Role of the Cytoskeleton in Calcium Signaling in NIH 3T3 Cells

AN INTACT CYTOSKELETON IS REQUIRED FOR AGONIST-INDUCED [Ca\textsuperscript{2+}], SIGNALING, BUT NOT FOR CAPACITATIVE CALCIUM ENTRY\textsuperscript{*}

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Treatment of NIH 3T3 cells with cytochalasin D (10 μM, 1 h at 37 °C) disrupted the actin cytoskeleton and changed the cells from a planar, extended morphology, to a rounded shape. Calcium mobilization by ATP or by platelet-derived growth factor was abolished, while the ability of thapsigargin (2 μM) to empty calcium stores and activate calcium influx was unaffected. Similar experiments with nocodazole to depolymerize the tubulin network yielded identical results. Platelet-derived growth factor induced an increase in inositol phosphates, and this increase was undiminished in the presence of cytochalasin D. Therefore, the blockade of agonist responses by this drug does not result from decreased phospholipase C. Injection of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) released calcium to the same extent in control and cytochalasin D-treated cells. Confocal microscopic studies revealed a significant rearrangement of the endoplasmic reticulum after cytochalasin D treatment. Thus, disruption of the cytoskeleton blocks agonist-elicited [Ca\textsuperscript{2+}], mobilization, but this effect does not result from a lower calcium storage capacity, impaired function of the IP\textsubscript{3} receptor, or diminished phospholipase C activity. We suggest that cytoskeletal disruption alters the spatial relationship between phospholipase C and IP\textsubscript{3} receptors, impairing phospholipase C-dependent calcium signaling. Capacitative calcium entry was not altered under these conditions, indicating that the coupling between depletion of intracellular calcium stores and calcium entry does not depend on a precise structural relationship between intracellular stores and plasma membrane calcium channels.

A variety of hormone and neurotransmitter agonists activate phospholipase C, promoting the breakdown of phosphatidylinositol, 4,5-bisphosphate into two intracellular messengers: inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (1). IP\textsubscript{3} binds to channel receptors located in the endoplasmic reticulum, promoting channel opening and increasing cytoplasmic calcium ([Ca\textsuperscript{2+}]), as a consequence of depletion of Ca\textsuperscript{2+} sequence...
cells were then incubated with fura-2/AM as described below.

Fura-2 Loading—The attached cells were mounted in a Teflon chamber (Bionique, Saranac Lake, NY) and incubated in DMEM containing 1 μM fura-2/AM (Molecular Probes, Eugene, OR) for 15 min at 37 °C and 5% CO₂. The cells were then washed and bathed in a HEPES-buffered physiological saline solution (HPSS, in mM: NaCl, 116; KCl, 5.4; MgSO₄, 0.8; HEPES, 20, and glucose, 10) at room temperature for at least 20 min before Ca²⁺ measurements were made.

Calcium Fluorescence Measurements—The fluorescence of the fura-2-loaded cells was monitored with a photomultiplier-based system, mounted on a Nikon Diaphot microscope equipped with a Nikon 40× (1.3 N.A.) Neofluor objective. The fluorescence light source was provided by a DeltaTec D101 (Photon Technology International Ltd., Monmouth Junction, NJ), equipped with a light path changer and dual excitation monochromators. The light path changer enabled rapid interchange between two excitation wavelengths (340 nm and 380 nm), and a photomultiplier tube monitored the emission fluorescence at 510 nm, selected by a barrier filter (Omega Optical, Brattleboro, VT). All experiments were carried out at 25 °C on a field of four to six cells. Calculation of [Ca²⁺], was carried out as described previously (7).

Cell Microinjection—NIH 3T3 cells (partially loaded with fura-2/AM to allow the measurement of baseline [Ca²⁺]) were microinjected with a solution containing 2 μM fura-2 and 1 μM or 1 μM (2,4,5)IP₃, via a glass micropipette attached to a WPI PV830 Micropump (World Precision Instruments, New Haven, CT) as described previously (8). [Ca²⁺]i, was measured immediately following microinjection as described above. 

NIH 3T3 cells grown on coverslips, were subjected to analysis of variance. Results were considered statistically significant of at least three separate experiments; the inositol phospholipid data depict the average of 10 experiments, performed in duplicate and repeated experiments. The inositol phospholipid mass was obtained. For the actin and tubulin double-labeling images, the fluorescein (representing tubulin) and BODIPY (representing actin) were excited simultaneously with the dye fluoro-515–565 nm. At a pinhole setting of 0.8 μm for both images. All cells were imaged across a plane as close as possible to where they were attached to the coverslip. Representative cells are shown in the figures.

Statistical Analysis—All individually shown experiments (fluorescence stainings, Ca²⁺ tracings, and electron micrographs) are representative of at least three separate experiments; the inositol phospholipid data depict the average of 10 experiments, performed in duplicate and subjected to analysis of variance. Results were considered statistically significant with p < 0.05.

RESULTS

NIH 3T3 cells treated with vehicle, 10 μM cytochalasin D to depolymerize the actin microfilaments, or 10 μM nocodazole to disrupt the tubulin microtubules, were visualized by differential interference contrast microscopy (Fig. 1). Control cells were characterized as being flat, extended, and elongated, express-
Cytophalaclin D or nocodazole treatment specifically disrupts the actin or the tubulin cytoskeleton, respectively, in NIH 3T3 cells. Cells were treated with vehicle (Me₂SO, control; A), cytochalasin D (B), nocodazole (C), or PMA (D) and double stained for actin (red) or tubulin (green) as described under “Materials and Methods.” A. Control fibroblasts express intact actin stress fibers and tubulin microtubules throughout the cytoplasm. B, cytophalaclin D treatment promotes disruption of the stress fibers, with the depolymerized actin shown as a punctate cytoplasmic accumulation, but the microtubules are left intact. C, nocodazole disrupts the microtubules, resulting in accumulation of the depolymerized tubulin on the cell periphery and in surface blebs, without altering the stress fibers. D, in PMA-treated cells, the stress fibers are disrupted but the tubulin cytoskeleton is spared. However, contrary to the effect of cytochalasin D, the PMA-depolymerized actin accumulates in the cell periphery toward membrane ruffles.

We next examined the effect of treatment with cytoskeletal-disrupting agents on [Ca²⁺]i mobilization. Only cells that expressed the spherical phenotype described above were selected for comparison with control fibroblasts. Fig. 3A depicts the Ca²⁺-mobilizing action of 1 mM adenosine triphosphate, ATP, an agonist that acts through purinergic receptors to couple to phospholipase Cβ activation. In control cells (Fig. 3A, solid line), ATP induced a rapid and transient increase in [Ca²⁺]i. This action was completely abolished in cells pretreated with 10 μM cytochalasin D (Fig. 3A, broken line). Activation of phospholipase Cγ by 50 ng/ml PDGF led, after a lag period of few hundred seconds, to a Ca²⁺ mobilization of longer duration compared with ATP in control cells (Fig. 3B, solid line) as previously reported (8). Disruption of the actin cytoskeleton with cytochalasin D also abolished the Ca²⁺ response to PDGF (Fig. 3B, broken line). Disruption of the tubulin network with nocodazole yielded similar results, with complete blockade of ATP- or PDGF-induced Ca²⁺ mobilization (Fig. 3, C and D, control, solid lines; nocodazole, broken lines). The failure of cytoskeleton-disrupted cells to respond to phospholipase C-activating agonists was not due to a loss of Ca²⁺ stores, because 2 μM thapsigargin released Ca²⁺ in cytochalasin D- or nocodazole-treated cells to the same extent as in control (Fig. 4, A and B, respectively); moreover, in cytochalasin D- or nocodazole-treated cells, the kinetics of activation and the magnitude of capacitative Ca²⁺ entry after thapsigargin-induced pool depletion were indistinguishable from those in control cells (Fig. 4, A and B, control, solid lines; cytochalasin D or nocodazole, broken lines). These findings indicate that inhibition of agonist-pro-
The failure of agonists to induce Ca\(^{2+}\) mobilization in cytoskeleton-disrupted cells could be accounted for by the following possibilities: (a) one or more of the steps involved in coupling cell surface receptor activation and phospholipase C stimulation may be affected by cytoskeletal disruption, the result being a failure of agonists to increase IP\(_3\) levels; (b) the activation of the IP\(_3\) receptor in the endoplasmic reticulum may be affected by cytoskeletal disruption, impairing IP\(_3\)-induced pool depletion; (c) cytoskeletal disruption-induced cell shape changes may alter the spatial-temporal relationship between phospholipase C activation and Ca\(^{2+}\) pool depletion, resulting in diminished Ca\(^{2+}\) mobilization. To address the first possibility, PDGF-induced phospholipase C activation was investigated in control and cytochalasin D-treated cells. As shown in Fig. 5, either PDGF or cytochalasin D pretreatment alone increased the levels of IP\(_3\) compared with control but, more importantly, when cells were pretreated with cytochalasin D, the effect of PDGF on IP\(_3\) production was not blocked and was, if anything, augmented. This suggested that the coupling between agonist receptor activation and phospholipase C stimulation was not affected by cytoskeletal disruption. These results are in agreement with studies in mast cells showing that actin or tubulin depolymerization did not inhibit agonist-increased IP\(_3\) levels (14).

The possibility that the function of the IP\(_3\) receptor could have been depressed by cytoskeletal disruption was addressed by experiments such as the one shown in Fig. 6. Fig. 6A (solid line) shows that microinjection of a single control NIH 3T3 cell incubated in nominally Ca\(^{2+}\)-free buffer with a maximal Ca\(^{2+}\)-mobilizing concentration (1 mM pipette concentration) of the nonhydrolyzable IP\(_3\) analogue, (2,4,5)IP\(_3\), led to a rapid and sustained rise in Ca\(^{2+}\) concentration.

The data shown in Fig. 5 represent the IP\(_3\) fraction from Dowex separations of inositol polyphosphates, and thus include both the 1,4,5 and 1,3,4 isomers of IP\(_3\). It is unlikely that the increases due to PDGF, cytochalasin D, or the combination of the two represent significant net increases in cellular content of IP\(_3\); we have previously shown that the increase of this isomer is too small in NIH 3T3 cells to be statistically detectable against the sizable background of basal IP\(_3\), whether assayed by high performance liquid chromatography-resolved radiolabeled inositol polyphosphates, or by mass assay of IP\(_3\) (25). We note that in addition to increases in the IP\(_3\) fraction from the Dowex columns, the fractions containing isomers of inositol diphosphate and of inositol tetraphosphate, were also significantly increased by PDGF, and in each case this was not prevented by treatment with cytochalasin D.

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Fig. 3. Disruption of the actin or the tubulin cytoskeleton blocks agonist-induced [Ca\(^{2+}\)]\(_i\) mobilization. NIH 3T3 cells grown on coverslips were treated with Me\(_2\)SO (control), 10 \(\mu\)M cytochalasin D, or 10 \(\mu\)M nocodazole as described under “Materials and Methods.” Cells were loaded with fura-2/AM, and [Ca\(^{2+}\)]\(_i\) mobilization was assessed after addition of 1 mM ATP (panels A and C) or 50 ng/ml PDGF (panels B and D) in HPSS buffer containing 1.8 mM Ca\(^{2+}\). A and B, control, solid lines; cytochalasin D-treated cells, broken lines. C and D, control, solid lines; nocodazole-treated cells, broken lines.

Fig. 4. Disruption of the actin or tubulin cytoskeleton does not block thapsigargin-induced [Ca\(^{2+}\)]\(_i\) mobilization. NIH 3T3 cells grown on coverslips were treated with Me\(_2\)SO (control), 10 \(\mu\)M cytochalasin D or 10 \(\mu\)M nocodazole and cells were loaded with fura-2/AM as described under “Materials and Methods.” To assess the capacity of the Ca\(^{2+}\) pool and the extent of Ca\(^{2+}\) entry in control, cytochalasin D or nocodazole-treated cells, 2 \(\mu\)M thapsigargin was added to nominally Ca\(^{2+}\)-free HPSS buffer, followed by restoration of the extracellular Ca\(^{2+}\) to 1.8 mM where indicated. Panel A, control, solid line; cytochalasin D, broken line. Panel B, control, solid line; nocodazole, broken line.

Motivated Ca\(^{2+}\) mobilization by cytoskeletal disruption does not result from a diminished Ca\(^{2+}\) pool storage capacity or capacitative entry or from increased Ca\(^{2+}\) pool buffering. In addition, they demonstrate that the signaling mechanism for capacitative Ca\(^{2+}\) entry is not rigorously dependent on cell morphology or on the integrity of the cytoskeleton.
transient Ca\(^{2+}\) mobilization as previously reported (8, 15); pretreatment with cytochalasin D did not affect this response (Fig. 6A, broken line). Likewise, the Ca\(^{2+}\)-mobilizing action of a submaximal concentration (1 \(\mu\)M pipette concentration) of (2,4,5)IP\(_3\) was the same in control and cytochalasin D-treated cells (Fig. 6B, control, solid line; cytochalasin D, broken line). To confirm that the Ca\(^{2+}\) stores were only partially depleted by the lower concentration of (2,4,5)IP\(_3\), 2 \(\mu\)M thapsigargin was subsequently applied; again, there was no difference in the response to thapsigargin in cytochalasin D-treated versus control cells (Fig. 6B). Thus, the ability of the IP\(_3\) receptor to bind IP\(_3\) and release Ca\(^{2+}\) was not altered by cytoskeletal disruption.

Since we found no evidence for alterations in the formation or action of IP\(_3\) under cytoskeletal-dismantled conditions, we tentatively concluded that the cell shape changes induced by cytochalasin D or nocodazole were altering the spatial relationship between phospholipase C and IP\(_3\) receptors, impairing phospholipase C-dependent Ca\(^{2+}\) signaling. Thus, we next investigated the effect of cytoskeletal depolymerization on the cellular distribution of the endoplasmic reticulum. To localize the endoplasmic reticulum in control and cytoskeleton-disrupted cells, this organelle was labeled in fixed cells with the fluorescent dihexaoxacarbocyanine dye, DIOC\(_6\)(3), as previously reported (10). By abolishing the mitochondrial membrane potential, cell fixation resulted in better labeling of the endoplasmic reticulum. Fig. 7 illustrates the fluorescent staining of the endoplasmic reticulum (orange) superimposed with the differential interference contrast image (green) in control (left) or cytochalasin D-treated (right) cells (DIOC\(_6\)(3) images were \(1 \mu\)m thick). In control cells, the endoplasmic reticulum appeared to be spread throughout the cytoplasm, although in the interior regions of the cells some labeling of mitochondria undoubtedly occurs, confusing the interpretation of the images. However, in the flattened regions in the cell periphery, single tubule-like structures were observed and confirmed to correspond to ribosome-studded endoplasmic reticulum in electron micrographs (not shown). As illustrated in Fig. 1, disruption of the actin cytoskeleton caused the cells to round up, and thus the flattened cellular regions containing diffuse endoplasmic reticulum strands were retracted into the more compacted cells. Although it is not possible from such images to determine the precise relationship between endoplasmic reticulum and plasma membrane effectors, it is nonetheless clear that the shape of the endoplasmic reticulum is dramatically changed in cytochalasin D-treated cells, at the very least to accommodate the drastic changes in cell shape. Thus, it is possible that this severe rearrangement of endoplasmic reticulum membranes alters the spatial relationship between the site for phospholipase C generation of IP\(_3\) and IP\(_3\)-sensitive sites on the endoplasmic reticulum.

**DISCUSSION**

Previous work from this laboratory provided evidence that the IP\(_3\) receptors in the endoplasmic reticulum are linked to the plasma membrane through the actin cytoskeleton (6). As a result, investigators from this laboratory (6) and elsewhere (5, 16) have suggested that the communication between endoplasmic reticulum and plasma membrane involved in signaling capacitative Ca\(^{2+}\) entry might depend on a structural connection between these two cellular sites. Thus, in the present work we investigated the effects of disruption of the actin and tubulin cytoskeletons on [Ca\(^{2+}\)]\(_i\) signaling in NIH 3T3 cells. Disruption of actin microfilaments with cytochalasin D or tubulin microtubules with nocodazole resulted in profound alterations of both cell morphology and physiology. Depolymerization of either the actin or tubulin networks caused the cells to assume a spherical shape in contrast to the planar, extended, and elongated control fibroblasts (Fig. 1). The specificity of the cytoskeleton-disrupting agents was confirmed by the use of a double-label fluorescence staining protocol; in the cytochalasin D-treated cells only the actin stress fibers were dismantled, while the microtubules were intact, whereas in the nocodazole-treated fibroblasts the microtubules were disrupted but the stress fibers were undisturbed (Fig. 2). When hormones coupled to either phospholipase C\(\beta\) or phospholipase C\(\gamma\) activation were tested for their ability to mobilize [Ca\(^{2+}\)], under the same cytoskeletal-dismantling conditions, it was found that agonist action was completely blocked (Fig. 3). This inhibition of ago-
nosit-induced \([\text{Ca}^{2+}]\), signaling appeared to be a general effect of cytoskeletal disruption and/or cell shape change, because it could be induced by depolymerization of either the actin or the tubulin network.

In contrast to the effects on agonist-induced \([\text{Ca}^{2+}]\), signaling, neither the release nor the entry of \([\text{Ca}^{2+}]\) in response to thapsigargin was significantly affected by cytoskeletal disruption. These findings rule out a number of causes for the block of the agonist response, including a loss of \([\text{Ca}^{2+}]\) stores, or augmented \([\text{Ca}^{2+}]\) buffering by \([\text{Ca}^{2+}]\) pumps. Additionally, the failure of cytoskeletal disruption to affect thapsigargin-induced \([\text{Ca}^{2+}]\) entry indicates that neither an intact cytoskeleton nor cytoskeletal-dependent processes are required for activation of capacitative \([\text{Ca}^{2+}]\) entry.

We also include data demonstrating disruption of the actin cytoskeleton by PMA. In an earlier work, we demonstrated that pretreatment of NIH 3T3 cells with PMA led to a significant loss of stored calcium without activation of capacitative calcium entry (8). Since the PMA pretreatment also caused the cells to round up and protein kinase C activation in fibroblasts can disrupt actin microfilaments (11–13), we speculated that PMA-induced calcium store loss may be a result of cytoskeletal rearrangement. The present studies confirmed that protein kinase C activation by PMA disrupts the actin stress fibers in NIH 3T3 cells; however, since dismantling of the actin cytoskeleton with cytochalasin D did not affect calcium pool storage capacity (even though the alteration of cellular shape due to cytochalasin D was more dramatic compared with PMA), we suggest that our earlier reported finding on the effect of PMA on calcium stores is independent of cytoskeletal rearrangement or cell shape. Furthermore, as reported in our earlier investigation (8) pretreatment of NIH 3T3 cells with PMA seemed to fragment the endoplasmic reticulum, whereas in the present work cytochalasin D appeared to induce a rearrangement rather than a fragmentation of this organelle.

The blockade of agonist-induced mobilization of \([\text{Ca}^{2+}]\) by cytoskeletal disruption was not a result of decreased phospholipase C activity as shown in Fig. 5. Interestingly, cytochalasin D treatment increased cellular levels of \(\text{PIP}_2\) (Fig. 5), but the mechanism for this effect is not known. It is known, however, that monomeric actin-binding proteins such as profilin also bind to phosphatidylinositol 4,5-bisphosphate, \(\text{PIP}_2\) (17), exerting an inhibitory constraint on the availability of this phospholipid for hydrolysis by phospholipase C. Therefore, it is possible that as the levels of monomeric actin are increased by filamentous actin disruption, the monomeric actin may compete with \(\text{PIP}_2\) for binding to profilin (or other monomeric actin-seques-tering proteins which bind \(\text{PIP}_2\)), thus releasing \(\text{PIP}_2\) from its inhibitory constraint and making more \(\text{PIP}_2\) available for phospholipase C breakdown. Regardless of the explanation for the cytochalasin D effect, when PDGF was added to cytochalasin D-treated cells, its effect on \(\text{IP}_3\) formation was clearly not blocked; rather, the action of the growth factor appeared additive with that of cytochalasin D.

The \(\text{IP}_3\) receptor has been shown to bind ankyrin (18–20), a cytoskeletal protein that connects integral plasma membrane proteins through spectrin or fodrin to the actin cytoskeleton (21, 22). If such an interaction with the actin cytoskeleton were essential for \(\text{IP}_3\) receptor function in NIH 3T3 cells, one would expect that, under actin-disrupted conditions, microinjection of (2,4,5)\(\text{IP}_3\) would not release sequestered \([\text{Ca}^{2+}]\) compared with control cells. However, cytoskeletal disruption with cytochalasin D (Fig. 6) did not affect \([\text{Ca}^{2+}]\) mobilization by (2,4,5)\(\text{IP}_3\), indicating that the blockade of agonist-induced \([\text{Ca}^{2+}]\), mobilization could not be attributed to an altered function of the \(\text{IP}_3\) receptor.

The findings in this study present something of a conundrum: the ability of phospholipase C-linked agonists to mobilize \([\text{Ca}^{2+}]\) is blocked by cytoskeletal disruption, but neither agonist-activated phospholipase C nor the ability of \(\text{IP}_3\) to mobilize \([\text{Ca}^{2+}]\) appears changed. This leads us to suggest that the blockade of agonist-mobilized \([\text{Ca}^{2+}]\), is a consequence of the structural changes imposed by cytoskeletal disruption, resulting in an altered spatial-temporal relationship between phospholipase C activation and \([\text{Ca}^{2+}]\) release sites. Earlier studies have shown that \(\text{IP}_3\) is a diffusible messenger capable of acting at a long range within the cytoplasm (23, 24). However, since the levels of agonist-increased \(\text{IP}_3\) are low and \(\text{IP}_3\) is rapidly metabolized in NIH 3T3 cells (25), an increased distance from phospholipase C to \(\text{IP}_3\) receptors may result in a failure of \(\text{IP}_3\) to reach the release sites. The proposal that \(\text{IP}_3\) may be rapidly degraded and thus cannot diffuse long distances in these cells is in agreement with a report in nasal epithelia in which the action of \(\text{IP}_3\) was found to be restricted to the regions of the cell where it was produced, inducing only a localized \([\text{Ca}^{2+}]\) release (26). The present data may indicate that hormone-sensitive \([\text{Ca}^{2+}]\) stores are confined to a cytoplasmic domain which is close to plasma membrane phospholipase C; such a distribution of \([\text{Ca}^{2+}]\) pools may be normally maintained by the structure of the cytoskeleton. This proposal is in agreement with previous subcellular fractionation findings from this laboratory (6): in rat liver cells, \(\text{IP}_3\)-binding vesicles co-purified
with a plasma membrane marker and such a distribution of IP$_3$-binding sites was altered by cytochalasin B, suggesting that the IP$_3$ receptor-containing organelles may be linked to the plasma membrane through the actin cytoskeleton. Furthermore, microtubular disruption with colchicine also dissociated the IP$_3$ binding sites from the plasma membrane (6), implicating a role for the tubulin cytoskeleton on the distribution of IP$_3$-containing organelles.

Several investigations have suggested the existence of a physical relationship between the endoplasmic reticulum and the actin or tubulin cytoskeletons. Studies on endoplasmic reticulum membrane subfractions after disruption of Krebs II ascites cells revealed that treatment with cytochalasin B increases the yield of all subfractions, suggesting an interaction between different endoplasmic reticulum domains and actin filaments (27). The rough endoplasmic reticulum of characean algal cells was shown to bind and slide along tracks of actin filaments (28) and, in photoreceptor cells, in the submicrovillar region where the endoplasmic reticulum codistributes with actin filaments, cytochalasin B disrupted this actin network leading to a disorganization and disintegration of the endoplasmic reticulum (29). Similarly, when the microtubular system of kidney cells was disrupted by colchicine or rotenone, the organization of the endoplasmic reticulum was found to be affected (30). Moreover, a subsequent study demonstrated that treatment of different cells with nocodazole leads to a simultaneous retraction of the endoplasmic reticulum and the microtubule networks toward the cell center (31).

The primary purpose of the present work was to address the role of the cytoskeleton in the coupling between Ca$^{2+}$ pool depletion and activation of the capacitative Ca$^{2+}$ entry pathway (for detailed reviews, see Putney (4) and Berridge (5)). Cytoskeletal depolymerization functionally uncoupled phospholipase C activity from Ca$^{2+}$ mobilization, but left unaltered the coupling between store depletion and activation of capacitative Ca$^{2+}$ entry. Thus, it is clear that the cytoskeleton does not play an obligatory role in signaling capacitative Ca$^{2+}$ entry. This would argue against the hypothesis that Ca$^{2+}$ pool depletion promotes an insertion of vesicles containing capacitative Ca$^{2+}$ entry channels (32), since such an exocytotic mechanism usually depends on an intact cytoskeletal network. Furthermore, as cytoskeletal disruption dramatically altered the shape of the cells and the endoplasmic reticulum, and appeared to disrupt the spatial relationship between plasma membrane and endoplasmic reticulum proteins, this would seem inconsistent with a coupling mechanism involving direct, spatially restricted interactions between endoplasmic reticulum and plasma membrane proteins. Rather, it would appear more likely that the signaling for capacitative Ca$^{2+}$ entry involves a diffusible messenger generated by or released from the endoplasmic reticulum acting at specific sites on the plasma membrane. Pettit and Hallett (33) reached a similar conclusion on the basis of confocal studies in neutrophils, showing that the sites of Ca$^{2+}$ release appeared to be located at a considerable distance from the plasma membrane.

We note that, in endothelial cells, Holda and Blatter (34) obtained essentially the opposite results as in the current study: cytochalasin D blocked Ca$^{2+}$ entry due to thapsigargin, but did not block Ca$^{2+}$ release due to ATP (acting on purinergic receptors). These investigators interpreted their findings as supportive of the conformational coupling model (5). The reason for these markedly discrepant results is not clear. It is possible that in endothelial cells capacitative calcium entry involves a diffusible messenger. However, alteration of the association between plasma membrane and endoplasmic reticulum may disrupt the spatial relationship required for signal-

In conclusion, the present studies demonstrate that in NIH 3T3 cells disruption of the cytoskeleton blocks agonist-elicted [Ca$^{2+}$], mobilization. This effect does not result from a lower calcium storage capacity, an impaired function of the IP$_3$ receptor or a diminished production of IP$_3$. Cytoskeletal disruption may alter the spatial relationship between phospholipase C and IP$_3$ receptors, impairing phospholipase C-dependent calcium signaling. Capacitative calcium entry was not altered by cytoskeletal dismantling, suggesting that the coupling between calcium pool depletion and capacitative calcium entry does not depend on a precise structural relationship between intracellular stores and plasma membrane calcium channels. Further experiments will be needed to address more precisely the cellular distribution of the agonist-sensitive Ca$^{2+}$ pools in control and cytochalasin D-treated cells.

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