Inositol trisphosphate analogues selective for types I and II inositol trisphosphate receptors exert differential effects on vasopressin-stimulated Ca^{2+} inflow and Ca^{2+} release from intracellular stores in rat hepatocytes

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Previous studies have shown that adenophostin A is a potent initiator of the activation of SOCs (store-operated Ca^{2+} channels) in rat hepatocytes, and have suggested that, of the two subtypes of Ins(1,4,5)P_3 receptor predominantly present in rat hepatocytes [Ins(1,4,5)P_3R_1 (type I receptor) and Ins(1,4,5)P_3R_2 (type II receptor)], Ins(1,4,5)P_3R_1s are required for SOC activation. We compared the abilities of Ins(1,4,6)P_3 [with higher apparent affinity for Ins(1,4,5)P_3R_1] and Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4 [with higher apparent affinities for Ins(1,4,5)P_3R_2] to activate SOCs. The Ins(1,4,5)P_3 analogues were microinjected into single cells together with fura 2, and dose–response curves for the activation of Ca^{2+} inflow and Ca^{2+} release from intracellular stores obtained for each analogue. The concentration of Ins(1,4,6)P_3 which gave half-maximal stimulation of Ca^{2+} inflow was substantially lower than that which gave half-maximal stimulation of Ca^{2+} release. By contrast, for Ins(1,3,6)P_3 and Ins(1,2,4,5)P_3, the concentration which gave half-maximal stimulation of Ca^{2+} inflow was substantially higher than that which gave half-maximal stimulation of Ca^{2+} release. The distribution of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 in rat hepatocytes cultured under the same conditions as those employed for the measurement of Ca^{2+} inflow and release was determined by immunofluorescence. Ins(1,4,5)-P_3R_1s were found predominantly at the cell periphery, whereas Ins(1,4,5)P_3R_2s were found at the cell periphery, the cell interior and nucleus. It is concluded that the idea that a small region of the endoplasmic reticulum enriched in Ins(1,4,5)P_3R_1 is required for the activation of SOCs is consistent with the present results for hepatocytes.

Key words: endoplasmic reticulum, inositol trisphosphate receptor, store-operated Ca^{2+} channel.

INTRODUCTION

SOCs (store-operated Ca^{2+} channels) in the plasma membrane are required for regulation of the $[Ca^{2+}]_{cyt}$ (cytoplasmic free Ca^{2+} concentration) and re-filling the ER (endoplasmic reticulum) Ca^{2+} stores in hepatocytes and in other non-excitable cells, and in some excitable cells [1,2]. Several studies have shown that SOCs are required for the maintenance of agonist-induced oscillations in $[Ca^{2+}]_{cyt}$ [3,4], and there is evidence that SOCs are more effective in re-filling the ER than non-selective cation channels [5]. The activation of SOCs is initiated by a decrease in the concentration of Ca^{2+} in the ER induced by the action of Ins(1,4,5)P_3 at Ins(1,4,5)P_3Rs [Ins(1,4,5)P_3 receptors] and by Ca^{2+} at ryanodine receptors [1,2]. There is some evidence which suggests there may be a direct interaction (conformational coupling) between some Ins(1,4,5)P_3Rs and some putative SOCs or other types of plasma membrane Ca^{2+} channels (reviewed in [1,2]). Evidence that the activation of SOCs involves, or requires, a specific region of the ER close to the plasma membrane in the vicinity of SOCs [6–9] and/or continuity of the whole ER [10,11] has been reported. While there have been numerous experiments designed to determine the relationship between the degree to which Ca^{2+} in the ER is decreased and the activation of SOCs, and the location of this decrease in ER Ca^{2+} [12,13], no clear answers have so far been obtained.

Rat hepatocytes *in situ* are polarized epithelial cells with clearly defined canalicular, basal and basolateral membrane regions (reviewed in [14]). Freshly isolated rat hepatocytes lose much of this polarity, but after culture for a few hours begin to regain some polarity [15]. Normal rat hepatocytes possess predominantly Ins(1,4,5)P_3R_1 (type I) and Ins(1,4,5)P_3R_2 (type II), with very little Ins(1,4,5)P_3R_3 (type III) [16,17]. Ins(1,4,5)P_3R_2s predominate, and may be responsible for the generation of $[Ca^{2+}]_{cyt}$ waves emanating from the canalicular region [17]. There is evidence that Ins(1,4,5)P_3R_1s are associated with a subregion of the ER which is close to the plasma membrane and attached to the peripheral F-actin [18,19].

The results of previous studies with rat hepatocytes, which employed adenophostin A, an Ins(1,4,5)P_3 analogue with a very high affinity for Ins(1,4,5)P_3R, Glc(2,3′,4′)P_3 (2-hydroxyethyl-α-D-glucopyranoside 2,3′,4′-trisphosphate), and an antibody against Ins(1,4,5)P_3R_1, which inhibits Ins(1,4,5)P_3R function, suggested that a subregion of the ER and the Ins(1,4,5)P_3R_1 are both required for the activation of SOCs [9]. Adenophostin A has been used to investigate the role of Ins(1,4,5)P_3R and the ER in the mechanism of activation of SOCs in several other cell types [7,20], leading, in some studies, to the conclusion that a small subregion of the ER is required for SOC activation [7].

The aim of the present study was to test further the roles of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 in the activation of SOCs in rat hepatocytes. This has been done using Ins(1,4,5)P_3 analogues with different apparent affinities for Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 [21] to initiate intracellular Ca^{2+} release and Ca^{2+} inflow. The intracellular locations of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 under

Abbreviations used: $[Ca^{2+}]_{cyt}$, cytoplasmic Ca^{2+} concentration; $Ca^{2+}{}_o$, extracellular Ca^{2+}; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; ER, endoplasmic reticulum; Ins(1,4,5)P_3R, Ins(1,4,5)P_3 receptor; SOC, store-operated Ca^{2+} channel.

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the same primary cell culture conditions as those employed for the measurement of Ca^{2+} inflow and release were also determined by immunofluorescence. The results provide evidence which indicates that a region of the ER (or another intracellular Ca^{2+} store) which is enriched in Ins(1,4,5)P_3R_1 is involved in the activation of SOCs.

MATERIALS AND METHODS

Materials

D-*myo*-Ins(1,3,6)P_3 [22], D-*myo*-Ins(1,4,6)P_3 [23], and D-*myo*-Ins(1,2,4,5)P_4 [24] were synthesized as described previously. D-*myo*-2-Deoxy-Ins(1,4,5)P_3 (A. M. Riley and B. V. L. Potter, unpublished work) was synthesized from D-3,6-di-*O*-benzyl-4,5-*O*-(2,3-dimethoxybutane-2,3-diyl)-*myo*-inositol [25]. All ligands were prepared as their tri-ethylammonium salts and were homogeneous by routine spectroscopic methods. The freeze-dried form of each Ins(1,4,5)P_3 analogue was reconstituted in water, washed through 0.5 ml of Chelex-100 resin (to replace the tri-ethylammonium cation with Na^+) and again freeze-dried. Stock solutions were prepared by dissolving the freeze-dried Ins(1,4,5)P_3 analogue in 125 mM KCl. Monoclonal antibodies KM1112 and KM1083 specific for Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 respectively [26] were kindly provided by Professor K. Mikoshiba, University of Tokyo, Tokyo, Japan. Horseradish-peroxidase-conjugated goat anti-mouse IgG was from Sigma. Nitrocellulose and PVDF membranes and ECL® (enhanced chemiluminescence) detection reagents were provided by Amersham Biosciences. SaOS-2 human osteosarcoma cells (A.T.C.C., Rockville, MD, U.S.A.) were kindly provided by Dr T. J. McCann, Babraham Institute, Cambridge, U.K., and L15 mouse fibroblasts [27] by Professor K. Mikoshiba. SaOS-2 cells [28] and L15 mouse fibroblasts [29] were cultured as described previously.

Isolation of hepatocytes and measurement of $[Ca^{2+}]_{cyt}$

The isolation of hepatocytes from Hooded Wistar rats, attachment of hepatocytes to collagen-coated coverslips, the microinjection of fura 2 and Ins(1,4,5)P_3 analogues and measurement of the fluorescence of single hepatocytes loaded with fura 2 (using a ratiometric technique) were carried out as described previously [9]. Changes in Ca^{2+} concentration are expressed as changes in the fluorescence ratio ($F_{340\,nm}/F_{380\,nm}$). The dilution factor for the microinjection of agents to hepatocytes was determined to be 1:75 [9] and was used to estimate the intracellular concentrations of the Ins(1,4,5)P_3 analogues microinjected 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 microinjected with fura 2 together with a given Ins(1,4,5)P_3 analogue, incubated for 10 min (to allow the cells to re-seal [9]), transferred to a medium containing no added Ca^{2+}, and the fluorescence ratios were measured. Rates of Ca^{2+} inflow in single hepatocytes were estimated following the addition of $Ca^{2+}{}_o$ (extracellular Ca^{2+}) to cells incubated in the absence of added $Ca^{2+}{}_o$ (the 'Ca^{2+} add-back' protocol) [9] from measurement of the initial rate of the Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ (expressed as change in fluorescence ratio units per min). Since the amount of Ca^{2+} which accumulates in the cytoplasmic space in a Ca^{2+} add-back protocol depends on both the rate of inflow across the plasma membrane and the rate of removal from the cytoplasmic space by transport into intracellular stores and the extracellular space, the initial rate of increase in $[Ca^{2+}]_{cyt}$ is a more accurate reflection of the rate of Ca^{2+} inflow through SOCs than the value of the subsequent plateau in $[Ca^{2+}]_{cyt}$ (underlying assumptions discussed in [30]).

To estimate the amount of Ca^{2+} released from intracellular stores by a given Ins(1,4,5)P_3 analogue, vasopressin (40 nM) was added to cells loaded with the Ins(1,4,5)P_3 analogue plus fura 2, or with fura 2 alone. The amount of Ca^{2+} released by the Ins(1,4,5)P_3 analogue was calculated as the difference between the vasopressin-induced release of Ca^{2+} measured in the absence and presence of the Ins(1,4,5)P_3 analogue. It has previously been shown, using EGTA to chelate extracellular Ca^{2+}, that the rapid increase in $[Ca^{2+}]_{cyt}$ induced by vasopressin in the presence of extracellular Ca^{2+} reflects the release of Ca^{2+} from intracellular stores with little contribution from Ca^{2+} inflow across the plasma membrane [9]. This was confirmed in the present series of experiments by measuring vasopressin-induced Ca^{2+} release (the height of the vasopressin-induced peak of $[Ca^{2+}]_{cyt}$) in the presence of $Ca^{2+}{}_o$ (1.5 mM) and in the presence of both $Ca^{2+}{}_o$ and EGTA (2 mM). Experiments were performed with cells microinjected with fura 2 plus Ins(1,4,6)P_3 (10 μM), Ins(1,2,4,5)P_4 (70 μM) and Ins(1,3,6)P_3 (110 μM). For each analogue, there was no significant difference in the amount of Ca^{2+} released in response to vasopressin (40 nM) measured in the presence and absence of EGTA. The amounts (fluorescence ratio units) of Ca^{2+} release induced by vasopressin were 0.28 ± 0.02 and 0.36 ± 0.04 (+ EGTA), 0.52 ± 0.05 and 0.51 ± 0.05 (+ EGTA), and 0.58 ± 0.04 and 0.53 ± 0.03 (+ EGTA) (means ± S.E.M., $n = 8$–15) for cells containing Ins(1,4,6)P_3, Ins(1,2,4,5)P_4, and Ins(1,3,6)P_3 respectively.

Western blot analysis

Tissue preparation, protein assays, SDS gels, and semi-dry blotting were performed essentially as described previously [29]. SDS-minigels (6 %) were run using the Bio-Rad Immunoblot assay kit. Standard homogenization, Western blotting and transfer buffers were used throughout, and either nitrocellulose or PVDF membranes were used for immunoblotting. Bands of Ins(1,4,5)-P_3R_1 and Ins(1,4,5)P_3R_2 were detected using mouse monoclonal anti-Ins(1,4,5)P_3R_1 KM1112 or mouse monoclonal anti-Ins-(1,4,5)P_3R_2 KM1083 [26], goat anti-mouse IgG conjugated to horseradish peroxidase as secondary antibody and ECL® detection. 'SeeBlue' pre-stained standards (4–250 kDa, Novex) were used as molecular-mass markers. On 6 % SDS-minigels, the myosin standard (250 kDa) ran very close to the Ins(1,4,5)P_3R_1 and Ins(1,4,5)-P_3R_2.

Immunolocalization of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2

Rat hepatocytes grown on collagen-coated coverslips were rinsed with PBS and fixed with 4 % (w/v) paraformaldehyde in PBS for 30 min, rinsed with PBS, then permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 10 min at room temperature. Cells were again rinsed with PBS, blocked with 10 % (w/v) foetal bovine serum in PBS for 1 h at room temperature, washed with PBS containing 0.05 % (v/v) Tween 20 (PBS-T), and incubated overnight at 4 °C with either mouse monoclonal anti-Ins(1,4,5)P_3R_1 KM1112 or mouse monoclonal anti-Ins(1,4,5)P_3R_2 KM1083 [26]. The cells were then washed with PBS-T and incubated for 1 h at room temperature in the dark with the secondary antibody, Cy3-conjugated anti-mouse IgG, at 1:1000 dilution in PBS-T containing 1 % (v/v) serum. After washing with PBS-T, then four times in PBS, the coverslips were mounted on glass slides and viewed using a Bio-Rad MRC-1000 confocal microscope, krypton–argon laser, and Chroma 31 002 (excitation 515–550 nm, emission 575–615 nm) filters, and 60× oil objective. The location of the ER

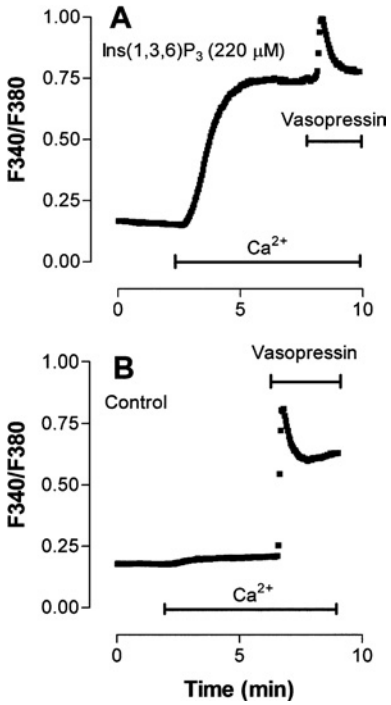

Figure 1 Effects of Ins(1,3,6)P_3 on Ca^{2+} inflow and the vasopressin-induced increase in $[Ca^{2+}]_{cyt}$ in single rat hepatocytes

Single hepatocytes were injected with fura 2 plus 220 μM Ins(1,3,6)P_3 (estimated intracellular concentration) (**A**) or fura 2 alone (**B**) 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 21–97 individual cells from two to seven separate hepatocyte preparations.

in fixed freshly isolated rat hepatocytes in primary culture was determined using $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) and confocal microscopy as described previously [9].

RESULTS

Effects of Ins(1,4,6)P_3, Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4 on Ca^{2+} inflow and release

The strategy employed was to use Ins(1,4,5)P_3 analogues with different affinities for Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 as probes for the involvement of each of these receptor subtypes in the activation of SOCs. The analogues employed were D-*myo*-Ins(1,4,6)P_3 [with a higher affinity for Ins(1,4,5)P_3R_1] and D-*myo*-Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4 [with a higher affinity for Ins(1,4,5)P_3R_2] [21]. The abilities of D-*myo*-Ins(1,4,6)P_3, Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4 to activate Ca^{2+} release and Ca^{2+} inflow in rat hepatocytes were investigated by microinjecting each analogue into hepatocytes together with fura 2. The cells were incubated in the absence of added $Ca^{2+}{}_o$, then $Ca^{2+}{}_o$ was added (to allow an estimate of the rate of Ca^{2+} inflow) followed by vasopressin (to release Ca^{2+} remaining in the intracellular stores). It has previously been shown that each of these analogues is resistant to metabolism by 5′-phosphatase and 3′-kinase activities [31–34]. The results obtained for a high intracellular concentration (estimated to be 220 μM) of Ins(1,3,6)P_3 are shown in Figure 1(A), and those for a control cell, microinjected with fura 2 alone, in Figure 1(B). The amount of Ca^{2+} released from the ER by the Ins(1,4,5)P_3 analogue was estimated by determining the difference between the Ca^{2+} released by vasopressin in the control cell and that released by vasopressin in the cell loaded with the Ins(1,4,5)P_3 analogue. (The rationale for this procedure is discussed in more detail in the Materials and methods section.)

The dose–response curve for the effect of Ins(1,3,6)P_3 on Ca^{2+} release and Ca^{2+} inflow is shown in Figure 2(A). This shows that the concentration of Ins(1,3,6)P_3 which gave half-maximal stimulation of Ca^{2+} inflow is substantially higher than that which gave half-maximal stimulation of Ca^{2+} release. A similar dose–response pattern, but with less pronounced differences, was observed with Ins(1,2,4,5)P_4 (Figure 2B). D-2-Deoxy-Ins(1,4,5)P_3 {with higher affinity for Ins(1,4,5)P_3R_2 than Ins(1,4,5)P_3R_1 [21]} was also tested. However, the microinjection of D-2-deoxy-Ins(1,4,5)P_3 had no effect on either Ca^{2+} release or Ca^{2+} inflow, most likely due to its rapid metabolism in liver cells [35].

In contrast with the results obtained with Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4, when the experiment was conducted with Ins(1,4,6)P_3 (Figure 2C), the concentration of Ins(1,4,6)P_3 which gave half-maximal stimulation of Ca^{2+} inflow was found to be lower than that which gave half-maximal stimulation of Ca^{2+} release. Thus relative to the ability to induce Ca^{2+} release, Ins(1,4,6)P_3 is more effective than Ins(1,3,6)P_3 in inducing Ca^{2+} inflow.

Intracellular distribution of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 under conditions employed for the measurement of Ca^{2+} inflow

The intracellular locations of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 were determined using antibodies specific for these proteins and immunofluorescence. The specificity of the antibodies for Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 was confirmed by Western blot analysis using extracts of L15 mouse fibroblasts [27], which express Ins(1,4,5)P_3R_1, but do not express significant levels of Ins(1,4,5)P_3R_2 [29], and extracts of SaOS-2 human osteoblasts, which predominantly express Ins(1,4,5)P_3R_2 and Ins(1,4,5)P_3R_3 [28]. A single band corresponding to the expected size of the Ins(1,4,5)P_3R was observed in each case (results not shown). In extracts of rat liver, each antibody also gave a single band corresponding to the expected size of the Ins(1,4,5)P_3R_1 or Ins(1,4,5)P_3R_2 (results not shown).

In immunofluorescence experiments with hepatocytes cultured for 2 h under conditions similar to those employed for the measurement of Ca^{2+} inflow and release, Ins(1,4,5)P_3R_1 was principally located in a band at the cell periphery, with little in the interior of the cytoplasmic space, and none in the nucleus (Figure 3A). Ins(1,4,5)P_3R_2 was also located in a clearly defined band at the cell periphery with some expression also in the cytoplasmic space (Figure 3B). In some experiments, labelling of the nucleus by anti-Ins(1,4,5)P_3R_2 was seen (results not shown). Panels b, f and j of each of Figures 3(A) and 3(B) show equatorial sections from three representative cells. Images were also obtained at 3 μm above (panels c, g, k) and below (panels d, h, l) the equatorial plane (the diameter of the cultured cells in the z axis was 10–20 μm). More intense staining was observed for the images obtained 3 μm above the equatorial plane (closer to the coverslip to which the cells were attached).

Freshly isolated hepatocytes exhibit little polarity, but when attached to a collagen-coated glass surface begin to regain polarity (assessed, for example, by formation of the cortical actin cytoskeleton) after about 4 h in culture [15,36]. To determine whether re-polarization affects the intracellular distribution of Ins(1,4,5)P_3R, the distribution of Ins(1,4,5)P_3R_2 was determined at 1 and 4 h after the initiation of cell culture. Ins(1,4,5)P_3R_2 was studied because it gave a more intense immunofluorescence signal than that generated by Ins(1,4,5)P_3R_1. Images obtained in the equatorial plane are shown in Figure 4. There was a significant increase

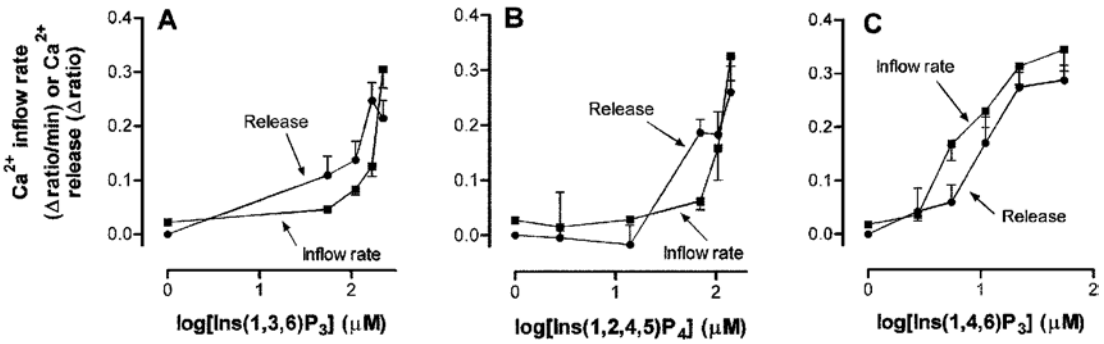

Figure 2 Comparison of concentration–response curves for the effects of Ins(1,3,6)P_3, Ins(1,2,4,5)P_4 and Ins(1,4,6)P_3 on the release of Ca^{2+} from intracellular stores and on Ca^{2+} inflow

Ins(1,3,6)P_3 (**A**), Ins(1,2,4,5)P_4 (**B**) or Ins(1,4,6)P_3 (**C**) was co-injected into 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 Ca^{2+} inflow (■) and amounts of Ca^{2+} release (●) were estimated as described in the Materials and methods section. Each data point is the mean ± S.E.M. of the values obtained from 17–97 individual hepatocytes from one to seven separate hepatocyte preparations.

in total immunofluorescence due to Ins(1,4,5)P_3R_2 at 4 h, possibly due to increased synthesis and/or decreased degradation of Ins(1,4,5)P_3R, although there was no change in the intracellular distribution of Ins(1,4,5)P_3R_2 (Figure 4).

The distribution of the ER in hepatocytes cultured under conditions similar to those employed for the measurement of Ca^{2+} inflow was also determined using $DiOC_6$(3). The results indicate that the ER is distributed throughout the cytoplasmic space and, at the periphery of the cell, extends to the plasma membrane (results not shown; and [15]).

DISCUSSION

The most interesting aspect of the present study is the observation that the relationship between the dose–response curves for Ca^{2+} inflow and Ca^{2+} release for each of Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4 differs from that for Ins(1,4,6)P_3. Thus Ins(1,4,6)P_3, which, on the basis of studies conducted with Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 expressed in insect Sf9 cells [21], has a higher affinity for Ins(1,4,5)P_3R_1 than Ins(1,4,5)P_3R_2, activated Ca^{2+} inflow at lower concentrations than those which induced significant Ca^{2+} release. In contrast, Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4, each of which has a higher affinity for Ins(1,4,5)P_3R_2 than Ins(1,4,5)P_3R_1 [21], induced Ca^{2+} release at lower concentrations than those which activated substantial Ca^{2+} inflow. These results suggest that, in intact hepatocytes, the binding of Ins(1,4,5)P_3 to Ins(1,4,5)P_3R_1s is more effective in activating SOCs than the binding of Ins(1,4,5)P_3 to Ins(1,4,5)P_3R_2s.

It is recognized that there are a number of assumptions made in the interpretation of the dose–response curves for the Ins(1,4,5)P_3 analogues reported in the present paper. First, the measured difference in apparent affinity of a given analogue for Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 is relatively small [21]. Secondly, it is assumed that Ins(1,3,6)P_3 and Ins(1,4,6)P_4 are resistant to metabolism [33,34] and diffuse throughout the cytoplasmic space [37], so that the cytoplasmic concentration of the Ins(1,4,5)P_3 analogue remains approximately constant during the period over which Ca^{2+} inflow and Ca^{2+} release are measured. Thirdly, it was not possible to raise the intracellular concentrations of Ins(1,3,6)P_3 and Ins(1,2,4,5)P_4 to saturate the Ins(1,4,5)P_3R. Fourthly, the amount of Ca^{2+} released was estimated using a somewhat indirect strategy. While these assumptions appear valid, some caution should be exercised in interpreting the dose–response curves. Nevertheless, the difference in the patterns of the dose–response curves for analogues with different affinities for Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 is quite striking.

Previously, the differences in affinities of Ins(1,3,6)P_3, Ins(1,4,6)P_3 and Ins(1,4,5)P_3 for Ins(1,4,5)P_3Rs have been rationalized by comparing their molecular structures in diagrams that align the phosphate groups of the three molecules [21,33,38]. Molecular docking experiments using the recently published X-ray crystal structure [39] of the Ins(1,4,5)P_3R_1 binding domain can be used to extend this model by suggesting how the relative affinities of Ins(1,4,6)P_3 and Ins(1,3,6)P_3 for Ins(1,4,5)P_3R_1 may be related to their abilities to mimic Ins(1,4,5)P_3 in the context of the Ins(1,4,5)P_3R_1-binding site (Figure 5). An equivalent crystal structure for an Ins(1,4,5)P_3R_2 domain is not yet available, but it is interesting to note that the residues of Ins(1,4,5)P_3R_1 that interact with Ins(1,4,5)P_3 are all conserved in Ins(1,4,5)P_3R_2, with the exception that Gly^{268} in Ins(1,4,5)P_3R_1 is replaced by leucine in the Ins(1,4,5)P_3R_2 sequence in both the mouse [40] and rat [41] (reviewed in [42]) Ins(1,4,5)P_3R_2 sequences. It is possible that the replacement of glycine with the sterically bulky and hydrophobic leucine residue at this position could influence the local environment at the Ins(1,4,5)P_3R_2-binding site, perhaps with consequences for the relative affinities of Ins(1,4,5)P_3, Ins(1,4,6)P_3, Ins(1,3,6)P_3 and also Ins(1,2,4,5)P_4.

Both Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 were found to be predominantly located at the periphery of the cell in rat hepatocytes

Figure 3 Localization by immunofluorescence of the type I (A) and type II (B) Ins(1,4,5)P_3Rs in rat hepatocytes grown for 2 h in primary culture

The culture of rat hepatocytes, permeabilization and cell fixation, staining with anti-Ins(1,4,5)P_3 antibody, and confocal microscopy were performed as described in the Materials and methods section. (**A**) Cells stained with mouse monoclonal anti-Ins(1,4,5)P_3R_1 antibody KM1112 and anti-mouse antibody conjugated to Cy3. (**B**) Cells stained with mouse monoclonal anti-Ins(1,4,5)P_3R_2 antibody KM1083 and anti-mouse antibody conjugated to Cy3. The results shown are those obtained for one of three experiments employing three separate rat hepatocyte preparations which each gave similar results. The scale bar represents 10 μm. Panels a–d, e–h and i–l each represent a different single cell, showing a bright-field image, an equatorial image, 3 μm above equatorial image (z plane) and 3 μm below equatorial respectively. Panels m and n are controls in which primary antibody has been omitted. All images in (**A**) and (**B**) were obtained with the same confocal gain setting.

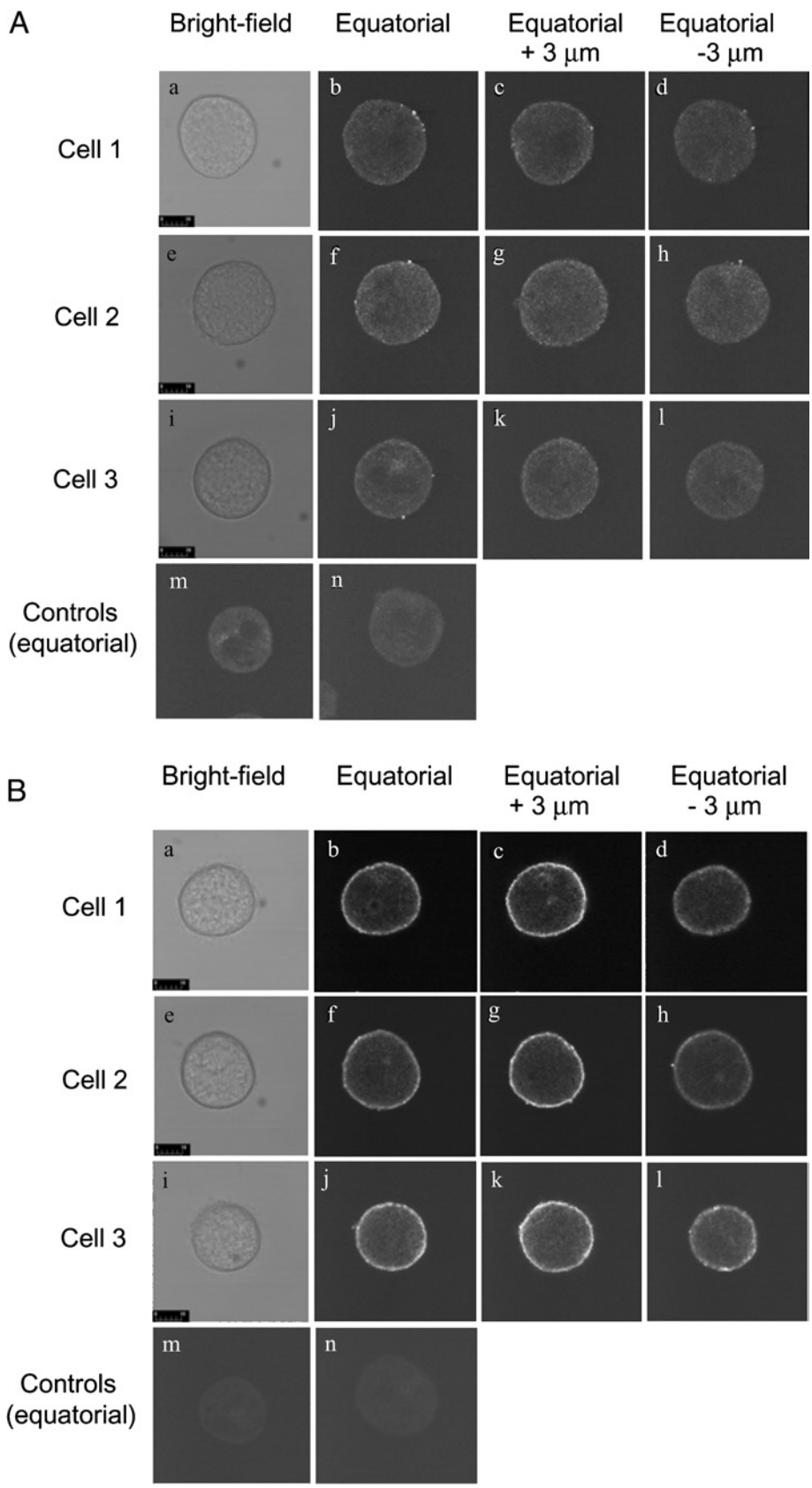

Figure 3 **For legend see facing page**

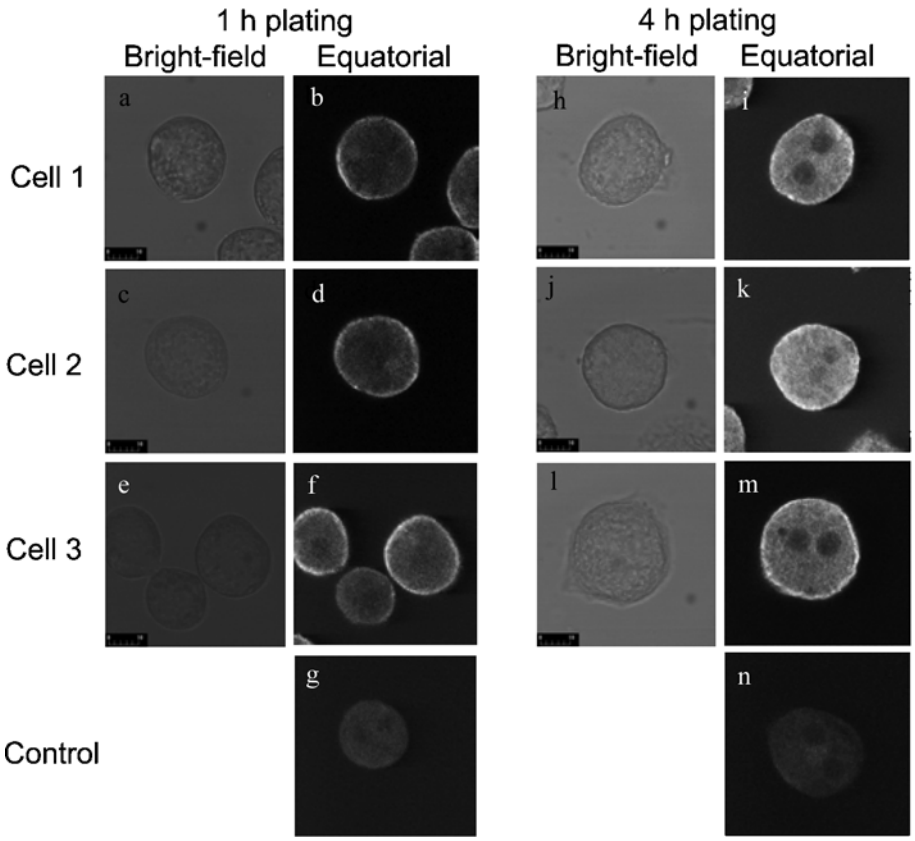

Figure 4 Localization by immunofluorescence of the Ins(1,4,5)P_3R_2 in rat hepatocytes grown for 1 and 4 h in primary culture

The culture of rat hepatocytes, permeabilization and cell fixation, staining with anti-Ins(1,4,5)P_3 antibody, and confocal microscopy were performed as described in the Materials and methods section. Cells were stained with mouse monoclonal anti-Ins(1,4,5)P_3R_2 antibody KM1083 and anti-mouse antibody conjugated to Cy3. The results shown are those obtained for one of three experiments employing three separate rat hepatocyte preparations which each gave similar results. The scale bar represents 10 μm. Panels a–f represent different cells showing the bright-field images (a, c and e) and immunofluorescence (equatorial sections) (b, d and f) for cells cultured at 1 h. Panels h–m represent different cells showing the bright-field images (h, j and l) and immunofluorescence (equatorial sections) (i, k and m) for cells cultured at 4 h. Panels g and n are controls in which the primary antibody has been omitted.

cultured under conditions similar to those employed for the measurement of Ca^{2+} inflow (single cells attached to collagen-coated glass slides and cultured for 2 h). At this stage of culture, the cells exhibit little polarity [15,36]. Some co-location of Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 at the periphery of the cell may be due to the formation of Ins(1,4,5)P_3R_1–Ins(1,4,5)P_3R_2 hetero-tetramers [44]. It is interesting to note that while Ins(1,4,5)P_3R_1 and Ins(1,4,5)P_3R_2 were both found concentrated near the plasma membrane in hepatocytes cultured for 2 h, the ER was found distributed throughout the cytoplasmic space. This suggests that, for cells in this *in vitro* condition, the majority of the ER has a very low density of Ins(1,4,5)P_3R. Previous histochemical studies with freshly isolated hepatocytes (unpolarized), hepatocyte couplets (partially polarized) and hepatocytes *in situ* (completely polarized) have shown that Ins(1,4,5)P_3R_1 is located principally near the plasma membrane, while Ins(1,4,5)P_3R_2 is located at the plasma membrane and bile canaliculus [17–19]. Moreover, Ins(1,4,5)P_3R_2 located around the bile canaliculus appears to be responsible for the initiation of Ca^{2+} waves [17]. Studies employing subcellular fractionation and electron microscopy have provided clear evidence that, in liver cells, some Ins(1,4,5)P_3R_1s are located in regions of the ER that are closely associated with the plasma membrane through F-actin [18,19]. The present immunofluorescence results showing Ins(1,4,5)P_3R_1 at the cell periphery are consistent with these results. Thus the role of Ins(1,4,5)P_3R_1 in apparently selectively activating Ca^{2+} inflow is associated with the location of Ins(1,4,5)P_3R_1 close to the plasma membrane.

Taken together, the present results with Ins(1,4,5)P_3 analogues selective for either Ins(1,4,5)P_3R_1 or Ins(1,4,5)P_3R_2, the previous results using an anti-Ins(1,4,5)P_3R_1 antibody to inhibit Ins(1,4,5)P_3 function and adenophostin A to activate Ca^{2+} inflow [9], and the evidence that some Ins(1,4,5)P_3R_1s are closely associated with the plasma membrane [18,19], suggest that, in hepatocytes, the activation of SOCs requires the release of Ca^{2+} from a small region of the ER close to the plasma membrane and enriched in Ins(1,4,5)P_3R_1. The requirement for Ins(1,4,5)P_3R_1 for the activation of SOCs in hepatocytes may simply reflect enrichment of those putative subregions of the ER involved in the activation of SOCs with Ins(1,4,5)P_3R_1. It is hypothesized in the present paper that the role of Ins(1,4,5)P_3R_1 is solely to mediate release of Ca^{2+} from the ER. At this stage, there is no evidence for direct interaction between the Ins(1,4,5)P_3R_1 protein and the SOC protein in hepatocytes. *In vivo*, where the activation of SOCs is initiated by Ins(1,4,5)P_3 generated by the hormone-induced activation of phospholipase C, additional factors will likely also influence the relative ability of Ins(1,4,5)P_3 to activate SOCs and release $Ca^{2+}$from intracellular stores. These include the rate of metabolism of Ins(1,4,5)P_3 [6], the effects of Ca^{2+} on the affinity of Ins(1,4,5)P_3 for Ins(1,4,5)P_3Rs [45] and the locations

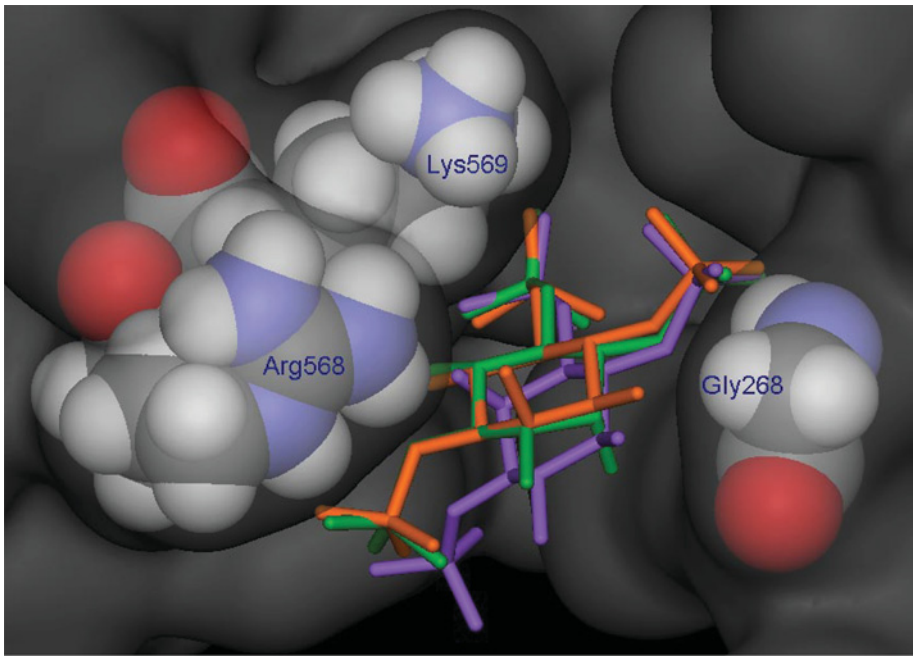

Figure 5 Structure of the Ins(1,4,5)P_3R$_1$-binding site based on the X-ray crystal structure of the mouse Ins(1,4,5)P_3R$_1$ core binding domain

The crystallographically observed position of bound Ins(1,4,5)P_3 [39] is shown in orange. Molecular docking experiments suggest that Ins(1,4,6)P_3 (green) may be a relatively effective mimic of Ins(1,4,5)P_3 at the Ins(1,4,5)P_3R$_1$-binding site because it can bind in an orientation that allows its phosphate groups to mimic the three phosphate groups of Ins(1,4,5)P_3, while its axial 2-hydroxy group is accepted by an open region close to Gly^{268}. However, Ins(1,3,6)P_3 (purple) may be prevented from adopting a similar binding mode due to unfavourable steric interactions of its axial 2-hydroxy group with Arg^{568} and Lys^{569}. Molecular-docking experiments were carried out using the X-ray crystal structure of the Ins(1,4,5)P_3-binding core of mouse type 1 InsP_3R in complex with Ins(1,4,5)P_3 (PDB code 1N4K [39]) according to methods previously described [43]. For clarity, six molecules of water included in the docking experiments, and the hydrogen atoms of Ins(1,4,5)P_3, Ins(1,4,6)P_3 and Ins(1,3,6)P_3 are not shown.

of hormone receptors and phospholipase C in relation to that of Ins(1,4,5)P_3Rs [46,47].

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