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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Short Title: Inositol trisphosphate receptors and store-operated Ca$^{2+}$ channels

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SUMMARY

Previous studies have shown that adenophostin A is a potent initiator of the activation of store-operated Ca\(^{2+}\) channels (SOCs) in rat hepatocytes, and have suggested that, of the two subtypes of inositol trisphosphate (Ins(1,4,5)P\(_3\)) receptor predominantly present in rat hepatocytes (types 1 and 2 (Ins(1,4,5)P\(_3\)R\(_{1}\) and Ins(1,4,5)P\(_3\)R\(_{2}\))), Ins(1,4,5)P\(_3\)R\(_{1}\)s are required for SOC activation. We compared the abilities of Ins(1,4,6)P\(_3\) (higher apparent affinity for Ins(1,4,5)P\(_3\)R\(_{1}\)) and Ins(1,3,6)P\(_3\) and Ins(1,2,4,5)P\(_4\) (higher apparent affinities for Ins(1,4,5)P\(_3\)R\(_{2}\)) 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\(_3\)R\(_{1}\) and Ins(1,4,5)P\(_3\)R\(_{2}\) 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\(_3\)R\(_{1}\)s were found predominantly at the cell periphery whereas Ins(1,4,5)P\(_3\)R\(_{2}\)s were found at the cell periphery, the cell interior and nucleus. It is concluded that the idea that a small region of the ER enriched in Ins(1,4,5)P\(_3\)R\(_{1}\) is required for the activation of SOCs is consistent with the present results for hepatocytes.

Keywords: Store-operated Ca\(^{2+}\) channels, endoplasmic reticulum, inositol trisphosphate receptors.

Abbreviations used: Ins(1,4,5)P\(_3\), inositol 1,4,5-trisphosphate; Ca\(^{2+}\)\(_{o}\), extracellular Ca\(^{2+}\); Ins(1,4,5)P\(_3\)R, InsP\(_3\) receptor; ER, endoplasmic reticulum; [Ca\(^{2+}\)]\(_{cyt}\), cytoplasmic Ca\(^{2+}\) concentration.
INTRODUCTION

Store-operated Ca\(^{2+}\) channels (SOCs) in the plasma membrane are required for regulation of the cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{cyt}}\)]) and re-filling the endoplasmic reticulum (ER) 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\(^{2+}\)\(_{\text{cyt}}\)] [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 inositol 1,4,5-trisphosphate (Ins(1,4,5)P\(_3\)) at Ins(1,4,5)P\(_3\) receptors (Ins(1,4,5)P\(_3\)R) 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\(_3\)Rs 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 polarised 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\(_3\)R\(_1\) and Ins(1,4,5)P\(_3\)R\(_2\) with very little type 3 Ins(1,4,5)P\(_3\) receptor [16,17]. Ins(1,4,5)P\(_3\)R\(_2\) predominate, and may be responsible for the generation of [Ca\(^{2+}\)\(_{\text{cyt}}\)] waves emanating from the canalicular region [17]. There is evidence that Ins(1,4,5)P\(_3\)R\(_1\)s 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\(_3\)R, 2-hydroxyethyl-\(\alpha\)-D-glucopyranoside 2,3',4'-trisphosphate (Glc(2,3',4')P\(_3\)), and an antibody against Ins(1,4,5)P\(_3\)R\(_1\) which inhibits Ins(1,4,5)P\(_3\)R function, suggested that a subregion of the ER and the Ins(1,4,5)P\(_3\)R\(_1\) are both required for the activation of SOCs [9]. Adenophostin A has been used to investigate the role of Ins(1,4,5)P\(_3\)R 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 sub-region of the ER is required for SOC activation [7].

The aim of the present studies was to test further the roles of Ins(1,4,5)P$_3$R$_1$ and Ins(1,4,5)P$_3$R$_2$ 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$_3$R$_1$ and Ins(1,4,5)P$_3$R$_2$ [21] to initiate intracellular Ca$^{2+}$ release and Ca$^{2+}$ inflow. The intracellular locations of Ins(1,4,5)P$_3$R$_1$ and Ins(1,4,5)P$_3$R$_2$ under 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$_3$R$_1$ is involved in the activation of SOCs.
MATERIALS AND METHODS

Materials

\(^{1,3,6}\)P3 \[22\], \(^{1,4,6}\)P3 \[23\], and \(^{1,2,4,5}\)P4 \[24\] were synthesised as described previously. \(^{2-}\)Deoxy-\(^{1,4,5}\)P3 (AM Riley and BVL Potter unpublished) was synthesized from \(^{3,6}\)-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 homogenous by routine spectroscopic methods. The lyophilised form of each \(^{1,4,5}\)P3 analogue was reconstituted in water, washed through 0.5 ml Chelex-100 resin (to replace the tri-ethylammonium cation with Na\(^+\)) and again lyophilised. Stock solutions were prepared by dissolving the lyophilised \(^{1,4,5}\)P3 analogue in 125 mM KCl. Monoclonal antibodies KM1112 and KM1083 specific for \(^{1,4,5}\)P3R1 and \(^{1,4,5}\)P3R2, respectively \[26\] were kindly provided by Professor K Mikoshiba, University of Tokyo. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Sigma. Nitrocellulose and PVDF membranes and ECL detection reagents were provided by Amersham. SaOS-2 human osteosarcoma cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were kindly provided by Dr T.J. McCann, Babraham Institute, Cambridgeshire, UK, and L15 mouse fibroblasts \[27\] by Professor K. Mikoshiba, University of Tokyo, Japan. SaOS-2 cells \[28\] and L15 mouse fibroblasts \[29\] were cultured as described previously.

Isolation of hepatocytes and measurement of the cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]c)

The isolation of hepatocytes from Hooded Wistar rats, attachment of hepatocytes to collagen-coated coverslips, the microinjection of fura-2 and \(^{1,4,5}\)P3 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\(_{340\text{nm}}\)/F\(_{380\text{nm}}\)). 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 \(^{1,4,5}\)P3 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 \(^{1,4,5}\)P3 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 measured.
Rates of Ca\textsuperscript{2+} inflow in single hepatocytes were estimated following the addition of extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}\textsubscript{o}) to cells incubated in the absence of added Ca\textsuperscript{2+}\textsubscript{o} (the “Ca\textsuperscript{2+} add-back” protocol) [9] from measurement of the initial rate of the Ca\textsuperscript{2+}-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} (expressed as change in fluorescent ratio units per min). Since the amount of Ca\textsuperscript{2+} which accumulates in the cytoplasmic space in a Ca\textsuperscript{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\textsuperscript{2+}]\textsubscript{cyt} is a more accurate reflection of the rate of Ca\textsuperscript{2+} inflow through SOCs than the value of the subsequent plateau in [Ca\textsuperscript{2+}]\textsubscript{cyt} (underlying assumptions discussed in [30]).

To estimate the amount of Ca\textsuperscript{2+} released from intracellular stores by a given Ins(1,4,5)P\textsubscript{3} analogue, vasopressin (40 nM) was added to cells loaded with the Ins(1,4,5)P\textsubscript{3} analogue plus fura-2, or with fura-2 alone. The amount of Ca\textsuperscript{2+} released by the Ins(1,4,5)P\textsubscript{3} analogue was calculated as the difference between the vasopressin-induced release of Ca\textsuperscript{2+} measured in the absence and presence of the Ins(1,4,5)P\textsubscript{3} analogue. It has previously been shown, using EGTA to chelate extracellular Ca\textsuperscript{2+}, that the rapid increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} induced by vasopressin in the presence of extracellular Ca\textsuperscript{2+} reflects the release of Ca\textsuperscript{2+} from intracellular stores with little contribution from Ca\textsuperscript{2+} inflow across the plasma membrane [9]. This was confirmed in the present series of experiments by measuring vasopressin-induced Ca\textsuperscript{2+} release (the height of the vasopressin-induced peak of [Ca\textsuperscript{2+}]\textsubscript{cyt}) in the presence of Ca\textsuperscript{2+}\textsubscript{o} (1.5 mM) and in the presence of both Ca\textsuperscript{2+}\textsubscript{o} and EGTA (2 mM). Experiments were performed with cells microinjected with fura-2 plus Ins(1,4,6)P\textsubscript{3} (10 µM), Ins(1,2,4,5)P\textsubscript{4} (70 µM) and Ins(1,3,6)P\textsubscript{3} (110 µM). For each analogue there was no significant difference in the amount of Ca\textsuperscript{2+} released in response to vasopressin (40 nM) measured in the presence and absence of EGTA. The amounts of Ca\textsuperscript{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 ± SEM, n= 8-15) for cells containing Ins(1,4,6)P\textsubscript{3}, Ins(1,2,4,5)P\textsubscript{4}, and Ins(1,3,6)P\textsubscript{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 Biorad Immunoblot assay kit. Standard homogenisation, 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\textsubscript{3}R\textsubscript{1} and Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} were detected using mouse monoclonal anti-Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} KM1112 or mouse monoclonal anti-Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} KM1083 [26], horse anti-mouse IgG
conjugated to Cy3 as secondary antibody and ECL detection. “SeeBlue” pre-stained standards (4-250 kDa, Novex) were used as molecular weight markers. On 6% SDS-minigels, the myosin standard (250 kDa) ran very close to the Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂.

**Immunolocalisation of Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂**

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 permeabilised 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) fetal 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)P₃R₁ KM1112 or mouse monoclonal anti-Ins(1,4,5)P₃R₂ 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 BioRad MRC-1000 confocal microscope, Krypton-Argon laser, and Chroma 31002 (excitation 515-550 nm, emission 575-615 nm) filters, and x60 oil objective. The location of the ER in fixed freshly-isolated rat hepatocytes in primary culture was determined using DiOC₆(3) and confocal microscopy as described previously [9].
RESULTS

Effects of Ins(1,4,6)P₃, Ins(1,3,6)P₃ and Ins(1,2,4,5)P₄ on Ca²⁺ inflow and release

The strategy employed was to use Ins(1,4,5)P₃ analogues with different affinities for Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂ 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)P₃ (higher affinity for Ins(1,4,5)P₃R₁) and D-myo-Ins(1,3,6)P₃ and Ins(1,2,4,5)P₄ (higher affinity for Ins(1,4,5)P₃R₂) [21]. The abilities of D-myo-Ins(1,4,6)P₃, Ins(1,3,6)P₃ and Ins(1,2,4,5)P₄, to activate Ca²⁺ release and Ca²⁺ 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 Ca²⁺o, then Ca²⁺o was added (to allow an estimate of the rate of Ca²⁺ inflow) followed by vasopressin (to release Ca²⁺ 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)P₃ are shown in Figure 1A, and those for a control cell, microinjected with fura-2 alone, in Figure 1B. The amount of Ca²⁺ released from the ER by the Ins(1,4,5)P₃ analogue was estimated by determining the difference between the Ca²⁺ released by vasopressin in the control cell and that released by vasopressin in the cell loaded with the Ins(1,4,5)P₃ analogue. (The rationale for this procedure is discussed in more detail in Materials and Methods.)

The dose-response curve for the effect of Ins(1,3,6)P₃ on Ca²⁺ release and Ca²⁺ inflow is shown in Figure 2A. This shows that the concentration of Ins(1,3,6)P₃ which gave half-maximal stimulation of Ca²⁺ inflow is substantially higher than that which gave half-maximal stimulation of Ca²⁺ release. A similar dose-response pattern but with less pronounced differences, was observed with Ins(1,2,4,5)P₄ (Figure 2B). D-2-deoxy-Ins(1,4,5)P₃ (higher affinity for Ins(1,4,5)P₃R₂ than Ins(1,4,5)P₃R₁ [21]) was also tested. However, the microinjection of D-2-deoxy-Ins(1,4,5)P₃ had no effect on either Ca²⁺ release or Ca²⁺ inflow, most likely due to its rapid metabolism in liver cells [35].

In contrast to the results obtained with Ins(1,3,6)P₃ and Ins(1,2,4,5)P₄, when the experiment was conducted with Ins(1,4,6)P₃ (Figure 2C), the concentration of Ins(1,4,6)P₃ which gave half-maximal stimulation of Ca²⁺ inflow was found to be lower than that which gave half-maximal stimulation of Ca²⁺ release. Thus, relative to the ability to induce Ca²⁺ release, Ins(1,4,6)P₃ is more effective than Ins(1,3,6)P₃ in inducing Ca²⁺ inflow.
Intracellular distribution of Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂ under conditions employed for the measurement of Ca²⁺ inflow

The intracellular locations of Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂ were determined using antibodies specific for these proteins and immunofluorescence. The specificity of the antibodies for Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂ was confirmed by Western blot analysis using extracts of L15 mouse fibroblasts [27], which express Ins(1,4,5)P₃R₁ but do not express significant levels of Ins(1,4,5)P₃R₂ [29], and extracts of SaOS-2 human osteoblasts, which predominantly express Ins(1,4,5)P₃R₂ and Ins(1,4,5)P₃R₃ [28]. A single band corresponding to the expected size of the Ins(1,4,5)P₃R 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)P₃R₁ or Ins(1,4,5)P₃R₂ (results not shown).

In immunofluorescence experiments with hepatocytes cultured for 2 h under conditions similar to those employed for the measurement of Ca²⁺ inflow and release, Ins(1,4,5)P₃R₁ 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)P₃R₂ 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)P₃R₂ was seen (results not shown). Panels b, f and j of each of Figures 3A and 3B 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 on 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-polarisation affects the intracellular distribution of Ins(1,4,5)P₃R, the distribution of Ins(1,4,5)P₃R₂ was determined at 1 and 4 h after the initiation of cell culture. Ins(1,4,5)P₃R₂ was studied because it gave a more intense immunofluorescence signal than that generated by Ins(1,4,5)P₃R₁. Images obtained in the equatorial plane are shown in Figure 4. There was a significant increase in total immunofluorescence due to Ins(1,4,5)P₃R₂ at 4 h, possibly due to increased synthesis and/or decreased degradation of Ins(1,4,5)P₃R, although there was no change in the intracellular distribution of Ins(1,4,5)P₃R₂ (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 this study is the observation that the relationship between the dose-response curves for Ca\textsuperscript{2+} inflow and Ca\textsuperscript{2+} release for each of Ins(1,3,6)P\textsubscript{3} and Ins(1,2,4,5)P\textsubscript{4} differs from that for Ins(1,4,6)P\textsubscript{3}. Thus Ins(1,4,6)P\textsubscript{3} which, on the basis of studies conducted with Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} and Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} expressed in insect Sf9 cells [21], has a higher affinity for Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} than Ins(1,4,5)P\textsubscript{3}R\textsubscript{2}, activated Ca\textsuperscript{2+} inflow at lower concentrations than those which induced significant Ca\textsuperscript{2+} release. By contrast, Ins(1,3,6)P\textsubscript{3} and Ins(1,2,4,5)P\textsubscript{4}, each of which has a higher affinity for Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} than Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} [21], induced Ca\textsuperscript{2+} release at lower concentrations than those which activated substantial Ca\textsuperscript{2+} inflow. These results suggest that, in intact hepatocytes, the binding of Ins(1,4,5)P\textsubscript{3} to Ins(1,4,5)P\textsubscript{3}R\textsubscript{1}s is more effective in activating SOCs than the binding of Ins(1,4,5)P\textsubscript{3} to Ins(1,4,5)P\textsubscript{3}R\textsubscript{2}s.

It is recognised that there are a number of assumptions made in interpretation of the dose-response curves for Ins(1,4,5)P\textsubscript{3} analogues reported here. Firstly, the measured difference in apparent affinity of a given analogue for Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} and Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} is relatively small [21]. Secondly, it is assumed that Ins(1,3,6)P\textsubscript{3} and Ins(1,4,6)P\textsubscript{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\textsubscript{3} analogue remains approximately constant during the period over which Ca\textsuperscript{2+} inflow and Ca\textsuperscript{2+} release are measured. Thirdly, it was not possible to raise the intracellular concentrations of Ins(1,3,6)P\textsubscript{3} and Ins(1,2,4,5)P\textsubscript{4} to saturate the Ins(1,4,5)P\textsubscript{3}R. Fourthly, the amount of Ca\textsuperscript{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\textsubscript{3}R\textsubscript{1} and Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} is quite striking.

Previously, the differences in affinities of Ins(1,3,6)P\textsubscript{3}, Ins(1,4,6)P\textsubscript{3} and Ins(1,4,5)P\textsubscript{3} for Ins(1,4,5)P\textsubscript{3}R\textsubscript{s} have been rationalised by comparing their molecular structures in diagrams which 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\textsubscript{3}R\textsubscript{1} binding domain can be used to extend this model by suggesting how the relative affinities of Ins(1,4,6)P\textsubscript{3} and Ins(1,3,6)P\textsubscript{3} for Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} may be related to their abilities to mimic Ins(1,4,5)P\textsubscript{3} in the context of the Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} binding site (Figure 5). An equivalent crystal structure for an Ins(1,4,5)P\textsubscript{3}R\textsubscript{2} domain is not yet available, but it is interesting to note that the residues of Ins(1,4,5)P\textsubscript{3}R\textsubscript{1} that interact with Ins(1,4,5)P\textsubscript{3} are all conserved in Ins(1,4,5)P\textsubscript{3}R\textsubscript{2}, with the
exception that Gly-268 in Ins(1,4,5)P₃R₁ is replaced by leucine in the Ins(1,4,5)P₃R₂ sequence in both the mouse [40] and rat [41] (reviewed in [42]) Ins(1,4,5)P₃R₂ 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)P₃R₂ binding site, perhaps with consequences for the relative affinities of Ins(1,4,5)P₃, Ins(1,4,6)P₃, Ins(1,3,6)P₃ and also Ins(1,2,4,5)P₄.

Both Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂ were found to be predominantly located at the periphery of the cell in rat hepatocytes cultured under conditions similar to those employed for the measurement of Ca²⁺ 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)P₃R₁ and Ins(1,4,5)P₃R₂ at the periphery of the cell may be due to the formation of Ins(1,4,5)P₃R₁/Ins(1,4,5)P₃R₂ heterotetramers [44]. It is interesting to note that while Ins(1,4,5)P₃R₁ and Ins(1,4,5)P₃R₂ 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)P₃R. Previous histochemical studies with freshly-isolated hepatocytes (unpolarised), hepatocyte couplets (partially polarised) and hepatocytes in situ (completely polarised) have shown that Ins(1,4,5)P₃R₁ is located principally near the plasma membrane while Ins(1,4,5)P₃R₂ is located at the plasma membrane and bile canaliculus [17-19]. Moreover, Ins(1,4,5)P₃R₂ located around the bile canaliculus appear to be responsible for the initiation of Ca²⁺ waves [17]. Studies employing subcellular fractionation and electron microscopy have provided clear evidence that in liver cells some Ins(1,4,5)P₃R₁ are located in regions of the ER which are closely associated with the plasma membrane through F-actin [18,19]. The present immunofluorescent results showing Ins(1,4,5)P₃R₁ at the cell periphery are consistent with these results. Thus, the role of Ins(1,4,5)P₃R₁ in apparently selectively activating Ca²⁺ inflow is associated with the location of Ins(1,4,5)P₃R₁ close to the plasma membrane.

Taken together, the present results with Ins(1,4,5)P₃ analogues selective for either Ins(1,4,5)P₃R₁ or Ins(1,4,5)P₃R₂, the previous results using an anti-Ins(1,4,5)P₃R₁ antibody to inhibit Ins(1,4,5)P₃ function and adenophostin A to activate Ca²⁺ inflow [9], and the evidence that some Ins(1,4,5)P₃R₁ are closely associated with the plasma membrane [18,19], suggest that, in hepatocytes, the activation of SOCs requires the release of Ca²⁺ from a small region of the ER close to the plasma membrane and enriched in Ins(1,4,5)P₃R₁. The requirement for Ins(1,4,5)P₃R₁ 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)P₃R₁. It is hypothesised here that the role of Ins(1,4,5)P₃R₁ is solely to mediate release of Ca²⁺ from the ER. At this stage there is no evidence for direct interaction between the Ins(1,4,5)P₃R₁ protein and the SOC protein in hepatocytes. *In vivo*, where the activation of SOCs is initiated by Ins(1,4,5)P₃ generated by the hormone-induced activation of phospholipase C, additional factors will likely also influence the relative ability of Ins(1,4,5)P₃ to activate SOCs and release Ca²⁺ from intracellular stores. These include the rate of metabolism of Ins(1,4,5)P₃ [6], the effects of Ca²⁺ on the affinity of Ins(1,4,5)P₃ for Ins(1,4,5)P₃Rs [45] and the locations of hormone receptors and phospholipase C in relation to that of Ins(1,4,5)P₃Rs [46,47].
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LEGENDS TO FIGURES

Figure 1  Effects of Ins(1,3,6)P₃ on Ca²⁺ inflow and the vasopressin-induced increase in [Ca²⁺]ₘₐₓ in single rat hepatocytes

Single hepatocytes were injected with fura-2 plus 220 µM Ins(1,3,6)P₃ (estimated intracellular concentration) (A) or fura-2 alone (B) 10 min before beginning the measurement of fluorescence. Additions of Ca²⁺ (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 2-7 separate hepatocyte preparations.

Figure 2  Comparison of concentration-response curves for the effects of Ins(1,3,6)P₃, Ins(1,2,4,5)P₄ and Ins(1,4,6)P₃ on the release of Ca²⁺ from intracellular stores and on Ca²⁺ inflow

Ins(1,3,6)P₃ (A), Ins(1,2,4,5)P₄ (B) or Ins(1,4,6)P₃ (C) was co-injected into single hepatocytes with fura-2 10 min before beginning the measurement of fluorescence, and Ca²⁺ and vasopressin were added to the incubation medium as described in Figure 1. Rates of Ca²⁺ inflow (■) and amounts of Ca²⁺ release (●) were estimated as described in Materials and Methods. Each data point is the mean ± S.E.M. of the values obtained from 17-97 individual hepatocytes from 1-7 separate hepatocyte preparations.

Figure 3  Localisation by immunofluorescence of the type I (A) and type II (B) Ins(1,4,5)P₃ receptors in rat hepatocytes grown for 2 h in primary culture

The culture of rat hepatocytes, permeabilisation and cell fixation, staining with anti-Ins(1,4,5)P₃ antibody, and confocal microscopy were performed as described in Materials and Methods. (A) Cells stained with mouse monoclonal anti-Ins(1,4,5)P₃R₁ antibody KM1112 and anti-mouse antibody conjugated to Cy3. (B) Cells stained with mouse monoclonal anti-Ins(1,4,5)P₃R₂ antibody KM1083 and anti-mouse antibody conjugated to Cy3. The results shown are those obtained for one of 3 experiments employing 3 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 bright field image, 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 4  Localisation by immunofluorescence of the type II Ins(1,4,5)P₃ receptor in rat hepatocytes grown for 1 and 4 h in primary culture

The culture of rat hepatocytes, permeabilisation and cell fixation, staining with anti-Ins(1,4,5)P₃ antibody, and confocal microscopy were performed as described in Materials and Methods. Cells were stained with mouse monoclonal anti-Ins(1,4,5)P₃R2 antibody KM1083 and anti-mouse antibody conjugated to Cy3. The results shown are those obtained for one of 3 experiments employing 3 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, e) and immunofluorescence (equatorial sections) (b, d, f) for cells cultured at 1 h. Panels h-m represent different cells showing the bright field images (h, j, l) and immunofluorescence (equatorial sections) (i, k, m) for cells cultured at 4 h. Panels g and n are controls in which the primary antibody has been omitted.

Figure 5  Structure of the Ins(1,4,5)P₃R₁ binding site based on the x-ray crystal structure of the mouse Ins(1,4,5)P₃R₁ core binding domain

The crystallographically-observed position of bound Ins(1,4,5)P₃ [39] is shown in orange. Molecular docking experiments suggest that Ins(1,4,6)P₃ (green) may be a relatively effective mimic of Ins(1,4,5)P₃ at the Ins(1,4,5)P₃R₁ 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)P₃, while its axial 2-hydroxyl group is accepted by an open region close to Gly-268. However, Ins(1,3,6)P₃ (purple) may be prevented from adopting a similar binding mode due to unfavourable steric interactions of its axial 2-hydroxyl 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)P₃-binding core of mouse type 1 InsP₃R in complex with Ins(1,4,5)P₃ (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)P₃, Ins(1,4,6)P₃ and Ins(1,3,6)P₃ are not shown.
Figure 2

Graph A: 
- X-axis: log[Ins(1,3,6)P_3] (µM)
- Y-axis: Inflow rate (Δratio/min) or Ca^{2+} release (Δratio)
- Arrows indicate Release and Inflow rate

Graph B: 
- X-axis: log[Ins(1,2,4,5)P_4] (µM)
- Y-axis: Inflow rate (Δratio/min) or Ca^{2+} release (Δratio)
- Arrows indicate Release and Inflow rate

Graph C: 
- X-axis: log[Ins(1,4,6)P_3] (µM)
- Y-axis: Inflow rate (Δratio/min) or Ca^{2+} release (Δratio)
- Arrows indicate Release
Figure 3A
Figure 3B

Bright-field | Equatorial | Equatorial + 3 µm | Equatorial - 3 µm
---|---|---|---
Cell 1 | ![Image](a) | ![Image](b) | ![Image](c) | ![Image](d)
Cell 2 | ![Image](e) | ![Image](f) | ![Image](g) | ![Image](h)
Cell 3 | ![Image](i) | ![Image](j) | ![Image](k) | ![Image](l)
Controls (equatorial) | ![Image](m) | ![Image](n)
Figure 4

1 h plating
Bright-field Equatorial

Cell 1

Cell 2

Cell 3

Control

4 h plating
Bright-field Equatorial

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Figure 5