Inositol 1:2(cyclic),4,5-trisphosphate is not a major product of inositol phospholipid metabolism in vasopressin-stimulated WRK1 cells

Nai Sum WONG, Christopher J. BARKER, Stephen B. SHEARS, Christopher J. KIRK and Robert H. MICHELL*

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

1. A method has been devised for quenching cell incubations with an aqueous phenol/chloroform/EDTA mixture of neutral pH, to allow the analysis of acid-labile cell components. 2. Using this method, we have searched for the appearance of Ins(1:2cyclic,4,5) P_3 [inositol 1:2(cyclic),4,5-trisphosphate] in WRK1 mammary tumour cells that were labelled to high specific radioactivity with [3H]inositol and then stimulated with 0.4 μM -vasopressin. 3. Vasopressin caused a very rapid accumulation of Ins(1,4,5) P_3 (inositol 1,4,5-trisphosphate), followed by a slower decline towards the original concentration. An acid-labile and inositol-labelled compound with the chromatographic properties of Ins(1:2cyclic,4,5) P_3 was present in unstimulated cells at less than 5% of the elevated concentration of Ins(1,4,5) P_3 . Its concentration rose 2–3-fold during stimulation for 3 min, at which time its concentration was about 5% of the elevated concentration of Ins(1,4,5) P_3 . 4. We conclude that Ins(1,4,5) P_3 is the major product of phosphoinositidase C-catalysed phosphatidylinositol 4,5-bisphosphate hydrolysis in vasopressin-stimulated WRK1 cells. Ins(1:2cyclic,4,5) P_3 is unlikely to be an important intracellular messenger in these cells, at least during the first few minutes of stimulation.

INTRODUCTION

PtdIns(4,5) P_2 is hydrolysed in response to the binding of Ca^{2+} -mobilizing hormones to their receptors on the plasma membranes of target cells. The hydrolysis of PtdIns(4,5) P_2 is catalysed by a receptor-controlled phosphoinositidase C, with the production of Ins(1,4,5) P_3 and 1,2-diacylglycerol, both of which are intracellular messenger molecules (reviewed by Berridge & Irvine, 1984; Nishizuka, 1984; Downes & Michell, 1985; Putney, 1987). Phosphoinositidase C is capable of attacking three substrates, namely PtdIns(4,5) P_2 , PtdIns4P and PtdIns, and it can be activated *in vitro* by unphysiologically elevated concentrations of Ca^{2+} (Wilson *et al.*, 1984; Irvine *et al.*, 1984; Wang *et al.*, 1986). Dawson *et al.* (1971) and Lapetina & Michell (1973) showed that the water-soluble products from the hydrolysis of PtdIns by soluble and membrane-associated forms of phosphoinositidase C consist of a mixture of Ins(1:2cyclic) P and Ins(1) P . More recently, Wilson *et al.* (1985a) extended this observation to PtdIns4P and PtdIns(4,5) P_2 by showing that a soluble phosphoinositidase C purified from sheep seminal vesicles produces Ins(1:2-cyclic,4) P_2 and Ins(1:2-cyclic,4,5) P_3 from these two lipids, in addition to the expected formation of Ins(1,4) P_2 and Ins(1,4,5) P_3 . There is evidence that Ins(1:2-cyclic,4,5) P_3 can liberate Ca^{2+} in permeabilized platelets and activates *Limulus* photoreceptors, suggesting that it might serve as an intracellular signal (Wilson *et al.*, 1985b).

Although these studies have demonstrated that

Ins(1:2-cyclic,4,5) P_3 can be formed by phosphoinositidase C-catalysed hydrolysis of PtdIns(4,5) P_2 , they do not tell us whether the cyclic phosphoester is formed in stimulated cells. In studies before 1986 which used acid to extract inositol phosphates from stimulated cells, Ins(1:2cyclic,4,5) P_3 would not have been detected because it would have been hydrolysed to Ins(1,4,5) P_3 and, to a much lesser extent, Ins(2,4,5) P_3 during extraction.

We have approached this question by measuring the relative amounts of Ins(1:2cyclic,4,5) P_3 and Ins(1,4,5) P_3 that are produced in stimulated WRK1 mammary tumour cells. In order to do this, we developed a new method for extracting inositol phosphates from cellular material at neutral pH without destroying Ins(1:2-cyclic,4,5) P_3 . WRK1 cells were used in these experiments because they possess V_{1a} -vasopressin receptors that activate inositol lipid breakdown and they can be labelled to very high specific radioactivity with [3H]inositol.

MATERIALS AND METHODS

Materials

Eagle's modified minimal essential medium was purchased from Gibco. [^{32}P] P_i (10 mCi/ml) and [2-^3H]inositol (16.6 Ci/mmol) were bought from Amersham. Human erythrocytes were from human blood obtained from the National Blood Transfusion Centre within 8 days of donation.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; Ins P , Ins P_2 , Ins P_3 and Ins P_4 , inositol mono-, bis-, tris- and tetrakis-phosphate, with locants designated where appropriate; Ins(1:2cyclic,4,5) P_3 , inositol 1:2(cyclic),4,5-trisphosphate; Ins(1:2cyclic,4) P_2 , inositol 1:2(cyclic),4-bisphosphate; Ins(1:2cyclic) P , inositol 1:2(cyclic)-monophosphate. Enantiomeric phosphates are numbered by reference to D-*myo*-inositol 1-phosphate.

* To whom correspondence and reprint requests should be addressed.

Growth and stimulation of [^3H]inositol-labelled cells

WRK1 cells were maintained in Eagle's modified minimal essential medium supplemented with 5% (v/v) foetal calf serum and 2% (v/v) rat serum and with penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were first grown to confluence and then replated into small dishes; routinely 6×10^4 cells were used for one (35 mm-diam.) dish. The replated cells were grown to confluence again in a medium composed of inositol-free Eagle's modified minimal essential medium supplemented with [2- ^3H]inositol (Amersham, 16.6 Ci/mmol) at 10 $\mu\text{Ci}/\text{ml}$, 5% dialysed foetal calf serum and 2% dialysed rat serum. Cells were grown in the above medium for 5 days, with one change of medium, on day 3.

After the 5-day labelling period, the radioactive culture medium was removed and the cells were incubated for 30 min in serum-free Eagle's modified minimal essential medium containing 0.56 μM non-radioactive inositol equivalent to the chemical amount of [^3H]inositol used during labelling of the cells. The medium was removed and was replaced with 1 ml of Dulbecco's phosphate-buffered saline (Gibco, Paisley, Scotland, U.K.) at 37 °C, containing 5.5 mM-glucose, 0.44 mM- CaCl_2 and 0.5 mM- MgCl_2 . This medium was removed after 1 min and the cells were then stimulated with 0.4 μM -[arginine]-vasopressin in 0.5 ml of Dulbecco's phosphate-buffered saline, supplemented as above, at 37 °C.

Quenching of cells and extraction of inositol phosphates

The quenching buffer was prepared from a solution of 5 mM- KH_2PO_4 /10 mM-EDTA, pH 6.3. This solution was saturated with chloroform and then mixed with half its own volume of phenol (prepared by melting crystalline phenol at 57 °C). When the mixture was left at room temperature for a few hours, two phases developed. The upper phase, designated as phenol/chloroform/EDTA (the quenching buffer), is used for quenching cells. Phenol/chloroform/3-methylbutan-1-ol (Slater *et al.*, 1973) was prepared by mixing the three in the proportions 38:24:1 (by vol.).

The cells were quenched by adding 1 ml of quenching buffer to the dish, which was subsequently left on ice for 15 min. The cell debris was scraped off with a rubber policeman and transferred to a plastic vial. The dish was washed with a further 0.5 ml of quenching buffer. The two portions were combined and added to 2 ml of

phenol/chloroform/3-methylbutan-1-ol and then shaken vigorously for 30 s. After centrifugation at 3600 rev./min for 15 min at 4 °C, the upper phase was withdrawn for analysis.

Separation of inositol phosphates by h.p.l.c.

Two h.p.l.c. procedures were employed in the determination of the inositol phosphates in cell extracts. The separation of InsP , InsP_2 , $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ was performed essentially by the method described by Batty *et al.* (1985) on a Partisil 10SAX column coupled to a guard column packed with pellicular anion exchanger. After injection of the sample, the column was washed with distilled water for 10 min to remove any unbound ^3H -labelled material from the column, most of which was free inositol. The gradient was then started and 0.37 ml fractions were collected from 10 until 34 min. In this system, the amount of $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$ cannot be accurately quantified, because the compound is not separated from $\text{Ins}(1,3,4)\text{P}_3$. Therefore, to quantify only the cyclic species, another portion of the same sample was eluted continuously at 1.25 ml/min with 2 M-ammonium formate (adjusted to pH 5.5 with H_3PO_4), and 0.5 ml fractions were collected. By this method, $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$ was fully separated from $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$, but the last two were only partially separated; see the Results section and Fig. 1.

Portions of cell extracts were acid-treated to open five-membered cyclic phosphate rings, such as that of $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$, by adding 27 μl of 72% (w/v) HClO_4 to 0.4 ml of cell extract and incubating for 15 min at 37 °C. Samples were then left on ice for 15 min and neutralized (checked with pH paper) by addition of ice-cold 1 M-KOH. KClO_4 was removed by centrifugation in a MSE MicroCentaur microfuge. The resulting acid-treated cell extracts were analysed by h.p.l.c. (isocratic elution at 2 M-ammonium formate, as described above).

Preparation of $\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$ and other inositol phosphate standards

Human erythrocytes were labelled with [^{32}P]P $_i$ and the erythrocyte membranes were isolated as described by Downes & Michell (1981). They were incubated with 0.8 mM- Ca^{2+} for 30 min at 37 °C in 30 mM-Tris/acetate buffer, pH 5.0. The membranes were sedimented in a MSE MicroCentaur microfuge and the supernatant was kept and stored frozen at -20 °C. The supernatant contained [^{32}P]P $_i$, [^{32}P]Ins(1,4)P $_2$, [^{32}P]Ins(1,4,5)P $_3$

Table 1. Recoveries of [^{32}P]Ins(1:2cyclic,4,5)P $_3$ and [^{32}P]Ins(1,4,5)P $_3$ after extraction with phenol/chloroform/EDTA

Human erythrocytes were labelled with [^{32}P]P $_i$ for 2 h and the erythrocyte membranes were harvested, washed and incubated at pH 5.0 in the presence of 0.8 mM- Ca^{2+} to produce both cyclic and non-cyclic Ins(1,4,5)P $_3$, as described in the Materials and methods section. The mixture of ^{32}P -labelled inositol phosphates in a volume of 0.5 ml was then added to a culture dish containing adherent and confluent WRK-1 cells. The cells were killed by adding 1 ml of phenol/chloroform/EDTA. After extraction, the amount of each inositol phosphate was quantified by h.p.l.c. separation using isocratic elution at 2 M-ammonium formate. Values represent averages of duplicate measurements in one of two similar experiments.

	Radioactivity added (d.p.m.)	Radioactivity after extraction (d.p.m.)	Recovery (%)
$\text{Ins}(1:2\text{cyclic},4,5)\text{P}_3$	1610	1390	86
$\text{Ins}(1,4,5)\text{P}_3$	5280	5360	101

and [4,5- ^{32}P]Ins(1:2cyclic,4,5) P_3 . Ins(1:2cyclic,4,5) P_3 in the supernatant was located by (a) separating the inositol [^{32}P]phosphates by h.p.l.c. on Partisil 10SAX (2 M-ammonium formate, pH 5.5, isocratic elution; see above), and then (b) identifying the labelled peak that migrated with the polarity of an Ins P_3 and was converted into a ^{32}P -labelled peak that was eluted with Ins(1,4,5) P_3 after acid treatment (Fig. 1). When labelled erythrocyte ghosts were incubated under these conditions, approx. 20% of the Ins P_3 released by phosphoinositidase C action was found to be the cyclic isomer. [^3H]Ins(1,3,4) P_3 was prepared as described by Shears *et al.* (1987).

RESULTS

Cell quenching, extraction of inositol phosphates and analysis by h.p.l.c.

In order to prevent acid hydrolysis of Ins(1:2cyclic,4,5) P_3 , the quenching of cellular reactions and the extraction of inositol phosphates have to be performed at neutral pH. Phenol was chosen because it denatures proteins, disrupts membranes and is soluble in water to give a near-neutral solution. The cells were therefore quenched by a low-ionic-strength potassium phosphate buffer containing 10 mM-EDTA and saturated with phenol and chloroform. This primary extract was re-extracted with a mixture of phenol, chloroform and 3-methylbutan-1-ol: this second extraction leaves all the inositol phosphates in an upper aqueous phase, and the cellular lipids are presumably dissolved in the lower organic phase. The amounts of inositol trisphosphates extracted in this way, as analysed by separation on an anion exchanger, were approximately the same as those extracted by 5% (w/v) HClO_4 . The recoveries of Ins(1:2cyclic,4,5) P_3 and Ins(1,4,5) P_3 during extraction were determined by adding known amounts of the two inositol phosphates to unlabelled cells, which were then quenched and extracted by the phenol method. The recoveries were 86% and 101% for the two inositol phosphates (Table 1).

By the h.p.l.c. method of Batty *et al.* (1985), Ins(1:2cyclic,4,5) P_3 could only be partially separated from Ins(1,3,4) P_3 , but Ins(1,4,5) P_3 was well separated from the other two inositol trisphosphates. However, we have found that, by eluting isocratically at pH 5.5 with 2 M-ammonium formate, Ins(1:2cyclic,4,5) P_3 can be baseline-separated from Ins(1,3,4) P_3 and Ins(1,4,5) P_3 , although the last two now run close together (Fig. 1). Thus a complete analysis of the Ins(1,4,5) P_3 , Ins(1,3,4) P_3 and Ins(1:2cyclic,4,5) P_3 contents of a sample requires both h.p.l.c. elution procedures and a comparison of neutral and acid-treated samples.

Inositol phosphates in stimulated cells

Separation of [^3H]inositol phosphates in stimulated WRK1 cells was achieved by h.p.l.c. firstly by the method of Batty *et al.* (1985). To identify the various inositol phosphates, a mixture of [^{32}P]P $_i$, [4- ^{32}P]Ins(1,4) P_2 , [4,5- ^{32}P]Ins(1:2cyclic,4,5) P_3 and [4,5- ^{32}P]Ins(1,4,5) P_3 was added to the extracts before injection on to the h.p.l.c. column. Peaks that co-chromatographed with the various standards were assumed to have the same identities as the standards. The sizes of the peaks after various periods of stimulation are shown in Table 2. The greatest changes were seen in Ins(1,4,5) P_3 and Ins(1,4) P_2 , both of which increased to approx. 4 times the control

Fig. 1. Separation of Ins(1:2cyclic,4,5) P_3 from other inositol phosphates

^{32}P -labelled inositol phosphates were produced from ^{32}P -labelled human erythrocyte membranes by incubation at pH 5.0 in the presence of 0.8 mM- Ca^{2+} . The supernatant of this incubation was then mixed with [^3H]Ins(1,3,4) P_3 (■) and injected on to the h.p.l.c. column, which was subsequently eluted with 2 M-ammonium formate, pH 5.5, at 1.25 ml/min. Fractions (0.5 min each) were collected from a neutral extract (●). A portion of the same supernatant was acid-treated and analysed by the same h.p.l.c. procedure (○). The disappearance of Ins(1:2cyclic,4,5) P_3 after acid treatment of the supernatant was balanced by an equivalent increment in the radioactivity of the Ins(1,4,5) P_3 peak.

values after 1 s of stimulation. During the next 9 s of stimulation, the amount of Ins(1,4,5) P_3 remained elevated, whereas Ins(1,4) P_2 continued to increase. The continued increase in Ins(1,4) P_2 is probably due to the specific dephosphorylation of Ins(1,4,5) P_3 by a 5-phosphomonoesterase (Downes *et al.*, 1982; Seyfred *et al.*, 1984; Storey *et al.*, 1984), though some PtdIns4P may undergo hydrolysis directly, yielding Ins(1,4) P_2 . It is also possible that by 120 s this peak may contain some [^3H]Ins(1,3) P_2 (Irvine *et al.*, 1987).

When cells were stimulated for between 1 and 5 s,

Table 2. Production of [³H]-inositol bisphosphates and -inositol trisphosphates in WRK-1 cells stimulated with vasopressin for various times

WRK-1 cells were grown in the presence of [2-³H]inositol for 5 days and then transferred to serum-free Eagle's medium for 30 min (see the Materials and methods section). The cells were then stimulated with 0.4 μ M-[arginine]vasopressin in 0.5 ml of phosphate-buffered saline. After various times of incubation, the cells were quenched with phenol/chloroform/EDTA and the inositol phosphates extracted. A 0.4 ml portion of the extract, together with the ³²P-labelled standards [P_i , Ins(1,4) P_2 , Ins(1,4,5) P_3 and Ins(1:2cyclic,4,5) P_3] were analysed by h.p.l.c. using the gradient of Batty *et al.* (1985).

Period of stimulation (s) ...	Radioactivity (d.p.m.)					
	0	1	3	5	10	180
Ins(1,4) P_2	11 600	45 000	40 000	57 300	82 200	105 000
Ins(1,3,4) P_3 + Ins(1:2cyclic,4,5) P_3	1 900	2 100	2 100	1 900	3 500	12 900
Ins(1,4,5) P_3	11 300	44 300	36 200	40 700	38 600	22 700

Table 3. Amount of radioactivity eluted with Ins(1:2cyclic,4,5) P_3 before and after acid treatment

Samples (0.4 ml) of cell extracts obtained as described in the legend to Table 2 were injected together with ³²P-labelled Ins(1,4,5) P_3 and Ins(1:2cyclic,4,5) P_3 on to the h.p.l.c. column, which was subsequently eluted with 2 M-ammonium formate, pH 5.5. The amount of ³H radioactivity that co-eluted with the ³²P-labelled Ins(1:2cyclic,4,5) P_3 was quantified. Samples of the same set of cell extracts (0.4 ml each) were then acid-treated and analysed in the same way by h.p.l.c. The amount of ³H radioactivity that still co-eluted with Ins(1:2cyclic,4,5) P_3 was again quantified. Values represent averages of duplicate measurements. The results are representative of those obtained in two similar experiments.

Period of stimulation (s) ...	Radioactivity (d.p.m.)					
	0	1	3	5	10	180
³ H radioactivity in the putative Ins(1:2cyclic,4,5) P_3 peak before acid treatment	550	890	940	930	1160	1540
³ H radioactivity in the putative Ins(1:2cyclic,4,5) P_3 peak after acid treatment	250	420	490	360	420	440
Acid-labile ³ H-labelled substance [Ins(1:2cyclic,4,5) P_3]	300	470	450	570	740	1100

there was very little formation of either Ins(1,3,4) P_3 or Ins(1:2cyclic,4,5) P_3 , which migrated close together. However, after 10 s of stimulation, inositol phosphates that were eluted in this part of the chromatogram began to accumulate, and had reached about 6 times the baseline value by 3 min (Table 2). This overall pattern of delayed rise is reminiscent of that usually shown by Ins(1,3,4) P_3 (Burgess *et al.*, 1985), which is formed from Ins(1,4,5) P_3 via Ins(1,3,4,5) P_4 (Downes *et al.*, 1986; Irvine *et al.*, 1986).

The same samples were analysed by the isocratic-elution procedure that resolves Ins(1:2cyclic,4,5) P_3 from both Ins(1,4,5) P_3 and Ins(1,3,4) P_3 (Fig. 1). About half as much radioactivity as was associated with Ins(1,3,4) P_3 /Ins(1:2cyclic,4,5) P_3 in the previous separation procedure was now found in the putative Ins(1:2cyclic,4,5) P_3 peak in unstimulated cells (cf. Tables 2 and 3), and this only increased 2–3-fold after 3 min of stimulation. When the samples were acid-treated and re-analysed, to determine the proportion of the radioactivity that had disappeared from this peak, and hence the amount of genuine Ins(1:2cyclic,4,5) P_3 , the peak was in each case decreased in size, to about half in the early samples and to about one-third in the samples stimulated for 3 min (Table 3). The small and relatively constant quantity of acid-stable

radioactivity associated with this peak presumably indicates the presence of some very minor, and unidentified, inositol derivative.

DISCUSSION

From the information presented above, it is clear that vasopressin stimulates a very rapid accumulation of Ins(1,4,5) P_3 in WRK1 cells, involving a 4-fold rise in the concentration of this compound when vasopressin was present for only approx. 1 s before the addition of the phenol-based quenching medium. In order to maximize the sensitivity of the method employed in our search for Ins(1:2cyclic,4,5) P_3 , we used cultured cells that had been labelled to isotopic equilibrium in a low-inositol medium and that therefore contained inositol phospholipids of very high specific radioactivity. As a result, the accumulation of inositol phosphates after stimulation was registered as much larger amounts of radioactivity than in most previous experiments (see below). The small amount of acid-labile material that was found in the putative Ins(1:2cyclic,4,5) P_3 peak, and which increased much more slowly than Ins(1,4,5) P_3 during stimulation of WRK1 cells, may indicate that a very small proportion of the phosphoinositidase C-catalysed hydrolysis of

PtdIns(4,5) P_2 in stimulated cells does indeed give rise directly to Ins(1:2cyclic,4,5) P_3 . However, the increase in the quantity of this material was no more than 1–2% of the total accumulation of all Ins P_3 species during the initial seconds of stimulation, and even after 3 min its concentration was only about 5% that of Ins(1,4,5) P_3 . Moreover, the quantity of putative Ins(1:2cyclic,4,5) P_3 formed was so small as to preclude any further experiments to substantiate its identity.

Ins(1:2cyclic,4,5) P_3 can undoubtedly be produced as a minor product during the hydrolysis of PtdIns(4,5) P_2 by phosphoinositidase C *in vitro*. Three other recent studies have addressed the question of whether Ins(1:2cyclic,4,5) P_3 is produced in cells in response to stimulation of receptors. In the first of these studies, Ishii *et al.* (1986) reported the formation of [32 P]Ins(1:2cyclic,4,5) P_3 during 10 s stimulation of 32 P-labelled platelets with thrombin. However, the h.p.l.c. separations shown in that study were not entirely convincing, and complex methods, giving poor final yields, were needed to separate putative 32 P-labelled inositol phosphates from the large quantities of other 32 P-labelled compounds present in the cells. More convincing evidence for the formation of Ins(1:2cyclic,4,5) P_3 was offered in a study of pancreatic minilobules that were labelled with [3 H]inositol and stimulated with carbamoylcholine for 40 min (Sekar *et al.*, 1987). In that study, Ins(1:2cyclic,4,5) P_3 represented 29% of the total extracted Ins P_3 species, but unfortunately no attempt was made to determine the relative efficiencies with which Ins(1:2cyclic,4,5) P_3 and Ins(1,4,5) P_3 were extracted from the stimulated tissue. Sekar *et al.* (1987) did not compare the time courses for the accumulation of the various Ins P_3 species, so did not determine whether Ins(1:2cyclic,4,5) P_3 was only a minor product of stimulated PtdIns(4,5) P_2 metabolism, but accumulated to a significant degree during prolonged stimulation because it is much more slowly metabolized than Ins(1,4,5) P_3 (Connolly *et al.*, 1986). However, in a study published since submission of the present paper, work from that same laboratory has shown that Ins(1:2cyclic,4,5) P_3 is formed in pancreatic minilobules much more slowly than Ins(1,4,5) P_3 , but does accumulate to a substantial concentration within the first few minutes of stimulation (Dixon & Hokin, 1987). Finally, Hawkins *et al.* (1987) reported experiments, using rigorously characterized extraction procedures, in which [3 H]-inositol-labelled parotid fragments were stimulated with carbamoylcholine for short periods. They concluded that Ins(1:2cyclic,4,5) P_3 could form no more than a few per cent of the accumulated products of receptor-stimulated PtdIns(4,5) P_2 hydrolysis at any time during brief stimulation.

Thus our results essentially concur with the conclusion of Hawkins *et al.* (1987), in that Ins(1:2cyclic,4,5) P_3 is formed in stimulated cells at only a few per cent of the rate of Ins(1,4,5) P_3 formation, at most. We cannot explain why our results, and those of Hawkins *et al.* (1987), contradict those of Ishii *et al.* (1986) on the amounts of Ins(1:2cyclic,4,5) P_3 generated in cells. However, it seems to us that the weight of present evidence, including that offered here, favours the view that Ins(1,4,5) P_3 alone, rather than either Ins(1:2cyclic,4,5) P_3 or a mixture of Ins(1,4,5) P_3 and Ins(1:2cyclic,4,5) P_3 , is likely to be responsible for the rapid mobilization of

intracellular Ca^{2+} that occurs in cells in response to many stimuli. For Ins(1:2cyclic,4,5) P_3 to have any important physiological effect in briefly stimulated cells, it would have to display the necessary activity at maybe one-tenth the minimum concentration at which Ins(1,4,5) P_3 mobilizes intracellular Ca^{2+} , and as yet there is no evidence for any such activity.

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