RESEARCH COMMUNICATION

Rapid kinetic measurements of $^{45}Ca^{2+}$ mobilization reveal that Ins(2,4,5)P_3 is a partial agonist at hepatic InsP_3 receptors

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Ins(2,4,5)P_3, a metabolically stable analogue of Ins(1,4,5)P_3, is widely used in analyses of Ca^{2+} signalling pathways, but its utility depends upon it faithfully mimicking the effects of the natural messenger, Ins(1,4,5)P_3, at InsP_3 receptors. To compare the kinetics of InsP_3-evoked $^{45}Ca^{2+}$ mobilization, Ins(1,4,5)P_3- and Ins(2,4,5)P_3-stimulated $^{45}Ca^{2+}$ release from the intracellular stores of permeabilized rat hepatocytes was measured using rapid superfusion. Both Ins(1,4,5)P_3 and Ins(2,4,5)P_3 caused concentration-dependent increases in the rate of $^{45}Ca^{2+}$ efflux, which accelerated towards a peak and then abruptly switched to a bi-exponentially decaying release rate. However, the peak rate of $^{45}Ca^{2+}$ mobilization evoked by maximal concentrations of Ins(2,4,5)P_3 was only $65 \pm 3\%$ ($n = 3$) of that evoked by Ins(1,4,5)P_3. Furthermore, Ins(2,4,5)P_3 inhibited the peak rate of $^{45}Ca^{2+}$ efflux evoked by Ins(1,4,5)P_3. These results indicate that Ins(2,4,5)P_3 is a partial agonist at hepatic Ins(1,4,5)P_3 receptors. Additionally, responses to Ins(2,4,5)P_3 were less positively co-operative [Hill coefficient (h) = 1.9 ± 0.3] than were those to Ins(1,4,5)P_3 ($h = 3.0 \pm 0.2$) and the kinetics of termination of $^{45}Ca^{2+}$ mobilization were slower. The lesser efficacy of Ins(2,4,5)P_3 may account for the lower cooperativity in the responses it evokes, the slower inactivation of InsP_3 receptors and the characteristic patterns of Ca^{2+} spiking it evokes in intact cells.

INTRODUCTION

Ins(1,4,5)P_3 receptors are intracellular Ca^{2+} channels that mediate the initial mobilization of intracellular Ca^{2+} stores evoked by receptors that stimulate polyphosphoinositide hydrolysis, and the subsequent cycles of Ca^{2+} release that generate Ca^{2+} waves and spikes [1]. In keeping with its role as an intracellular messenger, Ins(1,4,5)P_3 is rapidly metabolized by enzymes that either dephosphorylate it to Ins(1,4)P_2, which is inactive, or phosphorylate it to Ins(1,3,4,5)P_4, the role of which is uncertain [2,3]. Active analogues of Ins(1,4,5)P_3 that are resistant to inactivation by these enzymes [4,5] are widely used and are of proven utility. Both Ins(1,4,5)PS_3 (a trisphosphorothioate analogue of InsP_3) and Ins(2,4,5)P_3 have been used to distinguish the roles of InsP_3 and InsP_4 in regulating Ca^{2+} entry [2,6–8] and to provide stable levels of InsP_3 receptor activation in both intact [9–11] and permeabilized cells [12–14]. Although these analogues bind to InsP_3 receptors and evoke Ca^{2+} mobilization, they differ subtly from Ins(1,4,5)P_3 in both their characteristics of binding [15] and in the patterns of Ca^{2+} spiking they evoke [6,8,16]. The reasons for these differences are not understood, but one possibility is that Ins(2,4,5)P_3 and Ins(1,4,5)PS_3 are partial agonists of the InsP_3 receptor [15]. Previous measurements of the extent of Ca^{2+} release evoked by these analogues do not resolve their relative efficacies, because even partial agonists may completely empty the InsP_3-sensitive Ca^{2+} stores, but at slower rates than Ins(1,4,5)P_3 [17]. Measurements of initial rates of Ca^{2+} mobilization are essential to resolve the true efficacy of Ins(1,4,5)P_3 analogues. We have optimized a superfusion method that allows the effects of InsP_3 on unidirectional $^{45}Ca^{2+}$ efflux from the intracellular stores of permeabilized hepatocytes to be measured with high temporal resolution under conditions that effectively clamp the cytosolic [Ca^{2+}] [18]. This technique was used to compare the initial rates of Ca^{2+} release evoked by Ins(1,4,5)P_3 and Ins(2,4,5)P_3.

MATERIALS AND METHODS

Materials

D-Ins(1,4,5)P_3 was from Calbiochem. D-Ins(2,4,5)P_3, purified from bovine brain [19], was used for the experiments shown in Figure 3 and Table 2. D,L-Ins(2,4,5)P_3 was synthesized as previously reported [20] and used for the experiments shown in Figures 1 and 2. The substantial amounts of Ins(2,4,5)P_3 needed to establish concentration–effect relationships by superfusion necessitated the use of synthetic enantiomeric D,L-Ins(2,4,5)P_3 (Figures 1 and 2). To allow comparison with results obtained with D-Ins(2,4,5)P_3 alone (Figure 3 and Table 2), all concentrations are expressed in terms of the D-Ins(2,4,5)P_3 concentration. L-Ins(2,4,5)P_3 [= D-Ins(2,5,6)P_3] is at least 30-fold less active than D-Ins(2,4,5)P_3 [21] and contributes minimally to the observed responses. $^{45}Ca^{2+}$ and [^{3}H]inulin were from NEN–DuPont. All other reagents were from suppliers reported previously [22].

Measurement of $^{45}Ca^{2+}$ efflux by rapid superfusion

Isolated rat hepatocytes [22] were permeabilized by incubation with saponin (10 μg/ml) in cytosol-like medium (CLM: 140 mM KCl/20 mM NaCl/1 mM $MgCl_2$/1 mM EGTA/20 mM Pipes,

Abbreviations used: CLM, cytosol-like medium; EC_{50}, concentration causing half-maximal effect; h, Hill coefficient; PS_3, a trisphosphorothioate analogue of InsP_3.

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pH 7.0 at 37 °C). The cells (10^7/ml) were washed and loaded to steady state (1–2 nmol of $Ca^{2+}/10^6$ cells) with $^{45}Ca^{2+}$ (45 μCi/ml) by incubating them for 5 min at 37 °C in CLM supplemented with Ca^{2+} (free $[Ca^{2+}] = 200$ nM), ATP (7.5 mM), phosphocreatine (15 mM), creatine kinase (15 units/ml) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM).

Unidirectional $^{45}Ca^{2+}$ efflux from the intracellular stores of permeabilized hepatocytes was measured using a superfusion apparatus [18]. Briefly, permeabilized cells were immobilized on a filter array and mounted in a superfusion chamber that allowed rapid changes of medium (half time = 30 ± 3 ms, $n = 6$) and collection of the effluent into fractions (9–3000 ms) by means of a variable-speed fraction collector. Inclusion of [^{3}H]inulin in appropriate media allowed changes in superfusion media to be precisely related to changes in the rate of $^{45}Ca^{2+}$ efflux. The entire apparatus was maintained at 20 °C in a temperature-controlled cabinet. The steps between immobilization of the cells and initiation of the experimental protocol were complete within 20 s. Since the half-time for passive $^{45}Ca^{2+}$ efflux from the intracellular stores was 162 ± 13 s at 20 °C ($n = 3$), the $^{45}Ca^{2+}$ content of the stores at the start of the experiment was $\geqslant 92\%$ of their steady-state level. At the end of each experiment, $^{45}Ca^{2+}$ remaining within the intracellular stores was released by superfusion with CLM containing Triton X-100 (0.05 %), allowing the effects of InsP_3 to be expressed relative to the total $^{45}Ca^{2+}$ content of the intracellular stores. All results were corrected for the passive leakage of $^{45}Ca^{2+}$. The activity ($^{45}Ca^{2+}$ and ^{3}H) of each sample was determined by liquid scintillation counting in EcoScint-A scintillation cocktail.

Concentration–response relationships were fitted to a four-parameter logistic equation using a non-linear curve-fitting program (Kaleidagraph, Abeldeck Software) as previously described [23]. Computer-assisted curve-fitting (Kaleidagraph and Microsoft Excel) was used to fit exponential equations (> 200 iterations). The statistical significance of mono- and multi-exponential fits were assessed according to the 'extra sum of squares' principle [24]. All results are expressed as means ± S.E.M.

RESULTS

At each concentration of Ins(2,4,5)P_3, the rate of $^{45}Ca^{2+}$ efflux accelerated towards a peak, which was abruptly followed by a decrease in the mobilization rate (Figure 1). The time taken to attain the peak rate of $^{45}Ca^{2+}$ efflux decreased, while the maximal initial rate and the eventual extent of $^{45}Ca^{2+}$ mobilization increased with Ins(2,4,5)P_3 concentration. At all Ins(2,4,5)P_3 concentrations, the decay away from the peak rate of $^{45}Ca^{2+}$ mobilization was best fitted by a linear combination of two exponential functions ($P < 0.05$). The amplitude of the fast component (k_{fast}) increased with Ins(2,4,5)P_3 concentration from 33 ± 13 % to 70 ± 4 % ($n = 3$) at 2.5 μM and 150 μM Ins(2,4,5)P_3 respectively. The half-times of the two components (k_{fast} and k_{slow}) decreased to lower limits of 378 ± 42 ms and 2667 ± 472 ms ($n = 6$) as the Ins(2,4,5)P_3 concentration was increased to 150 μM. The maximal initial rate and the eventual extent of $^{45}Ca^{2+}$ mobilization increased with increasing concentration of Ins(2,4,5)P_3 or Ins(1,4,5)P_3 (Figure 2), indicating a quantal pattern of Ca^{2+} release [25]. Both measurements showed positive cooperativity and differed by only 2-fold in their sensitivity to each analogue. The kinetics of Ins(1,4,5)P_3- and Ins(2,4,5)P_3-stimulated $^{45}Ca^{2+}$ efflux are compared in Table 1.

Although some characteristics of Ins(2,4,5)P_3-evoked Ca^{2+} release are similar to those previously reported for Ins(1,4,5)P_3 [18], there were several significant differences. Responses to

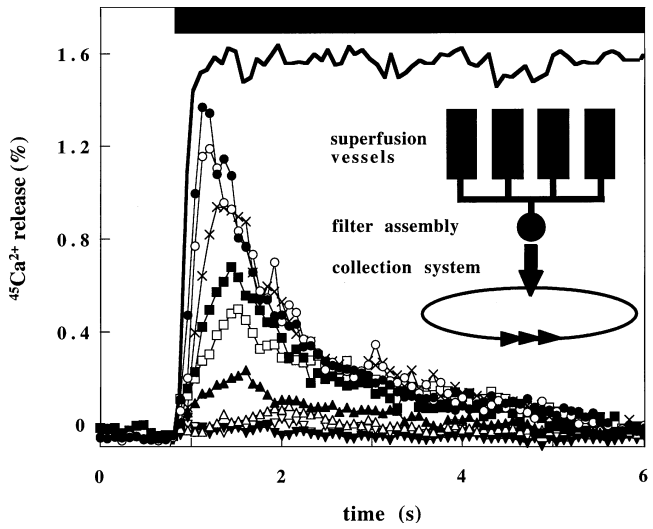

Figure 1 Rapid superfusion of permeabilized hepatocytes with Ins(2,4,5)P_3

Permeabilized hepatocytes were superfused with CLM (2 ml/s) which was switched to CLM containing various concentrations of Ins(2,4,5)P_3 for the period denoted by a solid horizontal bar. The time course of the exposure was reported by inclusion of [^{3}H]inulin in InsP_3-containing CLM (heavy line). Effluent containing the $^{45}Ca^{2+}$ released by the cells was collected at intervals of 80 ms. The concentrations of Ins(2,4,5)P_3 were: 150 μM (●), 50 μM (○), 25 μM (×), 15 μM (■), 5μ M (□), 4 μM (▲), 2.5 μM (△), 1.5 μM (▼), 0.5 μM (▽). The apparatus is depicted schematically in the inset.

Ins(2,4,5)P_3 were significantly less cooperative, both components of the decay phase were significantly slower, and the maximal peak rate of Ca^{2+} mobilization was less (Table 1). The peak rate of $^{45}Ca^{2+}$ efflux evoked by a maximal concentration of Ins(2,4,5)P_3 (300 μM) was only 65 ± 3 % ($n = 3$; $P < 0.05$) of that evoked by a maximal concentration of Ins(1,4,5)P_3 (10 μM) (Figure 3). The lesser response evoked by Ins(2,4,5)P_3 was not a consequence of a lesser receptor occupancy. By assuming that the concentration causing half-maximal effect (EC_{50}) for the peak rate of $^{45}Ca^{2+}$ efflux reflects the affinity of the active conformation of the InsP_3 receptor for its agonist, and then substituting those measurements together with our measurements of *h* into a logistic equation, we estimate that 300 μM Ins(2,4,5)P_3 and 10 μM Ins(1,4,5)P_3 will each cause > 99.8 % occupancy of InsP_3 receptors. Furthermore, increasing the Ins(2,4,5)P_3 concentration to 400 μM had no further effect (results not shown). Despite the substantially lower peak rate of $^{45}Ca^{2+}$ efflux, evoked by a maximal concentration of Ins(2,4,5)P_3, its effect on the eventual extent of $^{45}Ca^{2+}$ release was only slightly smaller than that evoked by Ins(1,4,5)P_3 (89 ± 2 %) (Figure 3, inset). These results suggest that Ins(2,4,5)P_3 is a partial agonist of InsP_3 receptors.

In order to confirm that Ins(2,4,5)P_3 is less efficacious than Ins(1,4,5)P_3, its effects on the rate of $^{45}Ca^{2+}$ mobilization evoked by a maximal concentration of Ins(1,4,5)P_3 were examined. Simultaneous application of Ins(2,4,5)P_3 (300 μM) with Ins(1,4,5)P_3 (10 μM) reduced the peak rate of $^{45}Ca^{2+}$ mobilization to 80 ± 5 % ($n = 3$) of that evoked by Ins(1,4,5)P_3 (10 μM) alone (Table 2).

DISCUSSION

Previous studies of InsP_3 analogues have almost invariably concluded that they are either full agonists or inactive [26]. The

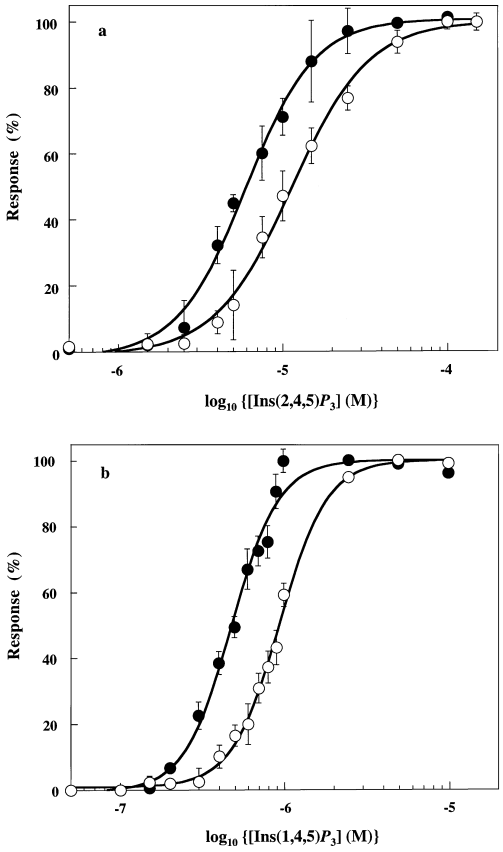

Figure 2 Concentration-dependent effects of Ins(2,4,5)P_3 and Ins(1,4,5)P_3 on the maximal rate and extent of $^{45}Ca^{2+}$ mobilization

Experiments similar to those shown in Figure 1 were used to establish the maximal rates (○) and, by integration, the eventual extents (●) of $^{45}Ca^{2+}$ release evoked by Ins(2,4,5)P_3 (**a**) and Ins(1,4,5)P_3 (**b**). Results (means ± S.E.M. of three independent experiments) are expressed as percentages of maximal responses. The absolute magnitudes of the maximal responses, which differ for the two analogues, are shown in Table 1.

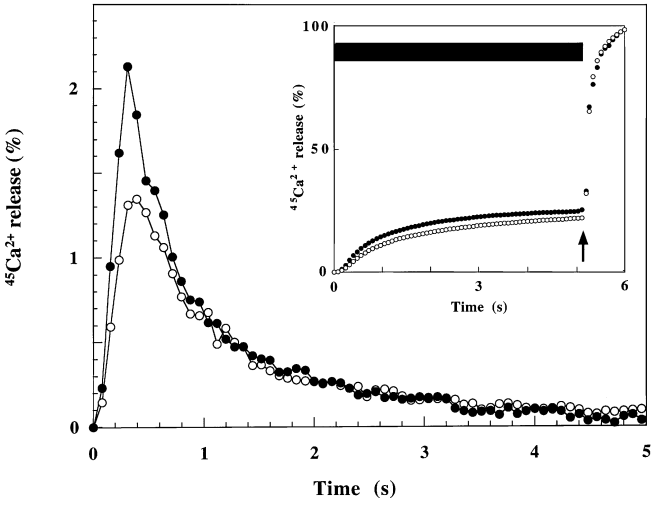

Figure 3 Comparison of the kinetics of Ins(1,4,5)P_3- and Ins(2,4,5)P_3-stimulated $^{45}Ca^{2+}$ mobilization

$^{45}Ca^{2+}$ efflux was measured at 80 ms intervals from cells superfused with CLM containing maximally effective concentrations of Ins(1,4,5)P_3 (10 μM, ●) or Ins(2,4,5)P_3 (300 μM, ○) for the entire period shown. The inset shows the cumulative $^{45}Ca^{2+}$ release evoked by each InsP_3 (solid bar) with time. Triton X-100 (0.05%) was added at the arrow.

Table 1 Kinetics of Ins(1,4,5)P_3- and Ins(2,4,5)P_3-evoked Ca^{2+} mobilization

The amount of $^{45}Ca^{2+}$ mobilized per fraction was expressed relative to the total $^{45}Ca^{2+}$ content of the intracellular stores determined by addition of Triton X-100 (see Materials and methods section). A maximally effective concentration of Ins(1,4,5)P_3 (10 μM) released 30 ± 5% (n = 10) of the intracellular stores. Both the times to the peak rate of Ca^{2+} release and the half-times of k_{fast} and k_{slow} are for maximally effective concentrations of Ins(1,4,5)P_3 (10 μM) and Ins(2,4,5)P_3 (150 μM). Results are means ± S.E.M. of at least three independent experiments.

	Ins(1,4,5)P_3	Ins(2,4,5)P_3
Extent of Ca^{2+} release		
Amplitude	100%	89 ± 2%
Amplitude, EC_{50}	477 ± 21 nM	6.0 ± 0.3 μM
h	3.0 ± 0.3	2.1 ± 0.1
Peak rate of Ca^{2+} release		
Amplitude	2.0 ± 0.2%	1.3 ± 0.1%
Peak rate, EC_{50}	941 ± 21 nM	11.3 ± 2.3 μM
h	3.0 ± 0.2	1.9 ± 0.3
Time to peak rate of Ca^{2+} release	370 ± 85 ms	314 ± 74 ms
Half-time of k_{fast}	244 ± 27 ms	378 ± 42 ms
Half-time of k_{slow}	1498 ± 280 ms	2667 ± 472 ms

Table 2 Rates and extents of $^{45}Ca^{2+}$ mobilization evoked by Ins(1,4,5)P_3 and Ins(2,4,5)P_3

Experiments similar to those shown in Figure 1 were used to assess the maximal rates and ultimate extents of $^{45}Ca^{2+}$ mobilization evoked by Ins(1,4,5)P_3 (10 μM), Ins(2,4,5)P_3 (300 μM) or by the addition of both. Results (means ± S.E.M. of three independent experiments) are expressed as percentages of those evoked by 10 μM Ins(1,4,5)P_3 alone.

	Ins(2,4,5)P_3	Ins(1,4,5)P_3 with Ins(2,4,5)P_3
Relative rate of Ca^{2+} release (%)	65 ± 3	80 ± 5
Relative extent of Ca^{2+} release (%)	89 ± 2	91 ± 8

only exceptions are the phosphorothioate analogues L-*chiro*-Ins(2,3,5)PS_3 and D-6-deoxy-Ins(1,4,5)PS_3 [17], which release 40% of the Ca^{2+} released by Ins(1,4,5)P_3, and D-3-amino-3-deoxy-Ins(1,4,5)P_3, which is a pH-dependent partial agonist [27]. Because of the quantal nature of InsP_3-evoked Ca^{2+} release, it is more likely that agonists with intermediate efficacy will be identified by measurement of the initial rates of InsP_3-evoked Ca^{2+} release [17]. By superfusion of immobilized hepatocytes, we were able to deliver InsP_3 to the intracellular Ca^{2+} stores with a half-time of 30 ms and measure unidirectional $^{45}Ca^{2+}$ efflux with a temporal resolution of 80 ms. These measurements revealed three significant differences between the kinetics of $^{45}Ca^{2+}$ mobilization evoked by Ins(1,4,5)P_3 and its commonly used stable analogue Ins(2,4,5)P_3 (Table 1). Firstly, the maximal rate of $^{45}Ca^{2+}$ efflux evoked by maximal concentrations of Ins(2,4,5)P_3 was only ~ 65% of that evoked by Ins(1,4,5)P_3 (Figure 3, Table 2), a difference that cannot be attributed to lesser receptor occupancy by the analogue. The lesser efficacy of Ins(2,4,5)P_3 was confirmed by its ability to reduce the response to a maximally effective concentration of Ins(1,4,5)P_3 (Table 2). Secondly, res-

ponses to Ins(2,4,5)P_3 were significantly less positively cooperative than those to Ins(1,4,5)P_3 (Figure 2; Table 1). Finally, both the fast and the slow component of the decay phase of the response, which may reflect InsP_3 receptor inactivation, were slower with Ins(2,4,5)P_3 than with Ins(1,4,5)P_3 (Table 1).

Hepatocytes express type II (81%) and type I (19%) Ins(1,4,5)P_3 receptors [28], but the lesser effect of Ins(2,4,5)P_3 is unlikely to reflect a selective interaction with particular subtypes. Firstly, in hepatocytes, Ins(2,4,5)P_3 completely displaces specific [^{3}H]Ins(1,4,5)P_3 binding with a Hill slope of 1 [29]. Secondly, the relative sensitivity of the rate of Ca^{2+} release to the two isomers [EC_{50} Ins(2,4,5)P_3/EC_{50} Ins(1,4,5)P_3 = 12] is similar to their relative effectiveness at displacing [^{3}H]Ins(1,4,5)P_3 from its receptor (K_d Ins(2,4,5)P_3/K_d Ins(1,4,5)P_3 = 15). Thirdly, in the cerebellum, where 99% of Ins(1,4,5)P_3 receptors are type I, the complex characteristics of Ins(2,4,5)P_3 and Ins(1,4,5)PS_3 binding have been interpreted as evidence in favour of these analogues being partial agonists [15].

Several lines of evidence have previously suggested that Ins(1,4,5)P_3 and Ins(2,4,5)P_3 exert subtly different effects on the Ins(1,4,5)P_3 receptor. Most conspicuous are the different patterns of cytosolic Ca^{2+} oscillation that each evokes after microinjection into *Xenopus* oocytes [6,8,16], an effect which is not attributable to their different susceptibilities to phosphorylation. Furthermore, whereas prior injection of Ins(1,4,5)P_3 prevented a subsequent response to Ins(2,4,5)P_3, the converse was not true [6]. These differences are consistent with our observation that Ins(2,4,5)P_3 is a partial agonist, because the rate of Ca^{2+} release is likely to profoundly influence feedback regulation of Ins(1,4,5)P_3 receptors by cytosolic Ca^{2+} [1] and may thereby mould the shape of Ca^{2+} spikes.

In conclusion, our rapid kinetic measurements have succeeded where more conventional methods have failed, by demonstrating that Ins(2,4,5)P_3 is a partial agonist of hepatic Ins(1,4,5)P_3 receptors. The lesser efficacy of Ins(2,4,5)P_3 may be responsible for the lower cooperativity of the responses, the slower rate of InsP_3 receptor inactivation and the different patterns of Ca^{2+} spiking evoked by Ins(1,4,5)P_3 and Ins(2,4,5)P_3 in intact cells.

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