Maintenance of the filamentous actin cytoskeleton is necessary for the activation of store-operated Ca^{2+} channels, but not other types of plasma-membrane Ca^{2+} channels, in rat hepatocytes

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The roles of the filamentous actin (F-actin) cytoskeleton and the endoplasmic reticulum (ER) in the mechanism by which store-operated Ca^{2+} channels (SOCs) and other plasma-membrane Ca^{2+} channels are activated in rat hepatocytes in primary culture were investigated using cytochalasin D as a probe. Inhibition of thapsigargin-induced Ca^{2+} inflow by cytochalasin D depended on the concentration and time of treatment, with maximum inhibition observed with 0.1 μM cytochalasin D for 3 h. Cytochalasin D (0.1 μM for 3 h) did not inhibit the total amount of Ca^{2+} released from the ER in response to thapsigargin but did alter the kinetics of Ca^{2+} release. The effects of cytochalasin D (0.1 μM) on vasopressin-induced Ca^{2+} inflow were similar to those on thapsigargin-induced Ca^{2+} inflow, except that cytochalasin D did inhibit vasopressin-induced release of Ca^{2+} from the ER. Cytochalasin D (0.1 μM) inhibited vasopressin-induced Mn^{2+} inflow (predominantly through intracellular messenger-activated non-selective cation channels), but the degree of inhibition was less than that of vasopressin-induced Ca^{2+} inflow (predominantly through Ca^{2+}-selective SOCs). Maitotoxin- and hypotonic shock-induced Ca^{2+} inflow were enhanced rather than inhibited by 0.1 μM cytochalasin D. Treatment with 0.1 μM cytochalasin D substantially reduced the amount of F-actin at the cell cortex, whereas 5 μM cytochalasin D increased the total amount of F-actin and caused an irregular distribution of F-actin at the cell cortex. Cytochalasin D (0.1 μM) caused no significant change in the overall arrangement of the ER {monitored using 3′,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] in fixed cells} but disrupted the fine structure of the smooth ER and reduced the diffusion of $DiOC_6(3)$ in the ER in live hepatocytes after photobleaching. It is concluded that (i) the concentration of cytochalasin D is a critical factor in the use of this agent as a probe to disrupt the cortical F-actin cytoskeleton in rat hepatocytes, (ii) a reduction in the amount of cortical F-actin inhibits SOCs but not intracellular messenger-activated non-selective cation channels, and (iii) inhibition of the activation of SOCs and reduction in the amount of cortical F-actin is associated with disruption of the organization of the ER.

Key words: cytochalasin D, endoplasmic reticulum, hypotonic shock, maitotoxin, Mn^{2+} inflow.

INTRODUCTION

In hepatocytes, metabolism, contraction of the bile canaliculus, and many other processes are regulated by hormones such as vasopressin, adrenaline and angiotensin II through hormone-induced changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and in the concentration of Ca^{2+} in the mitochondrial matrix [1]. Maintenance of these intracellular Ca^{2+} signals requires hormone-initiated Ca^{2+} inflow across the plasma membrane through store-operated Ca^{2+} channels (SOCs), intracellular messenger-activated non-selective cation channels, and stretch-activated non-selective cation channels [1–3]. The predominant type of SOCs in liver cells are the Ca^{2+} release-activated Ca^{2+} channels which are indistinguishable from Ca^{2+} release-activated Ca^{2+} channels in mast cells and lymphocytes [4]. The liver cell intracellular messenger-activated non-selective cation channels include $Ins(1,4,5)P_3$-activated [5,6] and Ca^{2+}-activated [7] channels, and the channels activated in response to maitotoxin [8–10]. The activation of SOCs requires a decrease in the concentration of Ca^{2+} in the lumen of the endoplasmic reticulum (ER). Under physiological conditions this is induced by $Ins(1,4,5)P_3$ generated in response to hormone–receptor interaction. SOCs can also be activated in the absence of hormone by inhibitors of the ER ($Ca^{2+}+Mg^{2+}$)ATPase, such as thapsigargin [11]. In hepatocytes, there is evidence that hormones and thapsigargin can initiate the activation of putative intracellular messenger-activated non-selective cation channels, as well as the activation of Ca^{2+}-specific SOCs [1,12].

The mechanism by which SOCs in liver cells and in other types of non-excitable animal cells are activated is not well understood [1,3,13–15]. Studies with hepatocytes have provided evidence that the activation of SOCs requires the normal function of filamentous actin (F-actin), a trimeric (G_{i2}) and a monomeric (one of the ARF proteins; where ARF stands for ADP-ribosylation factor) GTP-binding regulatory protein, the maintenance of a region of the ER near the plasma membrane, and normal communication within the lumen of the ER [16–19]. There is also evidence from other cell types that the cortical band of the F-actin plays a significant role in the activation mechanism [15,20]. However, the precise role played by F-actin in the activation of SOCs, and whether the activation of other plasma-membrane Ca^{2+} channels exhibits a requirement for normal F-actin function is not known. Cytochalasins D and B, fungal metabolites that inhibit the polymerization of F-actin [21], have been used to investigate the role of F-actin in the activation of SOCs. Cytochalasin D is more potent and has fewer additional effects than cytochalasin B [21]. However, results obtained with cytochalasins and other actin-disrupting agents have been variable. They range from nearly complete inhibition of SOC activation [22] through partial inhibition [23–26] to no inhibition [27,28]. These changes are associated with varying degrees of F-actin disruption.

Abbreviations used: $[Ca^{2+}]_{cyt}$, cytoplasmic free Ca^{2+} concentration; $DiOC_6(3)$, 3′,3′-dihexyloxacarbocyanine iodide; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; F-actin, filamentous actin; fura 2/AM, fura 2 acetoxymethyl ester; SOC, store-operated Ca^{2+} channel.

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The present experiments have employed cytochalasin D as a probe to disrupt F-actin in rat hepatocytes. The aims were to determine the optimal conditions (concentration and time) under which cytochalasin D inhibits the activation of SOCs in rat hepatocytes in primary culture, to determine the effect of this concentration of cytochalasin D on the activation of intracellular messenger- and stretch-activated non-selective cation channels, and to correlate the effects on Ca^{2+} channel activation with the degree of disruption of the cortical F-actin cytoskeleton and the ER. The results have shown that treatment with 0.1 μM cytochalasin D for 3 h disrupts the cortical F-actin cytoskeleton, decreases the movement of the ER marker 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and inhibits thapsigargin-induced Ca^{2+} inflow, but does not substantially inhibit thapsigargin-induced release of Ca^{2+} from intracellular stores, vasopressin-induced Mn^{2+} inflow, or maitotoxin- or hypotonic shock-induced Ca^{2+} inflow. It is concluded that F-actin plays a specific role in allowing the activation of SOCs, but is not required for activation of the other plasma-membrane Ca^{2+} channels tested.

MATERIALS AND METHODS

Materials

Collagenase Type 4 was obtained from Worthington Biochemical Corporation (Lakewood, NJ, U.S.A.); fura 2 acetoxymethyl ester (fura 2/AM), pluronic F-127, $DiOC_6(3)$ and Texas Red-X phalloidin from Molecular Probes (Eugene, OR, U.S.A.); [Arg^8]vasopressin, OsO_4, cytochalasin D, colchicine and BSA from Sigma–Aldrich (Castle Hill, NSW, Australia); and maitotoxin from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals and reagents were of the highest grade commercially available.

Loading of hepatocytes with fura 2

Rat hepatocytes were isolated and cultured on collagen-coated coverslips as described previously [18,19]. Cells were loaded with fura 2/AM by placing the coverslips (with a density of approx. 2.6×10^4 hepatocytes/cm^2) in 1 ml of fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 μM fura 2/AM and 0.0075 % (w/v) pluronic F-127 at 37 °C in a humidified atmosphere of air/CO_2 (19:1). The loading normally lasted for 1–2 h and started either before or after the addition of agents for treatment, depending on the duration of the treatment. All the cells were used within 5 h of plating. Approx. 70 % of the intracellular fura 2 was released by 20 μM digitonin, indicating that the major proportion of the fura 2 was located in the cytoplasmic space.

Measurement of Ca^{2+} inflow and Ca^{2+} release from the intracellular stores in fura 2-loaded hepatocytes

The measurement of fura 2 fluorescence commenced with coverslips in a thermostatted (34 °C) incubation chamber containing Ca^{2+}-free Hanks medium (137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4.7H_20$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.5 mM glucose and 20 mM Hepes, pH 7.4) and was performed using a Nikon Diaphot epifluorescence microscope, intensified CCD camera (Photonic Science ISIS-3/S20) and Axon Imaging Workbench Software, as described previously [18]. Routinely, the fluorescence ratio values (F_{340}/F_{380}) from ten cells on each coverslip were averaged and plotted as a single trace, and Ca^{2+} release and inflow were quantified as described below and reported as a single determination.

In experiments in which vasopressin- or thapsigargin-initiated Ca^{2+} changes in $[Ca^{2+}]_{cyt}$ were measured, 40 nM vasopressin or 10 μM thapsigargin was added to the incubation chamber in the absence of extracellular Ca^{2+} to elicit the release of Ca^{2+} from the intracellular stores. Ca^{2+} (final concentration of 1.5 mM) was subsequently added when the decrease in $[Ca^{2+}]_{cyt}$ had reached a steady state. The maximum rate of increase in fluorescence ratio observed after the addition of Ca^{2+} (final concentration of 1.5 mM) was taken as a measure of Ca^{2+} inflow. To estimate the amount of Ca^{2+} released from intracellular stores by thapsigargin or vasopressin, the difference between the maximal fluorescence ratio value induced by vasopressin or thapsigargin and the minimum fluorescence ratio value after $[Ca^{2+}]_{cyt}$ had decreased and reached a steady-state value was measured, and multiplied by the width of the Ca^{2+} release peak at half height to yield a measure of Ca^{2+} release.

In experiments in which maitotoxin-initiated changes in $[Ca^{2+}]_{cyt}$ were measured, 150 pM maitotoxin was added to the incubation chamber containing Ca^{2+}-free Hanks solution supplemented with 0.1 % (w/v) BSA, and 1.5 mM Ca^{2+} was added after approx. 7 min. Hepatocytes were exposed to hypotonic shock by diluting the Ca^{2+}-free Hanks medium with an equal volume of 3 mM Ca^{2+}.

Measurement of Mn^{2+} inflow in fura 2/AM-loaded hepatocytes

The measurement of Mn^{2+} inflow was performed in Ca^{2+}-free Hanks medium in the absence of added sulphate and phosphate, which are known to chelate Mn^{2+}. An excitation wavelength of 360 nm was employed and the initial rate of decrease of fluorescence intensity (initial rate of fluorescence quench by Mn^{2+}) immediately after the addition of 2 mM $MnCl_2$ was taken as a measure of Mn^{2+} inflow.

Treatment of hepatocytes with cytochalasin D and colchicine

Stock solutions of cytochalasin D or colchicine in DMSO were directly added into medium or buffer solutions with a dilution factor of 1:1000. A similar volume of vehicle (DMSO) was added as a control. Cytochalasin D, colchicine or DMSO was present throughout the Ca^{2+} and Mn^{2+} measuring processes as well.

Localization of F-actin and the ER in hepatocytes

The F-actin and ER in paraformaldehyde-fixed hepatocyte samples were labelled with Texas Red-X phalloidin and $DiOC_6(3)$ respectively, and confocal microscopy was performed in a similar manner as described previously [18,19], except that cells were viewed using a Bio-Rad MRC-1024 laser-scanning confocal microscope system in combination with an Olympus AX 70 microscope and a $\times$100 NA 1.40 oil-immersion objective lens. Images were analysed using the Bio-Rad LaserSharp program.

Photobleaching of $DiOC_6(3)$-stained living hepatocytes

Hepatocytes treated with vehicle, 0.1 μM cytochalasin D and 5 μM cytochalasin D for 2–3 h were stained with 1 μM $DiOC_6(3)$ for 2 min, washed briefly with DMEM twice, then placed in fresh DMEM supplemented with the same concentrations of the treatment agents for confocal microscopy, which was performed as described above with excitation and emission wavelengths set at 488 nm and 510 nm respectively for $DiOC_6(3)$. Before photobleaching, images of cells were taken using the following settings: laser power, 1 %; zoom, 2.5; iris, 2.5; gain, 900–1100 (low signal setting to enhance the weakly-stained structure); and black level,

−1. For photobleaching, by using a larger zoom setting, a small area of the cell (normally about one-eighth of the total cellular area) was exposed to higher laser energy (laser power, 30%; zoom, 20; and other settings were the same as above) for 15 s. Images were taken immediately and 5 min after photobleaching under the original settings. To assess the recovery of ER fluorescence after photobleaching, a square (2 μm × 2 μm) which contained no bright fluorescence spots (presumably mitochondria) was selected within the region to be bleached and the total fluorescence (pixels) in this square, before bleaching, immediately following bleaching and 5 min after bleaching, was counted using the Bio-Rad LaserSharp program. The difference between the fluorescence values at 5 and 0 min after photobleaching was taken as a measure for the recovery of fluorescence within 5 min.

Electron microscopy

Hepatocytes plated on coverslips and treated with vehicle or cytochalasin D were fixed with 1% (w/v) OsO_4 for 2 h at 23 °C and subsequently with 2.5% (w/v) glutaraldehyde overnight at 4 °C. Fixed cells were detached from coverslips using a rubber-tipped cell scraper, and further processed for electron microscopy as described previously [19].

RESULTS

Effects of cytochalasin D on thapsigargin- and vasopressin-induced Ca^{2+} inflow and Ca^{2+} release from intracellular stores

The effects of treatment of hepatocytes for 1 h with cytochalasin D at concentrations ranging from 0.05 to 10 μM on thapsigargin-induced Ca^{2+} inflow were investigated. A significant inhibition of thapsigargin-induced Ca^{2+} inflow was observed at 0.1 μM cytochalasin D (Figure 1B, cf. Figure 1A). However, 5 μM cytochalasin D gave no inhibition (Figure 1C, cf. Figure 1A). Similar results were obtained for a 3 h exposure of the cells to cytochalasin D (Figures 1E and 1F, cf. Figure 1D). Figure 2(A) shows the rate of thapsigargin-induced Ca^{2+} inflow as a function of the concentration of cytochalasin D for cells exposed to the cytochalasin for 1 h. Maximum inhibition (approx. 60% of the control value) was observed at 0.1 μM cytochalasin D. The time course for the effect of 0.1 μM cytochalasin D is shown in Figure 2(B). Near maximal inhibition of Ca^{2+} inflow (80% of the control value) was observed at 3 h. At any of the concentrations and times employed, cytochalasin D had no detectable effect on cell viability, assessed by the absence of an increase in the basal value of $[Ca^{2+}]_{cyt}$ (results not shown).

No significant inhibition of thapsigargin-induced Ca^{2+} release from intracellular stores (assessed by measuring the area under the thapsigargin-induced Ca^{2+} release curve) was observed when cells were incubated for 1 h at any of the concentrations of cytochalasin D tested [Figures 1B and 1C (cf. Figure 1A), and Figure 2A]. Moreover, no inhibition of thapsigargin-induced release of Ca^{2+} was observed when cells were incubated with 0.1 μM cytochalasin D for longer times (up to 3 h) [Figures 1E and 1F (cf. Figure 1D), and Figure 2B]. However, treatment with 0.1 μM cytochalasin D for 1 or 3 h slowed the onset of the thapsigargin-induced increase in $[Ca^{2+}]_{cyt}$ and increased the time required for $[Ca^{2+}]_{cyt}$ to return to the resting level (Figures 1B and 1E, cf. Figures 1A and 1D respectively). Similar results were observed at 0.05 μM cytochalasin D (results not shown). These effects were not seen in cells treated with 5 μM (Figures 1C and 1F) or 10 μM (results not shown) cytochalasin D. The results indicate that treatment with 0.1 μM cytochalasin D for 3 h causes maximal inhibition of thapsigargin-induced Ca^{2+} inflow while having little effect on the amount of Ca^{2+} released from intracellular stores.

Treatment with 0.1 μM cytochalasin D for 1, 2 or 3 h also inhibited vasopressin-induced Ca^{2+} inflow (Figure 3). Maximal

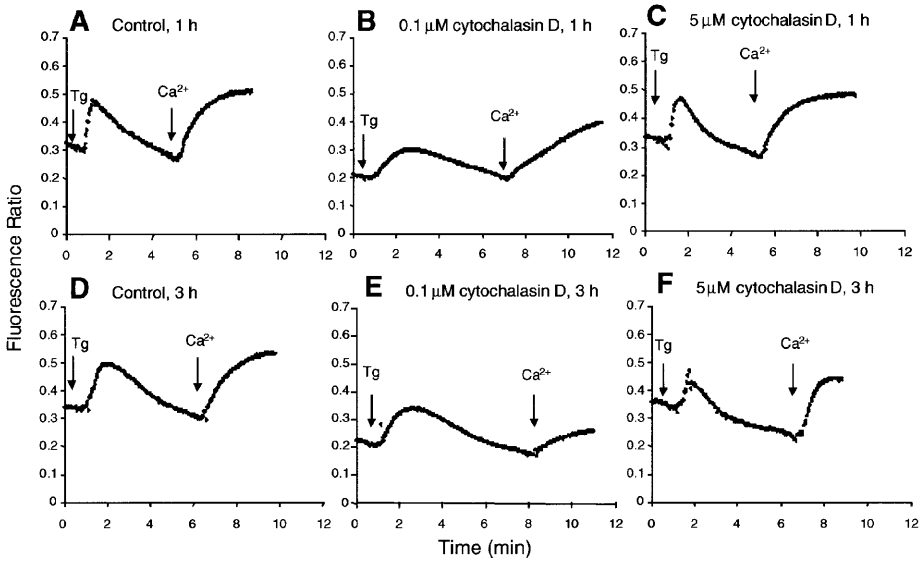

Figure 1 Effects of different concentrations of cytochalasin D and times of exposure to cytochalasin D on thapsigargin-induced Ca^{2+} inflow and Ca^{2+} release from intracellular stores

Hepatocytes were isolated from rat liver, plated, loaded with fura 2/AM and treated with DMSO (as control) (**A** and **D**), 0.1 μM cytochalasin D (**B** and **E**) or 5 μM cytochalasin D (**C** and **F**) for either 1 h (**A**–**C**) or 3 h (**D**–**F**) as described in the Materials and methods section. The times of addition of thapsigargin (Tg; 10 μM) and $CaCl_2$ (1.5 mM) are indicated by arrows. Each trace shown is an average of values from ten individual hepatocytes on a coverslip from one of three experiments which gave similar results.

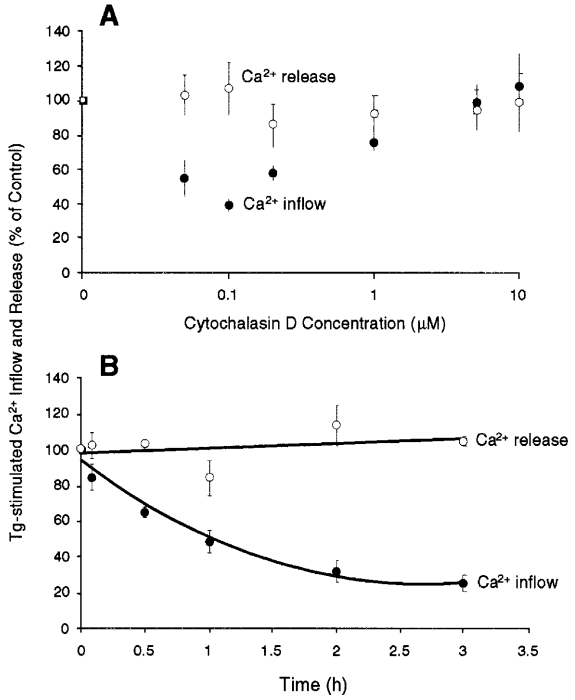

Figure 2 Dose–response curve and the time course for the effects of cytochalasin D on thapsigargin-induced Ca^{2+} inflow and Ca^{2+} release from intracellular stores

(**A**) The dose–response curve for thapsigargin (Tg)-induced Ca^{2+} release and Ca^{2+} inflow. Hepatocytes were isolated from rat liver, plated, loaded with fura 2/AM and treated with 0.05, 0.1, 0.2, 1, 5 or 10 μM cytochalasin D as well as DMSO (as control) for 1 h, and Ca^{2+} release and Ca^{2+} inflow initiated by 10 μM thapsigargin were measured as described in the Materials and methods section. Data for Ca^{2+} release and Ca^{2+} inflow in the cytochalasin D-treated groups are expressed as percentages of those of the control group, and the data are presented as means $\pm$ S.E.M. for four independent experiments involving separate cell preparations in each of which the values from ten individual hepatocytes on a coverslip were averaged and taken as a single determination. (**B**) The time course for the effects of 0.1 μM cytochalasin D on thapsigargin-induced Ca^{2+} release from intracellular stores and Ca^{2+} inflow in hepatocytes. Hepatocytes were isolated from rat liver, plated, loaded and treated with either 0.1 μM cytochalasin D or DMSO (as control) for 5 min, 0.5 h, 1 h, 2 h or 3 h, and Ca^{2+} release and Ca^{2+} inflow initiated by 10 μM thapsigargin were measured as described in the Materials and methods section. The data for the 0.1 μM cytochalasin D-treated group are expressed as percentages of those of the control group, and the data are presented as means $\pm$ S.E.M. for three independent experiments involving separate cell preparations in each of which the values from ten individual hepatocytes on a coverslip were averaged and taken as a single determination.

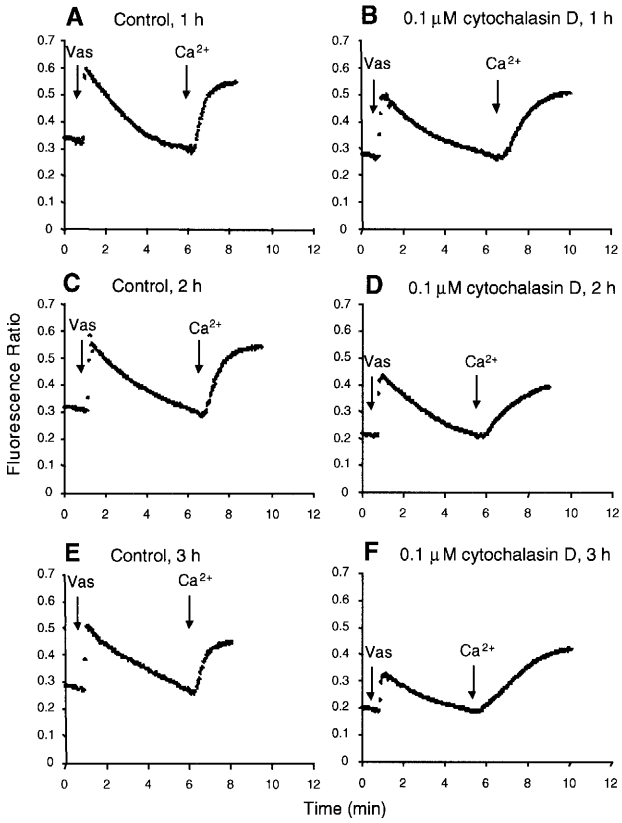

Figure 3 Effects of 0.1 μM cytochalasin D on vasopressin-induced Ca^{2+} inflow and Ca^{2+} release from intracellular stores at different times of exposure to cytochalasin D

Hepatocytes were isolated from rat liver, plated, loaded with fura 2/AM and treated with DMSO (as control) (**A**, **C** and **E**) or 0.1 μM cytochalasin D (**B**, **D** and **F**) for 1 h (**A** and **B**), 2 h (**C** and **D**) or 3 h (**E** and **F**) as described in the Materials and methods section. The times of addition of vasopressin (Vas; 40 nM) and $CaCl_2$ (1.5 mM) are indicated by arrows. Each trace shown is an average of values from ten individual hepatocytes on a coverslip from one of three experiments which gave similar results.

inhibition of approx. 80 % was observed at 3 h of exposure to 0.1 μM cytochalasin D (Figures 3 and 4). In contrast with the results obtained with thapsigargin, treatment with cytochalasin D (0.1 μM) for 3 h caused substantial inhibition of vasopressin-induced Ca^{2+} release (assessed by measuring the area under the vasopressin-induced Ca^{2+} release curve) (Figures 3 and 4). However, there was no detectable delay in the onset of vasopressin-induced release of Ca^{2+} from intracellular stores and nor was there a change in the time taken for $[Ca^{2+}]_{cyt}$ to return to the resting level (Figure 3).

Treatment with 5 μM cytochalasin D for 1, 2 or 3 h inhibited both vasopressin-induced Ca^{2+} inflow and vasopressin-induced release of Ca^{2+} from intracellular stores. At 1, 2 and 3 h of treatment with 5 μM cytochalasin D, the rates of Ca^{2+} inflow were 99 ± 11, 51 ± 4 and 48 ± 8 % ($n = 3$) of the control value respectively, and the amounts of Ca^{2+} released were 34 ± 4, 46 ± 4

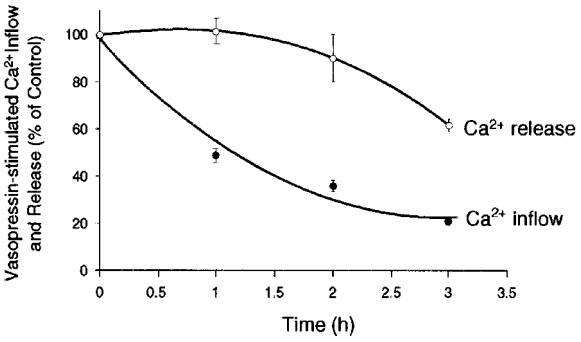

Figure 4 Time course for the effects of 0.1 μM cytochalasin D on vasopressin-induced Ca^{2+} inflow and Ca^{2+} release from intracellular stores

Hepatocytes were isolated from rat liver, plated, loaded with fura 2/AM and treated with either 0.1 μM cytochalasin D or DMSO (as control) for 1 h, 2 h and 3 h, and Ca^{2+} release and Ca^{2+} inflow initiated by 40 nM vasopressin were measured as described in the Materials and methods section. The data for the 0.1 μM cytochalasin D-treated group are expressed as percentages of those of the control group, and the data are presented as means $\pm$ S.E.M. for three independent experiments involving separate cell preparations in each of which the values from ten individual hepatocytes on a coverslip were averaged and taken as a single determination.

Table 1 Comparison of the effects of cytochalasin D on vasopressin- and thapsigargin-induced Mn^{2+} and Ca^{2+} inflow in hepatocytes

Hepatocytes were isolated, plated, loaded and treated with 0.1 μM cytochalasin D or DMSO for either 1 or 3 h, and Ca^{2+} and Mn^{2+} inflow were measured as described in the Materials and methods section. Basal rates of Mn^{2+} inflow have been subtracted. Data are presented as means $\pm$ S.E.M. for three independent experiments involving separate cell preparations in each of which the values from ten individual hepatocytes on a coverslip were averaged and taken as a single determination. The degree of significance, determined using the two-tailed Student's *t* test for unpaired samples, for a comparison of the values between the cytochalasin D groups and the control groups was $^*P \leqslant 0.05$, and between vasopressin- and thapsigargin-induced Mn^{2+} inflow was $\dagger P \leqslant 0.05$.

		Rate of agonist-induced Mn^{2+} inflow		Rate of agonist-induced Ca^{2+} inflow	
Agonist	Treatment	Fluorescence intensity units/min	Percentage of control (DMSO-treated cells)	Fluorescence ratio units/min	Percentage of control (DMSO-treated cells)
Thapsigargin (10 μM)	DMSO (1 h)	24 ± 3	–	0.22 ± 0.03	–
	Cytochalasin D (1 h)	24 ± 6	97 ± 15	$0.09 \pm 0.01^*$	44 ± 9
	DMSO (3 h)	23 ± 2	–	0.20 ± 0.03	–
	Cytochalasin D (3 h)	$12 \pm 1^*$	52 ± 4	$0.05 \pm 0.01^*$	24 ± 4
Vasopressin (40 nM)	DMSO (1 h)	$37 \pm 4\dagger$	–	0.32 ± 0.01	–
	Cytochalasin D (1 h)	$45 \pm 3\dagger$	125 ± 6	$0.14 \pm 0.01^*$	46 ± 5
	DMSO (3 h)	30 ± 3	–	0.30 ± 0.01	–
	Cytochalasin D (3 h)	$24 \pm 1\dagger$	87 ± 16	$0.06 \pm 0.01^*$	21 ± 3

and $62 \pm 15\%$ ($n = 3$) of the control value respectively (results not shown).

Effects of cytochalasin D on thapsigargin- and vasopressin-induced Mn^{2+} inflow

Previous studies with hepatocytes have shown that vasopressin and other hormones initiate the activation of intracellular messenger-activated non-selective cation channels in addition to the activation of SOCs [12,29,30]. SOCs in hepatocytes and in other cell types appear to admit little Mn^{2+} under the experimental conditions usually employed [13,30]. By contrast, there is evidence that hepatocyte intracellular messenger-activated non-selective cation channels admit considerable amounts of Mn^{2+} as well as Ca^{2+} [29,30]. Therefore Mn^{2+} was used to monitor bivalent cation inflow through vasopressin-activated non-selective cation channels.

In control cells treated with vehicle (DMSO), the rate of vasopressin-induced Mn^{2+} inflow was 1.5-fold greater than that of thapsigargin-induced Mn^{2+} inflow (Table 1), suggesting that, in addition to the activation of SOCs, vasopressin induces the activation of intracellular messenger-activated non-selective cation channels which admit Mn^{2+}. Treatment with cytochalasin D (0.1 μM) for 1 h did not inhibit thapsigargin-induced Mn^{2+} inflow, whereas treatment for 3 h caused 50% inhibition (Table 1). Cytochalasin D treatment for either 1 or 3 h caused no inhibition of vasopressin-induced Mn^{2+} inflow (Table 1). [Cytochalasin D (0.1 μM for 1 or 3 h) had no effect on the basal (no agonist) rate of Mn^{2+} inflow. The rates of basal Mn^{2+} inflow were 21 ± 1 ($n = 3$) and 26 ± 3 ($n = 3$) fluorescence intensity units/min at 1 and 3 h after treatment with cytochalasin D respectively, compared with rates of 19 ± 2 ($n = 3$) and 22 ± 1 ($n = 3$) for control cells (no cytochalasin D) cultured for 1 and 3 h respectively.]

Comparison of the effects of cytochalasin D treatment on Mn^{2+} inflow with those on Ca^{2+} inflow indicates that, for both thapsigargin and vasopressin, the inhibition of Ca^{2+} inflow caused by treatment with cytochalasin D (0.1 μM for 3 h) was considerably greater than that of Mn^{2+} inflow (Table 1). These results suggest that in hepatocytes (i) Mn^{2+} can be used to partially discriminate between Ca^{2+}-selective SOCs and intracellular messenger-activated non-selective cation channels and (ii) Ca^{2+}-selective SOCs are more susceptible to inhibition by treatment with 0.1 μM cytochalasin D.

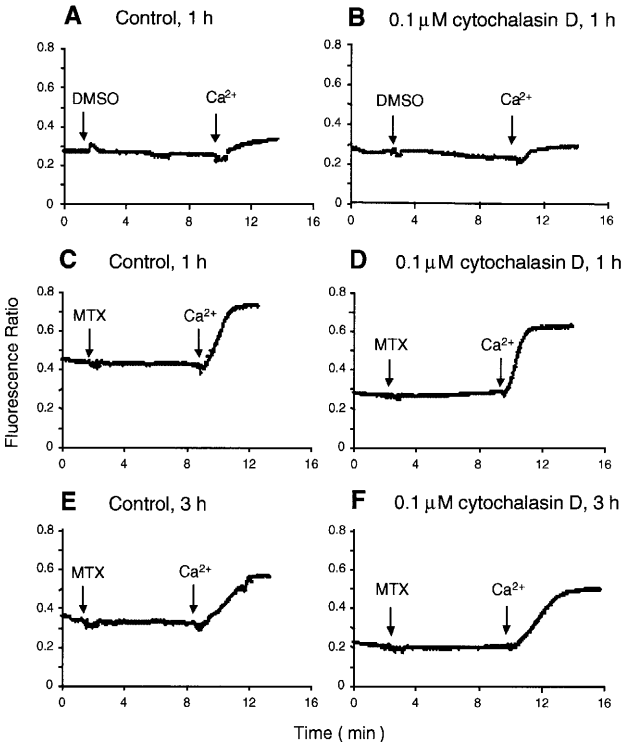

Figure 5 Effects of treatment with 0.1 μM cytochalasin D on maitotoxin-initiated Ca^{2+} inflow

Hepatocytes were isolated from rat liver, plated, loaded with fura 2/AM and treated with either DMSO (as control) (**A**, **C** and **E**) or 0.1 μM cytochalasin D (**B**, **D** and **F**) for either 1 h (**A**–**D**) or 3 h (**E** and **F**) as described in the Materials and methods section. $CaCl_2$ (1.5 mM) was added back approx. 7 min after the addition of either DMSO (**A** and **B**) or 150 pM maitotoxin (MTX) (**C**–**F**), and the rate of the increase of the fluorescence ratio was calculated as described in the Materials and methods section. Each trace shown is an average of values from ten individual hepatocytes on a coverslip from one of three experiments which gave similar results.

Effects of cytochalasin D on Ca^{2+} inflow induced by maitotoxin and hypotonic shock

In rat hepatocytes and in the H4-IIE rat liver cell line, maitotoxin initiates the activation of a non-selective cation channel that admits Na^+, Ca^{2+} and Mn^{2+} through a mechanism which does

Table 2 Effects of cytochalasin D on maitotoxin- and hypotonic shock-induced Ca^{2+} inflow in hepatocytes

Hepatocytes were isolated, plated, loaded with fura 2/AM and treated with 0.1 μM cytochalasin D or DMSO (as control) for either 1 or 3 h. Ca^{2+} inflow initiated by maitotoxin or hypotonic shock was measured as described in the Materials and methods section. Data are presented as the means $\pm$ S.E.M. for three independent experiments involving separate cell preparations in each of which the values from ten individual hepatocytes on a coverslip were averaged and taken as a single determination. The degree of significance for a comparison of the values between the cytochalasin D groups and the control groups was determined using the Mann–Whitney U test ($^*P \leqslant 0.05$).

Agonist	Treatment	Rate of maitotoxin- or hypotonic shock-induced Ca^{2+} inflow (fluorescence ratio units/min)
Maitotoxin (150 pM)	DMSO (1 h)	0.166 ± 0.020
	Cytochalasin D (1 h)	0.231 ± 0.047
	DMSO (3 h)	0.111 ± 0.004
	Cytochalasin D (3 h)	$0.162 \pm 0.021^*$
Hypotonic shock + 1.5 mM Ca^{2+}	DMSO (1 h)	0.021 ± 0.005
	Cytochalasin D (1 h)	$0.061 \pm 0.024^*$
	DMSO (3 h)	0.010 ± 0.005
	Cytochalasin D (3 h)	0.013 ± 0.002

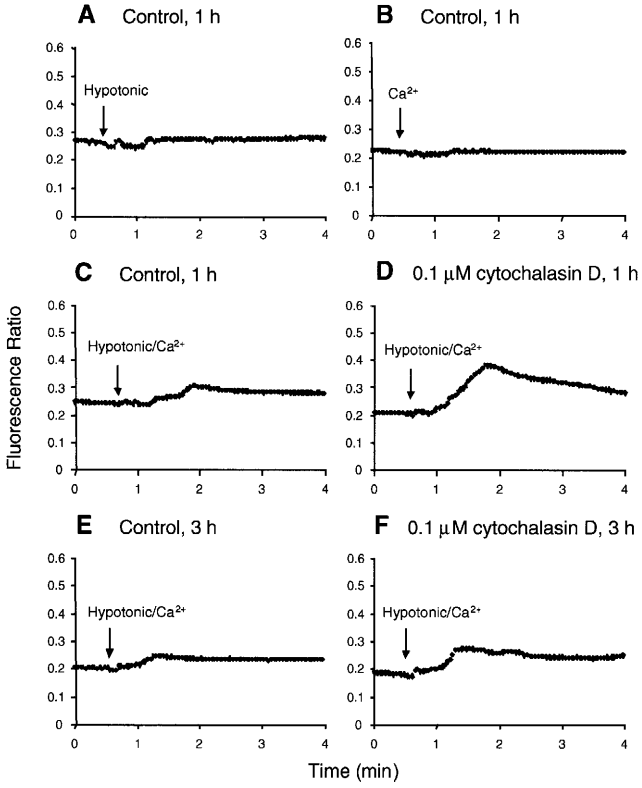

Figure 6 Effects of treatment with 0.1 μM cytochalasin D on hypotonic shock-initiated Ca^{2+} inflow

Hepatocytes were isolated from rat liver, plated, loaded with fura 2/AM and treated with either DMSO (as control) (**A**, **B**, **C** and **E**) or 0.1 μM cytochalasin D (**D** and **F**) for either 1 h (**A**–**D**) or 3 h (**E** and **F**), incubated in Ca^{2+}-free Hanks solution and the fluorescence ratio was measured as a function of time, as described in the Materials and methods section. Cells were exposed to hypotonic shock in the absence of added extracellular Ca^{2+} by diluting the extracellular medium with an equal volume of water (Hypotonic) or exposed to hypotonic shock in the presence of 1.5 mM extracellular Ca^{2+} by diluting the extracellular medium with an equal volume of 3 mM $CaCl_2$ in water (Hypotonic/Ca^{2+}). Each trace shown is an average of values from ten individual hepatocytes on a coverslip from one of three experiments which gave similar results.

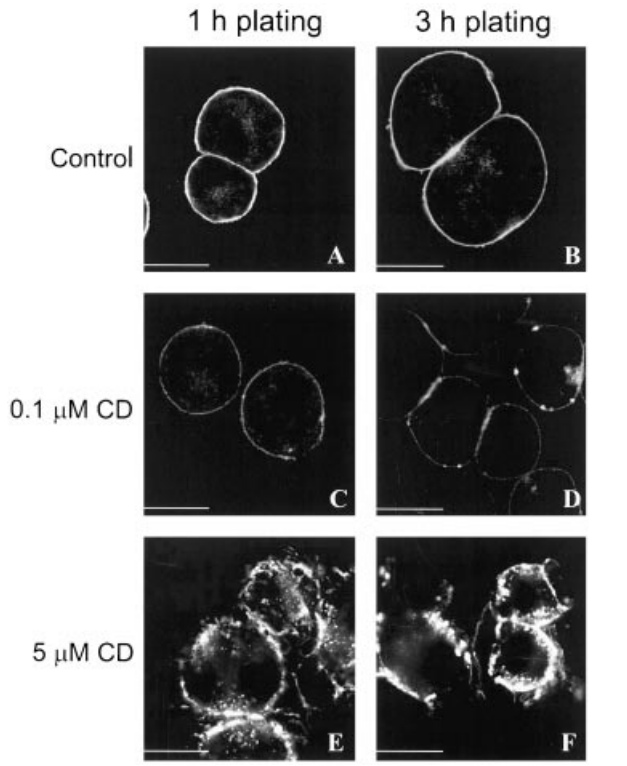

Figure 7 Effects of treatment with cytochalasin D on the distribution of F-actin in paraformaldehyde-fixed cells

Hepatocytes were isolated from rat liver, plated and treated with DMSO (control), 0.1 μM cytochalasin D (CD) or 5 μM cytochalasin D for either 1 h (1 h plating) or 3 h (3 h plating) as described in the Materials and methods section. Cells were fixed with paraformaldehyde and the distribution of F-actin was determined using Texas Red-X phalloidin. Data shown are representative of more than 60 cells examined in three independent experiments. Scale bars = 20 μm.

not appear to involve a decrease in Ca^{2+} in the ER [8,10]. Treatment of hepatocytes with cytochalasin D for 1 h caused no inhibition of maitotoxin-induced Ca^{2+} inflow, whereas treatment for 3 h caused a small enhancement [Figure 5F (cf. Figure 5E) and Table 2]. In the absence of maitotoxin, cytochalasin D treatment caused no activation of Ca^{2+} inflow (Figure 5B, cf. Figure 5A).

Hypotonic shock has been shown to induce the activation of Ca^{2+}-permeable non-selective cation channels in liver cells. These channels have been designated stretch-activated Ca^{2+} channels [31]. In the presence of 1.5 mM extracellular Ca^{2+}, hypotonic shock (induced by decreasing the osmolarity of the extracellular medium by 50%) caused a significant increase in the fura 2 fluorescence ratio (Figure 6C). The addition of 1.5 mM extracellular Ca^{2+} in the absence of hypotonic shock (Figure 6B) or the application of hypertonic shock in the absence of extracellular Ca^{2+} (Figure 6A) caused undetectable changes in the fluorescence ratio. These results confirm that hypotonic-shock treatment induces Ca^{2+} inflow in hepatocytes [31]. For cells cultured for 3 h, the rate of Ca^{2+} inflow induced by hypotonic shock was less than that observed for cells cultured for 1 h [Figure 6E (cf. Figure 6C) and Table 2]. Treatment with 0.1 μM cytochalasin D for 1 h significantly enhanced Ca^{2+} inflow induced by hypotonic shock [Figure 6D (cf. Figure 6C) and Table 2]. A smaller enhancement was induced by cytochalasin D treatment for 3 h [Figure 6F (cf. Figure 6E) and Table 2].

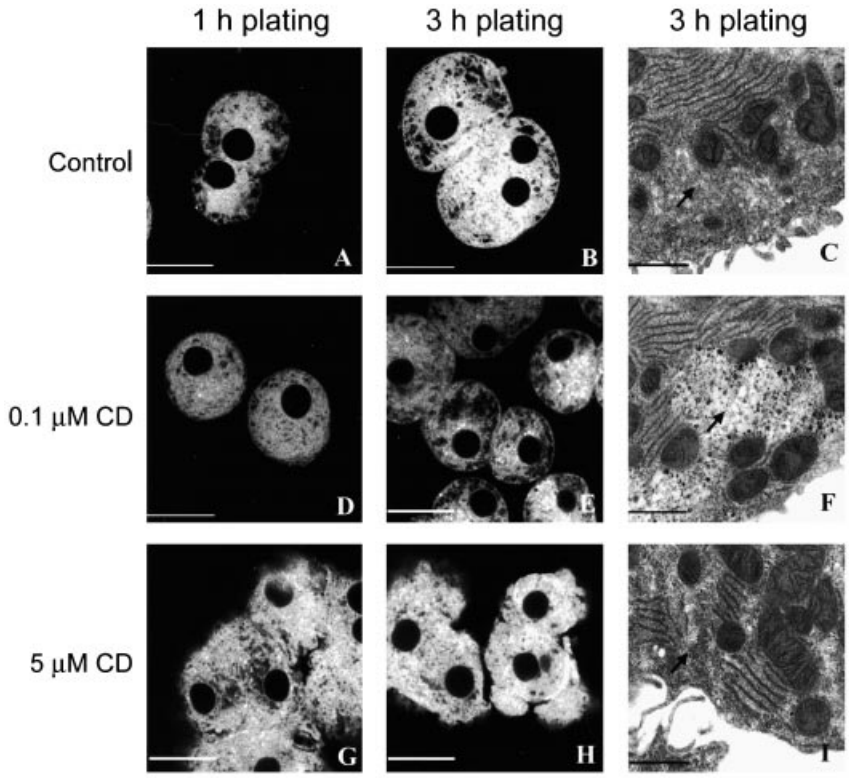

Figure 8 Effects of treatment with cytochalasin D on the distribution of the ER in fixed hepatocytes determined by fluorescence microscopy and electron microscopy

Hepatocytes were isolated from rat liver, plated and treated with DMSO (control), 0.1 μM cytochalasin D (CD) or 5 μM cytochalasin D for either 1 h (1 h plating) or 3 h (3 h plating) as described in the Materials and methods section. In (**A**, **B**, **D**, **E**, **G** and **H**) cells were fixed with paraformaldehyde and the distribution of the ER was determined using $DiOC_6(3)$. Data shown are representative of more than 60 cells examined in three independent experiments. Scale bars = 20 μm. In (**C**, **F** and **I**) cells were fixed with OsO_4 and glutaraldehyde, and processed and examined by electron microscopy as described in the Materials and methods section. Data shown are representative of more than 20 cells examined in two independent experiments. The regions of smooth ER are indicated by arrows. Scale bars = 1 μM.

Effects of colchicine on thapsigargin- and vasopressin-initiated Ca^{2+} inflow

To investigate whether the microtubule network is involved in the mechanism of activation of SOCs, hepatocytes were treated with 10 μM colchicine for 3 h. It has previously been shown that this causes complete disruption of the microtubule network in rat hepatocytes with little effect on F-actin [32]. Colchicine caused no inhibition of either the rate of thapsigargin-induced Ca^{2+} inflow ($103 \pm 1\%$ of control, $n = 3$) or the amount of Ca^{2+} released from intracellular stores by thapsigargin ($107 \pm 16\%$ of control, $n = 3$). In contrast, colchicine inhibited vasopressin-induced Ca^{2+} inflow and vasopressin-induced release of Ca^{2+} from the ER. In the presence of colchicine, the rate of vasopressin-induced Ca^{2+} inflow was $36 \pm 8\%$ of control ($n = 2$) and the amount of Ca^{2+} released from the ER by vasopressin was $63 \pm 1\%$ of control ($n = 2$).

Effects of cytochalasin D on the organization of F-actin and the ER

In order to gain some insight into the unexpected difference between the effects of low (0.1 μM) and high (5–10 μM) concentrations of cytochalasin D on thapsigargin-induced Ca^{2+} inflow, experiments were conducted to determine the effect of cytochalasin D on the distribution of F-actin (assessed using Texas Red-X phalloidin [19]) and on the organization of the ER (assessed using $DiOC_6(3)$ [19]). As shown previously [19,33], hepatocytes grown on a glass coverslip for 1 h exhibited a thick cortical band of F-actin (Figure 7A). A similar band, with slightly reduced intensity, was observed after 3 h of culture (Figure 7B). At 3 h, some cells had flattened out (indicated by an increased diameter) and had regained some polarity of the F-actin distribution (indicated by the concentration of actin at the site of the bile canaliculus) (Figure 7B). Treatment with 0.1 μM cytochalasin D for 1 h caused a significant reduction in the thickness of the cortical F-actin band (Figure 7C, cf. Figure 7A). The thickness of this band was further reduced in cells treated with cytochalasin D for 3 h, which also blocked cell spreading (Figure 7D, cf. Figure 7B). As reported previously [34], in cells treated with 5 μM cytochalasin D for 1 or 3 h, the total amount of F-actin was increased (compare with the decrease caused by 0.1 μM cytochalasin D). The cortical F-actin band appeared discontinuous and wider with some aggregates or patches at the cell cortex and in the cell interior (Figures 7E and 7F).

The organization of the ER in paraformaldehyde-fixed hepatocytes (fixed cells) and in hepatocytes in culture (live cells) was monitored using $DiOC_6(3)$, a lipophilic short-chain carbocyanine fluorochrome [35]. In fixed cells, $DiOC_6(3)$ principally binds to the membrane of the ER [36,37], whereas in live cells this dye diffuses into membranes of the mitochondria as well as those of the ER [38,39]. In cells treated with 0.1 μM cytochalasin D for

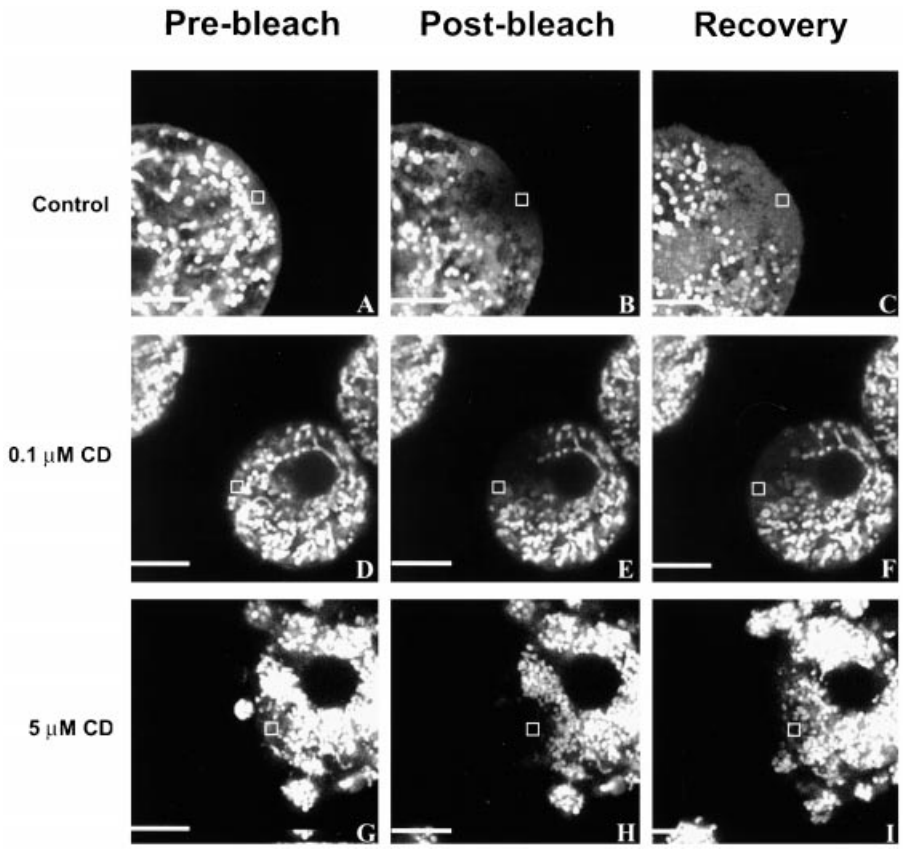

Figure 9 Effects of treatment with cytochalasin D on the recovery of $DiOC_6(3)$ fluorescence after photobleaching in live hepatocytes

Hepatocytes were isolated from rat liver, plated and treated with DMSO (control), 0.1 μM cytochalasin D (CD) or 5 μM cytochalasin D for 2–3 h. Cells were stained with $DiOC_6(3)$, and photobleaching experiments were performed as described in the Materials and methods section. Before photobleaching (Pre-bleach), images of cells were taken under original settings as described in the Materials and methods section. For photobleaching, by using a larger zoom setting, a small area of the cell was exposed to higher laser energy (laser power, 30%; zoom, 20; and other settings were the same as the original settings) for 15 s. Images were taken under the original settings immediately (Post-bleach) and 5 min (Recovery) after photobleaching. The square boxes labelled on the images indicate the areas in which the recovery of ER-representing fluorescence after photobleaching was quantified. Data shown are representative of 5–10 cells examined in three independent experiments. Scale bars = 10 μm.

1 or 3 h before being fixed, the $DiOC_6(3)$ fluorescence appeared to be more homogeneous than in control cells (Figures 8D and 8E, cf. Figures 8A and 8B respectively). Strikingly, although 5 μM cytochalasin D treatment induced the formation of cell blebs and altered cell shape profoundly, it did not dramatically change the distribution of the ER (Figures 8G and 8H, cf. Figures 8A and 8B respectively). Examination by electron microscopy revealed that in hepatocytes treated with 0.1 μM cytochalasin D, but not 5 μM cytochalasin D, the smooth ER was less dense compared with control cells (Figures 8F and 8I, cf. Figure 8C). The sheets of rough ER appeared to be unaffected.

The effects of cytochalasin D on luminal continuity and movement of the ER were investigated using live hepatocytes by monitoring the recovery of $DiOC_6(3)$ fluorescence after photobleaching. Figure 9(A) shows the distribution of $DiOC_6(3)$ in a hepatocyte cultured for 3 h in the presence of vehicle (control cells). The granule-like structures which exhibit the highest fluorescence intensity are areas enriched in mitochondria (cf. [39]). In order to monitor $DiOC_6(3)$, which is principally located in the ER, a region of the cell near the cortex (enriched in ER with few mitochondria) was chosen for analysis. When this region of the cell was photobleached, the fluorescence intensity recovered substantially after 5 min (Figure 9C, cf. Figures 9B and 9A). For cells treated with 0.1 μM cytochalasin D for 2–3 h, the recovery after photobleaching was slower (Figure 9F, cf. Figures 9E and 9D). For cells treated with 5 μM cytochalasin D, the fluorescence signal recovered substantially within 5 min after the photobleaching (Figure 9I, cf. Figures 9G and 9H).

For each of the three situations examined (control cells, and cells treated for 2–3 h with 0.1 or 5 μM cytochalasin D), the recovery from photobleaching was quantified as follows. A small region of the cytoplasm was defined within the photobleached area (indicated by the white squares in Figure 9). The value of the fluorescence intensity immediately after photobleaching was subtracted from the value of fluorescence intensity at 5 min after photobleaching to give the recovery in fluorescence intensity. The values obtained were 22 ± 5, 10 ± 2 and 20 ± 4 pixels/square (n = 5–10 cells from three separate cell preparations) for control cells, cells treated with 0.1 μM cytochalasin D, and cells treated with 5 μM cytochalasin D respectively. The value obtained for cells treated with 0.1 μM cytochalasin D is significantly different ($P < 0.05$, one way ANOVA, Dunnett test) from that for control cells. A similar analysis was performed for a region of the cell enriched in mitochondria. In this case, treatment with 0.1 or 5 μM cytochalasin D had no significant effect on the recovery of fluorescence after photobleaching (results not shown).

DISCUSSION

The results show that cytochalasin D has a biphasic effect on thapsigargin- and vasopressin-induced Ca^{2+} inflow, with maximal inhibition observed within a relatively narrow concentration range, around 0.1 μM cytochalasin D. Cytochalasin D-induced inhibition of Ca^{2+} inflow was time-dependent, with maximal effect at 3 h. The inhibition of Ca^{2+} inflow was associated with a significant reduction in the amount of cortical F-actin. Cytochalasins are known to have several actions on actin dynamics [40]: although they can cap the barbed end of the F-actin thereby causing F-actin de-polymerization, there is also evidence that they can promote the formation of actin dimers or enhance the initial rate of actin polymerization. It is also reported that they can sever actin filaments [41]. Effects of cytochalasin D on these different steps are exerted at different cytochalasin D concentrations [40]. Thus the overall effect of cytochalasin D on F-actin, and the concentration at which this is observed, is likely to depend on the sum of the effects of cytochalasin D on the various individual steps. A similar biphasic effect of cytochalasin D on the amount of F-actin in toad bladder epithelial cells and Madin–Darby canine kidney epithelial cells has been reported [40,42]. The present results demonstrate the importance of determining, for any given cell type, a concentration–response curve and a time course for the effects of cytochalasin D on the disruption of F-actin and on the activation of SOCs.

The following observations indicate that maintenance of the integrity of the F-actin cytoskeleton is necessary for the activation of SOCs but that it is unlikely to play such a role for stretch-activated and some intracellular messenger-activated non-selective cation channels. (i) Cytochalasin D (0.1 μM for 3 h) inhibited thapsigargin- and vasopressin-induced Ca^{2+} inflow by approx. 75 %. (ii) These effects of cytochalasin D were associated with substantial disruption of cortical F-actin. (iii) Thapsigargin- and vasopressin-induced Mn^{2+} inflow (likely to be predominantly mediated by intracellular messenger-activated non-selective cation channels) was less susceptible to inhibition by cytochalasin D than Ca^{2+} inflow (likely to be predominantly mediated by SOCs) induced by these agents. (iv) Cytochalasin D enhanced, rather then inhibited, maitotoxin- and hypotonic shock-induced Ca^{2+} inflow. The observation that colchicine treatment had no inhibitory effect on thapsigargin-induced Ca^{2+} inflow suggests that microtubules are not required for the activation of SOCs in hepatocytes (cf. [25,28]).

The results also provide further evidence that thapsigargin and vasopressin each induce the activation of SOCs and at least one type of intracellular messenger-activated non-selective cation channel. It has previously been shown that these channels can be distinguished on the basis of their different sensitivities to inhibition by Gd^{3+} [12] and permeability to Mn^{2+} [30]. The present study indicates that they can also be distinguished on the basis of different sensitivities to cytochalasin D.

Although cytochalasin D treatment did not substantially alter the total amount of Ca^{2+} released from intracellular stores by thapsigargin (assessed by the area under the Ca^{2+} release curve), it did modify the kinetics of thapsigargin-induced Ca^{2+} release. The profile of the transient increases in $[Ca^{2+}]_{cyt}$ is influenced by several Ca^{2+} transporters and channels in the ER and plasma membrane [13]. In particular, the action of the plasma membrane $(Ca^{2+}+Mg^{2+})$ATPase, the activation of which depends on $[Ca^{2+}]_{cyt}$, is expected to contribute to the profile of thapsigargin-induced Ca^{2+} release and Ca^{2+} inflow [43]. In the present work, rates of thapsigargin- and vasopressin-induced Ca^{2+} inflow, determined following the addition of extracellular Ca^{2+}, were assessed as the initial rate of increase in $[Ca^{2+}]_{cyt}$. Under these conditions, the activity of the plasma membrane $(Ca^{2+}+Mg^{2+})$ATPase is expected to be minimal [43]. If cytochalasin D treatment did modify the plasma membrane $(Ca^{2+}+Mg^{2+})$ATPase, the resultant effect on the initial rate of Ca^{2+} inflow would be small. Furthermore, we are not aware of any evidence that the activity of the plasma membrane $(Ca^{2+}+Mg^{2+})$ATPase is influenced by cytochalasin D. Therefore the altered kinetics of thapsigargin-induced Ca^{2+} release in the presence of cytochalasin D suggest that cytochalasin D treatment may have some effect on Ca^{2+} outflow from the ER and/or on the ER $(Ca^{2+}+Mg^{2+})$ATPase or the distribution of Ca^{2+} in the cytoplasmic space. The observation that cytochalasin D treatment altered the kinetics of thapsigargin- but not vasopressin-induced Ca^{2+} release may reflect the involvement of different ER Ca^{2+} outflow channels in thapsigargin- and vasopressin-induced Ca^{2+} outflow {Ins(1,4,5)P_3 receptor Ca^{2+} channels in the case of vasopressin and another type of Ca^{2+} outflow channel in the case of thapsigargin [44]} as well as different densities and different distributions of these two types of channels in the ER.

The inhibition by cytochalasin D (0.1 μM for 3 h) of the activation of SOCs induced by thapsigargin and vasopressin was associated with a significant disruption of cortical F-actin and an inhibition of the re-distribution of $DiOC_6(3)$ after photobleaching. $DiOC_6(3)$ is a lipophilic short-chain carbocyanine fluorochrome which can penetrate the plasma membrane and bind to intracellular membranes in live cells [35]. It has been extensively used to stain the ER in both fixed and live cells [36–38,45]. The $DiOC_6(3)$ involved in the recovery of the bleached ER-associated fluorescence could come from three sources: (i) diffusion within continuous ER membrane, as proposed for long-chain dialkylcarbocyanines, such as 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ('DiI') [46]; (ii) the movement of neighbouring unbleached subregions of the ER into the bleached area through vesicular trafficking; and (iii) the diffusion of $DiOC_6(3)$, which partitions in both the aqueous and membrane phases, through the cytoplasmic space. Therefore the observation that 0.1 μM cytochalasin D significantly slowed down the recovery of photobleached fluorescence indicates that this treatment impairs the integrity (and hence the luminal communication) of the ER either by disruption of the membrane or by inhibition of continual ER reorganization which may involve local vesicular trafficking processes [47]. This conclusion is consistent with the observation that cytochalasin D treatment alters the kinetics of thapsigargin-induced Ca^{2+} release from intracellular stores (principally the ER).

Taken together, the results indicate that the inhibition by cytochalasin D of SOC activation is due to disruption of the cortical F-actin cytoskeleton which, in turn, disrupts the luminal continuity and/or movement of the ER. The idea that an appropriate intracellular location of the ER and/or ER luminal continuity are necessary prerequisites for the activation of SOCs [18,19] is consistent with the present results. The results obtained for the effects of cytochalasin D on vasopressin-induced Mn^{2+} inflow and maitotoxin- and hypotonic-shock-induced Ca^{2+} inflow indicate that a specific location of the ER and/or ER luminal communication are unlikely to be required for the activation of intracellular messenger-activated and stretch-activated non-selective cation channels.

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