0026-895X/94/020271-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:271-276

DL-*myo*-Inositol 1,2,4,5-Tetrakisphosphate, a Potent Analog of D-*myo*-Inositol 1,4,5-Trisphosphate

MASATO HIRATA, NAOTO NARUMOTO, YUTAKA WATANABE, TAKASHI KANEMATSU, TOSHITAKA KOGA, and SHOICHIRO OZAKI

Department of Biochemistry, Faculty of Dentistry, Kyushu University, Fukuoka 812, Japan (M.H., T.K., T.K.) and Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790, Japan (N.N., Y.W., S.O.)

Received May 18, 1993; Accepted October 28, 1993

SUMMARY

Synthetic DL-*myo*-inositol 1,2,4,5-tetrakisphosphate (DL-Ins(1,2,4,5)P_4) functioned as a full agonist, with only 3-fold less potency than D-Ins(1,4,5)P_3 in eliciting the release of Ca^{2+} from nonmitochondrial pools of permeabilized rat basophilic leukemic cells. DL-Ins(1,2,4,5)P_4 inhibited the binding of D-[^{3}H]Ins(1,4,5)P_3 to the purified D-Ins(1,4,5)P_3 receptor with almost the same potency as seen for the Ca^{2+} release. This compound inhibited the hydrolysis of D-[^{3}H]Ins(1,4,5)P_3 to D-[^{3}H]Ins(1,4)P_2 catalyzed by erythrocyte ghosts, with a K_i value of as low as 1.4 μM, but it could not serve as a substrate for the same enzyme. D-Ins(1,4,5)P_3 3-kinase in rat brain cytosol did not recognize the compound at concentrations up to 30 μM. Thus, it would appear that DL-Ins(1,2,4,5)P_4 can serve as a potent and long lasting experimental and pharmacological tool for stimulating D-Ins(1,4,5)P_3-mediating processes.

D-Ins(1,4,5)P_3 is an established intracellular messenger that mobilizes Ca^{2+} from nonmitochondrial store sites in a variety of cells (1, 2). D-Ins(1,4,5)P_3 is metabolized by two known routes; one is dephosphorylation, catalyzed by D-Ins(1,4,5)P_3 5-phosphatase, and the other is phosphorylation of the 3-hydroxyl group of D-Ins(1,4,5)P_3 by an ATP-dependent kinase to produce D-Ins(1,3,4,5)P_4 (3–5). Thus, three proteins can be listed as D-Ins(1,4,5)P_3-recognizing macromolecules, the D-Ins(1,4,5)P_3 receptors involved in the Ca^{2+} release and two types of enzymes relating to its metabolism. To characterize the D-Ins(1,4,5)P_3-recognizing domain of these proteins in relation to their function, we synthesized a series of D-Ins(1,4,5)P_3 analogs in which large substituents were introduced into the second hydroxyl group, and we examined events related to their recognition by receptor sites and by metabolic enzymes (6, 7). Using these analogs, we found that such modification reduced little the potential of the analogs to interact with the receptor and metabolic enzymes, thereby indicating that the 2-OH of D-Ins(1,4,5)P_3 may not be primarily involved in the recognition by the D-Ins(1,4,5)P_3-recognizing molecules (6, 7).

This work was supported by The Uehara Memorial Foundation, The Kato Memorial Bioscience Foundation, The Ichiro Kanehara Foundation, and by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture of Japan.

In this study, the substitution at the 2-OH of D-Ins(1,4,5)P_3 was done with a phosphate group. DL-Ins(1,2,4,5)P_4 could be synthesized with relatively few steps, in a large amount. This compound was examined for events related to the recognition by the receptor sites and by metabolic enzymes. We found that DL-Ins(1,2,4,5)P_4 evoked Ca^{2+} release with almost the same potency as D-Ins(1,4,5)P_3, without the influence of effects of metabolic enzymes.

Materials and Methods

Materials. D-[^{3}H]Ins(1,4,5)P_3 (specific radioactivity, 777 GBq/mmol) and $^{45}Ca^{2+}$ (specific radioactivity, 416.6 MBq/mg) were obtained from DuPont-New England Nuclear (Boston, MA). D-Ins(1,4,5)P_3 was chemically synthesized as described (8). All other reagents were of the highest grade available.

Chemical synthesis of DL-Ins(1,2,4,5)P_4·3,6-di-*O*-benzyl-1,2,4,5-tetra-*O*-dibenzyloxyphosphoryl-*myo*-inositol. To a suspension of 1,4-di-*O*-benzyl-*myo*-inositol (200 mg, 0.45 mmol) (9) and 1*H*-tetrazole (209 mg, 2.99 mmol) in CH_2Cl_2 (3 ml) was added dibenzyl *N*,*N*-diisopropylphosphoramidite (689 mg, 2.0 mmol) at 0°, the mixture was stirred at the same temperature for 2 hr, and mCPBA (517 mg, 2.99 mmol) was added at −78°. The cooling bath was removed, and the mixture was warmed to room temperature and diluted with AcOEt. The organic layer was washed successively with 10% aqueous Na_2SO_3, 1N HCl, saturated aqueous $NaHCO_3$, and brine, dried on Na_2SO_4 and evaporated to dryness. The residue was subjected to column chroma-

ABBREVIATIONS: $InsP_2$,$InsP_3$,$InsP_4$, inositol bis-, tris-, tetrakis-phosphate with isomeric positioning of phosphate groups, as indicated in parenthesis, and assumed to be *myo*-conformation of inositol; HPLC, high performance liquid chromatography; SAX, strong anion exchange; RBL, rat basophilic leukemic cells; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AcOEt, ethyl acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

tography on silica gel (AcOEt/C_6H_{14} 1:2) to afford the title compound (610 mg, 96% yield) *Anal.* Calc. for $C_{76}H_{76}O_{18}P_4$: C, 65.14; H, 5.47. Found: C, 65.06; H, 5.59. NMR data: δ_H (270 MHz, $CDCl_3$) 3.54 (1 H, dt, *J* 9.46 and 2.94 Hz H_3), 3.97 (1 H, *J* 9.46 Hz, H_6), 4.40 (1 H, ddt, *J* 9.46, 8.85, and 2.94 Hz, H_1), 4.59 (1 H, dt, *J* 9.46 and 8.85 Hz, H_5), 4.94 (1 H, complex owing to overlapping with benzylic protons, H_4), 4.97–5.11 (20 H, complex, benzylic H), 5.42 (1 H, td, *J* 8.85 and 2.94 Hz, H_2), and 6.93–7.42 (50 H, m, aromatic H); δ_P (109 MHz, $CDCl_3$) −0.30, 0.04, 0.08, and 0.60; δ_C (67.8 MHz proton-decoupled, $CDCl_3$) 68.60, 68.67, 68.69, 68.71, 68.79, 68.89, 68.98, 69.02, 69.10, 71.67, 73.76 (m), 74.06, 74.89 (m), 75.44 (m), 76.87 (m), 78.10 (m), 126.64, 127.02, 127.15, 127.17, 127.26, 127.27, 127.39, 127.52, 127.58, 127.73, 127.81, 127.87, 127.94, 128.10, 134.91, 135.02, 135.11, 135.16, 135.22, 135.26, 135.35, 135.45, 135.47, 135.90, 137.39.

***myo*-Inositol 1,2,4,5-tetrakis (sodium hydrogen phosphate).** The fully protected phosphorylation product (220 mg, 0.148 mmol), 5% Pd-C (100 mg), MeOH (3 ml), and H_2O (1 ml) were stirred together under hydrogen for 24 hr at room temperature. After filtration of the catalyst and addition of pyridine to the filtrate, the aqueous solution was concentrated to a small volume, which then was subjected to cation-exchange chromatography of Dowex-50 (Na^+-form) and elution with water. The resulting eluate and washings were concentrated to dryness under reduced pressure below 40°. The residue was recrystallized from H_2O and MeOH, and the crystals in distilled water were evaporated under reduced pressure to remove a trace of the MeOH to give the final product ($C_6H_{12}Na_3O_{18}P_4$) as putative tetrasodium salt (32 mg), judged from our salt-forming procedure (8). The second crop (39 mg) was obtained by the recrystallization of the mother liquid (77% yield); mp > 280°, *Rf* (cellulose, ^{n}PrOH/28% NH_4OH/H_2O 5:4:1) 0.1 NMR data: δ_H (270 MHz, D_2O, neutral) 3.59 (1 H, dd, *J* 9.46 and 3.96 Hz, H_3), 3.77 (1 H, t, *J* 9.46 Hz, H_6), 3.89 (1 H, br ddd, *J* unreadable, H_1), 3.89 (1 H, q, *J* 9.46 Hz, H_5), 4.19 (1 H, q, *J* 9.46 Hz, H_4), H_2 proton overlapped with HOD proton, which was supported by decoupling experiment; δ_P (109 MHz proton-decoupled, D_2O, pD 11.6 with cyclohexylamine) 4.21, 4.91, 5.10, 5.20: δ_C (67.8 MHz proton-decoupled, D_2O, pD 11.6 with cyclohexyl-amine) 72.88 (s), 73.04 (m), 74.11 (m), 75.96 (m), 76.59 (m), 78.74 (m). The purity of final product was >98%. (HNMR spectrum of the tetrakisphosphate showed that it was not contaminated with any impurity.)

Assay of D-Ins(1,4,5)P_3 5-phosphatase activity. Erythrocyte ghosts were prepared from whole human blood (10) and were assayed for phosphatase activity, as follows: the assay mixture (0.2 ml) contained 20 mM HEPES buffer (pH 7.0), 5 mM $MgCl_2$, 0.2 mg/ml saponin, and 10 μM D-[^{3}H]Ins(1,4,5)P_3. After preincubation at 37° for 2 min, the reaction was initiated by adding ~100 μg ghosts, it was continued for 10 min, and it was halted by adding 0.2 ml of 10% trichloroacetic acid. After centrifugation using a table-top centrifuge, the supernatant was treated with water-saturated diethyl ether (3 ml × 3) and then was applied on a SAX column mounted in an HPLC system (11). Fractions corresponding to D-[^{3}H]Ins(1,4)P_2 and D-[^{3}H]Ins(1,4,5)P_3 were collected, and the radioactivity was counted to calculate phosphatase activity. Conditions were determined so that less than 30% of the substrate was consumed during the period of incubation. Erythrocyte ghosts were also used to determine whether the synthetic compounds could serve as a substrate for the 5-phosphatase. For this purpose, DL-Ins(1,2,4,5)P_4 at 100 μM was incubated with erythrocyte ghosts (~100 μg) or rat brain cytosol (~10 μg) in the same mixture as described above, except that incubation without the radioactive D-Ins(1,4,5)P_3 was continued for 1 hr. After termination of the reaction with trichloroacetic acid, the supernatant was assayed for liberated inorganic phosphate according to the method of Youngburg and Youngburg (12).

Assay of D-Ins(1,4,5)P_3 3-kinase activity. Rat whole brain cytosol, prepared as described (6), was assayed for kinase activity. The assay mixture (0.5 ml) contained 50 mM HEPES buffer (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 2 mM EGTA with or without 2 mM $CaCl_2$, and 1 μM D-[^{3}H]Ins(1,4,5)P_3. After preincubation at 37° for 2 min, the incubation was initiated by adding ~10 μg of brain cytosol, continued for 5 or 10 min, with or without $CaCl_2$, respectively, and terminated with 0.5 ml of 10% trichloroacetic acid. After centrifugation and neutralization with diethylether treatment, the sample was analyzed by applying to a SAX column. Fractions to D-[^{3}H]Ins(1,4,5)P_3 and D-[^{3}H]Ins(1,3,4,5)P_4 were collected, and the radioactivity was counted. Under this condition, about 40% of the substrate was consumed.

Assay of D-Ins(1,4,5)P_3 binding. D-Ins(1,4,5)P_3 receptor was purified to apparent homogeneity from rat cerebellum as described (13). The purity was checked by SDS-PAGE (not shown). The binding of D-[^{3}H]Ins(1,4,5)P_3 to the receptor was determined by the polyethyleneglycol precipitation method as described (14). Briefly, the purified receptor was incubated with 1 nM D-[^{3}H]Ins(1,4,5)P_3 in a solution (0.45 ml) containing 50 mM Tris-HCl buffer (pH 8.3), 2 mM EDTA and 0.2% Triton X-100 for 15 min on ice. To the mixture 50 μl of bovine γ-globulin (10 mg/ml) was added, followed by the addition of 0.5 ml of 30% polyethyleneglycol 6000. After centrifugation, the precipitate was dissolved in 1 ml of 0.1 N NaOH, and the radioactivity was counted. Nonspecific binding (about 200 dpm) was determined in the presence of 10 μM D-Ins(1,4,5)P_3 and was subtracted from that in its absence to determine the specific binding (4500~5500 dpm).

Assay of Ca^{2+} release by DL-Ins(1,2,4,5)P_4. RBL were harvested after 4~5 days, then were permeabilized with saponin as described for peritoneal macrophages (15). Ca^{2+} uptake and release by saponin-permeabilized RBL were determined by essentially the same method described previously (16). Briefly, cells (1×10^6 cells/ml) were preincubated in solution containing 0.13 M KCl, 20 mM Tris-maleate buffer (pH 6.8), 10 mM NaN_3, 5 mM $MgCl_2$, 0.12 mM $CaCl_2$ (containing 18.5 kBq/ml $^{45}Ca^{2+}$), and 1 mM EGTA at 37° for 2 min. The concentration of free Ca^{2+} was calculated to be 140 nM. Ca^{2+} uptake was initiated by adding 5 mM ATP and was determined by passing an aliquot of the mixture through a Whatman GF/C filter, as described (15, 16). After the uptake of Ca^{2+} had reached a plateau (in about 10 min), DL-Ins(1,2,4,5)P_4 at various concentrations was added to test for their potential to release Ca^{2+}.

Results

Recognition of DL-Ins(1,2,4,5)P_4 by D-Ins(1,4,5)P_3 receptor and Ca^{2+} releasing activity. Fig. 1 shows the structures of DL-Ins(1,2,4,5)P_4 and D-Ins(1,4,5)P_3. The D-Ins(1,4,5)P_3 receptor purified from rat cerebellum was assayed for D-[^{3}H]Ins(1,4,5)P_3 binding to determine whether or not DL-Ins(1,2,4,5)P_4 would be effective in inhibiting the binding. As shown in Fig. 2, DL-Ins(1,2,4,5)P_4 was capable of inhibiting the D-[^{3}H]Ins(1,4,5)P_3 binding and required the half-maximal concentration of 11.3 ± 1.1 nM (n = 5), whereas that for D-Ins(1,4,5)P_3 was 7.1 ± 0.9 nM (n = 5).

DL-Ins(1,2,4,5)P_4 was then examined for the possible potential to evoke the release of Ca^{2+} from permeabilized RBL. Permeabilized RBL accumulated $^{45}Ca^{2+}$, when provided with ATP, into nonmitochondrial pools at a free Ca^{2+} concentration of 140 nM in the presence of NaN_3. Within 10 min, the accumulation reached a plateau of 0.94 ± 0.4 nmol/10^6 cells. Addition of D-Ins(1,4,5)P_3 or DL-Ins(1,2,4,5)P_4 at 3 or 10 μM, respectively stimulated the release of $^{45}Ca^{2+}$ by 50 to 55% of that releasable by 10 μM ionomycin within 1 min, and no appreciable re-accumulation of $^{45}Ca^{2+}$ was observed up to 7 min after ligand addition when cell density was 10^6 cells/ml (Fig. 3A). Smaller amount of D-Ins(1,4,5)P_3 evoked a partial release of $^{45}Ca^{2+}$, and subsequent addition of DL-Ins(1,2,4,5)P_4 released further to the same extent as that seen with higher concentration of D-Ins(1,4,5)P_3 and *vice-versa*, thereby indicating that DL-Ins(1,2,4,5)P_4 acts on the same store of Ca^{2+} as D-Ins(1,4,5)P_3 (Fig. 3A). Furthermore, heparin, an antagonist to D-

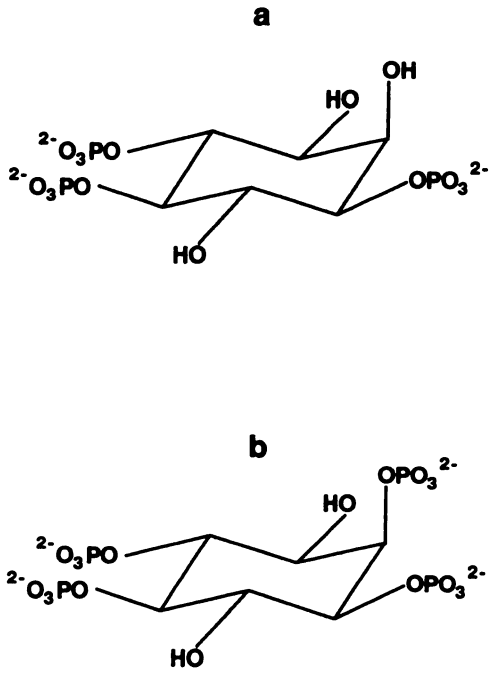


Fig. 1. Structure of D-Ins(1,4,5)P_3 (*a*) and DL-Ins(1,2,4,5)P_4 (*b*).

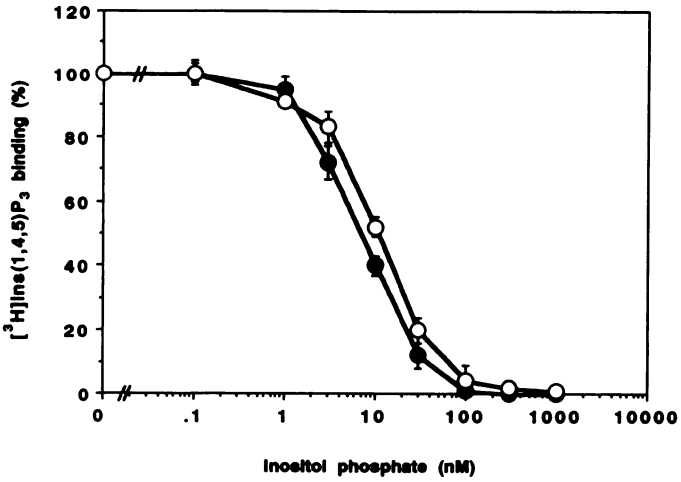


Fig. 2. Displacement of D-[^{3}H]Ins(1,4,5)P_3 from the purified receptor by DL-Ins(1,2,4,5)P_4. The purified receptor was incubated with 1 nM D-[^{3}H]Ins(1,4,5)P_3 in the presence of various concentrations of DL-Ins(1,2,4,5)P_4. Nonspecific binding (about 200 dpm) was determined in the presence of 10 μM D-Ins(1,4,5)P_3 and was subtracted from that in its absence to determine the specific binding. The specific binding in the absence of unlabeled ligand was taken as 100%. Each point is the mean ± standard error of four experiments. When error bar is absent, the size was smaller than the symbol. (●), D-Ins(1,4,5)P_3; (○), DL-Ins(1,2,4,5)P_4.

Ins(1,4,5)P_3, at 5 or 10 μg/ml, partially or totally inhibited the $^{45}Ca^{2+}$ release induced by 3 μM DL-Ins(1,2,4,5)P_4 (not shown). When cell density increased, the partial re-accumulation of $^{45}Ca^{2+}$ induced by 1 μM D-Ins(1,4,5)P_3 was observed; however, there was no re-accumulation of $^{45}Ca^{2+}$ induced by the same concentration of DL-Ins(1,2,4,5)P_4 (Fig. 3B). Fig. 3C shows the dose-dependence of $^{45}Ca^{2+}$ release; the half-maximal release occurred with 0.5 or 1.2 μM for D-Ins(1,4,5)P_3 or DL-Ins(1,2,4,5)P_4, respectively.

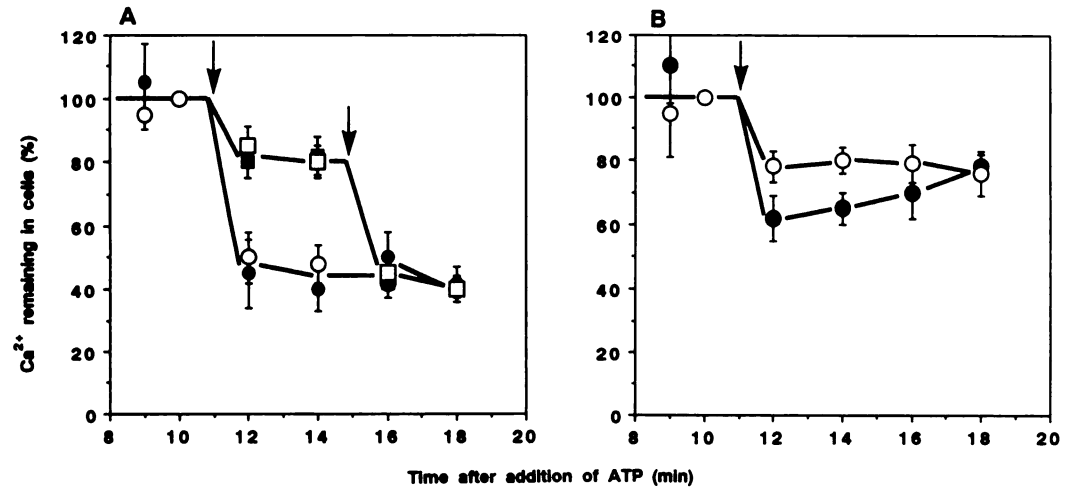
Recognition of DL-Ins(1,2,4,5)P_4 by D-Ins(1,4,5)P_3 metabolic enzymes. DL-Ins(1,2,4,5)P_4 at various concentrations was examined in the assay of D-Ins(1,4,5)P_3 5-phosphatase to determine possible recognition by the phosphatase of erythrocyte ghosts. These ghosts had a specific activity of 0.49 ± 0.05 nmol hydrolyzed/mg of protein/min at 10 μM D-[^{3}H]Ins(1,4,5)P_3. As shown in Fig. 4, DL-Ins(1,2,4,5)P_4 dose-dependently inhibited the hydrolysis of 10 μM [^{3}H]Ins(1,4,5)P_3 catalyzed by the ghosts, but the potency was greater than that seen with D-Ins(1,4,5)P_3. Dixon plot analysis revealed that DL-Ins(1,2,4,5)P_4 behaved like an apparent competitive inhibitor, and the K_i value was 1.4 ± 0.1 μM, whereas that for D-Ins(1,4,5)P_3 was 16.8 ± 2.1 μM (Table 1). We then examined whether DL-Ins(1,2,4,5)P_4 could serve as a substrate for the D-Ins(1,4,5)P_3 5-phosphatase in the ghosts, and for this purpose we measured the liberated inorganic phosphate. There was no appreciable hydrolysis of DL-Ins(1,2,4,5)P_4, compared to that observed with D-Ins(1,4,5)P_3 (Table 1). Rat brain cytosol, which contains various inositol polyphosphatases (5), was also used to examine whether Ins(1,2,4,5)P_4 could be hydrolyzed. This compound was found to be hydrolyzed to the least extent (Table 1). Thus, it appeared likely that the re-accumulation of Ca^{2+} induced by D-Ins(1,4,5)P_3, but not by DL-Ins(1,2,4,5)P_4, would be caused by the hydrolysis of the ligand. These results indicate that DL-Ins(1,2,4,5)P_4 could be recognized by the active center of the enzyme with a high affinity; however, the catalysis did not fully take place.

DL-Ins(1,2,4,5)P_4 at various concentrations was examined to determine whether this compound functions as an inhibitor in the assay of Ins(1,4,5)P_3 3-kinase in rat brain cytosol. Because the kinase is a Ca^{2+}/calmodulin-dependent enzyme (4, 5), the assays were carried out either in the presence or absence of Ca^{2+}. Rat brain cytosol possessed a specific activity at 1 μM D-[^{3}H]Ins(1,4,5)P_3 of 0.89 ± 0.07 or 0.49 ± 0.04 mmol/mg of protein/min in the presence or absence of Ca^{2+}, respectively. As shown in Fig. 5, DL-Ins(1,2,4,5)P_4 at 30 μM, the highest concentration examined, only slightly inhibited the phosphorylation of 1 μM D-[^{3}H]Ins(1,4,5)P_3 (<10%) when assayed at a free Ca^{2+} concentration of 30 μM. By decreasing the free Ca^{2+} concentration, DL-Ins(1,2,4,5)P_4 began to show some evidence of inhibition. On the other hand, D-Ins(1,4,5)P_3, at lower concentrations, similarly inhibited this process, independent of free Ca^{2+} concentration. Thus, DL-Ins(1,2,4,5)P_4 apparently was not recognized by the kinase in rat brain cytosol.

Discussion

DL-Ins(1,2,4,5)P_4 was capable of evoking the release of Ca^{2+} from nonmitochondrial pools in cells, with slightly less potency than D-Ins(1,4,5)P_3. The biological activity of DL-Ins(1,4,5)P_3 has been attributed entirely to the D-isomer and the L-isomer in the racemic mixture not interfering with the action of the D-isomer (17–19). The same was applicable to the 2-substituted analog of DL-Ins(1,4,5)P_3 (6, 7). Thus, it seems reasonable to assume that such may hold for DL-Ins(1,2,4,5)P_4; the half-maximal concentration for Ca^{2+} release with D-Ins(1,2,4,5)P_4 would then be 0.6 μM, thereby indicating that this compound is almost as potent as D-Ins(1,4,5)P_3.

DL-Ins(1,2,4,5)P_4 was found to be a strong inhibitor of D-Ins(1,4,5)P_3 5-phosphatase of erythrocyte ghosts. D-Ins(1,3,4,5)P_4 also was reported to be an inhibitor of the enzyme, with almost the same potency as DL-Ins(1,2,4,5)P_4 (3, 20, 21). If the D-isomer of DL-Ins(1,2,4,5)P_4 is assumed to be active in the inhibitory process, substitution with the phosphate moiety of the 2- or 3-hydroxyl site, a remote site from the site



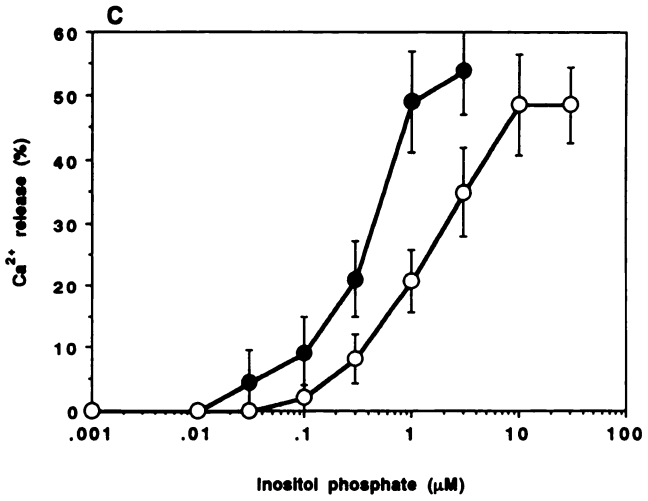


Fig. 3. $^{45}Ca^{2+}$-releasing activity of DL-Ins(1,2,4,5)P_4. Permeabilized RBL accumulated $^{45}Ca^{2+}$ at 37° for 10 min. Each point is the mean ± standard error of three (*A* and *B*) or four (*C*) independent determinations. A, At 11 min, as indicated by first *arrow*, D-Ins(1,4,5)P_3 at 0.3 μM/3 μM (■/●) or DL-Ins(1,2,4,5)P_4 at 1 μM/10 μM (□/○) was added to evoke the release of $^{45}Ca^{2+}$. At 15 min, as indicated by *second arrow*, the alternative ligand at maximal concentration was added, respectively. The cell density was 10^6 cells/ml. B, $^{45}Ca^{2+}$-releasing activity was assayed at cell density of 4×10^6 cells/ml. At 11 min, as indicated by an *arrow*, D-Ins(1,4,5)P_3 at 1 μM (●) or DL-Ins(1,2,4,5)P_4 at 1 μM (○) was added. C, At 11 min, DL-Ins(1,2,4,5)P_4 (○) as well as D-Ins(1,4,5)P_3 (●) at various concentrations given in the *abscissa* were added, and the $^{45}Ca^{2+}$ release was assayed at 12 min.

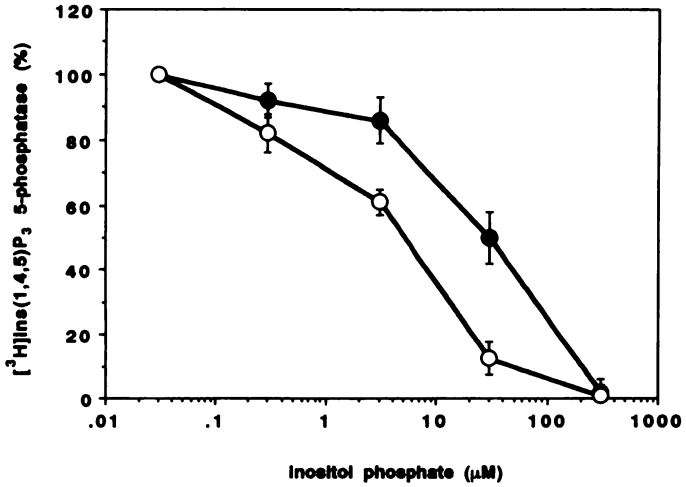


Fig. 4. Inhibition of D-[^{3}H]Ins(1,4,5)P_3 5-phosphatase activity by DL-Ins(1,2,4,5)P_4. The enzyme activity was assayed at 10 μM D-[^{3}H]Ins(1,4,5)P_3 in the presence of various concentrations of DL-Ins(1,2,4,5)P_4 given in the *abscissa*. The results are expressed relative to that in the absence of unlabeled D-Ins(1,4,5)P_3. Each value is the mean ± standard error of five independent determinations. (●), D-Ins(1,4,5)P_3; (○), DL-Ins(1,2,4,5)P_4.

TABLE I

Potency of DL-Ins(1,2,4,5)P_4 in D-Ins(1,4,5)P_3 5-phosphatase activity

Compounds	K_i[a]	Hydrolysis (nmol)[b]		
		By ghosts	By brain cytosol	
		+Mg^{2+}	+Mg^{2+}	−Mg^{2+}
D-Ins(1,4,5)P_3	16.8 ± 2.1	9.5 ± 1.7	33.7 ± 1.9	3.4 ± 0.1
DL-Ins(1,2,4,5)P_4	1.4 ± 0.1	0.9 ± 0.1	4.1 ± 0.8	2.0

[a] K_i value was determined by a Dixon plot analysis. The enzyme activity was assayed in the presence of various concentrations of inositol phosphates at 10, 25, or 50 μM D-[^{3}H]Ins(1,4,5)P_3. Each value is the mean ± standard error of three determinations.

[b] Hydrolysis was assayed by measuring inorganic phosphate. Each value is the mean ± standard error of four determinations.

of attack by the 5-phosphatase, may make the compound recognizable, with a higher affinity. DL-Ins(1,2,4,5)P_4 did not serve as a substrate for the enzyme, whereas D-Ins(1,3,4,5)P_4 was an active substrate for the same enzyme to produce D-Ins(1,3,4)P_3 (3, 20, 21). A proper explanation for this difference cannot be given, because catalytic mechanisms of the 5-phosphatase are not well understood (22). Alternatively, it is possible that the L-isomer of DL-Ins(1,2,4,5)P_4 functions as an inhibitor in the phosphatase assay system, because a number of compounds, even the L-Ins(1,4,5)P_3, were reported to be competitive inhibitors for the enzyme (23–25); and the L-isomer of 2-benzoyl analog of Ins(1,4,5)P_3 was found to be a strong

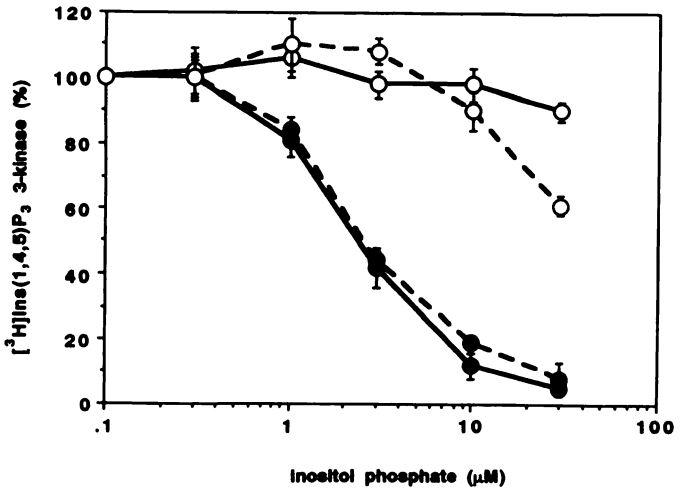


Fig. 5. Inhibition of [^{3}H]Ins(1,4,5)P_3 3-kinase activity by DL-Ins(1,2,4,5)P_4. The enzyme activity was assayed at 1 μM D-[^{3}H]Ins(1,4,5)P_3 in the presence of unlabeled DL-Ins(1,2,4,5)P_4 at concentrations given in the *abscissa* at free Ca^{2+} concentration of 30 μM (*solid line*) or less than 0.01 μM (*broken line*). The results are expressed relative to that in the absence of unlabeled D-Ins(1,4,5)P_3. Each value is the mean ± standard error of three independent determinations. (●), D-Ins(1,4,5)P_3; (○), DL-Ins(1,2,4,5)P_4.

inhibitor, but not a substrate (6, 7). The separation into individual enantiomers will clarify these proposals.

DL-Ins(1,2,4,5)P_4 up to 30 μM was not recognized by D-Ins(1,4,5)P_3 3-kinase of rat brain cytosol. In previous works, the introduction of a bulky substituent such as benzoyl or cyclohexanecarbonyl group to 2-OH of D-Ins(1,4,5)P_3 did not reduce the potential of the analog to interact with the kinase (6, 7). Therefore, the 2-phosphate group of D-Ins(1,2,4,5)P_4 does not seem to interfere sterically with the active sites of the enzyme; yet an electrically negative charge of the 2-phosphate may do so. On the other hand, the L-isomer of the analog with a bulky substituent as well as L-Ins(1,4,5)P_3 was not recognized by the kinase (6, 7, 23).

We propose that, in the presence of DL-Ins(1,2,4,5)P_4, D-Ins(1,4,5)P_3 produced upon cell stimulation would be phosphorylated to D-Ins(1,3,4,5)P_4, but would hardly be dephosphorylated to D-Ins(1,4)P_2, thereby facilitating the former process. D-Ins(1,3,4,5)P_4 thus produced, in some cases together with D-Ins(1,4,5)P_3, is expected to exert effects on Ca^{2+} homeostasis (26–29). DL-Ins(1,2,4,5)P_4 by itself is as potent as D-Ins(1,4,5)P_3 in causing the release of Ca^{2+}, without dephosphorylation. Therefore, DL-Ins(1,2,4,5)P_4 will be useful as a potent and long lasting experimental tool for stimulating intracellular Ca^{2+} homeostasis in cells. If this compound can be modified to a membrane-permeable type, as was done for D-Ins(1,4,5)P_3 by Schultz *et al.* (30), pharmacological events *in vivo* could also be examined.

DL-inositol 1,4,5-trisphosphorothioate (DL-Ins(1,4,5)P_3S_3) is known to affect the D-Ins(1,4,5)P_3 receptor site and metabolic enzymes in the same manner as does DL-Ins(1,2,4,5)P_4 presented here (17, 24, 25, 31, 32). However, the concentrations of DL-Ins(1,4,5)P_3S_3 required for affecting D-Ins(1,4,5)P_3-recognizable molecules are higher than those of DL-Ins(1,2,4,5)P_4, and DL-Ins(1,2,4,5)P_4 can be synthesized in a large amount with fewer steps required.

Acknowledgments

We thank M. Ohara for critical reading of the manuscript, and Dr. Tetsuro Ikebe for assistance with cell cultures.

References

1. Berridge, M. J. and R. F. Irvine. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**:315–321 (1984).
2. Berridge, M. J. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Ann. Rev. Biochem.* **53**:159–193 (1987).
3. Connoly, T. M., V. S. Bansal, T. E. Bross, R. F. Irvine, and P. W. Majerus. The metabolism of tris- and tetrakisphosphates of inositol by 5-phosphomonoesterase and 3-kinase enzymes. *J. Biol. Chem.* **262**:2146–2149 (1987).
4. Irvine, R. F., A. J. Letcher, J. P. Heslop, and M. J. Berridge. The inositol tris/tetrakisphosphate pathway-demonstration of Ins(1,4,5)P_3 3-kinase activity in animal tissues. *Nature* **320**:631–634 (1986).
5. Shears, S. B. Metabolism of inositol phosphates, in *Advances in Second Messengers and Phosphoprotein Research* (J. W. Putney, Jr., ed). Vol. 26. Raven Press, Ltd., New York, 63–92 (1992).
6. Hirata, M., Y. Watanabe, T. Ishimatsu, T. Ikebe, Y. Kimura, K. Yamaguchi, S. Ozaki, and T. Koga. Synthetic inositol trisphosphate analogs and their effects on phosphatase, kinase and the release of Ca^{2+}. *J. Biol. Chem.* **264**:20303–20308 (1989).
7. Hirata, M., F. Yanaga, T. Koga, T. Ogasawara, Y. Watanabe, and S. Ozaki. Stereospecific recognition of inositol 1,4,5-trisphosphate analogs by the phosphatase, kinase and binding proteins. *J. Biol. Chem.* **265**:8404–8407 (1990).
8. Ozaki, S., Kondo, Y., Shiotani, N., Ogasawara, T. and Watanabe, Y. Synthesis and some properties of D-*myo*-inositol 1,4,5-tris(dihydrogen phosphate). *J. Chem. Soc. Perkin Trans.* **1**:729–737 (1992).
9. Gigg, J., R. Gigg, S. Payne, and R. Conant. (±)-1,2:4,5-Di-*O*-isopropylidine-*myo*-inositol. *Carbohydr. Res.* **142**:132–134 (1985).
10. Downes, C. P., M. C. Mussat, and R. H. Michell. The inositol trisphosphate phosphomonoesterase of the human erythrocyte membrane. *Biochem. J.* **203**:169–177 (1982).
11. Yamaguchi, K., M. Hirata, and H. Kuriyama. Calmodulin activates inositol 1,4,5-trisphosphate 3-kinase activity in pig aortic smooth muscle. *Biochem. J.* **244**:787–791 (1987).
12. Youngburg, G. E. and M. V. Youngburg. Phosphorus metabolism; system of blood phosphorus analysis. *J. Lab. Clin. Med.* **16**:158–168 (1930).
13. Kanematsu, T., H. Takeya, Y. Watanabe, S. Ozaki, M. Yoshida, T. Koga, S. Iwanaga, and M. Hirata. Putative inositol 1,4,5-trisphosphate binding proteins in rat brain cytosol. *J. Biol. Chem.* **267**:6518–6525 (1992).
14. Hirata, M., Y. Watanabe, T. Ishimatsu, F. Yanaga, T. Koga, and S. Ozaki. Inositol 1,4,5-trisphosphate affinity chromatography. *Biochem. Biophys. Res. Commun.* **168**:379–386 (1990).
15. Hirata, M., E. Suematsu, T. Hashimoto, T. Hamachi, and T. Koga. Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. *Biochem. J.* **223**:229–236 (1984).
16. Hirata, M., E. Suematsu, and T. Koga. Effect of calmodulin and calmodulin antagonists on the Ca^{2+} uptake by the intracellular Ca^{2+}-accumulating system of the guinea pig peritoneal macrophages treated with saponin. *Mol. Pharmacol.* **23**:78–85 (1983).
17. Strupish, J., A. M. Cooke, B. V. L. Potter, R. Gigg, and S. R. Nahorski. Stereospecific mobilization of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate. *Biochem. J.* **253**:901–905 (1988).
18. Taylor, C. W., M. J. Berridge, A. M. Cooke, and B. V. L. Potter. Inositol 1,4,5-trisphosphorothioate, a stable analogue of inositol trisphosphate which mobilizes intracellular calcium. *Biochem. J.* **259**:645–650 (1989).
19. Willcocks, A. L., A. M. Cooke, B. V. L. Potter, and S. R. Nahorski. Stereospecific recognition sites for [^{3}H]inositol (1,4,5)trisphosphate in particulate preparations of rat cerebellum. *Biochem. Biophys. Res. Commun.* **146**:1071–1078 (1987).
20. Shears, S. B., W. H. Evans, C. J. Kirk, and R. H. Michell. Preferential localization of rat liver D-*myo*-inositol 1,4,5-trisphosphate/1,3,4,5-tetrakisphosphate 5-phosphatase in bile canalicular plasma membranes and "late" endosomal vesicles. *Biochem. J.* **256**:363–369 (1988).
21. Erneux, C., M. Lemos, B. Verjans, P. Vanderhaeghen, A. Delvaux, and J. E. Dumont. Soluble and particulate Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase in bovine brain. *Eur. J. Biochem.* **181**:317–322 (1989).
22. Safrany, S. T., R. A. Wilcox, C. Liu, B. V. L. Potter, and S. R. Nahorski. 3-Position modification of *myo*-inositol 1,4,5-trisphosphate: consequence for intracellular Ca^{2+} mobilization and enzyme recognition. *Eur. J. Pharmacol.* **226**:265–272 (1992).
23. Polokoff, M. A., G. H. Bencen, J. P. Vacca, S. J. deSolms, S. D. Young, and J. R. Huff. Metabolism of synthetic inositol trisphosphate analogs. *J. Biol. Chem.* **263**:11922–11927 (1988).
24. Cooke, A. M., N. J. Noble, R. Gigg, A. L. Willcocks, J. Strupish, S. R. Nahorski, and B. V. L. Potter. *myo*-Inositol 1,4-bisphosphate-5-phosphorothioate: chemical synthesis and biochemical properties. *Biochem. Soc. Trans.* **16**:992–993 (1988).
25. Cooke, A. M., S. R. Nahorski, and B. V. L. Potter. *myo*-Inositol 1,4,5-trisphosphorothioate is a potent competitive inhibitor of human erythrocyte 5-phosphatase. *FEBS Lett.* **242**:373–377 (1989).
26. Irvine, R. F. and R. M. Moor. Micro-injection of inositol 1,3,4,5-tetrakis-

phosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochem. J.* **240**:917–920 (1986).
27. Irvine, R. F. and R. M. Moor. Inositol(1,3,4,5)tetrakisphosphate-induced activation of sea urchin eggs requires the presence of inositol trisphosphate. *Biochem. Biophys. Res. Commun.* **146**:284–290 (1987).
28. Morris, A. P., D. V. Gallacher, R. F. Irvine, and O. H. Petersen. Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* **330**:653–655 (1987).
29. Lückhoff, A. and D. E. Clapham. Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel. *Nature* **355**:356–358 (1992).
30. Schultz, C., S. J. Pandol, M. Vajanaphanich, A. E. Traynor-Kaplan, K. E. Barrett, and R. Y. Tsien. Membrane-permeant acetoxymethyl esters of inositol phosphates: effects on REF-52 fibroblasts, pancreatic acinar cells, and T_{84} cells. in *8th International Conference on Second Messengers and Phosphoproteins*. L164W (1992).
31. Wojcikiewicz, R. H., A. M. Cooke, B. V. L. Potter, and S. R. Nahorski. Inhibition of inositol 1,4,5-trisphosphate metabolism in permeabilized SH-SY5Y human neuroblastoma cells by a phosphorothioate-containing analogue of inositol 1,4,5-trisphosphate. *Eur. J. Biochem.* **192**:459–467 (1990).
32. Willcock, A. L., B. V. L. Potter, A. M. Cooke, and S. R. Nahorski. *Myo*-inositol(1,4,5)trisphosphorothioate binds to specific [^{3H}inositol(1,4,5)trisphosphate sites in rat cerebellum and is resistant to 5-phosphatase. *Eur. J. Pharmacol.* **155**:181–183 (1988).

Send reprint requests to: Dr. Masato Hirata, Department of Biochemistry, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812 Japan.