Evidence for the involvement of a small subregion of the endoplasmic reticulum in the inositol trisphosphate receptor-induced activation of Ca^{2+} inflow in rat hepatocytes

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The roles of a subregion of the endoplasmic reticulum (ER) and the cortical actin cytoskeleton in the mechanisms by which $\text{Ins}(1,4,5)\text{P}_3$ induces the activation of store-operated Ca^{2+} channels (SOCs) in isolated rat hepatocytes were investigated. Adenophostin A, a potent agonist at $\text{Ins}(1,4,5)\text{P}_3$ receptors, induced ER Ca^{2+} release and the activation of Ca^{2+} inflow. The concentration of adenophostin A that gave half-maximal stimulation of Ca^{2+} inflow (10 nM) was substantially lower than that (20 nM) which gave half-maximal ER Ca^{2+} release. A low concentration of adenophostin A (approx. 13 nM) caused near-maximal stimulation of Ca^{2+} inflow but only 20% of maximal ER Ca^{2+} release. Similar results were obtained using another $\text{Ins}(1,4,5)\text{P}_3$ -receptor agonist, 2-hydroxyethyl- α -D-glucopyranoside 2,3',4'-trisphosphate. Anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor monoclonal antibody 18A10 inhibited vasopressin-stimulated Ca^{2+} inflow but had no observable effect on vasopressin-induced ER Ca^{2+} release. Treatment with cytochalasin B at a concentration that partially disrupted the cortical actin cytoskeleton inhibited Ca^{2+} inflow and ER Ca^{2+} release induced by vasopressin by 73 and 45%, respectively. However, it did not substantially

affect Ca^{2+} inflow and ER Ca^{2+} release induced by thapsigargin or 13 nM adenophostin A, intracellular Ca^{2+} release induced by ionomycin or $\text{Ins}(1,4,5)\text{P}_3\text{P}^{(5)}\text{-1-(2-nitrophenyl)ethyl ester}$ ['caged' $\text{Ins}(1,4,5)\text{P}_3$] or basal Ca^{2+} inflow. 1-(5-Chloronaphthalene-1-sulphonyl)homopiperazine, HCl (ML-9), an inhibitor of myosin light-chain kinase, also inhibited vasopressin-induced Ca^{2+} inflow and ER Ca^{2+} release by 53 and 44%, respectively, but had little effect on thapsigargin-induced Ca^{2+} inflow and ER Ca^{2+} release. Neither cytochalasin B nor ML-9 inhibited vasopressin-induced $\text{Ins}(1,4,5)\text{P}_3$ formation. It is concluded that the activation of SOCs in rat hepatocytes induced by $\text{Ins}(1,4,5)\text{P}_3$ requires the participation of a small region of the ER, which is distinguished from other regions of the ER by a different apparent affinity for $\text{Ins}(1,4,5)\text{P}_3$ analogues and is associated with the plasma membrane through the actin skeleton. This conclusion is discussed briefly in relation to current hypotheses for the activation of SOCs.

Key words: actin cytoskeleton, adenophostin A, hepatocyte, inositol trisphosphate, store-operated Ca^{2+} channel.

INTRODUCTION

Store-operated Ca^{2+} channels (SOCs) in animal cells are defined as plasma-membrane Ca^{2+} channels, the activation of which is initiated by a decrease in the concentration of Ca^{2+} in the lumen of the endoplasmic reticulum (ER) [1]. SOCs are present in most non-excitabile cells, including hepatocytes, and in some excitable cells [1–4]. The physiological pathway for the activation of SOCs, which is initiated by the binding of an agonist to its receptor, involves the generation of $\text{Ins}(1,4,5)\text{P}_3$, and a subsequent $\text{Ins}(1,4,5)\text{P}_3$ -induced decrease in the concentration of Ca^{2+} in the lumen of the ER ($[\text{Ca}^{2+}]_{\text{ER}}$) which, by an unknown mechanism, activates SOCs in the plasma membrane [1–4]. The major physiological role of SOCs is thought to be the replenishment of Ca^{2+} that flows out of the ER in response to the action of $\text{Ins}(1,4,5)\text{P}_3$ and is subsequently transported to the extracellular space by plasma-membrane ($\text{Ca}^{2+} + \text{Mg}^{2+}$)ATPases

[1–4]. Evidence for the presence of SOCs in hepatocytes has been obtained using inhibitors of the ER ($\text{Ca}^{2+} + \text{Mg}^{2+}$)ATPase, such as thapsigargin, to decrease $[\text{Ca}^{2+}]_{\text{ER}}$ [1], in conjunction with a 'Ca²⁺ add-back' protocol or the use of Mn^{2+} to monitor divalent cation inflow, or in conjunction with patch-clamp electrophysiological techniques (reviewed in [5]). Despite considerable study, the properties, subtypes, structures and mechanisms of activation of SOCs present in hepatocytes and in other animal cells are not well understood [1–4]. It has been proposed that SOCs are activated by interaction between the $\text{Ins}(1,4,5)\text{P}_3$ -receptor protein in the ER and the SOC protein in the plasma membrane (conformational coupling) or by an intracellular messenger ('calcium-influx factor') released by the ER (reviewed in [1–4]).

The results of previous work conducted in this laboratory, which employed guanosine 5'-[β -thio]diphosphate or an anti- $\text{G}_{q/11}$ antibody [inhibitors of the actions of $\text{G}_{q/11}$ and hence the

Abbreviations used: ER, endoplasmic reticulum; SOC, store-operated Ca^{2+} channel; $[\text{Ca}^{2+}]_{\text{c}}$, cytoplasmic free Ca^{2+} concentration; $[\text{Ca}^{2+}]_{\text{ER}}$, concentration of free Ca^{2+} in the ER lumen; $\text{Glc}(2,3',4')\text{P}_3$, 2-hydroxyethyl- α -D-glucopyranoside 2,3',4'-trisphosphate; 'caged' $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3\text{P}^{(5)}\text{-1-(2-nitrophenyl)ethyl ester}$; ML-9, 1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl; $\text{DiOC}_6(3)$, 3,3'-dihexyloxycarbocyanine iodide; $\text{S}_{0.5}$, agent concentration yielding half-maximal effect.

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activation of phospholipase C- β and formation of $\text{Ins}(1,4,5)\text{P}_3$, suggested that the $\text{Ins}(1,4,5)\text{P}_3$ receptors involved in activation of SOC in hepatocytes differ in some way from those responsible for Ca^{2+} release from the bulk of the ER [6,7]. Rat hepatocytes normally express type-1 [about 20 % of total $\text{Ins}(1,4,5)\text{P}_3$ receptors] and type-2 (about 80 %) $\text{Ins}(1,4,5)\text{P}_3$ receptors [8]. A substantial number of $\text{Ins}(1,4,5)\text{P}_3$ receptors are located close to the hepatocyte plasma membrane [9] and are maintained in this location by the actin network [10,11]. Studies of the roles of G-proteins in the activation of SOC in hepatocytes have shown that the α subunit of the trimeric G-protein, G_{12} , and a monomeric G-protein, possibly one of the Arf proteins, are required for hepatocyte SOC activation [5–7]. It was hypothesized that the G-proteins function permissively to maintain communication within the lumen of the ER and/or to maintain the location of a region of the ER near the plasma membrane [5]. Thomas and colleagues have provided evidence that the maintenance of communication between different regions of the lumen of the hepatocyte ER (assessed indirectly by measuring the extent to which Mn^{2+} quenched fura-2 located in the ER of permeabilized hepatocytes) requires the actions of both a G-protein and the actin cytoskeleton [12].

Taken together, these observations suggest that the activation of SOC in hepatocytes initiated by an increase in $\text{Ins}(1,4,5)\text{P}_3$ involves a distinct subregion of the ER and maintenance of the integrity of the actin cytoskeleton. The aim of the present experiments was to test these ideas. The involvement of a distinct subregion of the ER was tested using adenophostin A, a metabolically stable analogue of $\text{Ins}(1,4,5)\text{P}_3$ and a potent activator of $\text{Ins}(1,4,5)\text{P}_3$ -receptor Ca^{2+} channels [13], and an anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor antibody, which has been shown to inhibit the ability of $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from isolated microsomes [14]. Low concentrations of adenophostin A have been shown to activate SOC in *Xenopus laevis* oocytes with little detectable release of Ca^{2+} from the ER [15,16]. The role of the actin cytoskeleton was investigated using cytochalasin B, which inhibits actin polymerization [17], and 1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl (ML-9) [18,19], which, through its ability to inhibit myosin light-chain-kinase activity (a regulator of actin polymerization and depolymerization), can alter the structure of the actin cytoskeleton [19]. The results indicate that, in rat hepatocytes, the activation of SOC initiated by $\text{Ins}(1,4,5)\text{P}_3$ involves a subregion of the ER [distinguished from the bulk of the ER by a different apparent affinity for $\text{Ins}(1,4,5)\text{P}_3$ analogues], and, at least for a component of $\text{Ins}(1,4,5)\text{P}_3$ -activated Ca^{2+} inflow, maintenance of the normal arrangement of the actin cytoskeleton, which is located principally at the periphery of the cell.

EXPERIMENTAL

Materials

Anti-mouse type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor monoclonal antibody 18A10 (rat IgG2a) and anti-mouse type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor monoclonal antibody 4C11 (rat IgG2b) [14,20] were kindly provided by Professor Katsuhiko Mikoshiba (Institute of Medical Science, University of Tokyo, Tokyo, Japan), and 2-hydroxyethyl- α -D-glucopyranoside 2,3',4'-triphosphate [Glc-(2,3',4') P_3] was kindly provided by Dr. Roy Gigg, Mill Hill, London, U.K. Each batch of anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor antibody employed was shown to inhibit $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from brain microsomes (results not shown). Texas Red-X phalloidin and 3,3'-dihexyloxycarbocyanine iodide [$\text{DiOC}_6(3)$] were from Molecular Probes (Eugene, OR, U.S.A.), cytochalasin B was from Sigma-Aldrich (Castle Hill, NSW,

Australia) and ML-9 was from Calbiochem (Alexandria, NSW, Australia). Adenophostin A was synthesized as described by van Straten et al. [21]. All other chemicals were obtained from the sources described previously [5].

Isolation of hepatocytes and measurement of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

The isolation of hepatocytes from hooded Wistar rats, attachment of hepatocytes to collagen-coated coverslips, the micro-injection of fura-2, fluo-3, $\text{Ins}(1,4,5)\text{P}_3\text{P}^{(5)}\text{-1-(2-nitrophenyl)-ethyl ester}$ ['caged' $\text{Ins}(1,4,5)\text{P}_3$], adenophostin A and Glc(2,3',4') P_3 , measurement of the fluorescence of single hepatocytes loaded with fura-2 (using a ratiometric technique) or fluo-3, and the photolysis of 'caged' $\text{Ins}(1,4,5)\text{P}_3$ (introduced together with fluo-3 to the cytoplasmic space by micro-injection) were carried out as described previously [5]. Values of fluorescence ratio for single cells loaded with fura-2 were converted to values of $[\text{Ca}^{2+}]_i$ using an *in situ* calibration method [22] and a value of 224 nM for the K_d for the Ca^{2+} -fura-2 complex [23]. The dilution factor for the micro-injection of agents to hepatocytes was estimated to be 1:75 [24] and was used to estimate the intracellular concentrations of adenophostin A and Glc(2,3',4') P_3 micro-injected into hepatocytes.

For estimates of the amounts of Ca^{2+} released from intracellular stores and rates of Ca^{2+} inflow, hepatocytes attached to collagen-coated coverslips were micro-injected with fura-2 or fluo-3 and other agents under test as indicated, incubated for 10 min (to allow the cells to re-seal [6]), transferred to a medium containing no added Ca^{2+} , and the fluorescence measured. Rates of Ca^{2+} inflow in single hepatocytes were estimated using the Ca^{2+} add-back protocol [5] from measurement of the initial rate of the Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ (expressed as nM Ca^{2+}_i per min) in cells previously incubated in the absence of added Ca^{2+} . The amount of Ca^{2+} released from intracellular stores (in cells incubated in the absence of added Ca^{2+}) following the addition of vasopressin, thapsigargin or ionomycin was estimated by measuring the difference between the peak (maximum) value of $[\text{Ca}^{2+}]_i$ (nM) induced by the agent and the value of $[\text{Ca}^{2+}]_i$ before addition of the agent. The amount of Ca^{2+} released from the ER by the photolysis of 'caged' $\text{Ins}(1,4,5)\text{P}_3$ was estimated by integrating the area under the peak of plots of $[\text{Ca}^{2+}]_i$ as a function of time.

Measurement of cellular $\text{Ins}(1,4,5)\text{P}_3$ concentrations

Ice-cold trichloroacetic acid (7.5 %, w/v) was added to hepatocytes in suspension and $\text{Ins}(1,4,5)\text{P}_3$ assayed by competitive binding using [^3H] $\text{Ins}(1,4,5)\text{P}_3$ (Amersham Australia, Castle Hill, NSW, Australia) [25] as described by the manufacturers.

Cytochalasin B treatment

Hepatocytes were attached to collagen-coated coverslips and incubated in Petri dishes in Dulbecco's modified Eagle's medium supplemented with 2 % (w/v) BSA, 44 mM NaHCO_3 and 1 mM pyruvate at 37 °C in 5 % CO_2 /95 % O_2 . Cytochalasin B (40 μM) or vehicle [0.2 % (v/v) DMSO] were added and the incubation continued for 1.8–2.3 h. Cytochalasin B and DMSO were also included in the incubation medium employed for the micro-injection of agents to the cytoplasmic space, and during the 10-min incubation period (in CO_2 -independent medium; Gibco) between micro-injection and the beginning of the measurement of fluorescence. The total time of exposure of cells to cytochalasin B was 2–2.5 h.

Localization of the actin cytoskeleton and ER

The location of actin filaments was determined using Texas Red-X phalloidin [26]. Hepatocytes were fixed in 3.5% (v/v) formaldehyde, permeabilized with 0.1% (v/v) Triton X-100, and stained with Texas Red-X phalloidin (Molecular Probes) according to the manufacturer's instructions. The location of the ER was determined using $\text{DiOC}_6(3)$ as described by Terasaki et al. [27]. Hepatocytes were fixed in 3.7% formaldehyde. Images of Texas Red-X phalloidin- and $\text{DiOC}_6(3)$ -stained cells were obtained by confocal fluorescence microscopy using a krypton-argon laser and excitation and emission wavelengths set at 568 and 605 nm, respectively (Texas Red-X phalloidin) and at 488 and 522 nm, respectively [$\text{DiOC}_6(3)$] as described previously [7].

Expression of results

Results shown in the Figures are representative of the number of traces indicated, whereas those in the Tables and text are the means $\pm$ S.E.M. of those obtained for the number of individual cells indicated. Degrees of significance were determined using Student's *t* test for unpaired samples.

RESULTS

Effects of adenophostin A

Adenophostin A, a membrane-impermeant and metabolically stable $\text{Ins}(1,4,5)\text{P}_3$ analogue and a potent activator of $\text{Ins}(1,4,5)\text{P}_3$ -receptor Ca^{2+} channels [13], was co-injected with fura-2 (i.e. 10 min before transferring the hepatocytes to a medium that contained no added Ca^{2+} and beginning the measurement of fluorescence). Cells treated with 13 nM adenophostin A showed a greater rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ than did untreated cells and a higher plateau of $[\text{Ca}^{2+}]_c$ (Figure 1B, compare with Figure 1A), indicating that adenophostin A activates Ca^{2+} inflow. The initial rate of Ca^{2+} inflow and the magnitude of the plateau of $[\text{Ca}^{2+}]_c$ reached after the addition of Ca^{2+} increased as the concentration of injected

adenophostin A was increased (Figures 1B and 1C, compare with Figure 1A). The subsequent addition of vasopressin to cells pre-treated with 13 nM adenophostin A induced a rapid transient increase in $[\text{Ca}^{2+}]_c$ and established a new plateau of $[\text{Ca}^{2+}]_c$. The magnitude of the peak vasopressin-induced increase in $[\text{Ca}^{2+}]_c$ (net maximal value of the vasopressin-induced increase in $[\text{Ca}^{2+}]_c$) was less than that in untreated cells (Figure 1B, compare with Figure 1A) and decreased with an increase in the concentration of adenophostin A (Figure 1C, compare with Figure 1B), indicating that low concentrations of adenophostin A cause the partial depletion of $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores.

The intracellular origin of the Ca^{2+} responsible for the peak vasopressin-induced increase in $[\text{Ca}^{2+}]_c$ was verified using EGTA to chelate Ca^{2+} . CaCl_2 (1.5 mM) was added to hepatocytes injected with 26 nM adenophostin A. When the value of $[\text{Ca}^{2+}]_c$ reached a plateau, vasopressin (40 nM) was added with, or without, co-addition of 2 mM EGTA. The magnitude of the peak vasopressin-induced increase in $[\text{Ca}^{2+}]_c$ was 222 ± 92 nM (4) in the absence of EGTA compared with 212 ± 103 nM (4) in the presence of EGTA. This indicates that extracellular Ca^{2+} does not contribute to the rapid increase in $[\text{Ca}^{2+}]_c$ induced by vasopressin. The addition of a second bolus of vasopressin to cells that had been pre-treated with adenophostin A, and subsequently incubated in the presence of Ca^{2+} and vasopressin, did not induce further release of Ca^{2+} or further Ca^{2+} inflow (results not shown). The addition of 10 μM thapsigargin to cells that had been pre-treated with adenophostin A, and subsequently received additions of Ca^{2+} and vasopressin, induced a small transient increase in $[\text{Ca}^{2+}]_c$ (< 100 nM; results not shown). On the basis of all these results, it was concluded that the difference between the magnitudes of the peak vasopressin-induced increase in $[\text{Ca}^{2+}]_c$ for (i) untreated cells and (ii) adenophostin A-injected cells is a reasonable estimate of the amount of Ca^{2+} released from intracellular stores by pre-treatment with adenophostin A.

Plots of the rate of adenophostin A-induced Ca^{2+} inflow and adenophostin A-induced release of Ca^{2+} from intracellular stores as a function of the estimated intracellular concentration of

Figure 1 Effects of adenophostin A on Ca^{2+} inflow and the vasopressin-induced increase in $[\text{Ca}^{2+}]_c$ in rat hepatocytes

Single hepatocytes were injected with fura-2 alone (A), or fura-2 plus 13 nM adenophostin A (estimated intracellular concentration) (B), or fura-2 plus 130 nM adenophostin A (C) 10 min before beginning the measurement of fluorescence. Additions of Ca^{2+} (1.5 mM) and vasopressin (40 nM) were made as indicated by the horizontal bars. Each trace is representative of those obtained for 8–13 individual cells from 5–10 separate hepatocyte preparations.

Figure 2 Comparison of concentration–response curves for the effects of adenophostin A on the release of Ca^{2+} from intracellular stores and on Ca^{2+} inflow

Adenophostin A was co-injected to single hepatocytes with fura-2 10 min before beginning the measurement of fluorescence, and Ca^{2+} and vasopressin were added to the incubation medium as described in Figure 1. Rates of adenophostin A-stimulated Ca^{2+} inflow (●) and amounts of adenophostin A-induced Ca^{2+} release (■) were estimated as described in the text. Each data point is the mean $\pm$ S.E.M. of the values obtained from 6–13 individual hepatocytes from 3–10 separate hepatocyte preparations, except for one data point (hepatocytes from a single preparation).

adenophostin A are shown in Figure 2. The concentration of adenophostin A that gave half-maximal release of Ca^{2+} from intracellular stores (20 nM) was approximately twice that which gave half-maximal stimulation of Ca^{2+} inflow (10 nM). Furthermore, 13 nM adenophostin A gave near-maximal Ca^{2+} inflow but caused the release of only about 20 % of the total amount of $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} . The disparity between the abilities of 13 nM adenophostin A to stimulate Ca^{2+} inflow on the one

hand and release Ca^{2+} from intracellular stores on the other was not affected by increasing the time elapsed between the micro-injection of adenophostin A and beginning the measurement of Ca^{2+} inflow from 10 to 20 min. Thus for cells injected with 13 nM adenophostin A and pre-incubated for 10 or 20 min before the measurement of Ca^{2+} inflow, the magnitude of the net vasopressin-induced increase in $[\text{Ca}^{2+}]_i$ was 709 ± 108 (4) and 673 ± 27 nM (3), respectively.

Results similar to those for adenophostin A were obtained using $\text{Glc}(2,3',4')\text{P}_3$, a slowly metabolizable $\text{Ins}(1,4,5)\text{P}_3$ -receptor agonist with an affinity for the $\text{Ins}(1,4,5)\text{P}_3$ receptor [28] that is much lower than that of adenophostin A. For hepatocytes loaded by micro-injection with $\text{Glc}(2,3',4')\text{P}_3$ at an estimated intracellular concentration of $0.67 \mu\text{M}$, the amount of Ca^{2+} released from intracellular stores by $\text{Glc}(2,3',4')\text{P}_3$ was 82 ± 96 nM (about 10 % of maximal) and the initial rate of $\text{Glc}(2,3',4')\text{P}_3$ -induced Ca^{2+} inflow was 353 ± 37 nM/min (9) (about 60 % of maximal).

Actions of anti- $\text{Ins}(1,4,5)\text{P}_3$ -receptor antibody 18A10

The anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor monoclonal antibody, 18A10 [20], which has been shown previously to inhibit the ability of $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from isolated brain microsomes [14], inhibited vasopressin-stimulated Ca^{2+} inflow to rat hepatocytes when administered to the cytoplasmic space by micro-injection (Figure 3B, compare with control cells, Figure 3A). There was little effect on vasopressin-induced release of Ca^{2+} from intracellular stores. No inhibition of vasopressin-stimulated Ca^{2+} inflow was observed when the anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor monoclonal antibody 4C11 [14,20], which binds to the type-1 $\text{Ins}(1,4,5)\text{P}_3$ receptor but does not inhibit $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release [20], was employed (Figure 3C). The initial rates of the vasopressin-induced increase in fluorescence ratio were 1.02 ± 0.22 (8), 0.43 ± 0.14 (10) and 0.86 ± 0.16 (5) fluorescence ratio units/min for untreated cells (no antibody injected), cells loaded with antibody 18A10 and cells loaded with antibody 4C11, respectively ($P < 0.02$ for

Figure 3 Inhibition of vasopressin-stimulated Ca^{2+} inflow to rat hepatocytes by anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor antibody 18A10

Single hepatocytes were micro-injected with fura-2 together with solvent (PBS, pH 7.4; i.e. fura-2 alone, no antibody; **A**), 'inhibitory' anti- $\text{Ins}(1,4,5)\text{P}_3$ -receptor type-1 antibody 18A10 (**B**) or 'non-inhibitory' anti- $\text{Ins}(1,4,5)\text{P}_3$ -receptor type-1 antibody 4C11 (**C**). The terms 'inhibitory' and 'non-inhibitory' refer to the ability of these antibodies to inhibit the release of Ca^{2+} from isolated brain microsomes [14]. The intracellular concentrations of the injected antibodies were estimated to be 25–30 $\mu\text{g}/\text{ml}$. Additions of Ca^{2+} (1.5 mM) and vasopressin (40 nM) were made as indicated by the horizontal bars. Each trace is representative of those obtained for 5–10 individual cells from 1–3 separate hepatocyte preparations.

Figure 4 Effects of cytochalasin B on the arrangement of the actin cytoskeleton and the ER in rat hepatocytes

Hepatocytes were incubated for 2 h in the presence of either 0.2% (v/v) DMSO (**A, C**) or 40 μM cytochalasin B (**B, D**) before fixation and staining of actin with Texas Red-X phalloidin (**A, B**) or of ER with $\text{DiOC}_6(3)$ (**C, D**). The images, representative of cells from a single preparation, were obtained using a Bio-Rad confocal microscope with a 40 $\times$ objective lens. The bars represent 10 μm .

18A10-treated cells compared with 4C11-treated cells). The corresponding values for the peak vasopressin-induced increase in $[\text{Ca}^{2+}]_i$ were 0.76 ± 0.10 (8), 0.69 ± 0.08 (10) and 0.57 ± 0.11 (5) fluorescence ratio units, respectively. Inhibition of vasopressin-induced Ca^{2+} inflow was only observed with one batch of 18A10 antibody (prepared in 1993). Similar experiments conducted with two later batches showed no inhibition of vasopressin-stimulated Ca^{2+} inflow. The failure to observe inhibition of vasopressin-stimulated Ca^{2+} inflow with some batches of 18A10 antibody is likely to be due to an insufficiently high antibody titre or a failure of the antibody to reach type-1 $\text{Ins}(1,4,5)\text{P}_3$ receptors in hepatocytes. Incomplete inhibition of protein function by intracellular antibodies has been observed previously following the injection of anti-G-protein antibodies to hepatocytes [7,29] and fibroblasts [30].

Effects of agents that disrupt the actin cytoskeleton

Cytochalasin B, which inhibits actin polymerization [17], was employed to disrupt the actin cytoskeleton. In order to determine conditions under which hepatocytes could be treated with cytochalasin B without causing damage to the ion permeability of the plasma membrane, experiments were conducted using different concentrations and times of exposure to cytochalasin B. The pre-treatment of cells for 2 h with 40 μM cytochalasin B caused substantial rearrangement of the actin cytoskeleton but did not increase; the initial value of $[\text{Ca}^{2+}]_i$ observed in the absence of agonist in either the absence or presence of Ca^{2+} ; the rate of Ca^{2+} inflow measured following the addition of Ca^{2+} to cells incubated in the absence of an agonist; the leakage of fura-2 or lactate dehydrogenase; or the proportion of cells that took up Trypan Blue (results not shown). Cells treated with cytochalasin B exhibited significant morphological change as revealed by the appearance of numerous surface blebs (Figure 4B, compare with Figure 4A; compare with [31]). Actin was detected at the cell periphery but this appeared less organized than that observed

in untreated cells (compare with [32]), and a substantial amount of actin was detected in the cell interior (Figure 4B, compare with Figure 4A). Cytochalasin B did not grossly alter the distribution of the ER, as monitored using $\text{DiOC}_6(3)$. Compared with untreated (vehicle-only) cells, hepatocytes treated with cytochalasin B exhibited a more homogeneous distribution of the ER with fewer regions of the cytoplasmic space unoccupied by ER, and the ER appeared more punctate (Figure 4D, compare with Figure 4C).

Cytochalasin B caused a substantial decrease in the peak vasopressin-induced increase in $[\text{Ca}^{2+}]_i$ in cells incubated in the presence of Ca^{2+} . Thus for cells pre-treated with cytochalasin B (40 μM) or vehicle [0.2% (v/v) DMSO], the peak vasopressin-induced increases in $[\text{Ca}^{2+}]_i$ were 181 ± 35 (8) and 616 ± 134 (6) nM ($P < 0.001$), respectively. Cytochalasin B decreased the amount of Ca^{2+} released from intracellular stores by vasopressin in the absence of added Ca^{2+} by 45%, and decreased the rate of vasopressin-stimulated Ca^{2+} inflow by 73% (Figure 5 and Table 1). Cytochalasin B had no effect on: thapsigargin-induced release of Ca^{2+} from intracellular stores or on thapsigargin-stimulated Ca^{2+} inflow; the ability of 'caged' $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from intracellular stores; the ability of 13 nM adenophostin A to release Ca^{2+} from intracellular stores and stimulate Ca^{2+} inflow (Table 1), or on the ability of ionomycin (10 μM) to release Ca^{2+} in cells treated with adenophostin A. (The values of the peak ionomycin-induced increase in $[\text{Ca}^{2+}]_i$ were 273 ± 90 (7) and 270 ± 77 nM (8) for adenophostin A-loaded cells treated with cytochalasin B or vehicle, respectively.) Moreover, cytochalasin B caused no detectable change in the amount of Ca^{2+} released by ionomycin (in the absence of added Ca^{2+}) in cells incubated in the absence of an agonist, and no detectable change in the amount of Ca^{2+} released by ionomycin in cells previously treated with vasopressin (Table 2).

The amount of Ca^{2+} released by the photolysis of 'caged' $\text{Ins}(1,4,5)\text{P}_3$ in untreated cells was estimated to be 20% of that released by vasopressin in untreated cells. Moreover, the addition

Figure 5 Inhibition by cytochalasin B of vasopressin-stimulated Ca^{2+} inflow and the release of Ca^{2+} from intracellular stores

Hepatocytes were incubated for 2–2.5 h in the presence of either vehicle [0.2% (v/v) DMSO] (A) or 40 μ M cytochalasin B (B). Single cells were then injected with fura-2 and cell fluorescence measured. Additions of vasopressin (40 nM) and Ca^{2+} (1.5 mM) were made as indicated by the horizontal bars. Each trace is representative of those obtained for 7–14 individual cells from three separate hepatocyte preparations.

Table 1 The effects of pre-treatment with cytochalasin B on the release of Ca^{2+} from intracellular stores and Ca^{2+} inflow induced in rat hepatocytes by vasopressin, thapsigargin, adenophostin A and 'caged' $Ins(1,4,5)P_3$

Rat hepatocytes were incubated initially in the absence of Ca^{2+} . When present, 'caged' $Ins(1,4,5)P_3$ (estimated intracellular concentration 4 μ M) or adenophostin A (estimated intracellular concentration 13 nM) were co-injected with fluo-3 ['caged' $Ins(1,4,5)P_3$] or fura-2 (adenophostin A). The release of Ca^{2+} from intracellular stores was initiated by the addition of vasopressin (40 nM), thapsigargin (10 μ M) or the photolysis of 'caged' $Ins(1,4,5)P_3$, as indicated, or by addition of 10 μ M ionomycin in the case of adenophostin A. Ca^{2+} inflow was initiated by the addition of 1.5 mM Ca^{2+} . The degrees of significance for a comparison of the value obtained for cytochalasin B-treated cells with that for the corresponding untreated cells were: * $P < 0.05$, ** $P < 0.001$. Values in parentheses indicate numbers of individual cells. N. D., not determined.

Pre-treatment	Agonist	Agonist-induced Ca^{2+} release (nM Ca^{2+})	Initial rate of Ca^{2+} inflow (nM Ca^{2+} /min)
None	Vasopressin	465 $\pm$ 131 (7)	442 $\pm$ 116 (7)
Cytochalasin B	Vasopressin	258 $\pm$ 70 (14)*	118 $\pm$ 34 (14)**
None	Thapsigargin	161 $\pm$ 37 (9)	383 $\pm$ 88 (9)
Cytochalasin B	Thapsigargin	181 $\pm$ 57 (10)	445 $\pm$ 106 (10)
None	Adenophostin A	238 $\pm$ 144 (8)	294 $\pm$ 92 (6)
Cytochalasin B	Adenophostin A	273 $\pm$ 168 (7)	350 $\pm$ 69 (5)
None	Photolysed 'caged' $Ins(1,4,5)P_3$	318 $\pm$ 60 (7)	N. D.
Cytochalasin B	Photolysed 'caged' $Ins(1,4,5)P_3$	300 $\pm$ 43 (7)	N. D.

Table 2 Effect of pre-treatment with cytochalasin B on the ability of ionomycin to release Ca^{2+} from intracellular stores

Ionomycin or vasopressin followed by ionomycin (added after the vasopressin-induced increase in $[Ca^{2+}]_i$ had returned to the basal value), were added to rat hepatocytes incubated in the absence of added Ca^{2+} . Vasopressin- and ionomycin-induced Ca^{2+} release was estimated as the difference between the maximum value of $[Ca^{2+}]_i$ observed in the presence of the agonist and the value of $[Ca^{2+}]_i$ immediately before the addition of agonist. Values in parentheses indicate numbers of individual cells.

Addition	Agonist	Vasopressin-induced Ca^{2+} release (nM)	Ionomycin-induced Ca^{2+} release (nM)
None	Ionomycin (10 μ M)	—	472 $\pm$ 177 (5)
Cytochalasin B	Ionomycin (10 μ M)	—	502 $\pm$ 174 (5)
None	Vasopressin (40 nM) followed by ionomycin (10 μ M)	307 $\pm$ 99 (11)	27 $\pm$ 6 (11)
Cytochalasin B	Vasopressin (40 nM) followed by ionomycin (10 μ M)	158 $\pm$ 40 (12)	40 $\pm$ 19 (12)

Table 3 The effects of the myosin light-chain-kinase inhibitor, ML-9, on the release of Ca^{2+} from intracellular stores and Ca^{2+} inflow induced by vasopressin and thapsigargin

Following injection with fura-2, hepatocytes were incubated for 10 min in CO_2 -independent medium in the presence of the indicated concentration of ML-9 or vehicle [0.17% (v/v) ethanol]. The medium was then exchanged for Ca^{2+} -free Hank's solution, which also contained ML-9 or 0.17% (v/v) ethanol, and the measurement of fluorescence in the absence of added Ca^{2+} was begun. Ca^{2+} release was initiated by addition of vasopressin (40 nM) or thapsigargin (10 μM), and Ca^{2+} inflow by addition of 1.5 mM CaCl_2 . The degrees of significance for a comparison of the value obtained in the presence of a given concentration of ML-9 with that obtained in the absence of ML-9 are: * $P < 0.01$ and ** $P < 0.001$. Values in parentheses indicate sample sizes.

Concentration of ML-9 (μM)	Agonist	Agonist-induced Ca^{2+} release (nM Ca^{2+})	Initial rate of Ca^{2+} inflow (nM Ca^{2+} /min)
0	Vasopressin	254 ± 37 (8)	455 ± 34 (8)
1	Vasopressin	301 ± 132 (5)	309 ± 108 (5)
5	Vasopressin	142 ± 23 (8)*	214 ± 63 (8)**
100	Vasopressin	89 ± 50 (3)*	0 (3)
0	Thapsigargin	144 ± 40 (9)	436 ± 29 (9)
5	Thapsigargin	132 ± 27 (5)	367 ± 65 (5)
70	Thapsigargin	122 ± 30 (4)	0 (4)

of ionomycin (10 μM) to cells after the photolysis of 'caged' $\text{Ins}(1,4,5)\text{P}_3$ caused an increase in $[\text{Ca}^{2+}]_c$ that was about 70% of that observed following the addition of ionomycin to untreated cells (results not shown). These results indicate that the photolysis of 'caged' $\text{Ins}(1,4,5)\text{P}_3$ released only a fraction of the total $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} .

The pre-treatment of hepatocytes with 5 μM ML-9, an inhibitor of myosin light-chain kinase [18,19], inhibited vasopressin-induced release of Ca^{2+} from intracellular stores and vasopressin-stimulated Ca^{2+} inflow by 44 and 53%, respectively (Table 3). A lower concentration (1 μM) had no effect on vasopressin-induced Ca^{2+} release but inhibited vasopressin-stimulated Ca^{2+} inflow by about 30%. A substantially higher concentration (100 μM) caused 65% inhibition of vasopressin-induced release of Ca^{2+} from intracellular stores and completely inhibited vasopressin-stimulated Ca^{2+} inflow (Table 3). ML-9 did not inhibit thapsigargin-induced release of Ca^{2+} from intracellular stores and, at 5 μM , had no significant effect on thapsigargin-stimulated Ca^{2+} inflow. However, 70 μM ML-9 caused complete inhibition of thapsigargin-stimulated Ca^{2+} inflow (Table 3). ML-9 did not alter the permeability of the plasma membrane to ions in the basal state, as judged by examination of cell morphology and the measurement of basal values of $[\text{Ca}^{2+}]_c$, which were not affected by ML-9 (results not shown).

The treatment of hepatocytes with cytochalasin B or ML-9 caused no significant change in the ability of vasopressin to increase the concentration of $\text{Ins}(1,4,5)\text{P}_3$. The $\text{Ins}(1,4,5)\text{P}_3$ concentrations induced by addition of vasopressin (40 nM) for 30 s were 7.3 ± 2.1 (4) and 7.5 ± 3.3 pmol/mg of protein (4) for cells pre-treated with 40 μM cytochalasin B or vehicle [0.2% (v/v) DMSO], respectively, and 8.5 ± 2.6 (4) and 11 ± 3.7 pmol/mg of protein (4) for cells pre-treated with 5 μM ML-9 or vehicle [0.17% (v/v) ethanol], respectively.

DISCUSSION

Evidence for the involvement of a subregion of the ER in the activation of SOC

The observations that (i) the concentration of agent yielding a half-maximal effect ($S_{0.5}$) for the activation of Ca^{2+} inflow by adenophostin A was lower than the $S_{0.5}$ value for adenophostin A-induced release of Ca^{2+} from intracellular stores and (ii) anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor antibody 18A10 inhibited

vasopressin-stimulated Ca^{2+} inflow but had little effect on the ability of vasopressin, acting via $\text{Ins}(1,4,5)\text{P}_3$, to release Ca^{2+} from the bulk of the ER suggest that there is a functionally distinct subregion of the ER that is involved in the activation of SOC in hepatocytes. The results also suggest that this putative ER subregion has a different apparent affinity for, or accessibility to, adenophostin A.

For the following reasons, it is considered unlikely that the observed disparity between the $S_{0.5}$ values for the activation by adenophostin A of Ca^{2+} inflow and ER Ca^{2+} release is due to an apparent low rate of diffusion of adenophostin A through the cytoplasmic space. (i) The site of injection is likely to be randomly distributed within the cell. (ii) An increase in the time elapsed between micro-injection and the measurement of fluorescence from 10 to 20 min did not alter the results obtained with 13 nM adenophostin A. (iii) The results of tests of the ability of adenophostin A to release Ca^{2+} from the ER in permeabilized L15 and SH-SY5Y cells indicate that the apparent rate of diffusion of adenophostin A in the cytoplasmic space is comparable with that of $\text{Ins}(1,4,5)\text{P}_3$ (R. A. Wilcox, unpublished work). (iv) Similar results were obtained with $\text{Glc}(2,3',4')\text{P}_3$, which has a 5–30-fold lower affinity at $\text{Ins}(1,4,5)\text{P}_3$ receptors than $\text{Ins}(1,4,5)\text{P}_3$ [28]. An adenophostin A-induced desensitization of the $\text{Ins}(1,4,5)\text{P}_3$ receptors to $\text{Ins}(1,4,5)\text{P}_3$ is also considered unlikely since thapsigargin, added after adenophostin A and vasopressin, induced no substantial further release of Ca^{2+} , indicating that all available Ca^{2+} had been released from the ER by $\text{Ins}(1,4,5)\text{P}_3$ generated by vasopressin.

Two observations suggest that type-1 $\text{Ins}(1,4,5)\text{P}_3$ receptors play an important role in the activation of SOC in hepatocytes. First, the results reported here show that the anti-type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor antibody 18A10 caused 60% inhibition of vasopressin-stimulated Ca^{2+} inflow. Secondly, it has been shown by others that (i) hepatocyte $\text{Ins}(1,4,5)\text{P}_3$ receptors are composed predominantly of type-1 (20%) and type-2 (80%) proteins [8] and (ii) in freshly isolated rat hepatocytes cultured under conditions similar to those employed here, type-1 $\text{Ins}(1,4,5)\text{P}_3$ -receptor proteins are predominantly located at the plasma membrane [9].

The conclusion that a functionally distinct subregion of the ER with a different apparent affinity for, or accessibility to, $\text{Ins}(1,4,5)\text{P}_3$ is involved in the activation of SOC in hepatocytes is consistent with results obtained recently in other laboratories with other cell types. These also suggest that a subregion of the

ER, distinguished from the bulk of the ER by a different apparent sensitivity to $\text{Ins}(1,4,5)P_3$ or $\text{Ins}(1,4,5)P_3$ analogues, is involved in the activation of SOC_s [15,16,33–36] (but see [37]).

Involvement of the actin cytoskeleton in the activation of SOC_s

The results obtained with cytochalasin B and ML-9 indicate that, under the conditions of the present experiments, considerable disruption of the actin cytoskeleton in hepatocytes does not affect the ability of a decrease in $[\text{Ca}^{2+}]_{\text{ER}}$, induced by thapsigargin or adenophostin A, to activate SOC_s. Others have also observed that in other cell types the activation of SOC_s is remarkably resistant to disruption of the cytoskeleton [38–41].

The results obtained for the measurement of $\text{Ins}(1,4,5)P_3$ concentrations and with 'caged' $\text{Ins}(1,4,5)P_3$ indicate that the inhibition by cytochalasin B and ML-9 of vasopressin-stimulated Ca^{2+} inflow and vasopressin-induced ER Ca^{2+} release is unlikely to be due to an inhibition by these agents of one or more reactions involved in the formation of $\text{Ins}(1,4,5)P_3$ or to inhibition of the action of $\text{Ins}(1,4,5)P_3$ on $\text{Ins}(1,4,5)P_3$ receptors. The inhibition by cytochalasin B and ML-9 of vasopressin-induced ER Ca^{2+} release may be due to inhibition of the ability of $\text{Ins}(1,4,5)P_3$ receptors to release Ca^{2+} from specific regions of the lumen of the ER. Such an impairment may reflect the ability of cytochalasin B to disrupt luminal Ca^{2+} communication within the ER, as demonstrated in permeabilized hepatocytes [12].

The results reported here suggest that a functionally identifiable subregion of the ER, which is closely associated with the plasma membrane, is required for the activation of SOC_s in hepatocytes. Such an arrangement is a pre-requisite for a conformational coupling hypothesis for the activation of SOC_s [1–4], and such an hypothesis would be consistent with the observations reported here. However, further experiments are required to distinguish between three main possibilities concerning the roles of a putative ER subregion and the actin skeleton. These possibilities are that the cytoskeleton serves to maintain a subregion of the ER sufficiently close to the plasma membrane to allow direct interaction between the $\text{Ins}(1,4,5)P_3$ receptor (or another ER protein) and SOC proteins [1–4], that the role of the cytoskeleton is to allow close association of a subregion of the ER and the plasma membrane in order to limit the path required for the diffusion of a 'calcium influx factor' [1–4] or to create a subplasmalemmal space that may play a role in the activation of SOC_s [42].

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