki *et al*., 1986, 1990). Envisaging that the drug-induced disassembly and mitotic remodeling could alter the microtubule intracellular localization and hence that of the ER, we colocalized the filaments and the ER with fluorescence and DIC imaging to delineate cell outlines. In a phenomenon that mimics natural mitosis in control conditions, we found that the nocodazole-induced disassembly of the

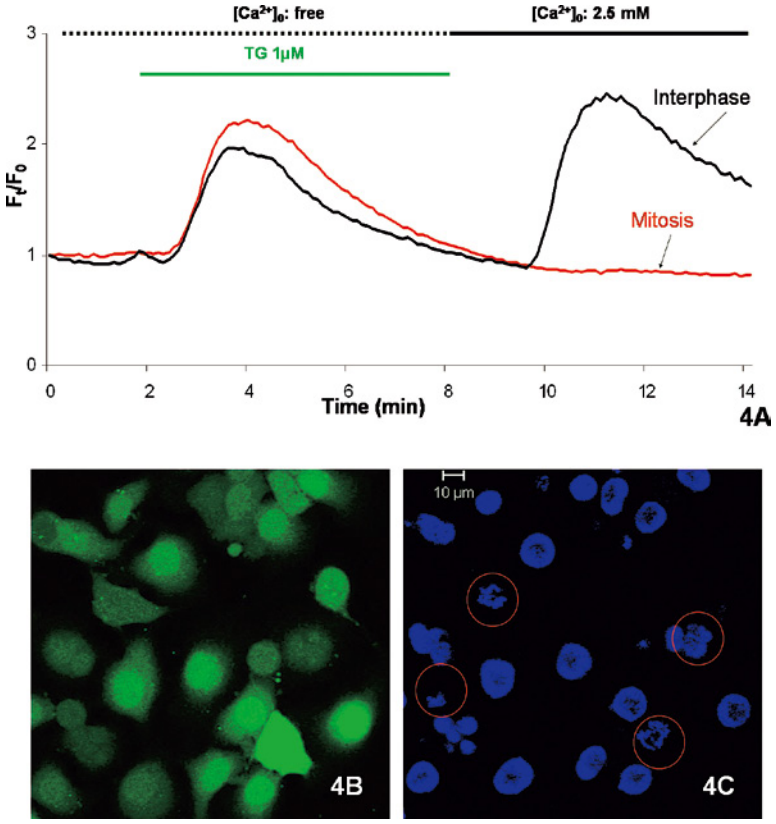


Fig. 4. A: Effect of thapsigargin (TG) on $[Ca^{2+}]_i$ dynamics in mitotic and interphase cells. Mean normalized fluorescence (F_1/F_0) profiles of mitotic (n=4) and interphase cells (n=14) are shown. Mitotic and interphase cells were first identified using differential interference contrast (DIC) microscopy based on morphological characteristics. **B, C:** Interphase and mitotic cells (red circles) stained with Fluo 4/AM at Peak 1 (**B**) and Hoechst 33342 (**C**) following $[Ca^{2+}]_i$ imaging.

microtubules began at sites farthest from the microtubule organizing centers. Additionally, in the treated cells, microtubules and the ER were seen to retract away from the cell margins (Fig. 8).

To further assert the effect of microtubules on SOCE, we treated the cells with paclitaxel, which is an anti-neoplastic drug that, unlike nocodazole, stabilizes microtubules (Xiao *et al.*, 2006). Intriguingly, when applied for a short time (30 min to 1 h) paclitaxel triggered a more than two-fold increase in thapsigargin-invoked SOCE, with an amplitude of 3.9 ± 0.6 (SD) and a mean time to peak of 2.4 min ± 0.8 (SD) (Fig. 9A) that was accompanied by a hyper-stabilized microtubule structure (Fig. 9B). When applied for extended time (1.5 h), paclitaxel caused SOCE suppression in about 25% of the cells (Fig. 9C). Cells with suppressed SOCE displayed a patchy pattern of microtubule disruption with multiple microtubule filament aggregations (Fig. 9D) that differed substantially from the uniform disruption pattern induced by nocodazole.

F-actin plays no role in mitotic SOCE suppression

An intact cortical actin is hypothesized to provide structural support in the interaction between the plasma membrane and the ER to facilitate SOCE signaling (Venkatachalan *et al.*, 2002; Lin *et al.*, 2003). Studies have shown that cells treated with serine/threonine

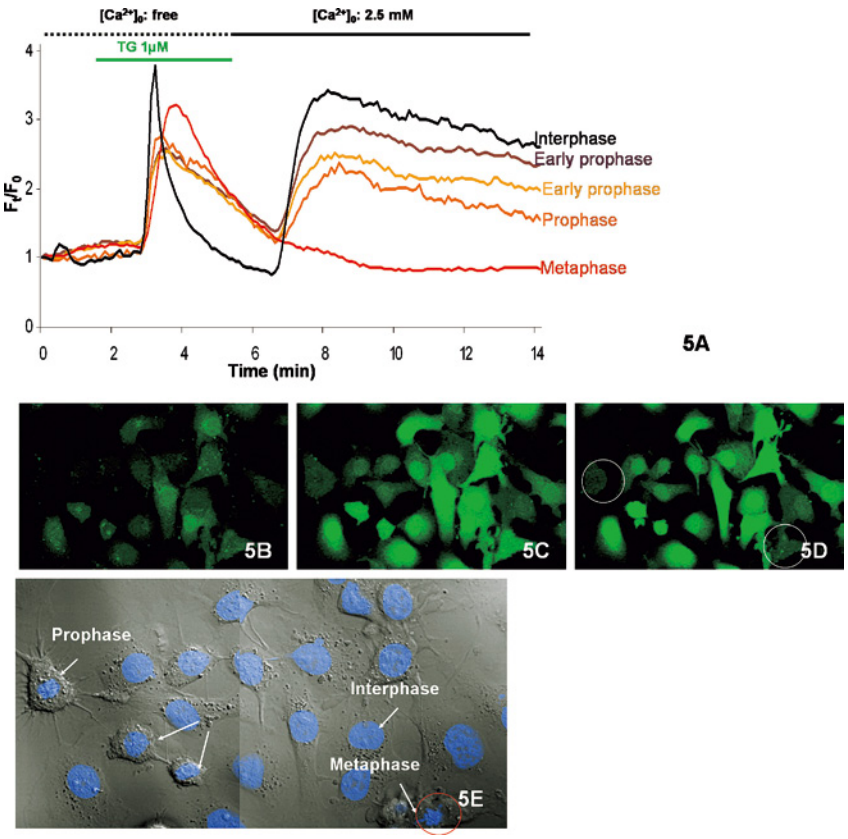


Fig. 5. A: Individual $[Ca^{2+}]_i$ dynamics of thapsigargin (TG)-treated cells, in different mitotic phases. An interphase cell shows a SOCE peak whereas a cell in metaphase shows a complete absence of the SOCE peak. Cells in early prophase and prophase show SOCE between these boundaries. **B–D:** Fluorescent images of the cells monitored in **A**, at resting (**C**), Peak 1 (**D**) and Peak 2 (**E**). Note that prophase and metaphase cells exhibit reduced fluorescence (white cycle). **E:** After the measurement of $[Ca^{2+}]_i$ dynamics, cells were stained with Hoechst 33342 and imaged with DIC to show the morphological differences of the cell cycle.

phosphatase inhibitors—such as calyculin A—exhibit a mitosis-like morphology that includes rounding off and reorganization of the filamentous actin into a thick cortical layer (Rosado *et al*., 2000; Lockwitch *et al*., 2001) that is structurally similar to that expressed by mitotic cells (Sanger, 1975; Jackson and Bellett, 1989). To determine if the thick mitotic actin layer could explain the apparent inhibition of SOCE during mitosis, we chemically induced actin condensation by incubating cells with 25 nM calyculin A, followed by the monitoring of ATP- and thapsigargin-induced $[Ca^{2+}]_i$ dynamics and F-actin labeling with a rhodamine-conjugated phalloidin. Cells were exposed to calyculin A for 20 min (longer time exposure or higher concentrations of the drug resulting

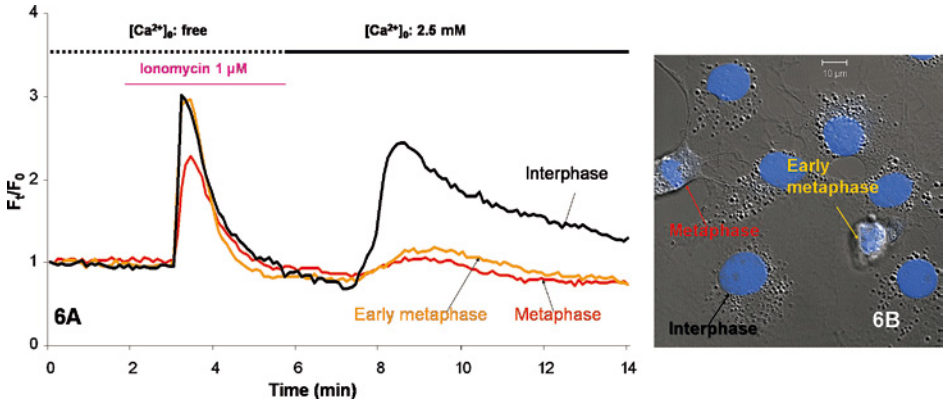


Fig. 6. A: Representative single cell tracings for ionomycin-induced SOCE in interphase and mitosis. Cells were perfused with 1 μM ionomycin in a $[Ca^{2+}]_0$ free buffer followed by the restoration of $[Ca^{2+}]_0$ to 2.5 mM. An interphase cell shows a typical SOCE profile whereas two cells in metaphase show severe suppression of the SOCE peak. **B:** After the measurement of $[Ca^{2+}]_i$ dynamics, cells were stained with Hoechst 33342 and imaged with DIC to show the different stages of the cell cycle.

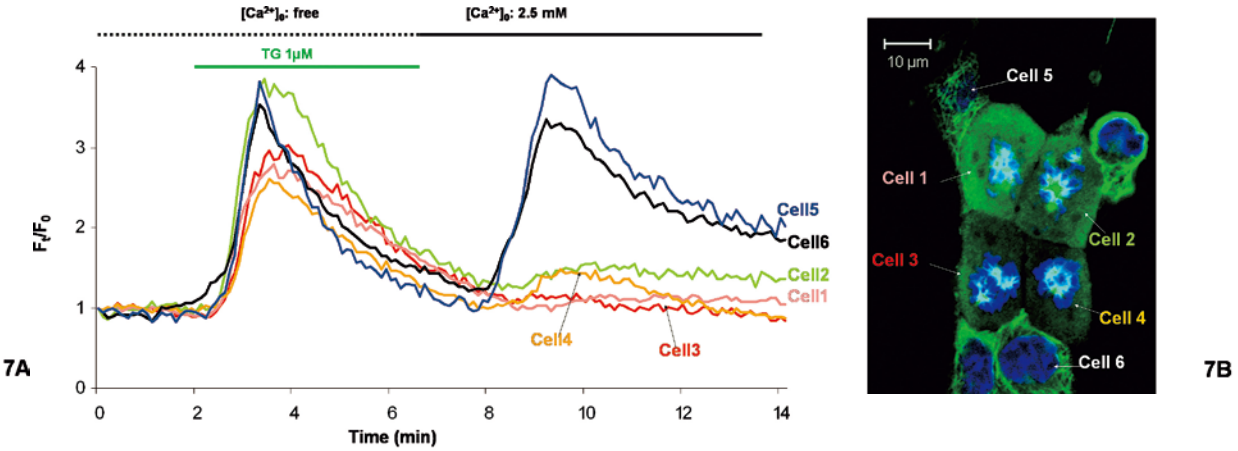


Fig. 7. Thapsigargin-induced $[Ca^{2+}]_i$ dynamics (**A**) and morphological changes (**B**) of individual cells after nocodazole treatment (5 μM for 15 min). Cells with disassembled microtubules, which also present a condensed chromatin, show a complete absence or a severely suppressed SOCE peak (Cells 1–4) whereas cells with intact microtubules (e.g., Cells 5, 6) show a typical SOCE profile. Microtubules were immunohistochemically stained (green), and chromatin was stained with Hoechst 33342 (blue).

in abnormal cell morphology and death). Intriguingly, thapsigargin-invoked SOCE was not affected by cortical actin thickening induced by calyculin A. To assess whether actin depolymerization could account for the observed SOCE suppression during mitosis, we used Cytochalasin D to depolymerize F-actin into monomeric globular actin proteins followed by the monitoring of $[Ca^{2+}]_i$ dynamics. Thapsigargin was able to induce SOCE in Cytochalasin D-treated cells more or less similar to that expressed in control cells (Fig. 10).

Discussion

Although Preston and co-workers (Preston *et al*., 1991) suggested an inhibition of SOCE in mitotic cells, this phenomenon has not been well established and the associated mechanisms are equally enigmatic. Questions remain as to whether SOCE is dysfunctional during mitosis despite being critical to the majority of cellular functions, and if so, through which pathways and mechanisms. The present work has, for the first time, empirically demonstrated that microtubule remodeling, among other possible causes, plays a significant role in the inhibition of SOCE during mitosis. We have shown that SOCE is progressively suppressed as cells enter mitosis and that maximal inhibition is attained in the metaphase, when there is virtually a complete absence of calcium influx.

ATP and thapsigargin are classical agonists that have been widely employed in *in vitro* settings to study

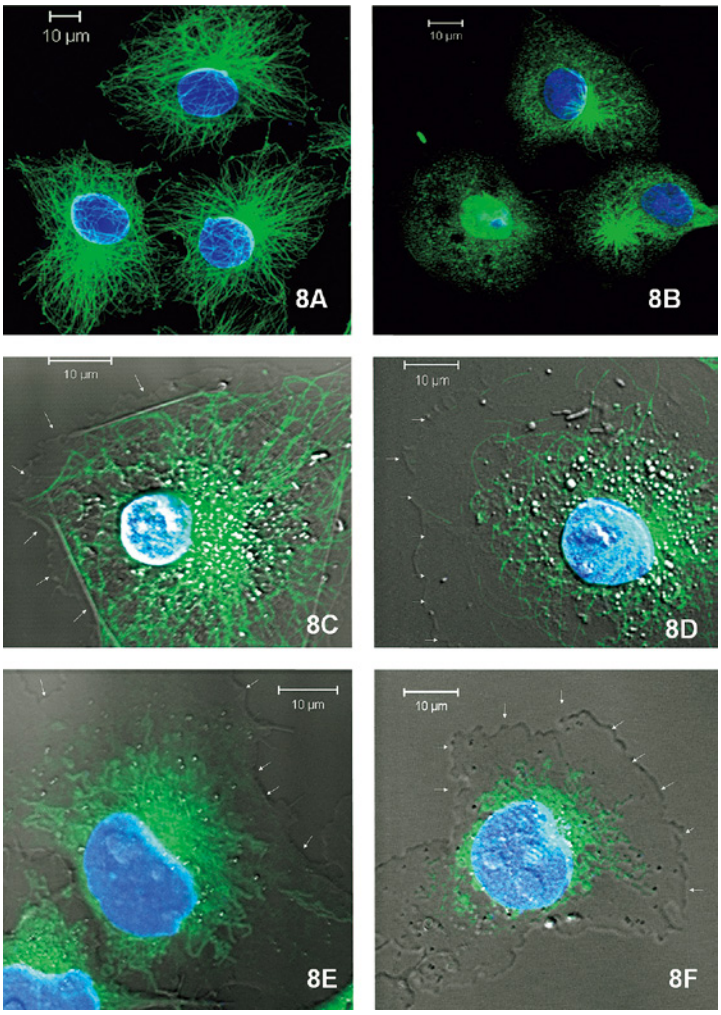


Fig. 8. Control (**A, C, E**) and nocodazole-treated cells (**B, D, F**). **A, B:** Microtubules (green) and nucleus (Hoechst 33342, blue). **C, D:** Microtubules (green), nucleus (blue) combined with a DIC image. **E, F:** ER (green), nucleus (blue) combined with a DIC image. Nocodazole-induced microtubule disassembly begins from sites farthest from the microtubule organizing center as compared with a normal microtubule. Retraction of microtubules (**D**) and the ER (**F**) from cell margin (arrowheads) following treatment of the cells with 5 μM nocodazole for 15 min. Untreated cells express a normal microtubule (**C**) and ER (**E**) that extend to the cell margins.

SOCE and other mechanisms of $[Ca^{2+}]_i$ dynamics. They traditionally elicit a biphasic $[Ca^{2+}]_i$ wave. In agreement with previous reports—though in different cell lines such as vascular endothelial cells (Holda and Blatter, 1997; Bishara *et al*., 2002), gliom C6 (Sabala *et al*., 1997); (Grimaldi *et al*., 2003) and astrocytes (Grimaldi *et al*., 2003), the ATP-triggered Peak 1 was more powerful, sharper, and returned to basal levels faster than that evoked by thapsigargin, which would imply a more exhaustive intracellular calcium store depletion by the former agonist and hence an equally robust Peak 2 (the plateau phase which signify SOCE). Contrary to expectations, thapsigargin caused a more vigorous SOCE. The reason for this apparent discrepancy is not clear, but it could be due to the fact that ATP downstream mechanisms actively open IP_3 receptors and hence release stored Ca^{2+} into the cytosol faster than thapsigargin, for which the passive release of stored Ca^{2+} would be slower. Alternatively, it could be due to the fact that ATP might stimulate Ca^{2+} uptake into other intracellular calcium compartments such as the mitochondria or efflux into the extracellular space, and

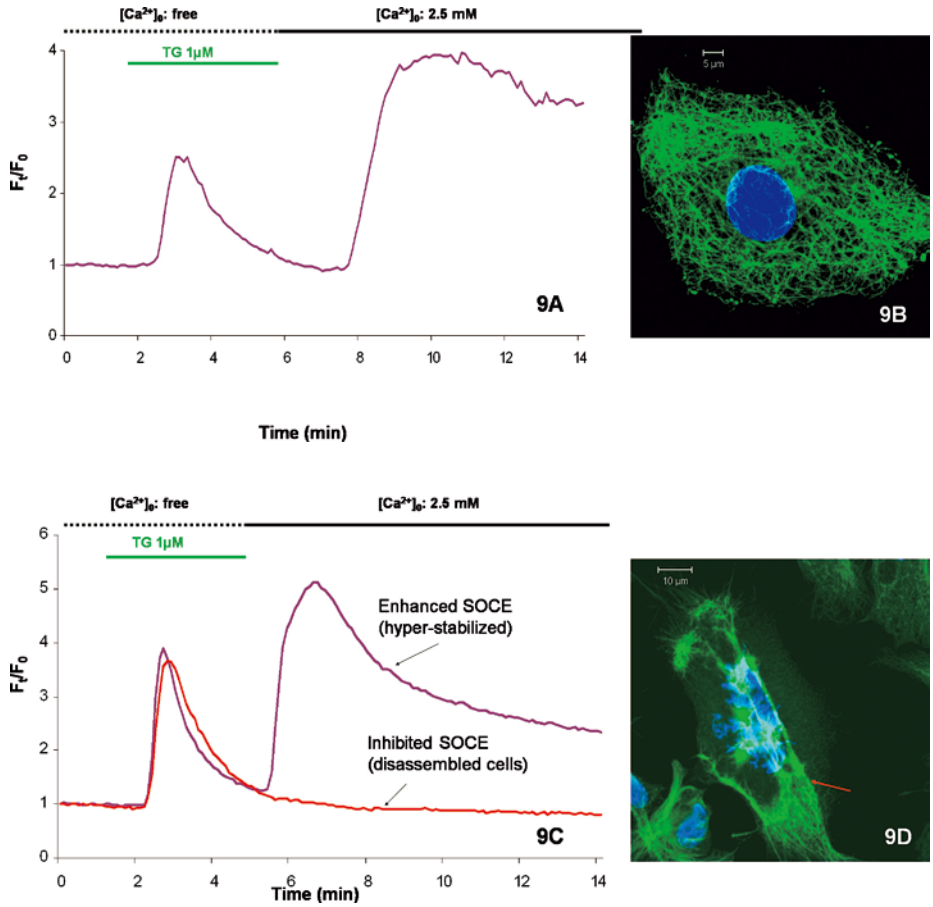
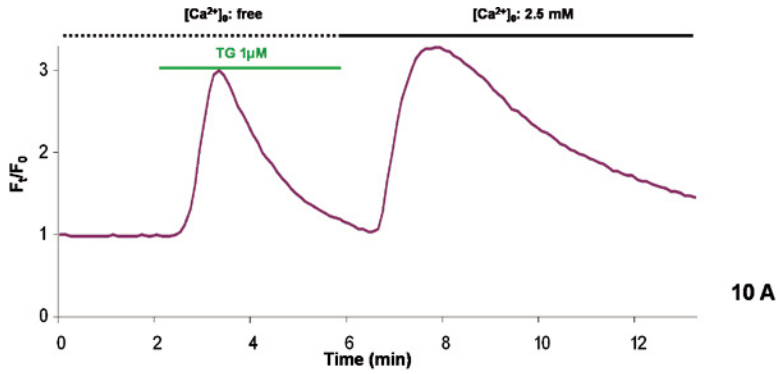
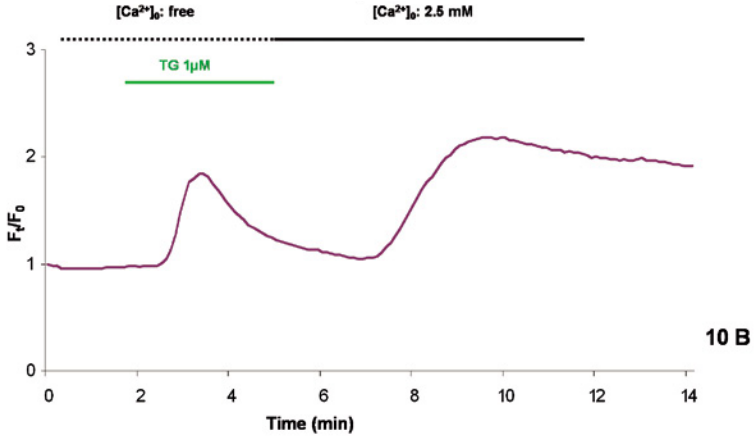


Fig. 9. A: An augmented SOCE peak in thapsigargin (TG)-induced $[Ca^{2+}]_i$ dynamics in cells treated with paclitaxel (n=14; 5 μM for 30 min). The SOCE peak increased approximately two-fold, compared with controls. **B:** Microtubles stained immunohistochemically. Note a dense network pattern of the microtubules. **C:** Long exposure of cells to paclitaxel (5 μM for 90 min) augmented the SOCE peak in thapsigargin-induced $[Ca^{2+}]_i$ dynamics in about 75% of cells (n=15) whereas about 25% (n=5) showed an inhibition of the SOCE that is comparable to that expressed in natural mitosis. **D:** Cells exposed to pacltaxel for 90 min; note a patchy pattern of microtubule disruption with multiple microtubule filament congregations (arrow).





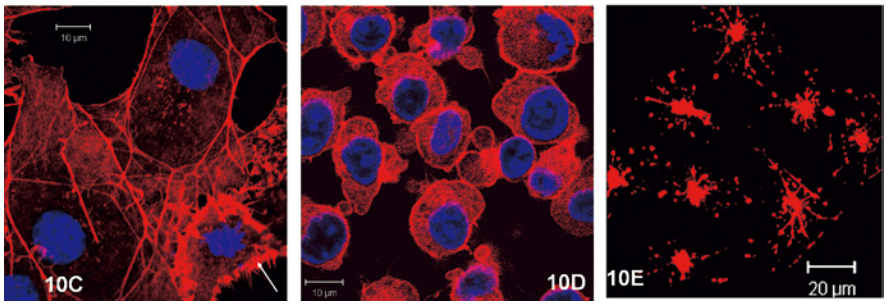


Fig.10. A: Thapsigargin-induced $[Ca^{2+}]_i$ dynamics in cells (n=19) treated with calyculin A (which enhances cortical actin polymerization; 25 nM for 20 min). **B:** Thapsigargin-induced $[Ca^{2+}]_i$ dynamics in cells (n=20) treated with Cytochalasin D (which depolymerizes F-actin; 5 μM for 1 hr). Both $[Ca^{2+}]_i$ peaks were unaffected by calyculin A and Cytochalasin D. **C–E:** An actin filament stained with rhodamine-conjugated phalloidin (red) and nuclei stained by Hoechst 33342 (blue). **C:** Control interphase cells show stress fibers whereas a mitotic cell portrays a thick, non-fibrous actin crust (arrow). **D:** Calyculin A-treated cells are characterized by a thick cortical actin filament layer similar to that expressed by natural mitotic cells. **E:** Cells treated with 5 μM Cytochalasin D show a disassembled F-actin structure.

—given the subtlety of intracellular calcium regulation—these other processes could replete cytoplasmic $[Ca^{2+}]_i$ without necessarily completely depleting the Ca^{2+} reservoir of the ER.

Nocodazole has been widely used in studies of the effect of microtubule disruption on various cellular functional and structural mechanisms, including SOCE (Hoebeke *et al*., 1976; Vasquez *et al*., 1997; Smyth *et al*., 2007). This report, however, is the first to link the mitosis-arresting effect of the drug and SOCE inhibition in mitosis while providing a breakthrough into the mechanism of SOCE regulation during mitosis. The fact that nocodazole was able to arrest interphase cells into a mitosis-like phenotype (including microtubule reorganization and chromosome condensation) in a mere 15 min exposure to the drug while expressing a dysfunctional SOCE profile provides direct evidence for the role of microtubules in SOCE signaling. To further support this view, DIC images showed a huge displacement of disassembled microtubule filaments adrift from the plasma membrane, which is a process that seems to culminate in the complete disassembly of microtubules and thus a mitosis-like chromosomal condensation. This agrees with recent reports that affirm the role of microtubules in regulating the ER position or its calcium sensor proteins stromal interaction molecule (STIM1) with plasma membrane-resident Orai1 proteins (presumed pore-forming subunits of SOCs) (Roos *et al*., 2005; Baba *et al*., 2006; Putney, 2007). Some studies also report a failure to inhibit SOCE following nocodazole treatment (Ribeiro *et al*., 1997; Bakowski *et al*., 2001). This could be due either to differences in the cells or tissues used, or to the methods employed, particularly to the agonist concentration or to the failure to detect individual cell response to the drugs. For example, in the present work, only about 30% of cells expressed a disassembled microtubule structure coupled with an inhibited SOCE.

Cells exposed for a short time to paclitaxel (or taxol, an antineoplastic drug with opposite effects to that of nocodazole) exhibited an augmented SOCE. Our results agree with those reported previously (Kidd *et al*., 2002) in which 10-20 μM paclitaxel applied for 10 min caused the elevation of $[Ca^{2+}]_i$ in pancreatic acinar cells—although this rise was interpreted to be of mitochondrial origin. Using human platelets, Redondo *et al*., (2007) essentially reached contrasting findings in which 10 μM paclitaxel applied for 30 min reduced the thapsigargin-induced SOCE by about 20%. Given that the time of exposure and the dosages were of the same ranges as those used in our work, the cause of this discrepancy is unclear though it could be due to the different cell types used. Despite displaying a microtubule-disassembly profile distinct from that produced by nocodazole, long exposure to paclitaxel expressed a severely suppressed SOCE profile, which further proves the significance of microtubules in the process. Whether or not this ability of paclitaxel to alter SOCE contributes to the antineoplastic effect of the drug remains to be elucidated.

Finally, we assessed the effect of actin cytoskeleton. Based on the conformational coupling model, a chemically-induced thick cortical actin crust has been postulated to physically block the signal transmission between the ER and store-operated channels of the plasma membrane and hence to inhibit SOCE (Patterson *et al*., 1999; Rosado *et al*., 2000; Lockwitch *et al*., 2001). We used calyculin A (a protein phosphatase 1 and 2A enzymes inhibitor) to produce a thick cortical actin layer structurally comparable to that produced during natural mitosis. In agreement with previous findings (Ribeiro *et al*., 1997), the induction of the thick cortical actin layer did not inhibit SOCE. However, other researchers have reached disparate outcomes (Patterson *et al*., 1999); (Rosado *et al*., 2000), in which calyculin A treatment blocked SOCE in A7r5 and DDT1-MF2 smooth muscle-derived cell lines and human platelets, respectively. This disparity could be due to differences in the distribution of the actin cytoskeleton in these cells (Rosado and Sage, 2000b). In addition, Cytochalasin D, which depolymerizes actin filaments, did not seem to affect SOCE. The failure of two agents with antagonistic actions on actin filaments to affect SOCE implies—albeit indirectly—that these filaments play no compulsory role in SOCE signaling in either mitotic or interphase COS-7 cells.

In conclusion, we have shown that the microtubule remodeling that accompanies mitosis plays a significant role in the inhibition of SOCE during mitosis. This role is likely to be purely physical, such that microtubule retraction or collapse causes an ER drift away from the plasma membrane, resulting in, the failure of SOCE signals to reach the cell surface calcium ion channels. The actin filament seems to play no apparent role in mitotic SOCE inhibition in COS-7 cells. As for intermediate filaments, due to their static structure, they are not as likely to exert an effect on ER displacement, and would thus, have no predictable task in mitotic SOCE inhibition. Receptor defects during mitosis, membrane potential, and ion permeability changes may well account for SOCE dysfunction. Further research in this area will shed more light on these unanswered questions. It would also be valuable to understand how the cell compensates for the lack of SOCE signals during mitosis.

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