Inositol 1,3,4,5-tetrakisphosphate-induced release of intracellular Ca^{2+} in SH-SY5Y neuroblastoma cells

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Inositol-polyphosphate-induced Ca^{2+} mobilization was investigated in saponin-permeabilized SH-SY5Y human neuroblastoma cells. $\text{Ins}(1,4,5)\text{P}_3$ induced a dose-related release from intracellular Ca^{2+} stores with an EC_{50} (concn. giving half-maximal effect) of $0.1\ \mu\text{M}$ and a maximal release of 70 %. $\text{Ins}(1,3,4)\text{P}_3$, $\text{DL-Ins}(1,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ did not evoke Ca^{2+} mobilization in these cells when used at concentrations up to $10\ \mu\text{M}$. However, $\text{Ins}(1,3,4,5)\text{P}_4$ was found to release Ca^{2+} in a dose-related manner, but the response was dependent on the source of $\text{Ins}(1,3,4,5)\text{P}_4$ used. When commercially available $\text{D-Ins}(1,3,4,5)\text{P}_4$ was used, the EC_{50} and maximal response values were $1\ \mu\text{M}$ and 50 % respectively, compared with values for chemically synthesized $\text{DL-Ins}(1,3,4,5)\text{P}_4$ of $2\ \mu\text{M}$ and 25 %. The enhanced maximal response of commercial $\text{D-Ins}(1,3,4,5)\text{P}_4$ was decreased by pretreatment with rat brain crude $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase and was therefore concluded to be indicative of initial $\text{Ins}(1,4,5)\text{P}_3$ contamination of the $\text{Ins}(1,3,4,5)\text{P}_4$ preparation. When metabolism of $\text{DL-Ins}(1,3,4,5)\text{P}_4$ ($10\ \mu\text{M}$) in these cells at $25\ ^\circ\text{C}$ was investigated by h.p.l.c., substantial amounts of $\text{Ins}(1,4,5)\text{P}_3$ ($0.2\ \mu\text{M}$) and $\text{Ins}(1,3,4)\text{P}_3$ ($0.8\ \mu\text{M}$) were found to be produced within 3 min. Analysis of $\text{DL-Ins}(1,3,4,5)\text{P}_4$ incubation with cells at $4\ ^\circ\text{C}$, however, indicated that metabolism had been arrested [^3H] $\text{Ins}(1,4,5)\text{P}_3$ detection limits were estimated to be approx. $0.01\ \mu\text{M}$. When chemically synthesized $\text{DL-Ins}(1,3,4,5)\text{P}_4$ and incubation conditions of low temperature were used, the Ca^{2+} -releasing properties of this compound were established to be $1\ \mu\text{M}$ and 19 % for the EC_{50} and maximal response values respectively. The results obtained strongly suggest that $\text{Ins}(1,3,4,5)\text{P}_4$ alone has the ability to release intracellular Ca^{2+} . However, in the presence of sub-maximal concentrations of $\text{Ins}(1,4,5)\text{P}_3$, Ca^{2+} release appears to be synergistic with $\text{Ins}(1,3,4,5)\text{P}_4$, but at supramaximal concentrations not even additive effects are observed.

INTRODUCTION

It is now generally accepted that $\text{D-Ins}(1,4,5)\text{P}_3$, a second messenger produced upon agonist stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis, mobilizes Ca^{2+} from intracellular stores in a variety of cell types [1,2]. This molecule is metabolized either by dephosphorylation to $\text{Ins}(1,4)\text{P}_2$ or by phosphorylation to $\text{Ins}(1,3,4,5)\text{P}_4$ via 5-phosphatase or 3-kinase activity respectively [3,4]. The former metabolite is generally believed to be inactive on Ca^{2+} homeostasis, but there is much current interest in potential messenger roles for InsP_4 .

Previous work using mouse lacrimal cells [5], Swiss-mouse 3T3 cells [6], hepatocytes [7] and sea urchin eggs [8] has suggested that $\text{Ins}(1,3,4,5)\text{P}_4$ alone has no Ca^{2+} -mobilizing activity, but in the presence of Ca^{2+} -mobilizing inositol trisphosphates it can stimulate extracellular Ca^{2+} entry. Other work, however, has demonstrated that extracellular Ca^{2+} is not necessary for this synergistic action and that $\text{Ins}(1,3,4,5)\text{P}_4$ in the presence of inositol trisphosphates may be capable of mobilizing intracellular Ca^{2+} stores [9,10]. More recently $\text{Ins}(1,3,4,5)\text{P}_4$ alone has been shown to release Ca^{2+} from an $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store in rat cerebellar and adrenal-cortical microsomes [11,12]. However, other workers have suggested that the Ca^{2+} -mobilizing activity associated with $\text{Ins}(1,3,4,5)\text{P}_4$ could in fact be due to $\text{Ins}(1,4,5)\text{P}_3$, which is produced upon $\text{Ins}(1,3,4,5)\text{P}_4$ dephosphorylation by a 3-phosphatase activity. However, this dephosphorylation appeared to be decreased or abolished when the detergent digitonin was used to permeabilize cells [13].

Here we demonstrate that $\text{Ins}(1,3,4,5)\text{P}_4$ has the ability to release intracellular Ca^{2+} from SH-SY5Y neuroblastoma cells in a dose-dependent manner. This Ca^{2+} -mobilizing activity appears to be independent of $\text{Ins}(1,3,4,5)\text{P}_4$ metabolism and is not believed

to be due to $\text{Ins}(1,4,5)\text{P}_3$ contamination of the inositol phosphate preparation.

EXPERIMENTAL

Materials

SH-SY5Y cells were initially a gift from Dr. J. L. Biedler, Sloan Kettering Institute (New York, NY, U.S.A.). Cells were cultured in 175 cm^2 flasks in Gibco minimum essential medium supplemented with 10 % (v/v) foetal-calf serum, penicillin (100 i.u./ml), streptomycin (100 $\mu\text{g}/\text{ml}$), fungizone (5 $\mu\text{g}/\text{ml}$) and 2 mM-glutamine. Cells were used when confluent.

$\text{D-Ins}(1,4,5)\text{P}_3$ was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). $\text{D-Ins}(1,3,4,5)\text{P}_4$ was purchased from Calbiochem and Boehringer Mannheim. $\text{DL-Ins}(1,3,4,5)\text{P}_4$ was synthesized from 2,6-dibenzyl-*myo*-inositol (generously given by Dr. D.C. Billington, Merck Sharp and Dohme Research, Harlow, Essex, U.K.), by using phosphorylation and deprotection methods reported for $\text{Ins}(1,4,5)\text{P}_3$ [14]. $\text{DL-Ins}(1,4,5,6)\text{P}_4$ was synthesized from $\text{DL-1,2-isopropylidene-}myo\text{-inositol}$ in a similar fashion (M. R. Hamblin & B. V. L. Potter, unpublished work). Purification of both $\text{DL-Ins}(1,3,4,5)\text{P}_4$ and $\text{DL-Ins}(1,4,5,6)\text{P}_4$ was by DEAE-Sephadex A-25 anion-exchange chromatography using a linear triethylammonium bicarbonate elution gradient (0.1–1 M, pH 8) followed by preparative h.p.l.c. using an ammonium formate gradient elution of the inositol phosphate as described by Stephens *et al.* [15]. The purified $\text{DL-Ins}(1,3,4,5)\text{P}_4$ and $\text{DL-Ins}(1,4,5,6)\text{P}_4$ were then extensively characterized by ^1H and ^{31}P n.m.r. spectroscopy. ATP, saponin, quin-2, oligomycin and Hepes were obtained from Sigma. $^{45}\text{CaCl}_2$ was purchased from Amersham International. [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ was a gift from

Abbreviation used: EC_{50} , concentration causing half-maximal effect.

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NEN DuPont U.K. D-Ins(1,3,4) P_3 and D-Ins(1,3,4,5,6) P_5 were purchased from Calbiochem. All other chemicals were obtained from Fisons or BDH.

$^{45}\text{Ca}^{2+}$ release

The method used was essentially a modification of that of Gershengorn *et al.* [16] as detailed in Strupish *et al.* [17]. Cells were harvested in 20 ml of Hepes-buffered saline (HBS), consisting of 10 mM-Hepes and 15 mM-NaCl supplemented with 0.02% (w/v) EDTA. The cell suspension was centrifuged at 500 *g* for 2 min and the resulting pellet resuspended in culture medium and incubated at 37 °C for 30 min. The cells were then centrifuged at 500 *g* for 2 min, washed twice with HBS and finally resuspended in 5 ml of 'cytosol-like' buffer, consisting of 120 mM-KCl, 5 mM-ATP, 6 mM-MgCl₂, 2 mM-KH₂PO₄, 5 mM-sodium succinate and 20 mM-Hepes. The free Ca^{2+} concentration of this solution was buffered to between 100 and 500 nM by addition of 5 μM quin-2 free acid. This subsequently allowed accurate fluorimetric monitoring of the final $[\text{Ca}^{2+}]$ to be performed.

After incubation at 25 °C for 15 min, the cells were collected by centrifugation and resuspended in 'cytosol-like' buffer containing 100 μg of saponin/ml and with a cell protein concentration of 5–7 mg/ml. After exactly 1 min the cells were centrifuged and resuspended in 'cytosol-like' buffer containing 2 μg of oligomycin/ml and 2 μCi of $^{45}\text{Ca}^{2+}$ /ml.

Cells were incubated at 25 °C for 20 min to allow loading of non-mitochondrial Ca^{2+} stores with $^{45}\text{Ca}^{2+}$; 100 μl of the cell suspension was then added to 100 μl of buffer containing inositol phosphates as appropriate. After 3 min (unless otherwise indicated) 500 μl of a silicone oil mixture [Dow-Corning 556/550 (3:2, v/v)] was added and the cells were then pelleted by centrifugation at 16000 *g* for 3 min. Buffer and oil were removed by aspiration, and tubes were allowed to drain for 30 min. The resulting cell pellets were dissolved in 100 μl of Lumasolve (May and Baker Chemicals, Dagenham, Essex, U.K.) and counted for radioactivity in 1 ml of Optiphase X scintillation fluid (Pharmacia).

Preparation of samples for h.p.l.c.

Samples for h.p.l.c. analysis were treated as detailed above for Ca^{2+} -release studies, with the substitution of unlabelled CaCl_2 for $^{45}\text{CaCl}_2$, and spiked with 60 nCi of $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$. Incubations were terminated by addition of 200 μl of 1 M-trichloroacetic acid, and the precipitate was removed by centrifugation at 16000 *g* for 3 min. A 0.3 ml sample of the supernatant was removed, washed with 5 $\times$ 1 vol. of water-saturated diethyl ether to remove excess acid, and the pH was then adjusted to 6–7 with 25 mM-NaHCO₃. Samples were diluted to 2 ml with water and subsequently analysed by h.p.l.c. Each 2 ml h.p.l.c. sample was spiked with 75 nmol of AMP, ADP, ATP, adenosine 5'-tetrphosphate and 25 nmol of GMP, GDP, GTP and guanosine 5'-tetrphosphate before analysis.

Separation of inositol phosphates was performed as described by Batty *et al.* [18]. Essentially, samples were loaded on to a Partisil SAX (10 μm) high-pressure anion-exchange column fitted with a Whatman pellicular anion-exchange precolumn. Nucleotide-marker elution was monitored by their u.v. absorption at 254 nm. Water was allowed to flow through the column for 10 min at a rate of 1 ml/min, and then over 45 min a linear gradient from 0 to 100% of 1.4 M-ammonium phosphate buffered to pH 3.7 with H₃PO₄ was passed through the column. The phosphate buffer was maintained at 100% for a further 5 min and then linearly returned to water over 1 min. The column was further washed with water for 5 min before injection of subsequent samples. Fractions of the eluate were collected at

0.2 min intervals after 39 min, and then at 1 min intervals after 45 min. Routinely, ATP was eluted with, or just before, Ins(1,3,4) P_3 , GTP just after Ins(1,3,4) P_3 , and guanosine 5'-tetrphosphate was eluted just before or with inositol tetrakis-phosphates.

Crude Ins(1,4,5) P_3 3-kinase preparation

Whole brains were removed from four male Wistar rats and homogenized for 3 $\times$ 30 s in 20 ml of preparation buffer, containing 100 mM-KCl, 25 mM-Hepes, 20 mM-NaCl and 2 mM-MgCl₂, pH 7.4. The homogenate was then filtered through cheesecloth and centrifuged at 23000 *g* for 90 min at 4 °C. The resultant supernatant was divided into 1 ml fractions, giving a stock crude kinase preparation of 6% (w/v), and stored at –70 °C until use.

Ins(1,4,5) P_3 3-kinase pretreatment of samples

Ins(1,3,4,5) P_4 was incubated in 50 mM-Tris/maleate/10 mM-ATP/20 mM-MgCl₂/0.1% (w/v) BSA, pH 7.5, at 37 °C for 30 min. The reaction was terminated by boiling for 3 min. Under these conditions, 0.5 μM -Ins(1,4,5) P_3 spiked with 6 nCi of $[^3\text{H}]\text{Ins}(1,4,5)P_3$ undergoes phosphorylation to Ins(1,3,4,5) P_4 (80 $\pm$ 10%) without significant production of inositol mono- or bis-phosphates as assessed by Dowex-formate chromatography (described by Batty *et al.* [19]).

RESULTS

Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -induced Ca^{2+} release

Fig. 1 shows the dose-response relationship of Ca^{2+} mobilization for Ins(1,4,5) P_3 and commercially obtained (D-) or chemically synthesized (DL-) Ins(1,3,4,5) P_4 . Ins(1,4,5) P_3 releases intracellular Ca^{2+} in SH-SY5Y cells, with an apparent EC₅₀ (concn. causing half-maximal effect) of approx. 0.1 μM and

Fig. 1. Ins(1,4,5) P_3 - and Ins(1,3,4,5) P_4 -induced $^{45}\text{Ca}^{2+}$ release

Permeabilized SH-SY5Y cells were preloaded with $^{45}\text{Ca}^{2+}$ and incubated for 3 min with Ins(1,4,5) P_3 (○), commercial D-Ins(1,3,4,5) P_4 (■) and chemically synthesized DL-Ins(1,3,4,5) P_4 (●). The $^{45}\text{Ca}^{2+}$ content of cells was determined with respect to cells incubated without inositol phosphates. Results are presented as means $\pm$ S.E.M. for triplicate determinations in four to eight independent experiments.

Fig. 2. Effect of 3-kinase pretreatment of $\text{Ins}(1,3,4,5)\text{P}_4$ on $^{45}\text{Ca}^{2+}$ release

Commercially obtained D- $\text{Ins}(1,3,4,5)\text{P}_4$ (□, ■) and chemically synthesized DL- $\text{Ins}(1,3,4,5)\text{P}_4$ (○, ●) were pretreated with active (■, ●) and boiled (□, ○) 3-kinase preparations before incubation with SH-SY5Y cells for assessment of $^{45}\text{Ca}^{2+}$ -releasing activity as outlined in Fig. 1. Results are presented as means $\pm$ S.E.M. for triplicate determinations in five independent experiments.

maximal release of 70% of stored Ca^{2+} . $\text{Ins}(1,3,4,5)\text{P}_4$, however, releases intracellular Ca^{2+} from these cells, but the EC_{50} and maximal response values for this compound were dependent on the source used. The maximal response to the commercially available D- $\text{Ins}(1,3,4,5)\text{P}_4$ is much greater than that for the synthetic DL compound (50% compared with 25% Ca^{2+} release respectively), although the half-maximal response is achieved by each at an $\text{Ins}(1,3,4,5)\text{P}_4$ concentration of approx. 1–2 μM . Pretreatment of commercially available D- $\text{Ins}(1,3,4,5)\text{P}_4$ with a crude 3-kinase preparation resulted in a decrease of the maximal response to about 37% Ca^{2+} release, with half-maximal response achievable at an inositol phosphate concentration of approx. 2 μM (Fig. 2). The Ca^{2+} -releasing ability of the chemically synthesized DL- $\text{Ins}(1,3,4,5)\text{P}_4$ was unaltered after pretreatment of the compound with the kinase preparation.

H.p.l.c. analysis of $\text{Ins}(1,3,4,5)\text{P}_4$ metabolism

Fig. 3 demonstrates that at 25 °C (under the experimental conditions used to study Ca^{2+} release from cells) $\text{Ins}(1,3,4,5)\text{P}_4$ is metabolized to two products that are co-eluted with $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,3,4)\text{P}_3$ appears to be the most abundantly

Fig. 3. Analysis of $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ metabolism in SH-SY5Y cells

Permeabilized cells were incubated with 10 μM unlabelled DL- $\text{Ins}(1,3,4,5)\text{P}_4$ spiked with $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ for 3 min at 25 °C (●) or 4 °C (○). $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ removal (a) and $[\text{^3H}]\text{inositol trisphosphate}$ production (b) were determined by h.p.l.c. Control $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ and $[\text{^3H}]\text{inositol trisphosphate}$ levels were assessed by pretreatment of cells with 10% (w/v) trichloroacetic acid before their incubation with the spiked $\text{Ins}(1,3,4,5)\text{P}_4$ (■). Data shown are representative of results obtained for three individual experiments.

produced inositol trisphosphate under these conditions, but $\text{Ins}(1,4,5)\text{P}_3$ is also produced in significant amounts. It can be estimated that after 3 min incubation approx. 2% of the DL- $\text{Ins}(1,3,4,5)\text{P}_4$ present is converted into $\text{Ins}(1,4,5)\text{P}_3$ and 8% to $\text{Ins}(1,3,4)\text{P}_3$, thus giving a concentration of each inositol trisphosphate of approx. 0.2 and 0.8 μM respectively. When incubations are carried out at 4 °C, however, there is no observable metabolism of DL- $\text{Ins}(1,3,4,5)\text{P}_4$ in this system (limit of detection 0.01 μM). No apparent accumulation of inositol mono- or bis-phosphate was observed under the experimental conditions used.

Effect of time and temperature on $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} mobilization

Fig. 4 shows the effect of changing incubation time on the DL- $\text{Ins}(1,3,4,5)\text{P}_4$ -dependent Ca^{2+} mobilization at 25 °C and 4 °C. Essentially, at 25 °C the inositol phosphate-induced Ca^{2+} release increases with time over 3 min and appears to reach a steady state between 3 and 4 min. At 4 °C, however, this inositol phosphate-induced Ca^{2+} release appears to be relatively independent of incubation time. Similarly, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release is unaltered by temperature and is maximal at the first time point (1 min) observed.

Fig. 5 demonstrates the concentration-dependent effects of DL- $\text{Ins}(1,3,4,5)\text{P}_4$ at 4 °C. Incubation of samples at 4 °C resulted in decreasing the maximally evoked response from 25 to 19% Ca^{2+} release, but did not significantly shift the concentration at which

Fig. 4. Time- and temperature-dependence of Ins(1,4,5) P_3 - and DL-Ins(1,3,4,5) P_4 -induced $^{45}\text{Ca}^{2+}$ release

Permeabilized cells were preloaded with $^{45}\text{Ca}^{2+}$ and incubated with 10 μM -Ins(1,4,5) P_3 (Δ , $\blacktriangle$) and 10 μM -DL-Ins(1,3,4,5) P_4 ($\circ$, $\bullet$) for between 1 and 5 min at 4 °C ($\bullet$, $\blacktriangle$) and 25 °C ($\circ$, Δ), and $^{45}\text{Ca}^{2+}$ -releasing activity was determined as given in Fig. 1. Results are presented as means $\pm$ S.E.M. of triplicate determinations in four individual experiments.

the half-maximal response occurred (2 μM) when compared with incubation of samples at 25 °C.

Comparison of effects of various inositol phosphates on Ca^{2+} mobilization

In order to investigate the possibility of synergistic Ca^{2+} mobilization between DL-Ins(1,3,4,5) P_4 and other inositol phosphates without the interference of metabolism, a range of inositol phosphates were incubated on ice in the absence or presence of DL-Ins(1,3,4,5) P_4 . Ins(1,4,5) P_3 in the absence of DL-Ins(1,3,4,5) P_4 gives a characteristic dose-dependent release of Ca^{2+} from these cells (see Table 1). However, with a maximal concentration of Ins(1,4,5) P_3 in the presence of DL-Ins(1,3,4,5) P_4 there appears to be mobilization which is not totally additive to that of the two inositol phosphates acting alone, but instead the Ca^{2+} mobilization observed is comparable with that for maximal response to Ins(1,4,5) P_3 alone. Ins(1,3,4) P_3 , the major product of DL-Ins(1,3,4,5) P_4 metabolism, DL-Ins(1,4,5,6) P_4 and Ins(1,3,4,5,6) P_5 appear to have no Ca^{2+} -mobilizing activity in these cells at 10 μM . Synergy between DL-Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 is, however, observed when a sub-maximal Ins(1,4,5) P_3 concentration (0.03 μM) is used.

DISCUSSION

It is well established that significant accumulation of intracellular Ins(1,3,4,5) P_4 can be induced by agonists in a wide variety of cell types, primarily as a result of 3-kinase-induced phosphorylation of Ins(1,4,5) P_3 [3,4,20]. The significance of this

Fig. 5. Effect of temperature on the dose-response relationship between DL-Ins(1,3,4,5) P_4 and $^{45}\text{Ca}^{2+}$ release

Permeabilized cells were preloaded with $^{45}\text{Ca}^{2+}$, incubated with various concentrations of DL-Ins(1,3,4,5) P_4 for 3 min at 4 °C ($\bullet$) or 25 °C ($\circ$), and $^{45}\text{Ca}^{2+}$ -releasing activity was determined as given in Fig. 1. Results are presented as means $\pm$ S.E.M. of triplicate determinations in four individual experiments.

Table 1. Comparison of effects of various inositol phosphates on $^{45}\text{Ca}^{2+}$ mobilization in SH-SY5Y cells

Permeabilized cells were preloaded with $^{45}\text{Ca}^{2+}$ and incubated at 4 °C for 3 min with a range of inositol phosphates in the presence (+) or absence (–) of DL-Ins(1,3,4,5) P_4 (5 μM). Results presented are means $\pm$ S.E.M. for triplicate determinations in three to five individual experiments: *significantly different as assessed by Student's t test ($P < 0.01$). N/D, not determined.

Addition		Ca^{2+} release (%)	
		– Ins P_4	+ Ins P_4
Ins(1,4,5) P_3	(3 μM)	71.5 $\pm$ 1.5	67.5 $\pm$ 3.5
Ins(1,4,5) P_3	(0.3 μM)	59.0 $\pm$ 2.0	63.5 $\pm$ 0.5
Ins(1,4,5) P_3	(0.03 μM)	23.5 $\pm$ 4.0	54.0 $\pm$ 8.0*
Ins(1,3,4,5) P_4	(5 μM)	—	19.0 $\pm$ 1.0
Ins(1,3,4,5,6) P_5	(10 μM)	4.0 $\pm$ 3.0	19.0 $\pm$ 1.0
Ins(1,3,4) P_3	(10 μM)	5.5 $\pm$ 2.0	N/D
Ins(1,4,5,6) P_4	(10 μM)	2.0 $\pm$ 5.0	N/D

is still unclear, but, considering the recent identification of specific receptor-binding sites for Ins(1,3,4,5) P_4 in several cell types [21,22], the belief that this molecule may have a role as an intracellular second messenger does not seem unreasonable.

Certainly, earlier reports have implicated $\text{Ins}(1,3,4,5)\text{P}_4$ as a trigger for Ca^{2+} entry across the plasma membrane and/or Ca^{2+} movement between intracellular Ca^{2+} stores [5,6,23].

However, with the exception of recent work on cerebellar and adrenal microsomes [11,12], $\text{Ins}(1,3,4,5)\text{P}_4$ in many systems appears to be unable to release Ca^{2+} from intracellular stores in the absence of Ca^{2+} -mobilizing inositol trisphosphates [5–8]. It was therefore important in our system to establish whether the Ca^{2+} mobilization observed was a direct action of $\text{Ins}(1,3,4,5)\text{P}_4$ alone, or alternatively was due to a combination of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$ action on intracellular Ca^{2+} stores.

Complications of this assessment were found to be twofold: first, commercially available D- $\text{Ins}(1,3,4,5)\text{P}_4$ (from more than one source) appears to be contaminated with $\text{Ins}(1,4,5)\text{P}_3$, since pretreatment with 3-kinase preparations resulted in a decreased capacity to release intracellular Ca^{2+} . Secondly, our results clearly show that saponin-permeabilized SH-SY5Y cells rapidly generate both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ from $\text{Ins}(1,3,4,5)\text{P}_4$ at 25 °C, indicating the presence of both 3- and 5-phosphatase activities under these conditions. Our data in this respect differ from those of Cullen *et al.* [13], who report 3-phosphatase activity only after electroporation, but not when detergent alone was used to permeabilize L1210 lymphoma cells. At present, little is known of the control or intracellular distribution of this enzyme, but it is possible that cell-type or detergent differences may explain these differences.

In order to circumvent these complications, we have used a chemically synthesized DL- $\text{Ins}(1,3,4,5)\text{P}_4$ source which we believe to be free of contamination, and we have substantially decreased the problem of metabolism by performing experiments at 4 °C. Using these conditions, we strongly believe that $\text{Ins}(1,3,4,5)\text{P}_4$ alone can release intracellular Ca^{2+} in SH-SY5Y cells. Indeed, time-dependent release of Ca^{2+} was only observed under conditions when metabolism was occurring, and this, taken together with the observation that $\text{Ins}(1,4,5)\text{P}_3$ contamination of $\text{Ins}(1,3,4,5)\text{P}_4$ gave additional Ca^{2+} release when compared with Ca^{2+} -releasing action of $\text{Ins}(1,3,4,5)\text{P}_4$ on its own, suggests that the two inositol phosphates display synergistic release. The latter data generally support the models of $\text{Ins}(1,3,4,5)\text{P}_4$ action proposed by Irvine & colleagues [3,24].

The ability of $\text{Ins}(1,3,4,5)\text{P}_4$ to release maximally a substantially smaller proportion of the intracellular Ca^{2+} stores in SH-SY5Y cells than that observed for $\text{Ins}(1,4,5)\text{P}_3$ suggests a distinct site of binding and/or action for $\text{Ins}(1,3,4,5)\text{P}_4$, since Ca^{2+} -releasing inositol trisphosphates produce identical maximal responses (although with different EC_{50} values) [25,26]. There are, to our knowledge, no examples of partial agonists at the $\text{Ins}(1,4,5)\text{P}_3$ receptor, and we do not believe that this behaviour of $\text{Ins}(1,3,4,5)\text{P}_4$ could simply reflect a saturation of the 3-phosphatase, since metabolism is severely arrested under the conditions of our assays. In view of the current knowledge regarding inositol trisphosphate structure–activity relationships [27], it is clear that the presence of a 4,5-vicinal bisphosphate is the major prerequisite for agonist activity. The presence of a 1-phosphate group appears to enhance this activity, and since $\text{Ins}(1,3,4,5)\text{P}_4$ possesses all of these structural features in addition to a 3-phosphate group, it is not surprising that this molecule should have Ca^{2+} -releasing activity. The effect of modifying the 3-position of $\text{Ins}(1,4,5)\text{P}_3$ has, as yet, not been studied with respect to structure–activity relationships, but it remains possible that modification of the 3-position could result in partial agonist activity at the InsP_3 receptor. Therefore, definite proof of an action independent of $\text{Ins}(1,4,5)\text{P}_3$ receptors would require selective inhibition by an antagonist, and since in our hands the potential antagonists heparin [28,29] and decavanadate [30] appear to interact with both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$

receptor-binding sites (R. A. J. Challiss, A. L. Willcocks & S. R. Nahorski, unpublished work) we still seek a selective antagonist. However, considering structure–activity relationships, it is apparent from our results that the presence of a 6-phosphate group adjacent to the 4,5-vicinal bisphosphate appears to abolish Ca^{2+} -releasing activity, irrespective of the presence or absence of the 3-phosphate group [$\text{Ins}(1,3,4,5,6)\text{P}_5$ and DL- $\text{Ins}(1,4,5,6)\text{P}_4$ respectively].

It is noteworthy that the ability of $\text{Ins}(1,3,4,5)\text{P}_4$ to mobilize Ca^{2+} may be cell-dependent. Thus in neuronal cells (cerebellar microsomes [11] and the present work) and adrenal cortex [12] this polyphosphate is active, and, after agonist stimulation, it accumulates particularly dramatically in brain slices [19], SH-SY5Y cells (D. Lambert & S. R. Nahorski, unpublished work) and adrenal glomerulosa cells [31]. Furthermore, it is significant that cerebellum [21] and adrenal cortex [32] possess a relatively high density of specific $\text{Ins}(1,3,4,5)\text{P}_4$ binding sites. Possibly the function of this $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} release may be to prolong the intracellular Ca^{2+} signal in those cells in which there is effective phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ and accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$. Perhaps certain cell types have developed mechanisms to accumulate and respond to this inositol polyphosphate, and it will be important to investigate if there are cell-specific functions associated with these responses.

In conclusion, we suggest that $\text{Ins}(1,3,4,5)\text{P}_4$ may be interacting with a specific receptor [given that DL- $\text{Ins}(1,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ are inactive up to 10 μM] to release Ca^{2+} from an intracellular store. Since the synergistic action of DL- $\text{Ins}(1,3,4,5)\text{P}_4$ with $\text{Ins}(1,4,5)\text{P}_3$ may be dependent upon the concentration of the latter compound, this observation may be explained either by $\text{Ins}(1,3,4,5)\text{P}_4$ triggering release of Ca^{2+} from the same intracellular store as $\text{Ins}(1,4,5)\text{P}_3$, or alternatively by the existence of two Ca^{2+} pools which are sensitive to $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$ respectively, but which communicate and are intimately controlled, possibly by the relative size of each pool. Further work is now required to identify definitively the intracellular store from which $\text{Ins}(1,3,4,5)\text{P}_4$ is releasing Ca^{2+} , and its functional significance.

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