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Inositol 1,4,5-trisphosphorothioate, a stable analogue of inositol trisphosphate which mobilizes intracellular calcium

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D-Ins(1,4,5)P_3 is now recognized as an intracellular messenger that mediates the actions of many cell-surface receptors on intracellular Ca^{2+} pools, but its complex and rapid metabolism in intact cells has confused interpretation of its possible roles in oscillatory changes in intracellular [Ca^{2+}] and in controlling Ca^{2+} entry at the plasma membrane. We now report the actions and metabolic stability of a synthetic analogue of Ins(1,4,5)P_3, DL-inositol 1,4,5-trisphosphorothioate [DL-Ins(1,4,5)P_3[S]$_3$]. In permeabilized hepatocytes, DL-Ins(1,4,5)P_3[S]$_3$ and synthetic DL-Ins(1,4,5)P_3 stimulated Ca^{2+} release from the same intracellular stores, though the concentration required for half-maximal release was 3-fold higher for DL-Ins(1,4,5)P_3[S]$_3$. Since L-Ins(1,4,5)P_3 neither antagonized the effects of D-Ins(1,4,5)P_3 nor itself stimulated appreciable Ca^{2+} release, the activity of the racemic mixture of Ins(1,4,5)P_3, and presumably also of Ins(1,4,5)P_3[S]$_3$, is attributable to the D-isomer. Under conditions where there was negligible metabolism of D-[^{3}H]Ins(1,4,5)P_3, both DL-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3[S]$_3$ elicited rapid Ca^{2+} release from intracellular stores, and the stores remained empty during prolonged stimulation. When cells were incubated at high density, both compounds stimulated rapid Ca^{2+} release, but while the stores soon refilled as Ins(1,4,5)P_3 was degraded to Ins(1,4)P_2, there was no refilling of the pools after stimulation with DL-Ins(1,4,5)P_3[S]$_3$. When DL-Ins(1,4,5)P_3 or DL-Ins(1,4,5)P_3[S]$_3$ was treated with a crude preparation of Ins(1,4,5)P_3 3-kinase and ATP, and the Ca^{2+}-releasing activity of the products subsequently assayed, DL-Ins(1,4,5)P_3 was completely inactivated by phosphorylation, but there was no loss of activity of the phosphorothioate analogue. In additional experiments, DL-Ins(1,4,5)P_3[S]$_3$ (10 μM) did not affect the rate of phosphorylation of D-[^{3}H]Ins(1,4,5)P_3 (1 μM). We conclude that Ins(1,4,5)P_3[S]$_3$ is a full agonist and only 3-fold less potent than Ins(1,4,5)P_3 in mobilizing intracellular Ca^{2+} stores, but unlike the natural messenger it is resistant to both phosphorylation and dephosphorylation. We propose that this stable analogue will allow the direct actions of Ins(1,4,5)P_3 to be resolved from those that require its metabolism.

INTRODUCTION

Activation of many cell-surface receptors evokes both hydrolysis of PtdIns(4,5)P_2 (Berridge & Irvine, 1984; Downes & Michell, 1985) and an increase in cytosolic Ca^{2+} concentration. The latter response is frequently a series of transient oscillations (Woods *et al.*, 1986; Berridge *et al.*, 1988) that are often independent of extracellular Ca^{2+} and in some cells are mimicked by intracellular application of D-Ins(1,4,5)P_3 (Parker & Miledi, 1986). D-Ins(1,4,5)P_3 is unusual among intracellular messengers in the complexity of its metabolism (Majerus *et al.*, 1988); it may either be dephosphorylated to D-Ins(1,4)P_2 (Storey *et al.*, 1984) and probably thereby inactivated, or phosphorylated to D-Ins(1,3,4,5)P_4 (Irvine *et al.*, 1986), a product that may regulate Ca^{2+} transport at the plasma membrane (Irvine & Moor, 1986; Morris *et al.*, 1987).

Phosphorothioate compounds have found wide use in mechanistic enzymology and molecular biology as analogues of naturally-occurring substrates (Eckstein, 1985). DL-*myo*-Inositol 1,4,5-trisphosphorothioate (DL-Ins(1,4,5)P_3[S]$_3$), in which each phosphate group of Ins(1,4,5)P_3 has been replaced by a phosphorothioate group, was recently synthesized (Cooke *et al.*, 1987*a*,*b*) as a potential phosphatase-resistant analogue of Ins(1,4,5)P_3. We have earlier shown that Ins(1,4,5)P_3[S]$_3$ mobilizes Ca^{2+} from the intracellular stores of both *Xenopus* oocytes and permeabilized Swiss 3T3 cells (Taylor *et al.*, 1988), and more recent studies have confirmed our observations (Strupish *et al.*, 1988). We now report that in permeabilized hepatocytes, DL-Ins(1,4,5)P_3[S]$_3$ mobilizes intracellular Ca^{2+} stores, that it is a full agonist, only 3-fold less potent than DL-Ins(1,4,5)P_3, and furthermore its actions are prolonged because it is resistant to both phosphorylation and dephosphorylation. We expect this stable analogue of Ins(1,4,5)P_3 to find widespread use in analyses of inositol phosphate actions and metabolism, both as a long-lived analogue of the natural messenger and as a compound that cannot be further metabolized to other active messengers.

MATERIALS AND METHODS

Measurement of ^{45}Ca release from intracellular pools of permeabilized hepatocytes

Hepatocytes were prepared by collagenase digestion of livers removed from male Wistar rats (about 200 g) (Taylor & Putney, 1985). The cells were kept in cold

Abbreviations used: Ins(1,4,5)P_3[S]$_3$, inositol 1,4,5-trisphosphorothioate. Isomeric configurations are defined in the text.

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Eagle's medium supplemented with 2% (w/v) bovine serum albumin until required. Cells were permeabilized by incubation with saponin (75 μg/ml) for 10 min at 37 °C in a Ca^{2+}-free cytosol-type medium (140 mM-KCl/20 mM-NaCl/2 mM-$MgCl_2$/1 mM-EGTA/20 mM-Pipes, pH 6.8/2% bovine serum albumin. After permeabilization, the cells were washed and resuspended at low cell density (0.2–0.4 mg of protein/ml) in the same medium with Ca^{2+} added to give a free [Ca^{2+}] of about 120 nM (Berridge *et al.*, 1984). Permeabilized cells were added to $^{45}CaCl_2$ (2 μCi/ml), oligomycin (10 μM) and antimycin (10 μM), and 1 min later $^{45}Ca^{2+}$ uptake into non-mitochondrial pools was initiated by addition of ATP (1.5 mM), creatine phosphate (5 mM) and creatine phosphokinase (1 unit/ml). At 20 min, Ins(1,4,5)P_3 or Ins(1,4,5)P_3[S]$_3$ was added, and 5 min later samples of cells (200 μl) were diluted into 2 ml of cold iso-osmotic sucrose (310 mM) containing EGTA (4 mM) and either [^{3}H]mannose or ^3H_2O (0.3 μCi/ml) to correct for trapped volume. The samples were rapidly filtered through Whatman GF/C filters (Burgess *et al.*, 1983) and the filters were washed with 5 ml of sucrose/EGTA. After correction for trapped volume, the $^{45}Ca^{2+}$ contents of cells were calculated and the release stimulated by InsP_3 was expressed as a fraction of ATP-dependent $^{45}Ca^{2+}$ uptake.

Treatment of inositol phosphates with Ins(1,4,5)P_3 3-kinase

A rat-brain homogenate (4% in 500 mM-Tris/maleate/150 mM-sucrose, pH 7.5) was centrifuged at 1400 *g* for 10 min at 4 °C (Irvine *et al.*, 1986; Tennes *et al.*, 1987), and the supernatant fraction was stored frozen until required. DL-Ins(1,4,5)P_3 or DL-Ins(1,4,5)-P_3[S]$_3$ (100 μM) were added to the supernatant (2%) in sucrose (150 mM) containing $MgSO_4$ (20 mM) and buffered with Tris/maleate (500 mM; pH 7.5). After 1 min, ATP (10 mM), creatine phosphate (10 mM) and creatine phosphokinase (5 units/ml) were added, and the incubation was continued for a further 60 min at 37 °C. Samples (20 μl) were transferred to tubes and the incubations were terminated by heating at 70 °C for 3 min. Earlier experiments established that this procedure for stopping incubations caused no loss of activity of either Ins(1,4,5)P_3 or Ins(1,4,5)P_3[S]$_3$. The activities of the inositol phosphate samples were subsequently determined by addition of 200 μl of permeabilized hepatocytes prelabelled to steady state with $^{45}Ca^{2+}$ as described above. Results, corrected for the small $^{45}Ca^{2+}$ release stimulated by incubation medium alone, are shown as a percentage of ATP-dependent ^{45}Ca uptake.

Separation of inositol phosphates

The metabolism of inositol phosphates was determined by including [^{3}H]D-Ins(1,4,5)P_3 (final activity in most experiments, 0.2 μCi/ml) with the incubations. Reactions were terminated by addition of 400 μl of cold 4.5% (w/v) $HClO_4$ to 200 μl of cells, followed by addition of 50 μl of phytic acid hydrolysate (about 1 mg of *P*/ml) (Wreggett & Irvine, 1987). After centrifugation, the supernatant was neutralized (Sharpes & McCarl, 1982), EDTA (5 mM) was added, and the sample was stored frozen before analysis by either h.p.l.c. or anion-exchange chromatography.

For h.p.l.c. analysis, samples were loaded on to a Partisil 10 SAX anion-exchange h.p.l.c. column, and [^{3}H]inositol phosphates were eluted with linear NH_4CO_2H gradients as follows (pH 3.7 with H_3PO_4): 0–5 min, water; 5–27.5 min, water–0.84 M; 27.5–40 min, 0.84–3.4 M; flow rate 1.2 ml/min. Fractions (30 s) were counted in Hi-salt Scint (Zinsser Analytic) scintillation cocktail. Peaks were identified by comparison with absorbance (259 nm) from added adenine nucleotides and by their co-elution with authentic standards.

In some experiments, [^{3}H]inositol phosphates were separated on 3 cm columns of AG1-X8 (formate form) anion-exchange resin in Pasteur pipettes. Samples (2 ml) were loaded on to the columns and [^{3}H]inositol phosphates were eluted: inositol, 8 ml of distilled water; InsP_1, 8 ml of 0.2 M-NH_4CO_2H in 0.1 M-HCO_2H; InsP_2, 8 ml of 0.5 M-NH_4CO_2H in 0.1 M-HCO_2H; InsP_3, 8 ml of 0.95 M-NH_4CO_2H in 0.1 M-HCO_2H; InsP_4, 8 ml of 1.25 M-NH_4CO_2H in 0.1 M-HCO_2H. With this procedure, inositol, InsP_1, InsP_2 and InsP_3 were completely resolved, and InsP_3 and InsP_4 were separated with only about 1.3% of the InsP_4 fraction collected in the InsP_3 fraction and only 3.4% of InsP_3 collected in the InsP_4 fraction. Since the method does not separate isomers of inositol phosphates, it was used only when h.p.l.c. analyses indicated that such separations were unnecessary (see the Results section).

Effects of Ins(1,4,5)P_3[S]$_3$ on phosphorylation of Ins(1,4,5)P_3

[^{3}H]D-Ins(1,4,5)P_3 (0.1 μCi/ml; final concentration of D-Ins(1,4,5)P_3, 10 μM) was incubated with the brain supernatant preparation under identical conditions to those used to determine its effects on the activity of inositol phosphates (see above). Samples of 200 μl were removed at various times and stopped with 400 μl of cold $HClO_4$ (4.5%), and the samples were then neutralized and the products separated as described above. Recovery of the added radiolabel was 90 ± 6% (n = 6) after the 60 min incubation.

Materials

D-Ins(1,4,5)P_3, [^{3}H]D-Ins(1,4,5)P_3 (1 Ci/mmol) and D-Ins(1,4,[^{32}P]5)P_3 (about 1000 Ci/mmol) were from Amersham. Collagenase, ATP, creatine phosphate and creatine phosphokinase were from Boehringer Mannheim. DL-Ins(1,4,5)P_3, L-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3[S]$_3$ were synthesized as described (Cooke *et al.*, 1987*a*,*b*). All other reagents were from the suppliers given in earlier publications.

RESULTS

Effects of InsP_3[S]$_3$ on intracellular Ca^{2+} pools

Permeabilized hepatocytes sequestered $^{45}Ca^{2+}$ into a non-mitochondrial pool when provided with ATP. Within 10 min a steady state was reached, and by 20 min the $^{45}Ca^{2+}$ content of the cells had risen from 0.36 ± 0.06 to 3.88 ± 0.57 nmol/mg of protein (mean ± S.E.M.; n = 14). Addition of a maximally effective concentration of D-Ins(1,4,5)P_3 (100 μM) stimulated release of 60% of that Ca^{2+} pool, and half-maximal release occurred with 0.4 μM (Fig. 1*a*). Since the phosphorothioate analogue of Ins(1,4,5)P_3 was prepared as a racemic mixture of the two enantiomers, we examined the effect of the synthetic enantiomer of D-Ins(1,4,5)P_3. L-Ins(1,4,5)P_3 failed to stimulate Ca^{2+} release at concentrations of up to 10 μM and even at 100 μM it stimulated release of only 15% of

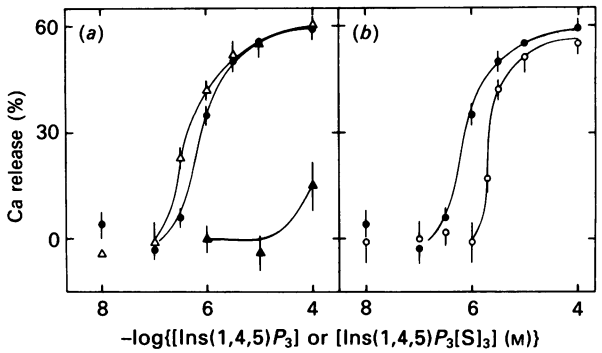

Fig. 1. Concentration–effect relationships for Ins(1,4,5)P_3- and Ins(1,4,5)P_3[S]$_3$-induced Ca^{2+} release from permeabilized hepatocytes

Permeabilized cells at low cell density were labelled to steady state with ^{45}Ca in the presence of mitochondrial inhibitors and then incubated with various inositol phosphates for 5 min. The release of $^{45}Ca^{2+}$ evoked by the inositol phosphates, as a percentage of ATP-dependent uptake, is shown as the mean ± S.E.M. of duplicate determinations from between four and nine independent experiments. ▲, L-Ins(1,4,5)P_3; △, D-Ins(1,4,5)P_3; ●, DL-Ins(1,4,5)P_3; ○, DL-Ins(1,4,5)P_3[S]$_3$.

the ATP-dependent Ca^{2+} pool (Fig. 1*a*). The responses to near-maximal (10 μM) or submaximal (0.3 μM) concentrations of D-Ins(1,4,5)P_3 were unaffected by the simultaneous presence of L-Ins(1,4,5)P_3 (10 μM) (results not shown). As expected from these results, a maximally effective concentration of the wholly synthetic racemic mixture of Ins(1,4,5)P_3 released the same amount of Ca^{2+} as did D-Ins(1,4,5)P_3, though the concentration–response relationship was shifted such that half-maximal release occurred at an approx. 2-fold higher concentration (0.75 μM) (Fig. 1*a*).

Fig. 1(*b*) compares the effects of DL-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3[S]$_3$ on intracellular Ca^{2+} pools. The responses to maximal concentrations of each were similar, i.e. release of about 60% of accumulated Ca^{2+}, and a combined application of maximal concentrations of each evoked no greater release. Half-maximal responses occurred at 0.75 μM for DL-Ins(1,4,5)P_3 and 2.1 μM for DL-Ins(1,4,5)P_3[S]$_3$. The results presented in Fig. 1(*a*) demonstrate that the biological activity of DL-Ins(1,4,5)P_3 is entirely attributable to the D-isomer. By analogy, it seems likely that only the D-isomer of DL-Ins(1,4,5)P_3[S]$_3$ is capable of stimulating Ca^{2+} release.

Under the conditions used to determine these concentration–effect relationships (the cells were washed after permeabilization and then resuspended at low cell density), there was negligible metabolism of [^{3}H]D-Ins(1,4,5)P_3 (Fig. 2*b*). The relative activities of Ins(1,4,5)P_3 at high cell density with DL-Ins(1,4,5)P_3 (10 μM) (*c*) degraded [^{3}H]D-Ins(1,4,5)P_3 with a half-time of about 10 min. Most of that degradation occurred by the 5-phosphatase pathway [at 30 min, 83% of activity was recovered as Ins + Ins$P$ + Ins(1,4)P_2] with only a very minor contribution from the 3-kinase pathway [8% of activity recovered as Ins(1,3,4)P_3 + Ins(1,3,4,5)P_4]. These results demonstrate that under the conditions used to examine the time course of the actions of DL-Ins(1,4,5)P_3 at high cell density (Fig. 3*a*), degradation of D-Ins(1,4,5)P_3 is dominated by 5-phosphatase action.

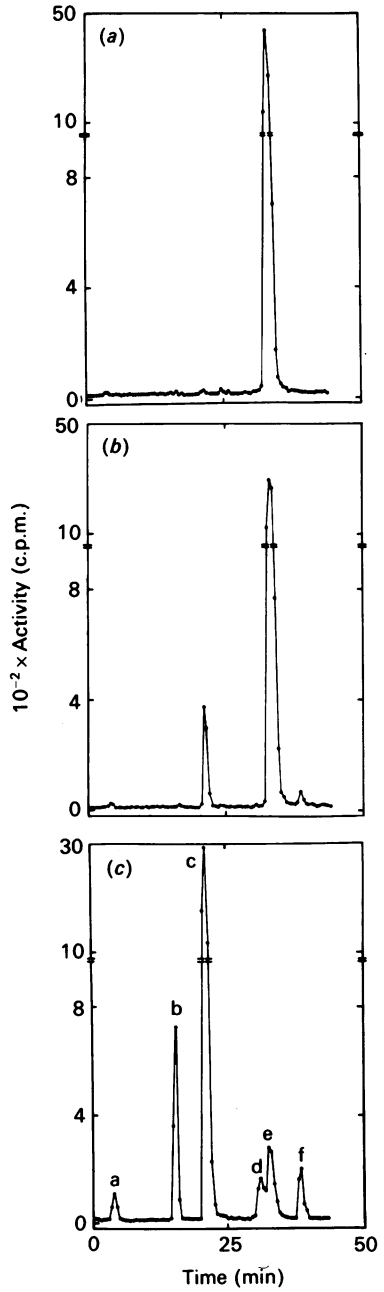

Fig. 2. Metabolism of [^{3}H]D-Ins(1,4,5)P_3 by permeabilized hepatocytes

Permeabilized hepatocytes were incubated at cell densities of 0.29 mg/ml (*b*) or 2.82 mg/ml (*c*). After 20 min, either [^{3}H]D-Ins(1,4,5)P_3 (Amersham, 1 Ci/mmol; 0.2 μCi/ml; final [D-Ins(1,4,5)P_3] = 0.2 μM) (*b*), or DL-Ins(1,4,5)P_3 (10 μM) labelled with [^{3}H]D-Ins(1,4,5)P_3 (*c*) was added. Reactions were terminated after a further 5 min (*b*) or 30 min (*c*). Peaks, identified by comparison with absorbance from added adenine nucleotides and by their co-elution with authentic standards, were as follows: a, Ins; b, InsP_1; c, Ins(1,4)P_2; d, Ins(1,3,4)P_3; e, Ins(1,4,5)P_3; f, Ins(1,3,4,5)P_4. Incubation of [^{3}H]D-Ins(1,4,5)P_3 in the absence of cells (*a*) caused no detectable degradation, while after a 5 min incubation with cells at low density (*b*), 91% of the activity was recovered as [^{3}H]Ins(1,4,5)P_3, 8% as [^{3}H]Ins(1,4)P_2 and 1% as [^{3}H]Ins(1,3,4,5)P_4. These results establish that under the conditions used to determine the concentration–effect relationships shown in Fig. 1, there is no appreciable metabolism of Ins(1,4,5)P_3. Cells incubated

and Ins(1,4,5)P_3[S]$_3$ therefore reflect their abilities to bind to and activate the receptor that regulates Ca^{2+} release from intracellular pools and are not a consequence of any difference in their relative rates of metabolism. We conclude that Ins(1,4,5)P_3[S]$_3$ is a full agonist for mobilization of intracellular Ca^{2+} pools, and is only about 3-fold less potent than the naturally occurring messenger, D-Ins(1,4,5)P_3.

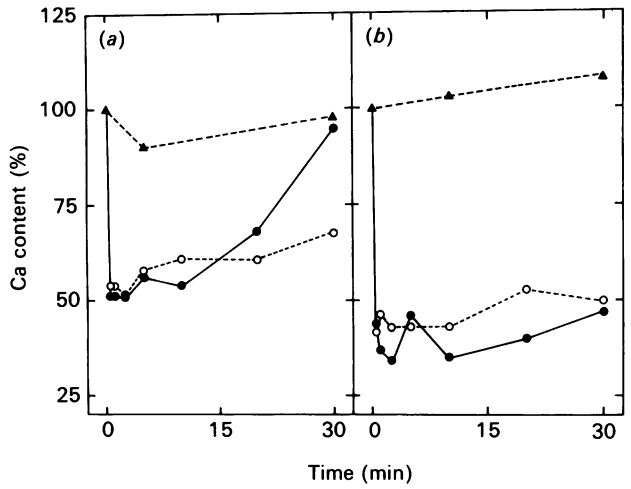

Fig. 3. Effects of 5-phosphatase on DL-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3[S]$_3$

Permeabilized cells were labelled to steady state with ^{45}Ca and after 20 min ($t = 0$ in Fig.) DL-Ins(1,4,5)P_3 (10 μM) or DL-Ins(1,4,5)P_3[S]$_3$ (10 μM) was added. Results are the means of duplicate determinations from a single experiment in which the cell densities were 2.82 mg of protein/ml (*a*) and 0.33 mg of protein/ml (*b*). ▲, Control; ●, DL-Ins(1,4,5)P_3; ○, DL-Ins(1,4,5)P_3[S]$_3$. Additional experiments provided similar results, a rapid release of Ca^{2+} after addition of DL-Ins(1,4,5)P_3 or DL-Ins(1,4,5)P_3[S]$_3$, and no subsequent re-accumulation of Ca^{2+} by cells incubated at low cell density (0.31 ± 0.11 mg of protein/ml, $n = 3$). However, when cells were incubated at high cell density (1.88 ± 0.47 mg of protein/ml, $n = 3$), they re-accumulated Ca^{2+} after its release by DL-Ins(1,4,5)-P_3 (52 ± 9 %, $n = 3$ at 30 s; 99 ± 4 %, $n = 3$ at 30 min) but not after its release by DL-Ins(1,4,5)P_3[S]$_3$ (61 ± 9 %, $n = 3$ at 30 s; 67 ± 2 % at 30 min).

Resistance of Ins(1,4,5)P_3[S]$_3$ to inactivation by dephosphorylation

Degradation of D-Ins(1,4,5)P_3 by permeabilized hepatocytes has been shown to coincide with re-uptake of Ca^{2+} (Joseph *et al.*, 1984). We therefore decided to examine the stability of DL-Ins(1,4,5)P_3[S]$_3$ by measuring Ca^{2+} re-uptake under conditions where D-Ins(1,4,5)P_3 is degraded. Hepatocytes incubated at high cell density (about 10-fold more dense than in the experiments described above) rapidly degraded [^{3}H]D-Ins(1,4,5)P_3 (half-time about 10 min) via the 5-phosphatase pathway with very little contribution from the 3-kinase pathway (Fig. 2*c*). We compared the effects of DL-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3[S]$_3$ under these conditions to see if the compounds differed in their susceptibility to inactivation by the 5-phosphatase. Almost maximal concentrations of each compound (10 μM) released very similar amounts of Ca^{2+}, but while the Ca^{2+} was soon re-sequestered after stimulation by Ins(1,4,5)P_3, the Ca^{2+} pool remained empty for up to 30 min after stimulation by Ins(1,4,5)P_3-[S]$_3$ (Fig. 3*a*). Fig. 3(*b*) demonstrates that when the same experiment was repeated at low cell density – conditions under which there is minimal degradation of [^{3}H]D-Ins(1,4,5)P_3 (Fig. 2*b*) – both compounds stimulated rapid release of Ca^{2+}, and for both the Ca^{2+} pool remained empty for the duration of the experiment (30 min). These results establish that the receptor that mediates the actions of these compounds on intracellular Ca^{2+} pools does not desensitize during prolonged stimulation, an observation that supports earlier findings (Prentki *et al.*, 1985). The prolonged actions of DL-Ins(1,4,5)P_3[S]$_3$ on the intracellular Ca^{2+} pools of cells incubated at high density must therefore result from its resistance to inactivation. Since under the conditions of these experiments 5-phosphatase-catalysed dephosphorylation is the major inactivation pathway, we conclude that

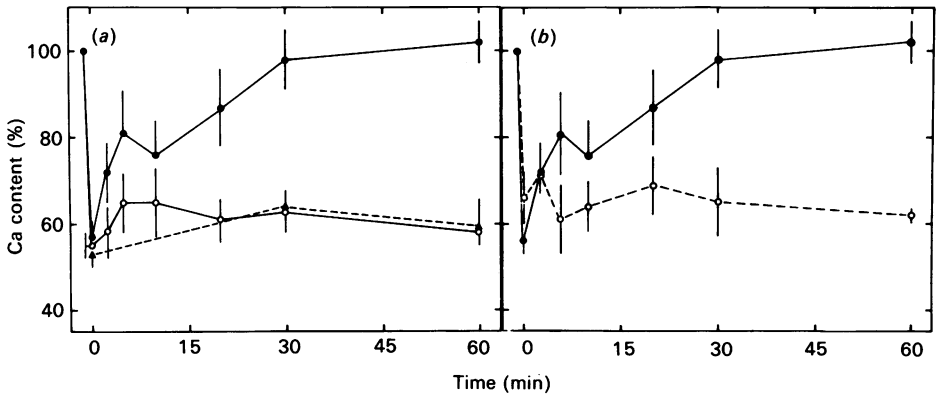

Fig. 4. Effects of 3-kinase on DL-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3[S]$_3$

DL-Ins(1,4,5)P_3 or DL-Ins(1,4,5)P_3[S]$_3$ were incubated with a rat brain supernatant under various conditions, and samples of the inositol phosphates removed at intervals for subsequent determination of their effects on the intracellular Ca^{2+} pools of permeabilized hepatocytes. Results, corrected for the small Ca^{2+} release stimulated by incubation medium alone, are shown as a percentage of ATP-dependent ^{45}Ca uptake (means ± S.E.M. of duplicate determinations from at least four independent experiments). (*a*) Incubations of DL-Ins(1,4,5)P_3. ●, Active kinase+ATP; ○, active kinase, no ATP; ▲, heat-inactivated kinase+ATP. (*b*) Incubations with active kinase and ATP. ●, DL-Ins(1,4,5)P_3; ○, DL-Ins(1,4,5)P_3[S]$_3$.

DL-Ins(1,4,5)P_3[S]$_3$ is substantially resistant to hydrolysis by that enzyme.

Resistance of Ins(1,4,5)P_3[S]$_3$ to inactivation by phosphorylation

D-Ins(1,3,4,5)P_4 (5 μM), the product of phosphorylation of D-Ins(1,4,5)P_3 (Irvine *et al.*, 1986), neither significantly stimulated Ca^{2+} release itself nor affected the release evoked by near-maximal concentrations (10 μM) of either DL-Ins(1,4,5)P_3 or DL-Ins(1,4,5)P_3[S]$_3$ (results not shown). This result provides an opportunity to compare the sensitivity of DL-Ins(1,4,5)P_3 and the phosphorothioate analogue to phosphorylation which should coincide with loss of their abilities to release Ca^{2+} from intracellular pools. Treatment of DL-Ins(1,4,5)P_3 with a crude preparation of D-Ins(1,4,5)P_3 3-kinase in the presence of ATP completely inactivated its Ca^{2+}-releasing activity within 30 min (half-time about 5 min) (Fig. 4*a*). In parallel experiments, there was no loss of activity when ATP was omitted from the incubation or when the kinase preparation was first heat-inactivated, suggesting that the loss of activity of DL-Ins(1,4,5)P_3 is entirely attributable to its phosphorylation. H.p.l.c. analysis of parallel incubations that included DL-Ins(1,4,5)P_3 (100 μM) labelled with [^{3}H]D-Ins(1,4,5)P_3 (1.5 μCi/ml) demonstrated that by 30–60 min, 90% of the D-Ins(1,4,5)P_3 had been phosphorylated to Ins(1,3,4,5)P_4, with negligible formation of Ins(1,3,4)P_3 and less than 3% metabolized to Ins, InsP_1 and InsP_2. These results demonstrate that under the conditions used for these incubations, the inactivation of Ins(1,4,5)P_3 is entirely attributable to its phosphorylation to Ins(1,3,4,5)P_4.

In complete contrast, even prolonged treatment (60 min) of DL-Ins(1,4,5)P_3[S]$_3$ with active kinase in the presence of ATP caused no loss of its ability to release Ca^{2+} (Fig. 4*b*). We conclude that while Ins(1,4,5)P_3 is phosphorylated and thereby inactivated by a D-Ins-(1,4,5)P_3 3-kinase, Ins(1,4,5)P_3[S]$_3$ is not a substrate for this inactivation pathway.

Lack of effect of Ins(1,4,5)P_3[S]$_3$ on phosphorylation of Ins(1,4,5)P_3

The resistance of Ins(1,4,5)P_3[S]$_3$ to phosphorylation prompted us to examine the possibility that it may nevertheless bind to the Ins(1,4,5)P_3 3-kinase and so inhibit phosphorylation of Ins(1,4,5)P_3. Under the conditions used to measure the activity of the 3-kinase, we have been unable to detect significant formation of Ins(1,3,4)P_3: h.p.l.c. analysis revealed no such product, and incubations of Ins(1,4,5)P_3 with the kinase in the absence of ATP failed to detect any Ins(1,4,5)P_3 5-phosphatase activity. We therefore pursued the competition studies by incubating [^{3}H]D-Ins(1,4,5)P_3 with the crude kinase preparation, and separated the products by anion-exchange chromatography. Further justification for using anion-exchange chromatography rather than h.p.l.c. is provided by our finding that under conditions where 60–70% of a mixture of [^{3}H]Ins(1,4,5)P_3 and Ins(1,4,[^{32}P]5)P_3 was phosphorylated to InsP_4, we found no change in the ratio of ^{32}P/^{3}H in the InsP_3 fraction, indicating that there can have been no significant formation of Ins(1,3,4)P_3.

During a prolonged incubation of [^{3}H]D-Ins(1,4,5)P_3 with the crude 3-kinase preparation, most of the Ins(1,4,5)P_3 was phosphorylated to Ins(1,3,4,5)P_4 as the concentration of substrate declined from 1 μM to 0.38 ± 0.1 μM (n = 6) (Fig. 5), well below the K_m of 0.6 μM (Irvine *et al.*, 1986). In parallel experiments, the rate of phosphorylation of [^{3}H]Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 was unaffected by the presence of a much higher concentration (10 μM) of DL-Ins(1,4,5)P_3[S]$_3$ (Fig. 5). These results demonstrate that Ins(1,4,5)P_3[S]$_3$ does not compete with Ins(1,4,5)P_3 for the Ins(1,4,5)P_3 3-kinase.

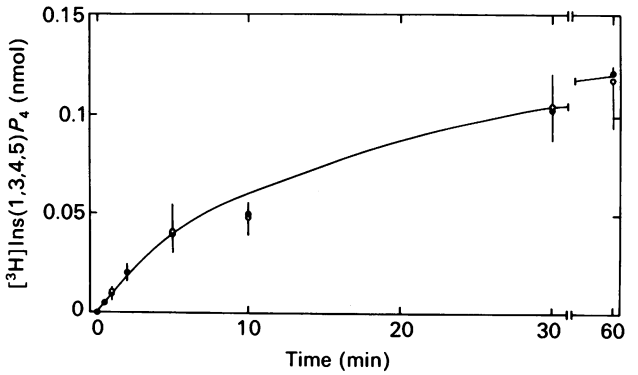

Fig. 5. Phosphorylation of [^{3}H]D-Ins(1,4,5)P_3 in the presence and absence of DL-Ins(1,4,5)P_3[S]$_3$

[^{3}H]D-Ins(1,4,5)P_3 (1 μM) was incubated with the rat brain supernatant preparation in the presence of ATP. Samples of 200 μl (containing 0.2 nmol of Ins(1,4,5)P_3 at the beginning of the incubation) were removed at intervals, and the products separated by anion-exchange chromatography. The rate of formation of [^{3}H]Ins(1,3,4,5)P_4 was unaffected by the presence of DL-Ins(1,4,5)P_3[S]$_3$ (10 μM). Results are the means ± S.E.M. of duplicate determinations from three independent experiments. ○, [^{3}H]D-Ins(1,4,5)P_3 alone; ●, [^{3}H]D-Ins(1,4,5)P_3 + DL-Ins(1,4,5)P_3[S]$_3$.

DISCUSSION

We have demonstrated that D-Ins(1,4,5)P_3[S]$_3$ is a potent analogue of the intracellular messenger D-Ins(1,4,5)P_3; it is a full agonist for mobilization of intracellular Ca^{2+} pools and only about 3-fold less potent than the natural messenger. Similar results have been reported in other cell types including *Xenopus* oocytes and Swiss 3T3 cells (Taylor *et al.*, 1988) and GH$_3$ cells (Strupish *et al.*, 1988). Consistent with these results is our recent finding that DL-Ins(1,4,5)P_3 and DL-Ins(1,4,5)P_3-[S]$_3$ also differ by a factor of three in their affinities for a high affinity D-Ins(1,4,5)P_3-binding site in permeabilized hepatocytes (D. L. Nunn, B. V. L. Potter and C. W. Taylor, unpublished work). However, in cerebellar membranes a 6-fold difference in affinities has been reported (Willcocks *et al.*, 1988), although it has not yet been possible to determine the effects of inositol phosphates on intracellular Ca^{2+} pools in this preparation.

Ins(1,4,5)P_3 is a substrate for both a 5-phosphatase enzyme and a 3-kinase enzyme, and metabolism by either route inactivates its ability to mobilize intracellular Ca^{2+} stores. However, our results suggest that Ins(1,4,5)P_3[S]$_3$ is not a substrate for either of these pathways. Using another bioassay protocol, Willcocks *et al.* (1988) have confirmed the resistance of Ins(1,4,5)P_3[S]$_3$ to inactivation by the 5-phosphatase. They showed that while treatment of Ins(1,4,5)P_3 with human erythrocyte 5-phosphatase abolished its ability to displace [^{3}H]D-Ins(1,4,5)P_3 from specific binding sites in cerebellar membranes, the activity of Ins(1,4,5)P_3[S]$_3$ was unaffected.

Although not a substrate for the 5-phosphatase enzyme, DL-Ins(1,4,5)P_3[S]$_3$ is a potent inhibitor of the enzyme ($K_i = 6\ \mu$M) (Cooke *et al.*, 1988). However, at the concentration used in our experiments (10 μM), Ins(1,4,5)P_3[S]$_3$ appears not to bind to the 3-kinase, since it is neither a substrate for the enzyme nor does it compete with the natural substrate.

While both the phosphorylation and dephosphorylation pathways inactivate the effects of the D-Ins(1,4,5)P_3 on intracellular Ca^{2+} pools, it has been suggested that at least one of the products, D-Ins(1,3,4,5)P_4, may itself be an intracellular messenger and regulate Ca^{2+} transport at the plasma membrane (Irvine & Moor, 1986; Morris *et al.*, 1987). The rapid turnover of D-Ins(1,4,5)P_3, and the possibility that it may be the source of other active intracellular messengers, have caused considerable experimental problems. Radioligand binding experiments and concentration–effect relationships, for example, may often be confused by metabolism of D-Ins(1,4,5)P_3. Furthermore, the direct actions of D-Ins(1,4,5)P_3 have not been readily distinguishable from those that require its metabolism. One model that seeks to explain hormone- and D-Ins(1,4,5)P_3-stimulated oscillations in cytosolic [Ca^{2+}] suggests that it reflects oscillatory changes in the intracellular concentration of D-Ins(1,4,5)P_3, while another suggests that the oscillations result from oscillatory release of Ca^{2+} from an overloaded intracellular store that remains overloaded only as long as D-Ins(1,4,5)P_3 is able to keep the InsP_3-sensitive store empty (Berridge *et al.*, 1988). We suggest that the activity of DL-Ins(1,4,5)P_3[S$_3$] in a variety of cell types and its metabolic stability will make it the ligand of choice in studying the actions of the natural messenger. In addition, it may allow the proposed mechanisms underlying oscillations in cytosolic [Ca^{2+}] to be distinguished, and the relative roles of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in regulating Ca^{2+} entry at the plasma membrane to be determined.

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REFERENCES

Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321

Berridge, M. J., Heslop, J. P., Irvine, R. F. & Brown, K. D. (1984) Biochem. J. **222**, 195–201

Berridge, M. J., Cobbold, P. H. & Cuthbertson, K. S. R. (1988) Philos. Trans. R. Soc. London B, **320**, 325–343

Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. W., Jr. (1983) J. Biol. Chem. **258**, 15336–15345

Cooke, A. M., Gigg, R. & Potter, B. V. L. (1987*a*) J. Chem. Soc. Chem. Commun. 1525–1526

Cooke, A. M., Gigg, R. & Potter, B. V. L. (1987*b*) Tetrahedron Lett. **28**, 2305–2308

Cooke, A. M., Nahorski, S. R. & Potter, B. V. L. (1988) FEBS Lett. **242**, 373–377

Downes, C. P. & Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signalling (Cohen, P. & Houslay, M. D., eds.), pp. 3–56, Elsevier, Amsterdam

Eckstein, F. (1985) Annu. Rev. Biochem. **54**, 367–402

Irvine, R. F. & Moor, R. M. (1986) Biochem. J. **240**, 917–920

Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) Nature (London) **320**, 631–634

Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) J. Biol. Chem. **259**, 3077–3081

Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S. & Lips, D. L. (1988) J. Biol. Chem. **263**, 3051–3054

Morris, A. P., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. (1987) Nature (London) **330**, 653–655

Parker, I. & Miledi, R. (1986) Proc. R. Soc. London B **228**, 307–315

Prentki, M., Corkey, B. E. & Matschinsky, F. M. (1985) J. Biol. Chem. **260**, 9185–9190

Sharpes, E. S. & McCarl, R. L. (1982) Anal. Biochem. **124**, 421–424

Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) Nature (London) **312**, 374–376

Strupish, J., Cooke, A. M., Potter, B. V. L., Gigg, R. & Nahorski, S. R. (1988) Biochem. J. **253**, 901–905

Taylor, C. W. & Putney, J. W., Jr. (1985) Biochem. J. **232**, 435–438

Taylor, C. W., Berridge, M. J., Brown, K. D., Cooke, A. M. & Potter, B. V. L. (1988) Biochem. Biophys. Res. Commun. **150**, 626–632

Tennes, K. A., McKinney, J. S. & Putney, J. W., Jr. (1987) Biochem. J. **242**, 797–802

Willcocks, A. L., Cooke, A. M., Potter, B. V. L. & Nahorski, S. R. (1987) Biochem. Biophys. Res. Commun **146**, 1071–1078

Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1986) Nature (London) **319**, 600–602

Wreggett, K. A. & Irvine, R. F. (1987) Biochem. J. **245**, 655–660

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