Interactions between inositol tris- and tetrakis-phosphates

Effects on intracellular Ca^{2+} mobilization in SH-SY5Y cells

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The potential Ca^{2+}-releasing activity of the inositol tetrakisphosphates Ins(1,3,4,6)P_4 and DL-Ins(1,4,5,6)P_4 and the inositol pentakisphosphate Ins(1,3,4,5,6)P_5 and their effect on Ins(1,4,5)P_3- and DL-Ins(1,3,4,5)P_4-mediated Ca^{2+} release were examined in permeabilized SH-SY5Y human neuroblastoma cells. Neither DL-Ins(1,4,5,6)P_4 nor Ins(1,3,4,5,6)P_5 exhibit Ca^{2+}-releasing activity at concentrations up to 10 μM, but Ins(1,3,4,6)P_4 releases Ca^{2+} dose-dependently, with an EC_{50} value (concn. giving half-maximal effect) of 5.92 ± 0.47 μM. Maximal response by this tetrakisphosphate (49 ± 2.5 %) is significantly less than that seen with Ins(1,4,5)P_3 (60 ± 3 %) and is achieved at a concentration of 30 μM. In the presence of this concentration of Ins(1,3,4,6)P_4 the EC_{50} value for Ins(1,4,5)P_3-mediated Ca^{2+} release increases from 0.12 ± 0.02 μM to 2.11 ± 0.51 μM, providing evidence that this naturally occurring inositol tetrakisphosphate may recognize and exhibit its Ca^{2+}-releasing activity via the Ins(1,4,5)P_3 receptor. DL-Ins(1,3,4,5)P_4, however, at its maximally effective concentration (10 μM) does not significantly affect Ins(1,4,5)P_3-mediated Ca^{2+} release, and therefore appears to mediate its Ca^{2+}-mobilizing action through a receptor distinct from that for Ins(1,4,5)P_3.

INTRODUCTION

It is well established that D-*myo*-inositol(1,4,5)P_3, produced upon receptor-stimulated activation of phosphoinositidase C, releases Ca^{2+} from intracellular vesicular stores by binding to specific receptors [1,2]. These receptors are closely associated with Ca^{2+} channels located, at least in part, with the endoplasmic reticulum and have been purified, reconstituted and very recently cloned and expressed in COS mouse fibroblast cells [3–6].

Phosphorylation of Ins(1,4,5)P_3 by 3-kinase activity produces Ins(1,3,4,5)P_4 [7]. The possible function of this inositol tetrakisphosphate is controversial, but evidence suggests a role for this metabolite in both Ca^{2+} entry [8,9] and intracellular Ca^{2+} release [10,11] from a variety of tissues and cell types. The effects of other inositol tetrakisphosphates, such as Ins(1,4,5,6)P_4 and Ins(1,3,4,6)P_4, on intracellular Ca^{2+} homoeostasis have not yet been established. However, we have recently demonstrated that Ins(1,3,4,5)P_4 has the ability to release intracellular Ca^{2+} from the human neuroblastoma SH-SY5Y cell line [12]. This action is independent of contamination with, or subsequent production of, Ins(1,4,5)P_3 via 3-phosphatase activity. Here we have investigated the potential Ca^{2+}-mobilizing activity of inositol tetrakisphosphates and inositol pentakisphosphate and the ability of these polyphosphates to influence Ins(1,4,5)P_3-receptor-mediated intracellular Ca^{2+} release in this cell line.

Implications for the involvement of these inositol phosphate metabolites in Ca^{2+} homoeostasis and the possibility for specific receptor-ligand development are discussed.

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 μg/ml), fungizone (5 μg/ml) and 2 mM-glutamine. Cells were used when confluent.

D-Ins(1,4,5)P_3 and [^{32}P]Ins(1,4,5)P_3 were gifts from NEN Dupont Biotechnology (Stevenage, Herts., U.K.). DL-Ins(1,3,4,5)P_4 was a gift from Dr. D. Billington, Merck Sharp & Dohme Research (Harlow, Essex, U.K.). DL-Ins(1,4,5,6)P_4 was synthesized from DL-1,2-isopropylidene-*myo*-inositol by the phosphorylation and deprotection procedure outlined by Cooke *et al.* [13] and purified by using a linear triethylammonium bicarbonate elution gradient (0.1–1 mM, pH 8) on a DEAE-Sephadex A25 anion-exchange chromatography column, followed by preparative h.p.l.c. with ammonium formate gradient elution as described by Stephens *et al.* [14]. The purified DL-Ins(1,4,5,6)P_4 was then extensively characterized by ^{1}H and ^{31}P n.m.r. spectroscopy.

Ins(1,3,4,6)P_4 was synthesized chemically by deprotection (alkaline hydrolysis followed by hydrogenolysis) of the crystalline analytically pure octa-2-cyanoethyl ester of 2,5-di-*O*-benzyl-*myo*-inositol 1,3,4,6-tetrakisphosphate [15] and treated by preparative ionophoresis as described by Dawson & Clarke [16] to remove the precursor. Ins(1,3,4,6)P_4 and DL-Ins(1,3,4,5)P_4 preparations were established to be free from Ins(1,4,5)P_3 contamination, as assessed by 3-kinase pretreatment performed as previously described by us [12].

D-Ins(1,3,4,5,6)P_5 was purchased from Boehringer Mannheim. ATP, saponin, quin-2, oligomycin and Hepes were obtained from Sigma. $^{45}CaCl_2$ was purchased from Amersham International. All other chemicals were obtained from Fisons or BDH.

Methods

$^{45}Ca^{2+}$ release. The method used was essentially a modification of Gershengorn *et al.* [17] as described in Strupish *et al.* [18].

Abbreviations used: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, DL-*myo*-inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4,6)P_4, *myo*-inositol 1,3,4,6-tetrakisphosphate; Ins(1,4,5,6)P_4, DL-*myo*-inositol 1,4,5,6-tetrakisphosphate; Ins(1,3,4,5,6)P_5, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate; EC_{50}, concentration causing half-maximal effect; IC_{50}, concentration causing 50 % displacement of specific binding.

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SH-SY5Y 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-$MgCl_2$ 2 mM-KH_2PO_4, 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 and monitored fluorimetrically. After incubation at 25 °C for 15 min, the cells were collected by centrifugation as above and resuspended in 'cytosol-like' buffer containing 100 μg of saponin/ml and 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 $^{45}Ca^{2+}$/ml.

Cells were incubated at 25 °C for 20 min to allow loading of non-mitochondrial Ca^{2+} stores with $^{45}Ca^{2+}$, cooled to 4 °C, and then 100 μl of the cell suspension was added to 100 μl of buffer containing inositol phosphates as appropriate. After a 3 min incubation at 4 °C, 500 μl of a silicone oil mixture [Dow–Corning 556/550 (3:2, v/v)] was added, and then the cells were 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 cerebellar membranes. This was done by a modification of the method described by Willcocks *et al.* [19].

Cerebella from adult Wistar rats (225–275 g) were homogenized (Polytron, setting 5 and 10 s) in 20 ml of ice-cold buffer A, consisting of 20 mM-Tris/HCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol, pH 7.7, and pelleted by centrifugation at 48000 *g* for 20 min at 4 °C. The pellet was resuspended in 20 ml of buffer A and the washing procedure repeated three times. The resulting final pellet was resuspended in buffer A to a protein concentration of 1–2 mg/ml and stored on ice until use. Membranes were freshly prepared before each experiment and used within 1 h of preparation.

[^{32}P]Ins(1,4,5)P_3 binding assays. These were performed at 4 °C in an assay cocktail consisting of buffer A, 0.45 nM-[^{32}P]Ins(1,4,5)P_3 (~ 16000–20000 d.p.m.), 0–100 μM displacing inositol phosphate and 40–60 μg of cerebellar-membrane protein. Samples were incubated on ice for 10 min and the assay was terminated by centrifugation at 16000 *g* for 3 min. Supernatants were removed by aspiration and the resultant pellets washed once with 200 μl of ice-cold buffer A. Samples were left to drain for 30 min, and then pellets were dissolved in 100 μl of Lumasolve and counted for radioactivity in 1 ml of Optiphase X scintillation fluid. Non-specific binding was defined as binding not displaced by 10 μM-Ins(1,4,5)P_3.

Curve fitting. EC_{50} and IC_{50} values were determined with a computer-assisted Allfit curve fitting program as described by DeLean *et al.* [20]. Significance testing of differences between EC_{50} values derived was performed by application of Student's *t* test using 95% confidence limits ($P < 0.05$).

RESULTS

$^{45}Ca^{2+}$-release experiments were performed at 4 °C, and all inositol tetrakisphosphates were pretreated with a crude kinase preparation as described by us [12]. These controlled conditions are crucial to prevent Ins(1,4,5)P_3 formation resulting from 3-phosphatase activity and to remove the possibility of endogenous Ins(1,4,5)P_3 contamination of inositol tetrakisphosphate preparations. Ins(1,4,5)P_3- and DL-Ins(1,3,4,5)P_4-induced Ca^{2+} release is maximal and sustained over an incubation period of 1–5 min under the conditions used [12].

Inositol phosphate-induced Ca^{2+} release

Fig. 1 demonstrates the dose–response relationship between intracellular Ca^{2+} release and Ins(1,4,5)P_3, DL-Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and DL-Ins(1,4,5,6)P_4 concentration in SH-SY5Y cells incubated at 4 °C. Ins(1,4,5)P_3, DL-Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 release intracellular Ca^{2+} in a dose-dependent manner, with apparent EC_{50} values of 0.12±0.02, 2.76±0.01 and 5.92±0.47 μM respectively. Ins(1,4,5)P_3 maximally releases a greater proportion of intracellularly stored Ca^{2+} than that released by either DL-Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 (60.5±3.3% of stored Ca^{2+}, compared with 37±3.0% and 49.5±3.5%). DL-Ins(1,4,5,6)P_4 has no significant Ca^{2+}-releasing activity in these cells under the conditions used.

Effect of inositol tetrakis- and pentakis-phosphates on Ins(1,4,5)P_3-induced Ca^{2+} release

Since Ins(1,3,4,6)P_4 and DL-Ins(1,3,4,5)P_4 have Ca^{2+}-releasing activity, but are maximally less efficacious than Ins(1,4,5)P_3, it is conceivable that these inositol tetrakisphosphates could be acting as partial agonists of the Ins(1,4,5)P_3 receptor. Similarly, as DL-Ins(1,4,5,6)P_4 (Fig. 1) and Ins(1,3,4,5,6)P_5 [12] at concentrations up to 10 μM have no Ca^{2+}-releasing activity in these cells, these compounds could be potential antagonists of the Ins(1,4,5)P_3 receptor. These possibilities were therefore investigated.

Fig. 2 shows the effect of DL-Ins(1,3,4,5)P_4 (10 μM),

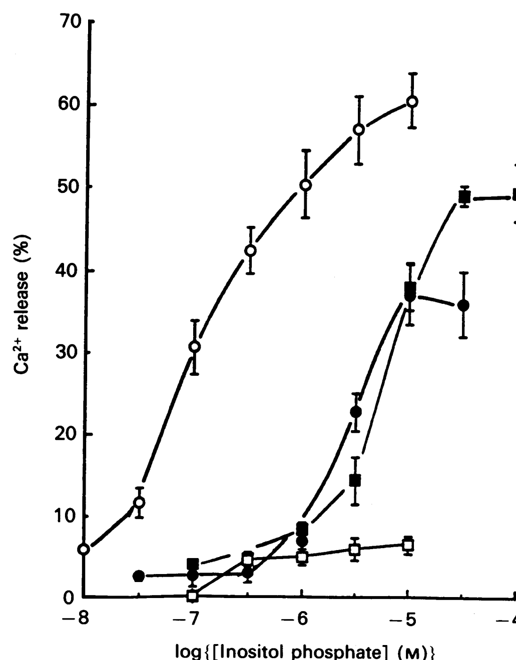

Fig. 1. Inositol phosphate-induced intracellular Ca^{2+} release

Permeabilized SH-SY5Y cells were preloaded with $^{45}Ca^{2+}$ and incubated for 3 min at 4 °C with Ins(1,4,5)P_3 (○), DL-Ins(1,3,4,5)P_4 (●), Ins(1,3,4,6)P_4 (■) and DL-Ins(1,4,5,6)P_4 (□). The $^{45}Ca^{2+}$ content of cells was determined with respect to cells incubated without inositol phosphates. Results are presented as means ± S.E.M. for triplicate determinations of 3–5 independent experiments.

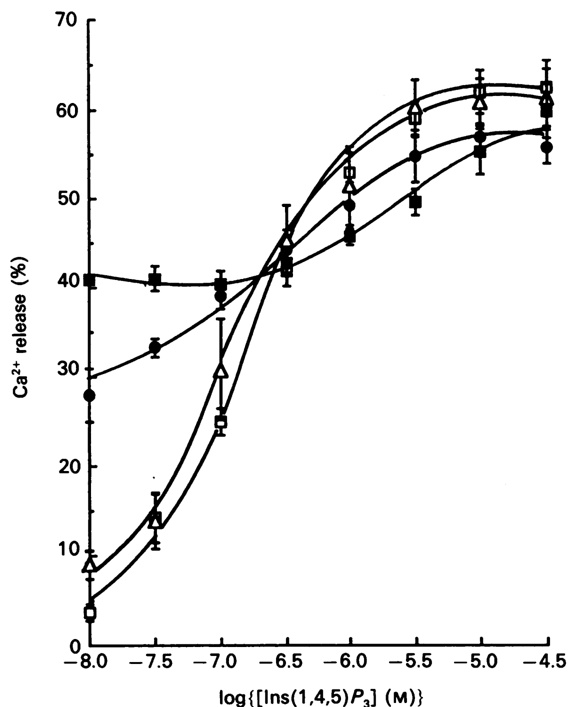

Fig. 2. Effect of inositol tetrakis- and pentakis-phosphate on Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release

SH-SY5Y cells were incubated at 4 °C with 30 μM-Ins(1,3,4,6)P_4 (■), 10 μM-DL-Ins(1,3,4,5)P_4 (●), 10 μM-DL-Ins(1,4,5,6)P_4 (□) and 10 μM-Ins(1,3,4,5,6)P_5 (△) in the presence of increasing concentrations of Ins(1,4,5)P_3. $^{45}Ca^{2+}$ release was assessed as outlined in Fig. 1, and results are presented as means ± S.E.M. for triplicate determinations of 4 independent experiments.

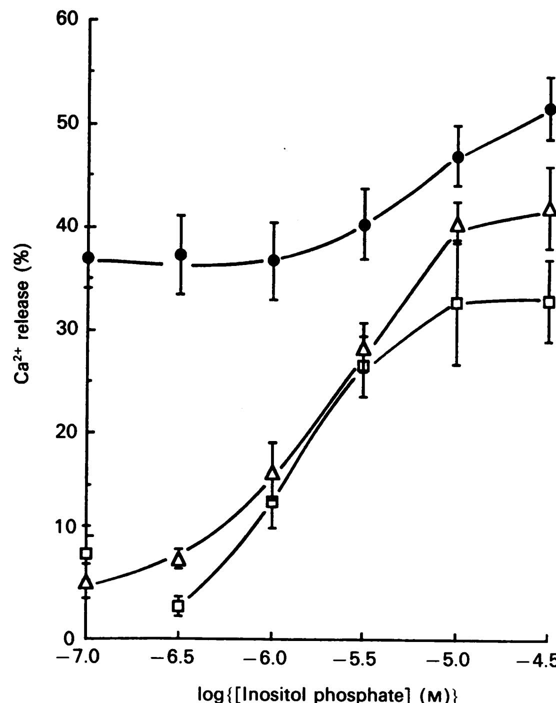

Fig. 3. Effect of DL-Ins(1,4,5,6)P_4, Ins(1,3,4,5,6)P_5 and DL-Ins(1,3,4,5)P_4 on inositol tetrakisphosphate-induced $^{45}Ca^{2+}$ release

SH-SY5Y cells were incubated at 4 °C with either 10 μM-DL-Ins(1,4,5,6)P_4 (□) or 10 μM-Ins(1,3,4,5,6)P_5 (△) in the presence of increasing concentrations of DL-Ins(1,3,4,5)P_4, or 10 μM-DL-Ins(1,3,4,5)P_4 (●) in the presence of increasing concentrations of Ins(1,3,4,6)P_4. $^{45}Ca^{2+}$ release was assessed as outlined in Fig. 1, and results are presented as means ± S.E.M. for triplicate determinations of 3–6 independent experiments.

Ins(1,3,4,6)P_4 (30 μM), DL-Ins(1,4,5,6)P_4 (10 μM) and Ins(1,3,4,5,6)P_5 (10 μM) on the dose–response curve for Ins(1,4,5)P_3-induced Ca^{2+} release. The presence of DL-Ins(1,3,4,5)P_4 does not appear to alter significantly the EC_{50} or maximal-response values of the D-Ins(1,4,5)P_3 dose–response curve (0.26 ± 0.08 μM and 57.66 ± 1.40 % respectively). However, the presence of Ins(1,3,4,6)P_4 significantly increased the EC_{50} value for Ins(1,4,5)P_3 to 2.11 ± 0.51 μM, but did not significantly alter the maximal response value (56.48 ± 2.60 %).

The Ins(1,4,5)P_3 dose–response curves in the presence of DL-Ins(1,4,5,6)P_4 (10 μM) and Ins(1,3,4,5,6)P_5 (10 μM) are superimposable with that for Ins(1,4,5)P_3 alone and have EC_{50} and maximal-response values of 0.15 ± 0.02 μM, 62 ± 2.4 % and 0.11 ± 0.02 μM, 61 ± 3.1 % respectively.

Effect of Ins(1,3,4,5,6)P_5 and DL-Ins(1,4,5,6)P_4 on DL-Ins(1,3,4,5)P_4-induced Ca^{2+} release

Fig. 3 illustrates the effect of DL-Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 on the Ca^{2+}-releasing activity of DL-Ins(1,3,4,5)P_4 in SH-SY5Y cells. The two curves are essentially superimposable, and maximal response is achieved at an Ins(1,3,4,5)P_4 concentration of 10 μM. The EC_{50} and maximal-response values are

Table 1. Comparison of EC_{50} and maximal-response values for inositol phosphate-induced $^{45}Ca^{2+}$ mobilization in SH-SY5Y cells

Dose–response curves for inositol phosphate-induced $^{45}Ca^{2+}$ release were curve fitted by using a computer-assisted Allfit program as described in the Experimental section and tested for significance by Student's *t* test (*$P < 0.05$). Values given are means ± S.E.M. for triplicate determinations for 3–6 individual experiments.

Incubation conditions	EC_{50} (μM)	Maximal response (%)
Ins(1,4,5)P_3	0.12 ± 0.02	60.5 ± 3.3
Ins(1,4,5)P_3 + 10 μM-DL-Ins(1,3,4,5)P_4	0.26 ± 0.08	57.7 ± 1.4
Ins(1,4,5)P_3 + 30 μM-Ins(1,3,4,6)P_4	2.11 ± 0.51*	56.5 ± 2.6
Ins(1,4,5)P_3 + 10 μM-DL-Ins(1,4,5,6)P_4	0.15 ± 0.02	62.0 ± 2.4
DL-Ins(1,3,4,5)P_4	2.76 ± 0.01	37.0 ± 3.0
DL-Ins(1,3,4,5)P_4 + 10 μM-DL-Ins(1,4,5,6)P_4	1.90 ± 0.43	36.5 ± 4.1
DL-Ins(1,3,4,5)P_4 + 10 μM-Ins(1,3,4,5,6)P_5	2.53 ± 0.20	40.4 ± 5.2
Ins(1,3,4,6)P_4	5.92 ± 0.47	49.5 ± 2.5
Ins(1,3,4,6)P_4 + 10 μM-DL-Ins(1,3,4,5)P_4	5.58 ± 1.14	50.6 ± 2.6

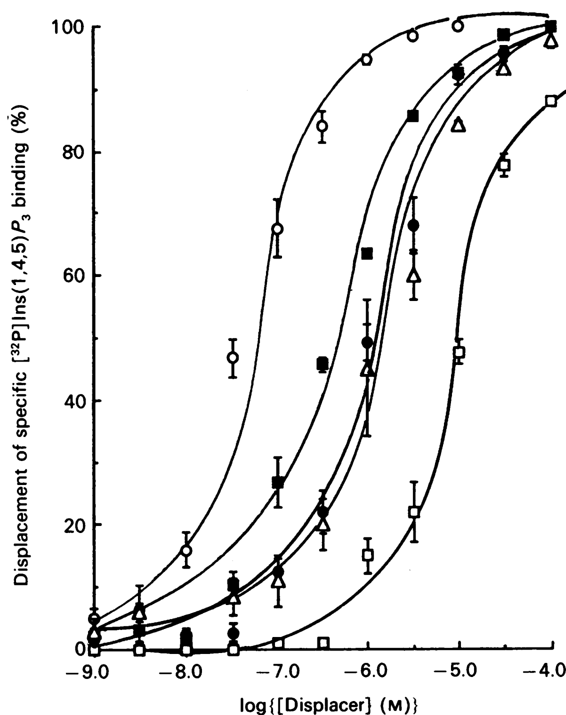

Fig. 4. Inositol phosphate displacement of specific [^{32}P]Ins(1,4,5)P_3 binding in rat cerebellar membranes

Displacement of specific [^{32}P]Ins(1,4,5)P_3 binding to rat cerebellar membranes by Ins(1,4,5)P_3 (○), Ins(1,3,4,6)P_4 (■), DL-Ins(1,3,4,5)P_4 (●), Ins(1,3,4,5,6)P_5 (△) and DL-Ins(1,4,5,6)P_4 (□) under incubation conditions as described in the Experimental section. Non-specific binding was defined as [^{32}P]Ins(1,4,5)P_3 binding not displaced by 10 μM-Ins(1,4,5)P_3. Results are presented as means ± S.E.M. for triplicate determinations of 4 independent experiments, each time with different membrane preparations.

Table 2. Comparison of IC_{50} values for displacement of specific [^{32}P]Ins(1,4,5)P_3 binding by inositol phosphates in rat cerebellar membranes

Displacement curves were fitted by using a computer-assisted Allfit program. Values given are means ± S.E.M. for triplicate determinations for 4 individual experiments, each using different membrane preparations.

Displacer	IC_{50} (μM)
Ins(1,4,5)P_3	0.04 ± 0.007
DL-Ins(1,3,4,5)P_4	1.12 ± 0.12
Ins(1,3,4,6)P_4	0.41 ± 0.03
DL-Ins(1,4,5,6)P_4	8.51 ± 1.52
Ins(1,3,4,5,6)P_5	1.59 ± 0.54

1.90 ± 0.43 μM, 36.5 ± 4.1 % and 2.53 ± 0.20 μM, 40.4 ± 5.2 % in the presence of DL-Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 respectively. There is no significant difference between these EC_{50} and maximal-response values and those obtained for DL-Ins(1,3,4,5)P_4-induced Ca^{2+} release in the absence of these compounds (Table 1).

Effect of DL-Ins(1,3,4,5)P_4 on Ins(1,3,4,6)P_4-induced Ca^{2+} release

Fig. 3 shows the dose–response curve for Ins(1,3,4,6)P_4-induced Ca^{2+} release in the presence of DL-Ins(1,3,4,5)P_4 (10 μM). Increasing concentrations of Ins(1,3,4,6)P_4 do not appear to show additional Ca^{2+}-releasing activity until a concentration > 1 μM is reached. Above this concentration the Ca^{2+}-releasing activity increases to give a maximal response of 50.6 ± 4.6 %. There is no significant difference between the EC_{50} and maximal response values for Ins(1,3,4,6)P_4 in the presence or absence of DL-Ins(1,3,4,5)P_4 (Table 1).

Relative potency of [^{32}P]Ins(1,4,5)P_3-binding displacement by inositol phosphates

To assess the order of relative affinity of each inositol phosphate for the Ins(1,4,5)P_3 receptor, the displacement of specific [^{32}P]Ins(1,4,5)P_3 binding was investigated. Owing to high (~ 50 %) non-specific binding in the SH-SY5Y cells, the displacement binding studies were performed in rat cerebellar membrane preparations, where non-specific binding was only about 10 % of the radioligand bound.

Fig. 4 demonstrates the displacement of specific [^{32}P]Ins(1,4,5)P_3-binding for each inositol phosphate investigated in the $^{45}Ca^{2+}$-release studies. The mean IC_{50} values derived from the displacement curves are given in Table 2. There is a clear order of potency for displacement of [^{32}P]Ins(1,4,5)P_3 bound to cerebellar membranes: Ins(1,4,5)P_3 > Ins(1,3,4,6)P_4 > DL-Ins(1,3,4,5)P_4 > Ins(1,3,4,5,6)P_5 > DL-Ins(1,4,5,6)P_4.

DISCUSSION

The potential Ca^{2+}-releasing activity of the naturally occurring inositol tetrakis- and pentakis-phosphates, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5, has been examined and their possible interaction with Ins(1,4,5)P_3-mediated intracellular Ca^{2+} release investigated in permeabilized SH-SY5Y neuroblastoma cells.

DL-Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 do not have Ca^{2+}-releasing activity and do not appear to influence the Ins(1,4,5)P_3- or DL-Ins(1,3,4,5)P_4-mediated Ca^{2+} release at concentrations of up to 10 μM. Ins(1,3,4,6)P_4, however, clearly displays significant Ca^{2+}-releasing activity, which is not believed to be due to Ins(1,4,5)P_3 contamination of the Ins(1,3,4,6)P_4 preparation, since it is resistant to 3-kinase pretreatment. This Ins(1,3,4,6)P_4-mediated Ca^{2+} release is less potent and more efficacious than that induced by DL-Ins(1,3,4,5)P_4, but is less potent and less efficacious than that induced by Ins(1,4,5)P_3.

In the presence of the maximally effective Ca^{2+}-releasing concentration of Ins(1,3,4,6)P_4 (30 μM), the EC_{50} value for the dose–response curve for Ca^{2+} release by Ins(1,4,5)P_3 is dramatically increased. This not only suggests that these inositol polyphosphates release Ca^{2+} from the same intracellular store, but also strongly indicates that they compete for the same receptor site. Thus Ins(1,3,4,6)P_4 appears to act as a classical partial agonist at a receptor for which Ins(1,4,5)P_3 is a full agonist. In the presence of Ins(1,3,4,6)P_4, responses to high concentrations of the agonist are shifted to the right, and less than additive effects are observed at concentrations of the agonist which produce a smaller response than that observed for Ins(1,3,4,6)P_4 alone.

Further evidence that Ins(1,3,4,6)P_4 is recognized with relatively high affinity by the Ins(1,4,5)P_3 receptor is demonstrated by the [^{32}P]Ins(1,4,5)P_3-binding experiments in cerebellar membranes. In these experiments Ins(1,3,4,6)P_4 possessed the highest affinity of the inositol tetrakisphosphates examined. The implication of a receptor shared by Ins(1,3,4,6)P_4 and Ins(1,4,5)P_3 would not be unexpected, on the basis of the potential similarity of the spacial distribution of the phosphate groups in these compounds [21].

In contrast, the ability of DL-Ins(1,3,4,5)P_4 to release Ca^{2+} from SH-SY5Y cells appears to be distinct from that released

by Ins(1,4,5)P_3 and Ins(1,3,4,6)P_4. Although this inositol tetrakisphosphate, like Ins(1,3,4,6)P_4, is less potent and less efficacious than Ins(1,4,5)P_3, at its maximal effective concentration (10 μM) it is unable to influence the Ca^{2+}-releasing activities of either Ins(1,4,5)P_3 or Ins(1,3,4,6)P_4. Therefore it is concluded that the Ca^{2+}-releasing action of DL-Ins(1,3,4,5)P_4 is not a result of interaction with the receptor for which Ins(1,4,5)P_3 and Ins(1,3,4,6)P_4 are agonists.

Since there is now substantial evidence for specific Ins(1,3,4,5)P_4-binding sites in several cell types [22–24], it would not seem unreasonable to assume that the Ca^{2+}-releasing activity of this polyphosphate may be exerted through such a receptor. As yet, we have not attempted to identify or characterize such binding sites in the SH-SY5Y cell line, and whether activation of such a receptor induces the release of Ca^{2+} from an intracellular store distinct from that accessible to Ins(1,4,5)P_3 has yet to be determined. Certainly, the lack of additivity between the effects of these two inositol polyphosphate would suggest that they release Ca^{2+} from a common or interconnecting pool.

Finally, the physiological significance of Ins(1,3,4,6)P_4-induced Ca^{2+} release seen here remains unknown. Ins(1,3,4,6)P_4 does accumulate in some cells (albeit relatively slowly) after agonist stimulation [25–27], and conceivably, if cellular levels are elevated sufficiently this polyphosphate could be responsible for a later Ca^{2+} release. Alternatively, in cells such as SH-SY5Y, in which Ins(1,4,5)P_3 accumulation is sustained after carbachol stimulation [28], Ins(1,3,4,6)P_4 may modulate the action of the former second messenger. The clear partial-agonist behaviour of Ins(1,3,4,6)P_4 at Ins(1,4,5)P_3 receptors suggests that a simple relationship between receptor occupancy and the opening of the Ca^{2+} channels does not exist. Possibly chemical modification of Ins(1,3,4,6)P_4 could lead to a selective antagonist of the Ins(1,4,5)P_3 receptor, which would be an invaluable tool for research in this area.

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