Capacitative calcium entry is inhibited in vascular endothelial cells by disruption of cytoskeletal microfilaments

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Abstract The role of an intact cytoskeleton for store-operated ('capacitative') Ca^{2+} influx was investigated in single cultured vascular endothelial cells. Capacitative Ca^{2+} entry was measured as changes of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by depletion of Ca^{2+} stores with thapsigargin. In cells pretreated with cytochalasin D, an agent that disrupts the microfilament network of the cytoskeleton, as confirmed with FITC-phalloidin staining, capacitative Ca^{2+} entry was inhibited. Cytochalasin D did not affect basal $[Ca^{2+}]_i$ nor ATP-induced increases of $[Ca^{2+}]_i$, indicating that release of Ca^{2+} from intracellular stores through the inositol-phosphate pathway was intact. These results suggest that microfilaments are an integral part of the mechanism for capacitative Ca^{2+} entry. The necessity for an intact cytoskeleton favors a conformational coupling model for store-operated Ca^{2+} influx.

Key words: ATP; Capacitative Ca^{2+} entry; Cytoskeletal microfilament; Intracellular calcium; Thapsigargin; Cytochalasin D

1. Introduction

In non-excitable cells, agonist-stimulated Ca^{2+} release typically occurs through generation of the Ca^{2+}-mobilizing second messenger inositol 1,4,5-trisphosphate (IP_3) and subsequent Ca^{2+} release from intracellular stores [1–3]. Elevated $[Ca^{2+}]_i$ levels return to baseline through two Ca^{2+} removal pathways involving the Ca^{2+}-ATPases at the endoplasmic reticulum (ER) and the plasma membrane. Release of Ca^{2+} and subsequent depletion of intracellular stores leads to the activation of a plasma membrane Ca^{2+} influx pathway termed capacitative Ca^{2+} entry [4]. Hoth and Penner [5] measured the Ca^{2+} current associated with store depletion and characterized it as a Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) to distinguish it from other plasma membrane Ca^{2+} influx pathways. Capacitative or store-operated Ca^{2+} entry has subsequently been demonstrated in numerous cell types (e.g. [6,7]), including endothelial cells (e.g. [8,9]). Over the past few years evidence has accumulated that capacitative Ca^{2+} entry plays a central role in many aspects of cellular Ca^{2+} signalling such as volume regulation, phototransduction, mitogenesis, regulation of adenylate cyclase and sustained calcium oscillations [7]. Although the ubiquitous importance of capacitative Ca^{2+} entry is generally recognized, until now no consensus has been reached about the cellular mechanism underlying this pathway.

Several hypotheses have evolved to explain the mechanism of capacitative Ca^{2+} entry [2,3,7]. The various models for capacitative Ca^{2+} entry can be divided into two fundamentally different mechanisms for the retrograde signal for Ca^{2+} entry: (1) models that consider a diffusible second messenger that is produced and released when stores become depleted and that subsequently activates Ca^{2+} influx, and (2) models that propose that information is signalled from an empty store to the surface membrane through conformational protein–protein interactions. Numerous second messengers have been proposed to initiate capacitative Ca^{2+} entry including G-proteins, cGMP, IP_3, and other metabolites of the inositol-phosphate pathway, elements of cytochrome P450 metabolism, and various lipids [3], but none of these have been proven convincingly to activate capacitative Ca^{2+} entry. Furthermore, it has been proposed that depletion of the intracellular stores causes the generation of a specific diffusible factor that in turn activates the plasma membrane Ca^{2+} influx pathway [10,11]. On the other hand the conformational-coupling models suggest that there is a direct (protein–protein) interaction between an intracellular Ca^{2+} storage compartment and the Ca^{2+} influx channel (CRAC channel) in the plasma membrane [7]. Specifically, the large cytoplasmic head of the ER IP_3 receptor, located in close proximity to the plasma membrane, integrates signals that regulate capacitative Ca^{2+} entry, and transmits this information directly to the CRAC channel [12,13]. There is a growing body of literature in favor of and against both of these pathways; however, to date, the specific mechanistic pathway for the activation of capacitative Ca^{2+} entry has not been determined unequivocally.

Nonetheless, any model of capacitative Ca^{2+} entry mandates that the intracellular compartment communicates the status of the store Ca^{2+} levels to the plasma membrane influx channels. The actin microfilaments of the cytoskeleton form a complex network, providing the structural basis for simultaneous interactions between multiple cellular structures. This network could be involved in capacitative Ca^{2+} entry activation by a number of different routes, most notably serving as a structural link between the ER and plasma membrane to promote direct protein–protein interactions between the compartments. Therefore, this study investigated the role of cytoskeletal microfilaments in the mechanism of capacitative Ca^{2+} entry. The data provide evidence, for the first time, that disruption of actin microfilaments eliminates capacitative Ca^{2+} entry without affecting basal $[Ca^{2+}]_i$ levels or agonist-induced Ca^{2+} release.

2. Materials and methods

2.1. Cultured vascular endothelial cells

Experiments were performed on single cultured CPAE vascular endothelial cells. The CPAE cell line was originally derived from

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bovine pulmonary artery endothelium and was purchased from American Type Culture Collection (ATCC, CCL-209, Rockville, MD). The cells were cultured in Eagle's Minimum Essential Medium, supplemented with 20% fetal bovine serum (GIBCO, Grand Island, NY) and L-glutamine (2 mM), and kept at 37°C in an atmosphere of 5% CO_2 and 95% air. Once a week the cells were dispersed using a Ca^{2+}-free (0.1% EDTA) 0.25% trypsin solution, and subcultured onto glass coverslips for later experimentation. Cells from passage 4 to 7 were used. Experiments were carried out within 1 week after plating the cells onto coverslips. All experiments were performed at room temperature (20–22°C) on single isolated cells.

2.2. Solutions and chemicals

The cells were superfused continuously with a physiological salt solution (standard Tyrode solution) composed of 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) titrated to pH 7.3 with NaOH. In the nominally Ca^{2+}-free Tyrode solution $CaCl_2$ was omitted. Stock concentrations of ATP (Sigma Chemical, St. Louis, MO) were dissolved in distilled water. Thapsigargin (Alexis, San Diego, CA) and cytochalasin D (Calbiochem, San Diego, CA) were solubilized in dimethyl sulfoxide (DMSO).

2.3. $[Ca^{2+}]_i$ measurements using the fluorescent Ca^{2+} indicator indo-1

Spatially averaged photometric $[Ca^{2+}]_i$ measurements from single isolated endothelial cells were obtained with the ratiometric Ca^{2+} indicator indo-1. Cultured endothelial cells were loaded at room temperature for 20 min with the cell permeant indo-1 acetoxymethyl ester (indo-1/AM; Molecular Probes, Inc., Eugene, OR). The cell loading

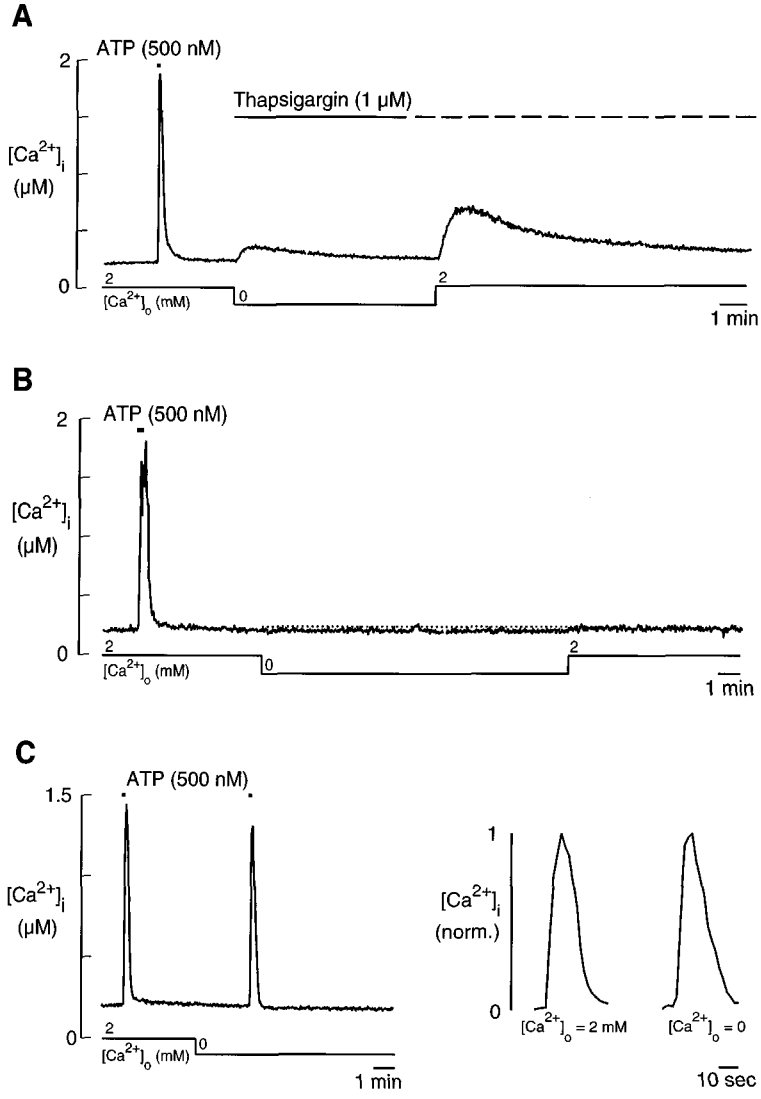

Fig. 1. Changes of $[Ca^{2+}]_i$ following exposure to ATP, thapsigargin and Ca^{2+}-free Tyrode. A: Protocol for activation of capacitative Ca^{2+} entry in vascular endothelial cells. Single, isolated vascular endothelial cells were superfused with standard Tyrode solution at room temperature. Cells were stimulated with ATP (500 nM) to generate an IP_3-dependent $[Ca^{2+}]_i$-transient and to ensure cell viability. The superfusion was switched to Ca^{2+}-free Tyrode solution containing 1 μM thapsigargin to induce store depletion. Following store depletion and the return of $[Ca^{2+}]_i$ to resting levels, Ca^{2+} was added to the extracellular environment (2 mM) and capacitative Ca^{2+} entry was observed as a large transient increase of $[Ca^{2+}]_i$. The dashed line marks the irreversible binding of thapsigargin, even though the agent was not present in the superfusate during the time indicated. B: Effect of short-term (~15 min) removal of extracellular Ca^{2+} on $[Ca^{2+}]_i$ and capacitative Ca^{2+} entry. Cells were superfused with standard Tyrode solution and stimulated with ATP (500 nM). The superfusion was then switched to Ca^{2+}-free Tyrode solution, in the absence of thapsigargin, for ~15 min. A slight decrease in baseline is shown by the dotted line. Ca^{2+} was then returned to the extracellular environment and no capacitative Ca^{2+} entry was observed. C: Comparison of brief ATP stimulation in presence and absence of extracellular Ca^{2+}. Left panel: Cells were superfused with standard Tyrode and stimulated with ATP (500 nM) for 10 s. Superfusion was switched to Ca^{2+}-free Tyrode and the cell was again stimulated for 10 s with ATP (500 nM). Right panel: Normalized ATP-stimulated $[Ca^{2+}]_i$-transients from left panel revealed similar kinetics of increase and decay of $[Ca^{2+}]_i$.

solution consisted of 2 ml of standard Tyrode solution, 5 μM indo-1/AM, 2.5 μl of 25% wt/wt Pluronic F-127 (Molecular Probes; solubilized in DMSO), and 75 μl of fetal calf serum (GIBCO). $[Ca^{2+}]_i$ was measured by exciting indo-1 fluorescence with light of 360 nm wavelength, and measuring emitted fluorescence signals simultaneously at 405 nm (F_{405}) and 485 nm (F_{485}). Single cell fluorescence signals were recorded with photomultiplier tubes (Model #R2693; Hamamatsu Corp., Bridgewater, NJ) by masking off individual cells with a pinhole positioned in the emission pathway. Superscope II software (GW Instruments, Somerville, MA) was used for data acquisition and analysis. $[Ca^{2+}]_i$ was calculated according to the formula [14,15] $[Ca^{2+}] = K_D \times \beta \times (R - R_{min}/R_{max} - R)$, where $R = F_{405}/F_{485}$. K_D was assumed to be 250 nM [14] and β was defined as $F_{485,zeroCa}/F_{485,saturatingCa}$. Values for R_{min} and R_{max} were obtained on the same experimental set-up from indo-1 salt containing solutions of intracellular ionic composition with 0 calcium (EGTA-buffered) and saturating $[Ca^{2+}]$, respectively.

2.4. *Localization of cytoskeletal microfilaments by confocal laser scanning fluorescence microscopy*

Laser scanning confocal microscopy (confocal microscope model LSM 410; Carl Zeiss, Germany) and a specific F-actin fluorescent probe were used to determine the subcellular localization and distribution of actin microfilaments. The microarchitecture of the F-actin network before and after cytochalasin D treatment was visualized [15,16] by staining endothelial cells with FITC-conjugated phalloidin (160 nM, 20 min; Molecular Probes). Prior to staining, the cells were fixed (10 min, room temperature) with 2% (w/v) paraformaldehyde in sodium phosphate-buffered saline (PBS), washed (15 min) in 1% (w/v) glycine in PBS, and permeabilized (15 min) with 0.5% (v/v) Triton X-100 in PBS. FITC-conjugated phalloidin was excited at 488 nm and emitted fluorescence was measured at wavelengths greater than 515 nm.

2.5. *Statistical analysis*

Results are reported as means ± SEM for the indicated number (n) of cells. Statistical significance was determined with the non-parametric 2-tailed Mann-Whitney U test.

3. Results

The protocol to activate capacitative Ca^{2+} entry in single, isolated vascular endothelial cells is illustrated in Fig. 1A. In the presence of 2 mM extracellular Ca^{2+} the cells were stimulated with a short exposure (10 s) to extracellular ATP (500 nM) in order to ensure cell viability and a functional pathway of Ca^{2+} release from IP_3-sensitive stores [9,17,18]. Subsequently, extracellular Ca^{2+} was removed and the cells were exposed to thapsigargin (1 μM), a plant alkaloid known to inhibit the Ca^{2+}-ATPase of the ER [19]. This inhibition of the Ca^{2+} pump leads to store depletion by spontaneous Ca^{2+} leakage from the ER followed by Ca^{2+} extrusion across the plasma membrane. After depletion of intracellular Ca^{2+} stores readmission of extracellular Ca^{2+} (2 mM) induced a large increase of $[Ca^{2+}]_i$, indicative of capacitative Ca^{2+} entry activation (Fig. 1A) [2,7,9,20]. This increase of $[Ca^{2+}]_i$ slowly recovered to baseline over the time course of several minutes. After exposure to thapsigargin subsequent stimulation with ATP failed to trigger Ca^{2+} release (see Fig. 3A), presumably due to the irreversible binding of thapsigargin to the ER Ca^{2+} pump (indicated by the dashed line Fig. 1A and 3), and continual inhibition of subsequent store refilling. Ca^{2+} removal alone (i.e. without exposure to thapsigargin) was unable to trigger capacitative Ca^{2+} entry as shown in Fig. 1B. Removing extracellular Ca^{2+} induced a small decrease in the resting $[Ca^{2+}]_i$ level (dotted line, Fig. 1B) presumably due to establishment of a new $[Ca^{2+}]_i$ equilibrium. Upon readmission of extracellular Ca^{2+} (2 mM) after ~15 min, $[Ca^{2+}]_i$ returned to the original basal level, but failed to produce a large increase of $[Ca^{2+}]_i$ (or capacitative Ca^{2+} entry). This experiment demonstrates that removal of extracellular Ca^{2+} alone, over the period of approximately 15 min, failed to activate capacitative Ca^{2+} entry.

To explore the possibility that brief ATP stimulations by themselves might activate capacitative Ca^{2+} entry, ATP-induced $[Ca^{2+}]_i$-transients were recorded in the same cell in the absence and presence of extracellular calcium. As illustrated in Fig. 1C short (10 s) exposures to ATP triggered $[Ca^{2+}]_i$-transients that were independent of extracellular Ca^{2+} and that were virtually identical in amplitude and duration. The normalized $[Ca^{2+}]_i$-transients (Fig. 1C, right panel) shown on an expanded time scale revealed very similar kinetics. From this experiment it was concluded that short exposures to ATP led to transient increases of $[Ca^{2+}]_i$ that were

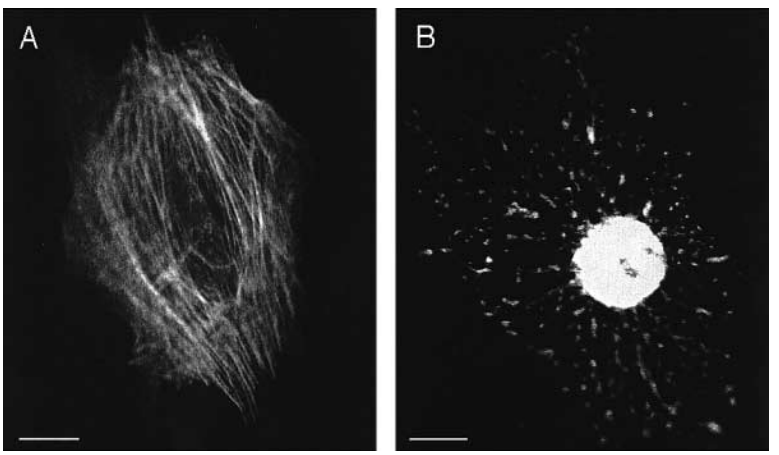

Fig. 2. Confocal images of endothelial cells stained with a microfilament-specific fluorescent probe. A: FITC-phalloidin stained control cell. Cells were fixed (see Section 2) and stained with 160 nM FITC-conjugated phalloidin (20 min) to visualize the organized F-actin microfilament network. B: FITC-phalloidin staining of cytochalasin D-treated cell. Cells were incubated with 20 μM cytochalasin D (to disrupt the microfilament network) for 3 h at 37°C, then fixed and stained as in (A). The image was recorded at high gain settings in order to visualize the outline of the cell and the faint remaining FITC-phalloidin fluorescence in the cell periphery after disruption of the actin filaments by cytochalasin. With these settings the signal recorded from the nuclear region was saturated. At gain settings similar to the ones used in (A) no actin staining could be detected in peripheral regions of the cell. Scale bar: 20 μm.

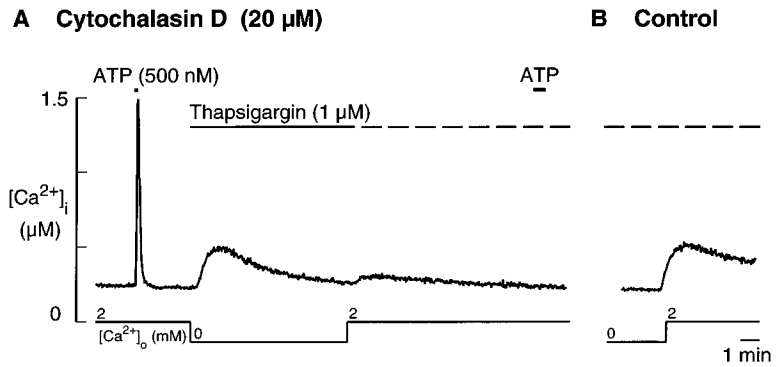

Fig. 3. Inhibition of capacitative Ca^{2+} entry in vascular endothelial cells by cytochalasin D. A: The coverslip with vascular endothelial cells was incubated for 3 h with 20 μM cytochalasin D, prior to experimentation. Single, isolated vascular endothelial cells were superfused with standard Tyrode solution at room temperature. Cells were stimulated with ATP (500 nM) to trigger IP_3-dependent Ca^{2+} release and to ensure cell viability. The superfusate was switched to Ca^{2+}-free Tyrode containing 1 μM thapsigargin to induce store depletion. Following store depletion and the return of $[Ca^{2+}]_i$ to resting levels, Ca^{2+} was added to the extracellular environment (2 mM). Only a small increase of $[Ca^{2+}]_i$ was observed indicating that capacitative Ca^{2+} entry was inhibited. The cell was again stimulated with ATP (500 nM); however, irreversible binding of thapsigargin (dashed line) did not allow for store refilling and therefore no $[Ca^{2+}]_i$-transient could be elicited. B: The same protocol as in Fig. 1A was followed for this control experiment. The control experiment was performed on the same day on cells from the same cell culture passage (due to the irreversible action of thapsigargin, the control and test experiment could not be performed on the same individual cell). A typical capacitative Ca^{2+} entry transient was recorded upon readmission of extracellular Ca^{2+}. The dashed line indicates prior treatment with thapsigargin to deplete Ca^{2+} stores.

due solely to release of Ca^{2+} from intracellular stores and that under these conditions capacitative Ca^{2+} entry was unlikely to contribute significantly to the rise of $[Ca^{2+}]_i$.

To test whether the cytoskeleton plays a role in capacitative Ca^{2+} entry, cytochalasin D was utilized to disrupt the cytoskeletal network by blocking formation of the microfilaments [21]. Endothelial cells were pretreated with 20 μM cytochalasin D for 3 h at 37°C. Staining the cells with FITC-conjugated phalloidin, to visualize F-actin [15,16], showed a significant disruption and degeneration of the organized microfilament network (Fig. 2B) in comparison to untreated cells (Fig. 2A).

Next we tested how disruption of the actin microfilament network affected ATP-induced $[Ca^{2+}]_i$-transients, intracellular Ca^{2+} stores and capacitative Ca^{2+} entry. Endothelial cells that were incubated with 20 μM cytochalasin D for 3 h at 37°C revealed identical $[Ca^{2+}]_i$-transients upon stimulation with extracellular ATP (Fig. 3A) as compared to untreated cells (Fig. 1A–C). This result clearly indicated that the IP_3-dependent release of Ca^{2+} from intracellular stores was unaffected by the cytochalasin treatment. Subsequently, exposure of the cells to thapsigargin (1 μM) to deplete the Ca^{2+} stores after prior removal of extracellular Ca^{2+} caused a typical transient increase of $[Ca^{2+}]_i$. The appearance of a thapsigargin-induced $[Ca^{2+}]_i$-transient further indicated that cytochalasin treatment did not affect the cells' ability to accumulate Ca^{2+} in the stores. In contrast to control cells (Fig. 1A and 3B) readdition of Ca^{2+} to the solution bathing the cytochalasin D-treated cells failed to induce a substantial increase in $[Ca^{2+}]_i$ (Fig. 3A), demonstrating inhibition of capacitative Ca^{2+} entry. Furthermore, subsequent stimulation with ATP (Fig. 3A) did not affect $[Ca^{2+}]_i$ confirming that the intracellular Ca^{2+} stores remained depleted. Control experiments, performed on the same day on cells of the same culture passage, revealed functional capacitative Ca^{2+} entry (Fig. 3B). In control cells readdition of extracellular Ca^{2+} after store depletion with thapsigargin resulted in a large increase of $[Ca^{2+}]_i$, indicative of capacitative Ca^{2+} entry. This increase of $[Ca^{2+}]_i$ slowly recovered to baseline after several minutes. On average, $[Ca^{2+}]_i$ was 584 ± 29 nM (n=13 cells) at the peak of capacitative Ca^{2+} entry under control conditions, whereas in the presence of cytochalasin D (n=8 cells) the maximum $[Ca^{2+}]_i$ upon restoration of extracellular Ca^{2+} was only 357 ± 17 nM, consistent with a significant inhibition of capacitative Ca^{2+} entry by cytochalasin D ($P < 0.001$, 2-tailed Mann-Whitney U test). Control experiments also revealed that the inhibitory effect of cytochalasin on capacitative Ca^{2+} entry depended on prolonged preincubation with the agent. When the cells were exposed to cytochalasin simultaneously with the readmission of extracellular Ca^{2+}, the typical large capacitative Ca^{2+} entry transient was observed (data not shown), indicating that a direct, acute, inhibitory effect of cytochalasin on the Ca^{2+} influx channel could be excluded.

4. Discussion

The aim of the present study was to investigate the role of an intact cytoskeleton in capacitative Ca^{2+} entry in vascular endothelial cells. These results clearly demonstrate that cytochalasin D, a drug that interferes with the microfilaments of the cytoskeletal architecture, blocked capacitative Ca^{2+} entry following store depletion. The cytochalasin-treated cells, however, maintained a stable resting $[Ca^{2+}]_i$ baseline and retained their ability to respond to Ca^{2+}-mobilizing agents such as thapsigargin and extracellular ATP. Normal resting $[Ca^{2+}]_i$ and an unaltered response to ATP stimulation indicated cell viability and suggested that important Ca^{2+} regulatory mechanisms remained functional in the presence of cytochalasin D. This was a particularly important observation for the response to ATP because it indicated that the release of Ca^{2+} from the ER (Fig. 1C), that depended on the generation of the diffusible messenger IP_3, was not affected. The blocking effect of cytochalasin on capacitative Ca^{2+} entry was only observed after prolonged incubation of the cells with the agent. Acute exposure to cytochalasin did not inhibit capacitative Ca^{2+} entry, thereby excluding a direct inhibitory effect on the influx pathway. This observation, however, is consistent with the

profound action of cytochalasin on the turnover of the microfilaments (see below) and their direct role in store-operated Ca^{2+} entry.

Cytochalasins are fungal metabolites that block the formation of actin microfilaments by preventing monomer addition at the growing end of the polymer [21]. Since microfilaments are dynamic structures, cytochalasin will cause the degeneration of this microfilament network by continued disassembly in the absence of growth. The cytoskeleton is a ubiquitous, complex network of filaments and tubules that extends throughout the cytoplasm of cells, including endothelial cells as confirmed in this study with FITC-phalloidin staining of F-actin (Fig. 2A). Along with its associated proteins the cytoskeleton is known to be important in many cell regulatory functions [22,23], including directing organelle movement within the cell, participating in the assembly and maintenance of the ER [24], communicating to intracellular stores [25], and maintaining functional membrane ion channels and transporters [26,27].

Since activation of capacitative Ca^{2+} entry requires that the status of the Ca^{2+} levels in the stores be communicated to the plasma membrane influx pathways, the microfilaments may provide this ability through linking the IP_3 receptor with the putative CRAC channel in the plasma membrane. IP_3 receptors have been localized in plasma membrane fractions [28], although treatment with cytochalasin shifted the subcellular distribution of the IP_3 receptors [29]. This suggests a possible microfilament coupling of the receptor to the plasma membrane. Morphological studies have provided evidence that in many cell types the ER is closely juxtaposed to the plasma membrane [7], including vascular endothelial cells [30], suggestive of surface couplings of ER membranes. Based on these ultrastructural and functional results one might propose that the cytoskeleton functions as a three-dimensional framework that holds the ER near the plasma membrane. The ability of the cytoskeleton, or more specifically microfilaments, to perform these functions is due in part to accessory proteins (for review see [31]) that permit the filaments to attach to the membrane and associated integral membrane proteins. From the standpoint of Ca^{2+} regulation, ankyrin, a microfilament adaptor protein, has been shown to associate with IP_3 receptors [32,33]. Furthermore, the *Drosophila* Trp protein (possibly a homolog to the mammalian CRAC channel) has been demonstrated to have an ankyrin binding motif [34,35]. In addition, studies on Ca^{2+} fluxes in mitotic cells have shown that store depletion-induced Ca^{2+} influx is absent [36] during the phase when the ER is retracted away from the surface membrane into a tightly packed mass concentrated around the spindles [37]. These results are similar to our observations that after cytochalasin treatment the actin microfilaments no longer extended throughout the entire cell (Fig. 2B). Taken together, the literature supports a crucial role for the cytoskeleton in these Ca^{2+} signalling processes.

Our results shed new light on the controversy about the mechanism of capacitative Ca^{2+} entry, particularly on the viability of a model based on diffusible factors. It is unlikely that microfilaments would be directly coupled to the production of a diffusible messenger within the stores and therefore the data are not consistent with that model. Our observation that IP_3-dependent release of Ca^{2+} remained intact in the presence of cytochalasin D provides further, albeit indirect evidence, that a signalling pathway that depends on a diffusible messenger (IP_3) was functional when the cytoskeleton became disrupted. Therefore, if the diffusible factor model was correct, one might assume that capacitative Ca^{2+} entry would not have been inhibited in the presence of cytochalasin D, since IP_3 diffusion did not appear altered. The present results, however, do support the conformational-coupling model for capacitative Ca^{2+} entry following store depletion. By this model, disruption of the microfilaments would suggest that either (1) the Ca^{2+} storage organelle is no longer held in close enough proximity to permit protein-protein interaction with the plasma membrane [12,13] or that (2) the microfilament itself had served as the direct link in the communication between the store and the plasma membrane. In either case our data show that microfilaments play a crucial role for functional capacitative Ca^{2+} entry.

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