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Synthetic Phosphorothioate-Containing Analogues of Inositol 1,4,5-Trisphosphate Mobilize Intracellular Ca^{2+} Stores and Interact Differentially with Inositol 1,4,5-Trisphosphate 5-Phosphatase and 3-Kinase

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SUMMARY

Intracellular Ca^{2+} stores in permeabilized SH-SY5Y neuroblastoma cells were mobilized by D-*myo*-inositol 1,4,5-trisphosphate [D-Ins(1,4,5)P_3] and two of its synthetic analogues, DL-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate (DL-InsP_3-5S) and DL-*myo*-inositol 1,4,5-trisphosphorothioate (DL-InsP_3S_3). The concentrations of D-Ins(1,4,5)P_3, DL-InsP_3-5S, and DL-InsP_3S_3 required for half-maximal release were 0.11, 0.8, and 2.5 μM, respectively. All agents were full agonists, releasing 55–60% of sequestered $^{45}Ca^{2+}$. D-Ins(1,4,5)P_3-induced mobilization of Ca^{2+} was transient, and Ca^{2+} reuptake followed D-Ins(1,4,5)P_3 metabolism closely. DL-InsP_3S_3-induced mobilization was persistent, consistent with the resistance of this analogue to metabolic enzymes. In contrast, DL-InsP_3-5S-induced Ca^{2+} mobilization was followed by reuptake of Ca^{2+}, albeit at a slower rate than that seen with D-Ins(1,4,5)P_3. DL-InsP_3-5S and DL-InsP_3S_3 were resistant to D-Ins(1,4,5)P_3 5-phosphatase and potently inhibited the enzyme, with K_i values of 6.8 and 1.7 μM, respectively. DL-InsP_3S_3 was resistant to D-Ins(1,4,5)P_3 3-kinase and was a very weak inhibitor of the enzyme (K_i = 230 μM). The ability of DL-InsP_3-5S to inhibit D-Ins(1,4,5)P_3 phosphorylation (apparent K_i = 5 μM) and its loss of Ca^{2+}-releasing ability on incubation with D-Ins(1,4,5)P_3 3-kinase suggest that this analogue may undergo phosphorylation to inositol 1,3,4-trisphosphate 5-phosphorothioate. These differential and complementary properties of DL-InsP_3-5S and DL-InsP_3S_3 may be useful in dissecting the roles of D-Ins(1,4,5)P_3 and D-*myo*-inositol 1,3,4,5-tetrakisphosphate in Ca^{2+} homeostasis.

There is now substantial evidence to suggest that D-Ins(1,4,5)P_3 (Fig. 1), generated from hydrolysis of phosphatidylinositol 4,5-bisphosphate (1–3), couples together the spatially separated events of cell surface receptor stimulation and mobilization of intracellular Ca^{2+}. D-Ins(1,4,5)P_3 is recognized by specific receptors associated with the endoplasmic reticulum, resulting in the opening of Ca^{2+} channels (4). The cerebellar D-Ins(1,4,5)P_3 receptor has been recently purified and sequenced (5), and its reconstitution into lipid vesicles or transfection into cells shows that it gates Ca^{2+} in a D-Ins(1,4,5)P_3-dependent manner (6, 7).

D-Ins(1,4,5)P_3 is readily metabolized by at least two discrete routes (8), which involve, at the primary step, either dephosphorylation by D-Ins(1,4,5)P_3 5-phosphatase to D-Ins(1,4)P_2 or phosphorylation by D-Ins(1,4,5)P_3 3-kinase to D-Ins(1,3,4,5)P_4. Although D-Ins(1,3,4,5)P_4 appears to be involved in Ca^{2+} homeostasis (2, 9, 10), all metabolites formed via the D-Ins(1,4,5)P_3 5-phosphatase pathway are inactive with respect to Ca^{2+} release, suggesting a requirement for a vicinal D-4,5-phosphate pairing in inositol phosphates with Ca^{2+}-releasing activity(11).

Definition of the cellular roles of inositol polyphosphates is, at present, limited by the lack of agents to manipulate their activity. It is clear that analogues of D-Ins(1,4,5)P_3 that are resistant to, or inhibit, the enzymes responsible for one or both of the routes of metabolism (11, 12) would facilitate understanding of the role of D-Ins(1,4,5)P_3 and its metabolites in the

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ABBREVIATIONS: Ins(1,4,5)P_3, *myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, *myo*-inositol 1,3,4,5-tetrakisphosphate; InsP_3S_3, *myo*-inositol 1,4,5-trisphosphorothioate; InsP_3-5S, *myo*-inositol 1,4-bisphosphate 5-phosphorothioate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; Ca^{2+}_{free}, free Ca^{2+} concentration; InsP_4-5S, *myo*-inositol 1,3,4-trisphosphate 5-phosphorothioate; Ins(1,4)P_2, *myo*-inositol 1,4-bisphosphate.

regulation of intracellular free Ca^{2+} concentrations. Thus, we have synthesised two phosphorothioate-containing analogues of D-Ins(1,4,5)P_3, namely, DL-InsP_3S_3 (Fig. 1) (13) and DL-InsP_3-5S (Fig. 1) (14). DL-InsP_3S_3 has already been shown to be an effective agonist at the D-Ins(1,4,5)P_3 receptor, in a variety of systems (15, 16), and a potent competitive inhibitor of D-Ins(1,4,5)P_3 5-phosphatase (17). The Ca^{2+}-releasing and biochemical properties of the novel DL-InsP_3-5S, however, have yet to be reported.

We describe here the interactions between the D-Ins(1,4,5)P_3 analogues and D-Ins(1,4,5)P_3 5-phosphatase and D-Ins(1,4,5)P_3 3-kinase and the ability of these compounds to release Ca^{2+} from permeabilized cells.

Materials and Methods

Cell culture. SH-SY5Y human neuroblastoma cells (passage 75–95) were grown as described (18). Swiss 3T3 (mouse fibroblast) cells were grown in Dulbecco's modified Eagle's medium supplemented with 110 μg/ml pyruvate, 1 mg/ml glucose, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 10% newborn calf serum. The cells, in 175-cm^2 tissue culture flasks with 30 ml of culture medium, were maintained at 37° in 5% CO_2/95% humidified air and subcultured twice weekly (split ratio, 1:4). Before use, the cells were harvested in 10 mM HEPES, 0.9% NaCl, pH 7.4, containing 0.02% EDTA, and were treated as described (15), before permeabilization.

Permeabilization and Ca^{2+} mobilization. SH-SY5Y cells (4–8 mg of protein/ml) were resuspended in "cytosol-like" medium, i.e., 120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM sodium succinate, 5 mM Na_2ATP, 2 mM KH_2PO_4, 10 μM quin2 free acid (to reduce free Ca^{2+} concentration to 100–300 nM), 0.2% dimethyl sulfoxide, pH 6.9. The cells were then permeabilized electrically, as described previously (19).

Swiss 3T3 cells were permeabilized (at a density of 0.5–1 mg of protein/ml) with saponin, as described (15). Cells were then loaded with $^{45}Ca^{2+}$ (15) at a density of 1–2 mg of protein/ml.

Aliquots of cells (100 μl) were then added to 100 μl of buffer containing D-Ins(1,4,5)P_3 or its analogues, in polypropylene microfuge tubes. After incubation at 20° for 2 or 15 min (to obtain dose-response curves) or 0.5–30 min (to obtain time-courses of $^{45}Ca^{2+}$ release and reuptake), 500 μl of a silicone oil mixture were added and the cells were separated from the medium and assayed for radioactivity as described (15).

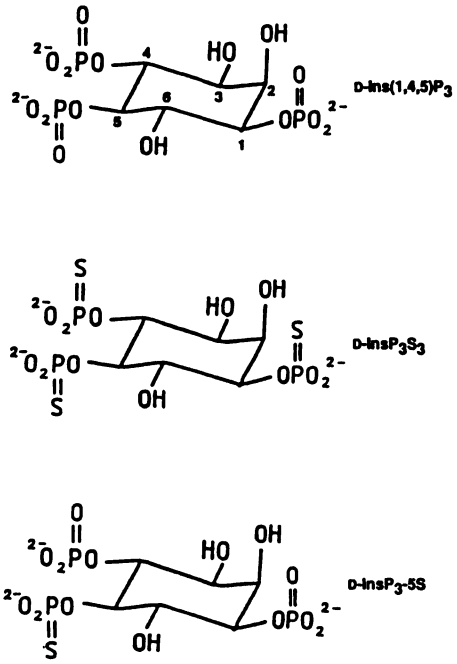

Fig. 1. Structures of Ins(1,4,5)P_3, InsP_3-5S, and InsP_3S_3. Only D-isomers are shown.

The temporal characteristics of Ca^{2+} mobilization from electroporated SH-SY5Y cells (3–4 mg of protein/ml) were monitored using a Ca^{2+}-sensitive electrode, as described (20).

D-[^{3}H]Ins(1,4,5)P_3 metabolism. Electroporated SH-SY5Y cells were resuspended in cytosol-like medium, at a cell density of 1.5–2.0 mg of protein/ml, and incubated at 20° for 20 min. Aliquots of cells (100 μl) were then added to 100 μl of buffer containing 2 μM Ins(1,4,5)P_3 and approximately 10,000 dpm, of D-[^{3}H]Ins(1,4,5)P_3. Incubations, for 0.5–30 min, were terminated with 200 μl of ice-cold perchloric acid (10%). After 20 min, the tubes were centrifuged at 10,000 × *g* for 2 min, and an aliquot (350 μl) of supernatant was mixed with 10 mM EDTA (73 μl) and 1:1 freon/octylamine (350μl). After centrifugation, 300 μl of the upper aqueous phase were removed and neutralized with $NaHCO_3$. Inositol trisphosphate was fractionated using ion exchange chromatography on Dowex AG1-X8 resin, as described (21, 22). A more detailed examination of the routes of D-Ins(1,4,5)P_3 metabolism was performed by incubation of electroporated SH-SY5Y cells (3–4 mg of protein/ml) with D-Ins(1,4,5)P_3 (5 μM) and approximately 100,000 dpm of D-[^{3}H]Ins(1,4,5)P_3. Incubations, for 0, 1, or 5 min, were terminated and inositol phosphates were prepared as described above. Samples were then analyzed by HPLC, using gradients comprising water and $(NH_4)H_2PO_4$, adjusted to pH 3.7 with H_3PO_4. We have previously validated this procedure using both internal standards and chemical identification (23).

D-Ins(1,4,5)P_3 and D-Ins(1,3,4,5)P_4 5-phosphatase activity. Human erythrocyte ghosts were prepared as described (24) and stored (7 mg of protein/ml) at −70°. D-Ins(1,4,5)P_3 or its synthetic analogues (60 μM) were incubated at 37° for 60 min in the presence of erythrocyte ghosts (1.7 mg of protein/ml) or inactivated (boiled) ghosts, in 30 mM HEPES, 2 mM $MgCl_2$, pH 7.2 (buffer A). Incubations were terminated by boiling, followed by centrifugation and removal of the supernatant. D-Ins(1,4,5)P_3 or its analogues were then stored at −20° until assayed for ability to release $^{45}Ca^{2+}$ from permeabilized Swiss 3T3 cells.

Inhibition of D-[5-^{32}P]Ins(1,4,5)P_3 metabolism by DL-InsP_3S_3 and DL-InsP_3-5S was carried out as described (17). Erythrocyte ghosts (0.7 mg of protein/ml) were incubated at 37° for 15 min in the presence of 30 μM D-Ins(1,4,5)P_3, approximately 10,000 dpm of D-[5-^{32}P]-Ins(1,4,5)P_3, and increasing amounts of DL-InsP_3S_3 or DL-InsP_3-5S. Under these conditions, no more than 20% of the substrate was consumed.

Inhibition of D-[5-^{32}P]Ins(1,3,4,5)P_4 metabolism by DL-InsP_3S_3 was analyzed using membranes obtained from SH-SY5Y cells. Cells were harvested and resuspended in buffer A. The cells were homogenized (Ultra-Turrax homogenizer; 6 × 2 sec) and centrifuged (50,000 × *g*, 20 min). The pellet was then rehomogenized and stored (12 mg of protein/ml) at −70°. SH-SY5Y membranes (0.1 mg of protein/ml) were incubated at 37° for 20 min in the presence of 3 μM D-Ins(1,3,4,5)P_4 (K_m = 1.24 μM), approximately 2000 dpm of D-[5-^{32}P]Ins(1,3,4,5)P_4, and increasing amounts of DL-InsP_3S_3. Under these conditions, no more than 20% of the substrate was consumed.

D-Ins(1,4,5)P_3 3-kinase activity. A supernatant preparation, high in D-Ins(1,4,5)P_3 3-kinase activity, was obtained from a crude homogenate of rat brain (25). DL-Ins(1,4,5)P_3 or its analogues (100 μM) were incubated at 37° for up to 30 min in the presence of the D-Ins(1,4,5)P_3 3-kinase preparation (0.3%, w/v) or inactivated (boiled) enzyme preparation, in a buffer consisting of 50 mM Tris maleate, 20 mM $MgCl_2$, 10 mM Na_2ATP, 5 mM 2,3-diphosphoglycerate, and 0.1% bovine serum albumin, pH 7.5. Incubations were terminated by boiling, followed by centrifugation and removal of the supernatant. Solutions containing DL-Ins(1,4,5)P_3 or its analogues were then stored at −20° until assayed for the ability to release $^{45}Ca^{2+}$ from permeabilized Swiss 3T3 cells.

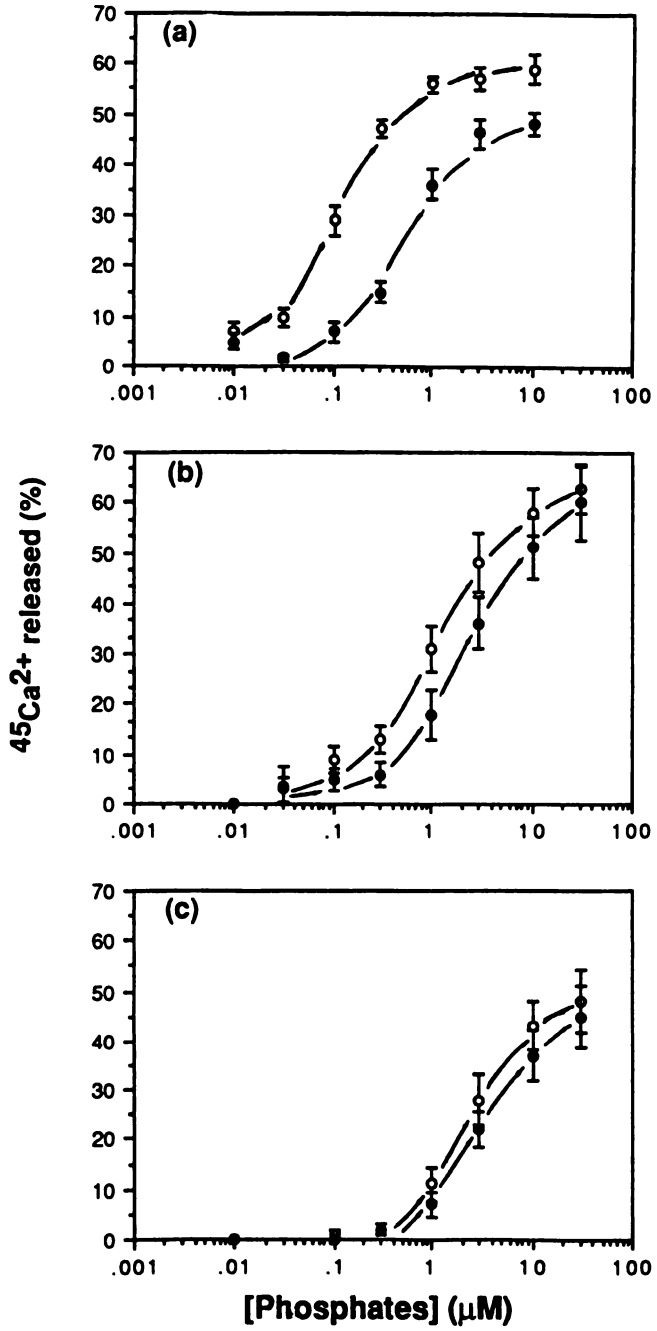

Fig. 2. Dose dependence of $^{45}Ca^{2+}$-releasing effects of D-Ins(1,4,5)P_3 (a), DL-InsP_3-5S (b), and DL-InsP_3S_3 (c) from permeabilized SH-SY5Y cells. Electrically permeabilized SH-SY5Y cells were loaded with $^{45}Ca^{2+}$ and then challenged with D-Ins(1,4,5)P_3 or its analogues. Incubations, at 20°, were terminated after either 2 (○) or 15 (●) min, at which points the amount of $^{45}Ca^{2+}$ released was assessed. Data shown are mean ± standard error from at least six independent experiments.

Inhibition of D-[^{3}H]Ins(1,4,5)P_3 phosphorylation by DL-InsP_3S_3 and DL-InsP_3-5S was carried out by incubation of the D-Ins(1,4,5)P_3 3-kinase preparation (0.1%, w/v) at 37° in the presence of 3, 10, or 30 μM D-Ins(1,4,5)P_3, approximately 10,000 dpm of D-[^{3}H]Ins(1,4,5)P_3, and increasing amounts of DL-InsP_3S_3 or DL-InsP_3-5S, under conditions where no more than 20% of D-Ins(1,4,5)P_3 was phosphorylated. Mono-, bis-, tris-, and tetrakisphosphate fractions were separated using ion exchange chromatography on Dowex AG1-X8 resin, as described (21, 22).

Miscellaneous. DL-Ins(1,4,5)P_3, DL-InsP_3, S_3 and DL-InsP_3-5S were

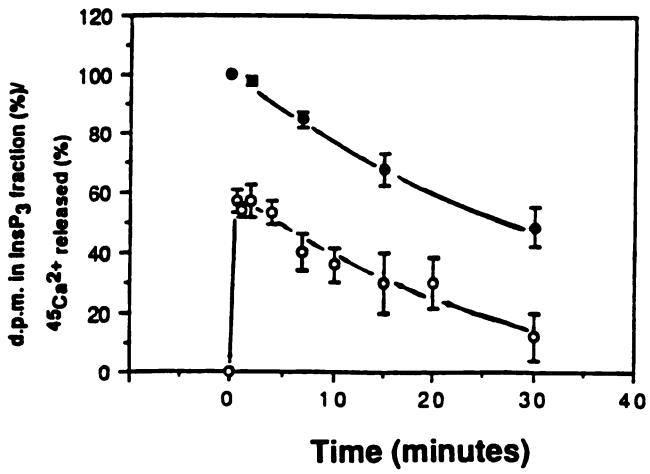

Fig. 3. Comparison of the rates of $^{45}Ca^{2+}$ reuptake and D-Ins(1,4,5)P_3 metabolism after addition of D-Ins(1,4,5)P_3 (1 μM) to permeabilized SH-SY5Y cells. For measurement of $^{45}Ca^{2+}$ reuptake after stimulation with D-Ins(1,4,5)P_3, electrically permeabilized SH-SY5Y cells were loaded with $^{45}Ca^{2+}$ and challenged with D-Ins(1,4,5)P_3 (1 μM), and released $^{45}Ca^{2+}$ was assessed at 0.5–30 min (○) (n = 6). The rate of D-Ins(1,4,5)P_3 metabolism was measured in parallel incubations (●) with cells not loaded with $^{45}Ca^{2+}$ but incubated with D-Ins(1,4,5)P_3 (1 μM) plus tracer D-[^{3}H] Ins(1,4,5)P_3 (~10,000 dpm). [^{3}H]Inositol trisphosphate was fractionated on Dowex AG1-X8 resin (four experiments).

synthesized as racemic mixtures (13, 14, 26) and purified by ion exchange chromatography on DEAE-Sephadex A-25, using linear gradients of triethylammonium bicarbonate, yielding triethyammonium salts. D-InsP_3S_3 was synthesized in an identical manner as the racemate, but from an optically resolved precursor. Protein concentration was determined using the assay described by Bradford (27). EC_{50} values, the concentration of agonist required to produce 50% of maximal response, were derived using ALLFIT computer-assisted curve fitting (28). Combined data from a number of independent experiments (n) are expressed as mean ± standard error, where $n > 3$. Statistical differences were determined using Student's t test and were considered significant when $p < 0.05$. SH-SY5Y cells and Swiss 3T3 cells were initially kind gifts from Drs. J. L. Biedler (Sloane-Kettering Institute, New York, NY) and C. Taylor (University of Cambridge, U.K.), respectively. All cell culture reagents were from GIBCO, Ltd.; D-Ins(1,4,5)P_3 was obtained from Calbiochem; $^{45}CaCl_2$ (~1000 Ci/mmol) was obtained from Amersham International, plc; Na_2ATP, quin2, and ionomycin were obtained from Sigma. Radiolabeled inositol polyphosphates were kind gifts from NEN-Du Pont, Ltd.

Results

Permeabilized SH-SY5Y cells exhibited rapid ATP-dependent sequestration of $^{45}Ca^{2+}$, with half-maximal uptake in less than 1 min (data not shown). Ionomycin (10 μM) released 88 ± 1.2% (n = 7) of sequestered $^{45}Ca^{2+}$, suggesting that the Ca^{2+} was intravesicular.

Addition of D-Ins(1,4,5)P_3, DL-InsP_3-5S, or DL-InsP_3S_3 caused rapid mobilization of sequestered $^{45}Ca^{2+}$, which was maximal by 30 sec, with no reuptake observed for at least 2 min (data not shown). The dose dependence of the response to D-Ins(1,4,5)P_3 and its analogues was examined in incubations terminated either 2 or 15 min after addition of the stimuli, in order to determine the relative potency and stability of the compounds. In 2-min incubations with SH-SY5Y cells (0.25–0.5 mg of protein/ml), D-Ins(1,4,5)P_3 and its analogues were equally efficacious in their ability to release sequestered $^{45}Ca^{2+}$ (Fig. 2). However, D-Ins(1,4,5)P_3 was a more potent stimulus

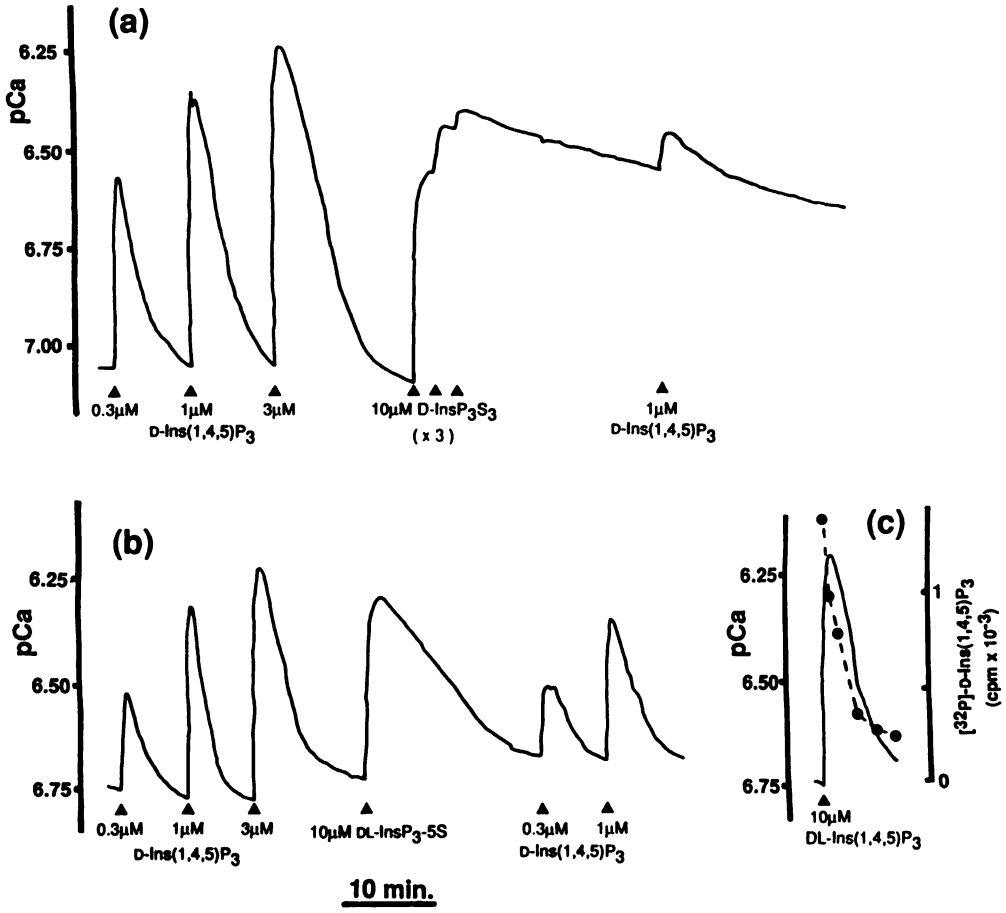

Fig. 4. Kinetics of Ca^{2+} release induced by D-Ins(1,4,5)P_3 and its analogues in permeabilized SH-SY5Y cells. Ca^{2+} release was stimulated by D-Ins(1,4,5)P_3 and InsP_3S_3 (D-isomer) (a), D-Ins(1,4,5)P_3 and DL-InsP_3-5S (b), or DL-Ins(1,4,5)P_3 plus D-[5-^{32}P]Ins(1,4,5)P_3 (c). $Ca^{2+}{}_{free}$ was monitored with a Ca^{2+}-sensitive electrode, and unmetabolized D-[5-^{32}P]Ins(1,4,5)P_3 (○) was isolated as in Fig. 3. The data obtained with D-InsP_3S_3 were essentially identical to those seen with DL-InsP_3S_3 (11). Data shown are representative of two independent experiments.

(EC_{50} = 0.11 ± 0.04 μM; n = 6), (Fig. 2a) than DL-InsP_3-5S (EC_{50} = 0.8 ± 0.17 μM; n = 6), (Fig. 2b) or DL-InsP_3S_3 (EC_{50} = 2.5 ± 0.29 μM; n = 6), (Fig. 2c). After 15-min incubations, however, the dose-response curves were shifted to the right, indicating that metabolism of the stimuli and reuptake of $^{45}Ca^{2+}$ had occurred (Fig. 2). This shift was more marked for D-Ins(1,4,5)P_3 (3.1-fold, p = 0.001) (Fig. 2a) than for DL-InsP_3-5S (1.75 fold, p = 0.006) (Fig. 2b). The shift for DL-InsP_3S_3 (0.3-fold) was not significant (p = 0.17) (Fig. 2c). These data suggest that, whereas D-Ins(1,4,5)P_3 and, to a lesser extent, DL-InsP_3-5S appear to be metabolized during incubations with the cells, DL-InsP_3S_3 is metabolized only very slowly, if at all.

To establish whether $^{45}Ca^{2+}$ reuptake was due to metabolism of the stimulus, SH-SY5Y cells were incubated with D-Ins(1,4,5)P_3 and tracer D-[^{3}H]Ins(1,4,5)P_3, and the rate of its metabolism was followed. It was found that $^{45}Ca^{2+}$ reuptake paralleled D-Ins(1,4,5)P_3 destruction (Fig. 3).

The differences between D-Ins(1,4,5)P_3, DL-InsP_3-5S, and DL-InsP_3S_3 with respect to reversibility of Ca^{2+} mobilization were indicated more clearly when $Ca^{2+}{}_{free}$ was monitored continuously, using a Ca^{2+}-sensitive electrode and a relatively high cell density (3–4 mg of protein/ml) (Fig. 4). D-Ins(1,4,5)P_3 increased $Ca^{2+}{}_{free}$ in a transient fashion (Fig. 4, a and b), because D-Ins(1,4,5)P_3 metabolism, monitored by measurement of destruction of D-[5-^{32}P]Ins(1,4,5)P_3 (Fig. 4c), was rapid due to the high concentrations of cellular enzymes present. DL-InsP_3S_3 released Ca^{2+} persistently, with only slow reuptake evident, again suggesting that it was resistant to metabolism (Fig. 4a) (11). In contrast, DL-InsP_3-5S-induced Ca^{2+} release was reversible, indicating that the analogue was being metabolized, albeit at a slower rate than D-Ins(1,4,5)P_3 (Fig. 4b).

A more detailed examination, using HPLC, of the routes of D-Ins(1,4,5)P_3 metabolism in permeabilized SH-SY5Y cells at high cell density (3–4 mg of protein/ml) showed that, although D-Ins(1,3,4,5)P_4 was formed (3.5 and 9.5% of total phosphates after 1 and 5 min, respectively), D-Ins(1,4)P_2 (44.1 and 61.4% of total phosphates after 1 and 5 min, respectively) was the major product of metabolism (Fig. 5). Thus, dephosphorylation by D-Ins(1,4,5)P_3 5-phosphatase was the predominant route of D-Ins(1,4,5)P_3 destruction. However, the contribution of D-Ins(1,4,5)P_3 3-kinase was significant and led to the formation of both D[^{3}H]Ins(1,3,4,5)P_4 and its metabolites (see Refs. 2 and 8). Little accumulation of inositol monophosphates, in contrast to the substantial accumulation seen at 37° (19, 29), was observed (Fig. 5). This suggests that the inositol bisphosphatase(s) present in SH-SY5Y cells are highly temperature sensitive and essentially inactive at 20°.

Treatment of DL-InsP_3-5S or DL-InsP_3S_3 with a human erythrocyte ghost preparation enriched in D-Ins(1,4,5)P_3 5-phosphatase (24), which caused a 5-fold loss of activity (rightward shift in EC_{50}) (p = 0.008) of D-Ins(1,4,5)P_3 in subsequent ability to release $^{45}Ca^{2+}$ from permeabilized Swiss 3T3 cells (Fig. 6a), caused no deactivation of the analogues (Fig. 6 b–c). Thus, DL-InsP_3-5S and DL-InsP_3S_3 are resistant to D-Ins(1,4,5)P_3 5-phosphatase. However, they do interact with D-Ins(1,4,5)P_3 5-phosphatase, because they both inhibited D-

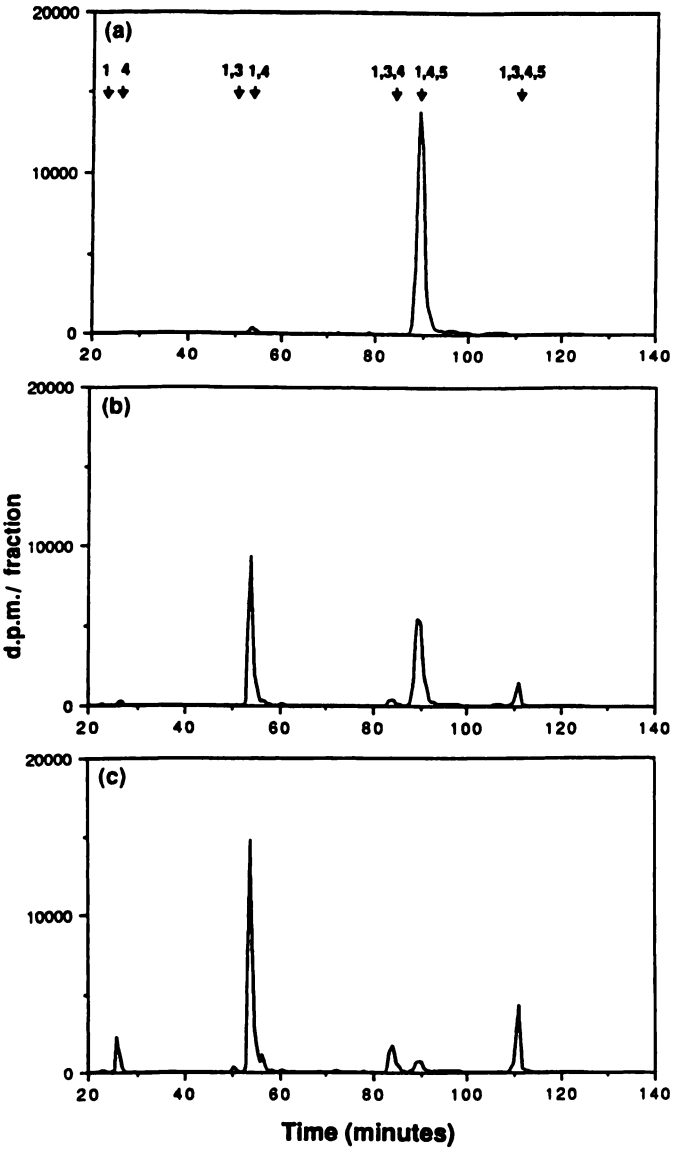

Fig. 5. Routes of D-Ins(1,4,5)P_3 metabolism in permeabilized SH-SY5Y cells. Electrically permeabilized SH-SY5Y cells (3–4 mg of protein/ml) were incubated, as in Fig. 3, with 5 μM D-Ins(1,4,5)P_3 plus D-[^{3}H] Ins(1,4,5)P_3 at 20°, for 0 (a), 1 (b), or 5 min (c). Incubations were terminated and extracts were prepared as described in Materials and Methods. Data shown are from one experiment, representative of three independent experiments with similar results.

Ins(1,4,5)P_3 dephosphorylation (Fig. 7). DL-InsP_3S_3 was found to be a more potent inhibitor (K_i = 1.7 ± 0.6 μM) than DL-InsP_3-5S (K_i = 6.8 ± 0.9 μM) (Fig. 7). DL-InsP_3S_3 also inhibited D-Ins(1,3,4,5)P_4 5-phosphatase (K_i = 1.33 ± 0.31 μM, n = 3; data not shown). Because both DL-InsP_3S_3 and DL-InsP_3-5S are resistant to D-Ins(1,4,5)P_3 5-phosphatase but DL-InsP_3-5S induces reversible Ca^{2+} release when incubated with suspensions of permeabilized SH-SY5Y cells (Figs. 2b and 4b), the interaction between D-Ins(1,4,5)P_3 3-kinase and the synthetic analogues was studied. DL-InsP_3-5S competitively inhibited D-Ins(1,4,5)P_3 phosphorylation (K_m = 3.2 μM), with an apparent K_i of 5 ± 2 μM (Fig. 8). In contrast, DL-InsP_3S_3 was a very weak inhibitor, with an apparent K_i of 230 ± 29 μM (n = 4). Thus, DL-InsP_3-5S, but not DL-InsP_3S_3, interacts potently with D-Ins(1,4,5)P_3 3-kinase. Furthermore, when DL-Ins(1,4,5)P_3 and its analogues were treated with the D-Ins(1,4,5)P_3 3-kinase preparation, the ability of DL-Ins(1,4,5)P_3 and DL-InsP_3-5S to release $^{45}Ca^{2+}$ from permeabilized Swiss 3T3 cells was attenuated (Fig. 6, d and e). Treatment of DL-Ins(1,4,5)P_3 with D-Ins(1,4,5)P_3 3-kinase for 5 min caused a 6-fold loss of activity (Fig. 6d) (p = 0.004). Prolonged treatment of DL-InsP_3-5S for 30 min caused a 4.3-fold loss of activity (Fig. 6e) (p = 0.01), suggesting that it was a substrate for the D-Ins(1,4,5)P_3 3-kinase. Identical treatment of DL-InsP_3S_3 caused no attenuation of subsequent Ca^{2+}-releasing activity (Fig. 6f). D-Ins(1,4,5)P_3 3-kinase treatment of DL-Ins(1,4,5)P_3 plus D-[^{3}H] Ins(1,4,5)P_3, followed by HPLC, showed that loss of Ca^{2+}-releasing activity was due entirely to the conversion of D-Ins(1,4,5)P_3 to D-Ins(1,3,4,5)P_4 (data not shown). Furthermore, the loss of activity seen after incubations with rat brain supernatant was blocked by heparin (10 μg/ml), an inhibitor of D-Ins(1,4,5)P_3 3-kinase but not D-Ins(1,4,5)P_3 5-phosphatase (30).

Discussion

The human neuroblastoma SH-SY5Y cell line has proven to be an excellent model system in which to examine phosphoinositide-mediated signaling. These cells express M_3 muscarinic receptors and show agonist-induced D-Ins(1,4,5)P_3 mass accumulation and Ca^{2+} responses that reflect release from intracellular stores and entry across the plasma membrane (18, 20). Furthermore, in the present studies we show that permeabilized SH-SY5Y cells possess substantial D-Ins(1,4,5)P_3-sensitive Ca^{2+} stores and the enzymes required for rapid degradation of the second messenger. These cells, together with permeabilized Swiss 3T3 cells, have enabled us to evaluate the properties of phosphorothioate-containing analogues of D-Ins(1,4,5)P_3.

We have demonstrated that both DL-InsP_3-5S and DL-InsP_3S_3 are full agonists for mobilization of Ca^{2+}, presumably from the D-Ins(1,4,5)P_3-sensitive store. The relative potencies of D-Ins(1,4,5)P_3 and its analogues with respect to Ca^{2+} release correlated closely with the rank order of their binding affinities for cerebellar membranes (31–33).[2] This correlation has been found by other groups and for other analogues of D-Ins(1,4,5)P_3 (33, 34), suggesting that all analogues of D-Ins(1,4,5)P_3 that bind to the D-Ins(1,4,5)P_3 receptor are full agonists (12).

The nature of the Ca^{2+} store mobilized after addition of D-Ins(1,4,5)P_3 or its analogues was not examined in detail here, but other studies with disrupted cells have shown quite clearly that a nonmitochondrial structure accumulates Ca^{2+} in an ATP-dependent manner (3, 35–39) and that D-Ins(1,4,5)P_3, through a specific receptor, facilitates emptying of this pool (2, 36–38). The balance between these activities constitutes only part of the mechanism by which cytosolic Ca^{2+}_{free} is regulated, however, because Ca^{2+} uptake into an D-Ins(1,4,5)P_3-insensitive nonmitochondrial pool can also occur (36–39). In the present study, D-Ins(1,4,5)P_3-induced increases in Ca^{2+}_{free} were transient, apparently due to metabolism of D-Ins(1,4,5)P_3 followed by rapid reuptake of the released Ca^{2+}. Use of DL-InsP_3S_3 showed that the Ca^{2+} released after activation of the D-Ins(1,4,5)P_3 receptor was not rapidly resequestered into an D-Ins(1,4,5)P_3-insensitive pool or mitochondria, because the stable D-Ins(1,4,5)P_3 analogue produced an increase in Ca^{2+}_{free} that decayed only very slowly. The longevity of InsP_3S_3 effects confirms that the function of the D-Ins(1,4,5)P_3 receptor does

[2] A.L. Willcocks and S.R. Nahorski, unpublished data.

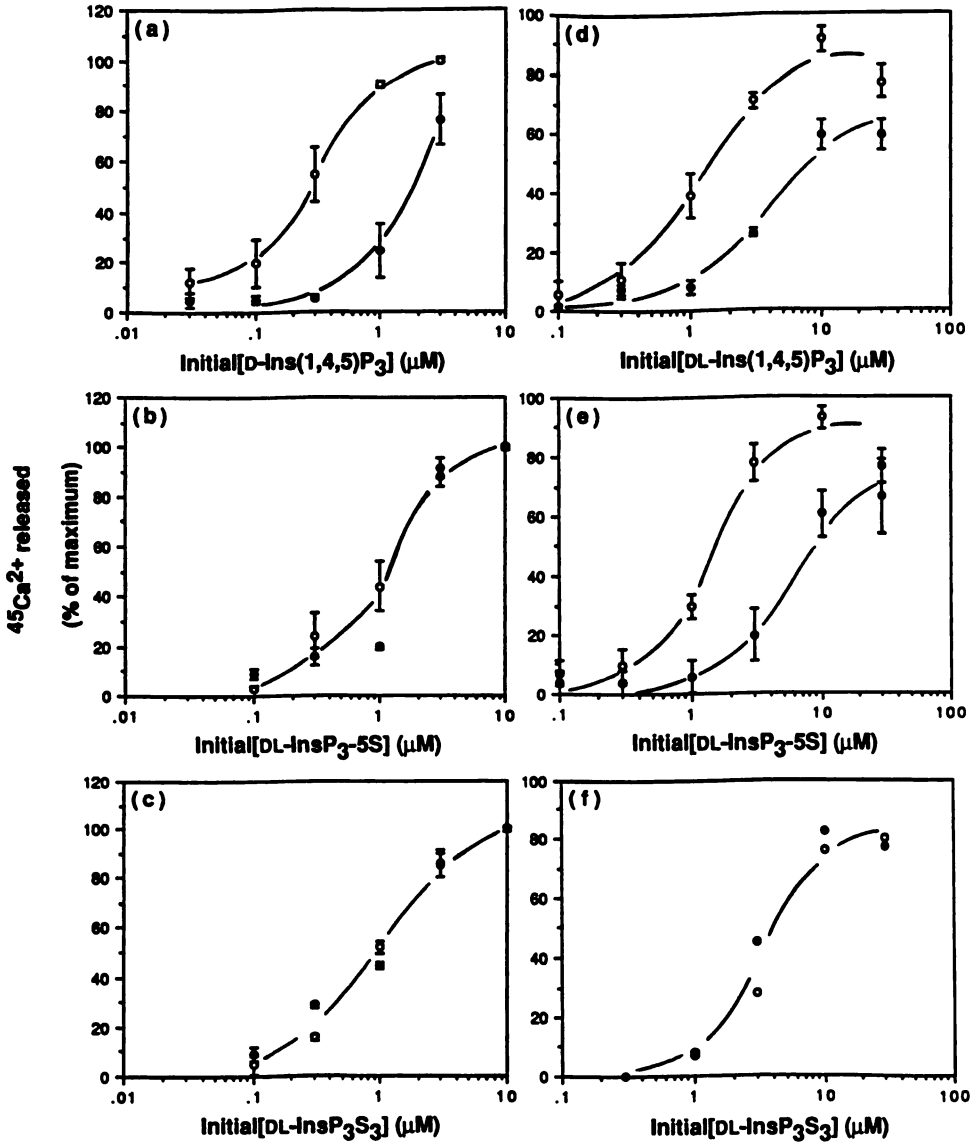

Fig. 6. Dose dependence of $^{45}Ca^{2+}$-releasing effects of D-Ins(1,4,5)P_3, DL-InsP_3-5S, and DL-InsP_3S_3 after treatment with D-Ins(1,4,5)P_3 5-phosphatase or 3-kinase. D-Ins(1,4,5)P_3 and its analogues (60 μM) were preincubated with either a boiled (○) or active (●) D-Ins(1,4,5)P_3 5-phosphatase preparation for 1 hr at 37° (n = 3) (a–c). Alternatively, DL-Ins(1,4,5)P_3 (d) and its analogues (e and f) were preincubated with either a boiled (○) or active (●) D-Ins(1,4,5)P_3 3-kinase preparation, for 5 min in the case of DL-Ins(1,4,5)P_3 (n = 3) (d) or 30 min in the cases of DL-InsP_3-5S (n = 3) (e) and DL-InsP_3S_3 (representative of three experiments) (f). The ability to mobilize $^{45}Ca^{2+}$ from saponized Swiss 3T3 cells was then tested using 2-min incubations.

not rapidly desensitize (16, 36). This contrasts with the finding that DL-InsP_3S_3-induced increases in Ca^{2+}_{free} are readily reversible in rat pancreatic acinar cells (37) and suggests that SH-SY5Y cells possess only a small D-Ins(1,4,5)P_3-insensitive pool or that such a pool rapidly equilibrates with the D-Ins(1,4,5)P_3-sensitive pool.

In addition to binding to the D-Ins(1,4,5)P_3 receptor, DL-InsP_3-5S and DL-InsP_3S_3 were able to interact differentially with D-Ins(1,4,5)P_3 5-phosphatase and D-Ins(1,4,5)P_3 3-kinase, the enzymes that are primarily responsible for D-Ins(1,4,5)P_3 metabolism (8). Although DL-InsP_3-5S and DL-InsP_3S_3 were resistant to D-Ins(1,4,5)P_3 5-phosphatase, they inhibited the enzyme potently (K_i = 6.8 and 1.7 μM, respectively). These values are much lower than that for L-Ins(1,4,5)P_3 [K_i = 39 μM (12) or K_i = 124 μM (17)] and >570-fold lower than that for the commonly used inhibitor D-2,3-diphosphoglycerate [K_i = 978 μM (17)]. Possible reasons for this high affinity of D-Ins(1,4,5)P_3 5-phosphatase for InsP_3S_3 have been discussed elsewhere (17). It appears that the phosphorothioate groups of an inositol phosphorothioate, having lower pK_a values than phosphate groups, can bind with higher affinity to D-Ins(1,4,5)P_3 5-phosphatase. This would also explain why DL-InsP_3S_3, which contains three phosphorothioate groups, is a more potent inhibitor of D-Ins(1,4,5)P_3 5-phosphatase than is DL-InsP_3-5S, which contains only one phosphorothioate group. In addition, because phosphorothioate groups are more hydrophobic than phosphate groups, the lower K_i for DL-InsP_3S_3, relative to that for DL-InsP_3-5S, may reflect enhanced hydrophobic interactions between the D-Ins(1,4,5)P_3 5-phosphatase and the analogue possessing the greater number of phosphorothioate groups. D-Ins(1,4,5)P_3 3-kinase appears to exhibit high stereo- and positional selectivity, resembling, in part, the specificity of the receptor associated with Ca^{2+} release (8, 11, 12). Although we find that DL-InsP_3S_3 is not a substrate for this enzyme, as has been shown elsewhere (16), DL-InsP_3S_3 does inhibit D-Ins(1,4,5)P_3 phosphorylation, albeit weakly. The precise reason for this low potency of DL-InsP_3S_3 inhibition is not yet clear, but an important factor may be the proximity of the unnatural 4-phosphorothioate group to the 3-hydroxyl group, which is phosphorylated by D-Ins(1,4,5)P_3 3-kinase.

Because L-Ins(1,4,5)P_3 has been shown to bind with low affinity to the D-Ins(1,4,5)P_3 receptor (31) and is, therefore,

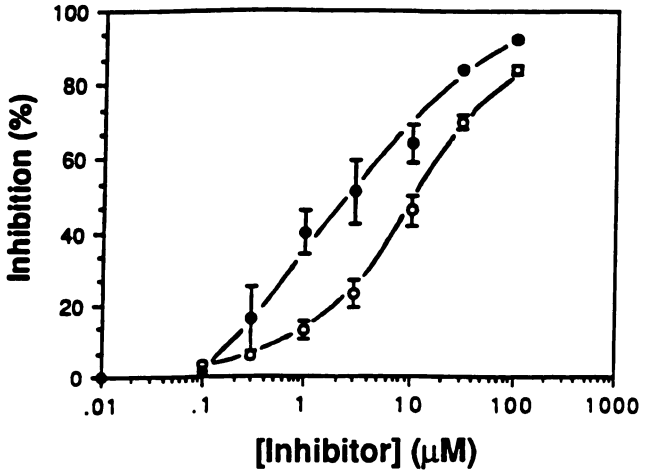

Fig. 7. Inhibition of D-Ins(1,4,5)P_3 5-phosphatase-catalyzed hydrolysis of D-Ins(1,4,5)P_3 by DL-InsP_3-5S and DL-InsP_3S_3. D-Ins(1,4,5)P_3 (30 μM), containing ~10,000 dpm of D-[5-^{32}P]Ins(1,4,5)P_3, was incubated at 37° for 15 min with D-Ins(1,4,5)P_3 5-phosphatase prepared from erythrocytes and 0.1–100 μM DL-InsP_3-5S (○) or DL-InsP_3S_3 (●) (n = 3). The rate of liberation of inorganic [^{32}P]phosphate was monitored as described in Materials and Methods.

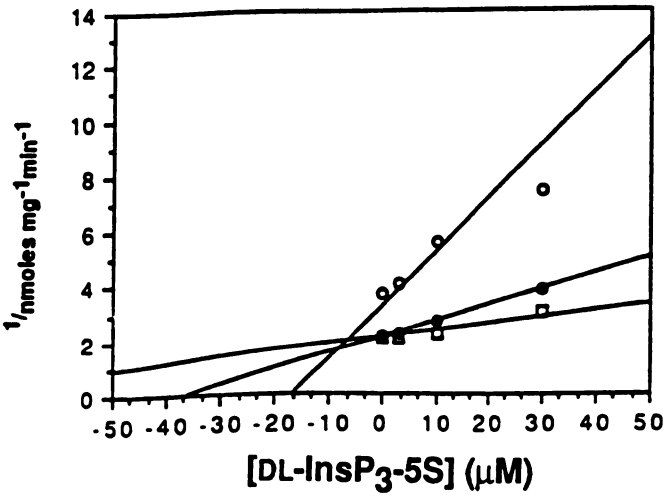

Fig. 8. Dixon plot showing competitive inhibition of D-Ins(1,4,5)P_3 3-kinase-catalyzed phosphorylation of D-Ins(1,4,5)P_3 by DL-InsP_3-5S. D-Ins(1,4,5)P_3 [3 (○), 10 (●), or 30 μM (□)], containing ~10,000 dpm of D-[^{3}H]Ins(1,4,5)P_3, was incubated at 37° for 15 min with the D-Ins(1,4,5)P_3 3-kinase preparation (0.1%, w/v) and 3–30 μM DL-InsP_3-5S (n = 6). The rate of formation of D-Ins(1,3,4,5)P_4 was monitored as described in Materials and Methods.

>150-fold less potent a stimulus of Ca^{2+} mobilization than D-Ins(1,4,5)P_3 in several systems (12, 15, 16), the L-isomers of the synthetic racemates can be expected to be relatively inactive towards Ca^{2+} mobilization. The difference in affinity between L- and D-isomers with respect to the metabolic enzymes is not as marked. The affinity of D-Ins(1,4,5)P_3 3-kinase for L-Ins(1,4,5)P_3 is ~100-fold lower than for D-Ins(1,4,5)P_3 (12), and the affinity of D-Ins(1,4,5)P_3 5-phosphatase for L-Ins(1,4,5)P_3 is ~3-fold lower than that for D-Ins(1,4,5)P_3 (12, 17). The L-isomer does not appear to be a substrate for D-Ins(1,4,5)P_3 5-phosphatase (12, 17). Therefore, if the same relative potencies are exhibited by the L-isomers of DL-InsP_3S_3 and DL-InsP_3-5S, then only a small proportion of the inhibitory effects on these enzymes will be due to the L-isomers.

We found DL-InsP_3-5S to be a potent inhibitor of D-Ins(1,4,5)P_3 phosphorylation, with D-Ins(1,4,5)P_3 3-kinase having a similar apparent affinity for both D-Ins(1,4,5)P_3 and DL-InsP_3-5S. Furthermore, treatment of DL-InsP_3-5S with D-Ins(1,4,5)P_3 3-kinase decreased its ability to release Ca^{2+} from internal stores, and DL-InsP_3-5S-induced Ca^{2+} mobilization from permeabilized SH-SY5Y cells was readily reversible. Because no phosphatase activity was observed in the D-Ins(1,4,5)P_3 3-kinase preparation and D-Ins(1,4,5)P_3 3-kinase activity is present in permeabilized SH-SY5Y cells, it seems most likely that these effects are due to the formation of D-InsP_4-5S. By comparison with the potencies of D-Ins(1,4,5)P_3 and D-Ins(1,3,4,5)P_4 on Ca^{2+} mobilization (9, 40), D-InsP_4-5S would be expected to be 20–50-fold less potent than DL-InsP_3-5S in the ability to release Ca^{2+}. It is to be expected that D-InsP_4-5S would be resistant to D-Ins(1,4,5)P_3 5-phosphatase, although readily attacked by D-Ins(1,3,4,5)P_4 3-phosphatase (8, 40).

We now have a tool with which to analyze the role of a D-Ins(1,4,5)P_3-like molecule in isolation (using DL-InsP_3S_3). Furthermore, DL-InsP_3-5S can be phosphorylated to an D-Ins(1,3,4,5)P_4-like molecule, albeit more slowly than seen with D-Ins(1,4,5)P_3 itself, but is resistant to D-Ins(1,4,5)P_3 5-phosphatase. DL-InsP_3S_3 is already proving to be a valuable compound for investigating a variety of D-Ins(1,4,5)P_3-associated events (11, 41). The biochemical and pharmacological characteristics reported here suggest that DL-InsP_3-5S will be complementary to DL-InsP_3S_3, particularly in the clarification of proposed roles (2, 9, 10) for D-Ins(1,3,4,5)P_4 in receptor-mediated events.

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