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KINETIC ANALYSIS OF NOVEL INHIBITORS OF INOSITOL POLYPHOSPHATE METABOLISM

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SUMMARY. The ability of the novel D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] analogues, L-*chiro*-inositol 1,4,6-trisphosphate [L-*chr* Ins(1,4,6)P_3] and the corresponding trisphosphorothioate compound [L-*chr* Ins(1,4,6)PS_3] to inhibit soluble inositol (1,4,5)P_3/(1,3,4,5)P_4-polyphosphate 5-phosphatase, potently and selectively, has been investigated. L-*chr* Ins(1,4,6)P_3 competitively inhibited 5-phosphate specific dephosphorylation of Ins(1,4,5)P_3 and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] with apparent K_i values of 6.35 and 1.76 μM, respectively. L-*chr* Ins(1,4,6)PS_3 competitively inhibited hydrolysis of Ins(1,4,5)P_3 and noncompetitively inhibited dephosphorylation of Ins(1,3,4,5)P_4 with apparent K_i values of 0.67 and 0.44 μM, respectively. L-*chr* Ins(1,4,6)PS_3 did not affect Ins(1,4,5)P_3 3-kinase activity. In the present investigation L-*chr* Ins(1,4,6)P_3 and L-*chr* Ins(1,4,6)PS_3 have been shown to be the most potent and selective inhibitors of inositol polyphosphate metabolism yet described.

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Abbreviations: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; Ins(1,(3,)4,5)PP 5-phosphatase, inositol (1,4,5)P_3/(1,3,4,5)P_4-polyphosphate 5-phosphatase; L-*chr* Ins(1,4,6)P_3, L-*chiro*-inositol 1,4,6-trisphosphate; L-*chr* Ins(1,4,6)PS_3, L-*chiro*-inositol 1,4,6-trisphosphorothioate; HPLC, high pressure liquid chromatography; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; DEAE-Sephacel, diethylaminoethyl-Sephacel; resin #204, 2-O-[4-(5-aminoethyl-2-hydroxyphenylazo)benzoyl]-1,4,5-tri-O-phosphono-*myo*-inositol-Sepharose 4B.

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Calcium signals generated by activation of the phosphoinositide pathway control many cellular processes (1). D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (Figure 1.(1)) is intimately involved in the generation of endoplasmic reticulum derived Ca^{2+} signals and the regulation of the complex spatiotemporal patterns of Ca^{2+} waves and oscillations which are coupled to inositol lipid hydrolysis (1). Ins(1,4,5)P_3 is deactivated by rapid dephosphorylation to D-*myo*-inositol 1,4-bisphosphate and by phosphorylation to D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] (2). Dephosphorylation is associated with both membrane and soluble extracts from cells, while phosphorylation occurs predominantly in the cytosol (4-11). The conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 may be a secondary pathway for Ins(1,4,5)P_3 metabolism, with the main function of Ins(1,4,5)P_3 3-kinase being to regulate the relationship between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, whose roles in Ca^{2+} signalling may be closely associated (12). Ins(1,3,4,5)P_4 may activate plasma membrane Ca^{2+} influx associated with the activation of the phosphoinositide signalling pathway (12). Ins(1,3,4,5)P_4 is also dephosphorylated by soluble and membrane associated 5-phosphate specific phosphatases (11-14). A number of forms of inositol (1,4,5)P_3/(1,3,4,5)P_4-polyphosphate 5-phosphatase [Ins(1,(3,)4,5)PP 5-phosphatase] may exist with different subcellular distributions and substrate specificities for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (3-11). The presence of a type or form of Ins(1,(3,)4,5)PP 5-phosphatase may be dependent on the metabolic function of the cell and role of the phosphoinositide cycle in Ca^{2+} signalling of that cell. Thus, Ins(1,(3,)4,5)PP 5-phosphatases play a pivotal role in the termination of Ca^{2+} signals and the regulation of a large array of agonist evoked cellular responses.

The development of potent and selective inhibitors of inositol polyphosphate metabolism will aid in the understanding of the complex mechanisms associated with agonist-induced, Ins(1,4,5)P_3-mediated Ca^{2+} mobilisation (15). In this investigation we have characterised the kinetics of L-*chiro*-inositol 1,4,6-trisphosphate [L-*chr* Ins(1,4,6)P_3] (Figure 1.(2)) and L-*chiro*-inositol 1,4,6-trisphosphorothioate [L-*chr* Ins(1,4,6)PS_3] (Figure 1.(3)) induced inhibition of inositol polyphosphate metabolism by soluble Ins(1,(3,)4,5)PP 5-phosphatase from skeletal muscle.

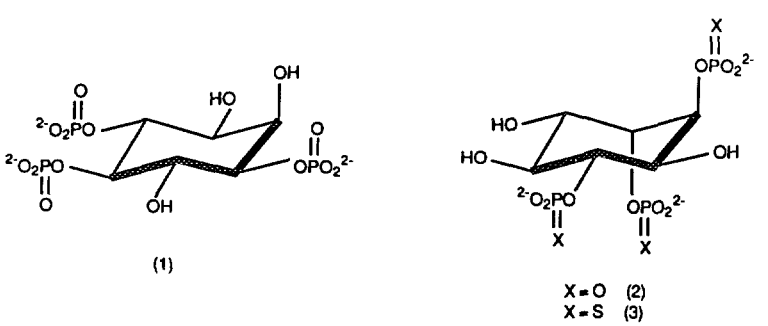

FIGURE 1. Structures of Ins(1,4,5)P_3 and synthetic analogues.

The structures of Ins(1,4,5)P_3 (1), L-*chr* Ins(1,4,6)P_3 (2) and L-*chr* Ins(1,4,6)PS_3 (3) are shown.

MATERIALS AND METHODS

Materials—[2-^{3}H]-Ins(1,4,5)P_3 (40 Ci/mmol) (Amersham International); [2-^{3}H]-Ins(1,3,4,5)P_4 (15-30 Ci/mmol) and Aquasol (Du Pont New-England Nuclear); Ins(1,4,5)P_3, Dowex 1 (100-200 mesh), ATP, ADP, AMP (Sigma); Ins(1,3,4,5)P_4 (Boehringer Mannheim); DEAE-Sephacel, blue-Sepharose (Pharmacia); Heparin-Agarose (Bio-Rad); 2-O-[4-(5-aminoethyl-2-hydroxyphenylazo)benzoyl]-1,4,5-tri-O-phosphono-*myo*-inositol-Sepharose 4B (resin #204) was a gift from Professor S. Ozaki (16).

Purification of Ins(1,(3,)4,5)PP 5-Phosphatase and Ins(1,4,5)P_3 3-Kinase from Porcine Skeletal Muscle—Ins(1,(3,)4,5)PP 5-phosphatase soluble type I and Ins(1,4,5)P_3 3-kinase were purified to homogeneity from porcine skeletal muscle by a combination of DEAE-Sephacel, blue-Sepharose, heparin-Agarose and resin #204 structural analogue affinity chromatography (17,18).

Assay of Ins(1,4,5)P_3 3-Kinase Activity—Ins(1,4,5)P_3 3-kinase activity was assayed at 37°C in 100 µl of 50 mM Hepes/HCl, pH 7.5/2 mM DTT/1 µM Ins(1,4,5)P_3/20 nCi [^{3}H]-Ins(1,4,5)P_3/1 µM free $CaCl_2$/3 µM CaM/1 mM ATP/5 mM $MgCl_2$ (18) in the presence or absence of L-*chr* Ins(1,4,6)P_3 or L-*chr* Ins(1,4,6)PS_3. The reaction was initiated by the addition of 10 µg of protein and terminated by adding 0.6 ml of ice cold 0.1 M ammonium formate/0.1 M formic acid. The resulting inositol phosphates were then separated and quantitated by HPLC and liquid scintillation (Aquasol) spectrometry.

Kinetic Characterisation of Ins(1,(3,)4,5)PP 5-Phosphatase Activity—Substrate/velocity relationships were determined using 2.5-80 µM Ins(1,4,5)P_3/20 nCi [2-^{3}H]-Ins(1,4,5)P_3 or 0.5-16 µM Ins(1,3,4,5)P_4/20 nCi [2-^{3}H]-Ins(1,3,4,5)P_4 in 10 mM Hepes/HCl, pH 7.2/2 mM $MgCl_2$ (buffer A). Reactions were initiated by the addition of the substrate and terminated as described above. The [^{3}H]-inositol polyphosphates were then separated on 2 ml Dowex 1 anion exchange chromatography columns (17) or by HPLC (18) and quantified by liquid scintillation spectrometry. Prior to kinetic analysis Ins(1,(3,)4,5)PP 5-phosphatase activity was shown to be linear with time displaying Michaelis-Menten behaviour and was 5-phosphate specific and Mg^{2+} dependent with maximal activity observed at 2 mM $MgCl_2$ (results not shown). Between 5-25% of substrate was metabolised in kinetic assays. K_m values for each substrate were determined from Lineweaver-Burk plots which were generated using the iterative UNIX computer programme (19).

Synthesis of the Inhibitors L-chr Ins(1,4,6)P_3 and L-chr Ins(1,4,6)PS_3—Inhibitors were synthesised from L-quebrachitol as previously described (20) and purified by ion-exchange chromatography on DEAE-Sepharose and used as their triethylammonium salts.

Kinetic Characterisation of the Inhibition of Ins(1,(3,)4,5)PP 5-Phosphatase Activity by L-chr Ins(1,4,6)P_3 and L-chr Ins(1,4,6)PS_3—Substrate/velocity relationships were determined in the presence of L-*chr* Ins(1,4,6)P_3 (40 and 80 µM) and L-*chr* Ins(1,4,6)PS_3 (1 and 2 µM) using 5-40 µM Ins(1,4,5)P_3/20 nCi [2-^{3}H]-Ins(1,4,5)P_3 or 1-8 µM Ins(1,3,4,5)P_4/20 nCi [2-^{3}H]-Ins(1,3,4,5)P_4 in buffer A. K_i values for each inhibitor were determined from Lineweaver-Burk plots which were generated using the iterative UNIX computer programme (19).

HPLC of [^{3}H]-Inositol Polyphosphates—The inositol polyphosphates produced from Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 metabolism were separated with a Partisil 10 SAX HPLC column (4.6 x 250 mm) (Whatman) using a 0-1.4 M ammonium dihydrogen

phosphate/orthophosphoric acid, pH 3.7 linear gradient. The gradients were developed over time as required, at a flow rate of 1.0 ml/min, and the column regenerated with high salt. The [2-^{3}H]-inositol polyphosphates were quantified using an on-line continuous flow scintillation counter (Flo-one A-250X, Packard Instruments). Unknown inositol polyphosphates were identified by comparison of retention times with those of known standards, separated under identical conditions. A mixture of ATP, ADP and AMP was included in all runs as an internal standard for gradient stability.

RESULTS AND DISCUSSION

Synthetic phosphorothioate analogues of Ins(1,4,5)P_3 are currently being used to investigate the complex mechanisms associated with the phosphoinositide signalling pathway (15,21). D-*myo*-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)PS_3], D-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate [Ins(1,4,5)P_3-5S] and other analogues are potent mobilisers of intracellular Ca^{2+} that are resistant to dephosphorylation by Ins(1,(3,)4,5)PP 5-phosphatases (15,21,22). Ins(1,4,5)PS_3 and Ins(1,4,5)P_3-5S also potently inhibit 5-phosphate specific dephosphorylation of Ins(1,4,5)P_3 with apparent K_i values of 1.7 and 6.8 μM, respectively (22). However, while both derivatives are much more potent than the commonly used Ins(1,4,5)P_3 5-phosphatase inhibitor 2,3-bisphosphoglycerate (K_i 350 μM) (23), their potent effect on intracellular Ca^{2+} stores excludes them from use in investigations designed to specifically modify inositol polyphosphate metabolism *in vitro*.

Recently L-*chr* Ins(1,4,6)P_3 and the corresponding inositol trisphosphorothioate have been synthesised from L-quebrachitol *via* L-*chiro*-2,3,5-tri-O-benzyl-inositol (20). L-*chr* Ins(1,4,6)P_3 competitively inhibited the 5-phosphate specific dephosphorylation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 by purified soluble Ins(1,(3,)4,5)PP 5-phosphatase from skeletal muscle with apparent K_i values of 6.35 and 1.76 μM, respectively (apparent K_m values for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 dephosphorylation were 8.97 and 0.98 μM, respectively) (Figures 2a and 2b). These results support preliminary findings which showed that both analogues competitively inhibit the dephosphorylation of Ins(1,4,5)P_3 by Ins(1,4,5)P_3 5-phosphatase of crude fragmented human erythrocyte membranes (20). L-*chr* Ins(1,4,6)PS_3 also competitively inhibited the dephosphorylation of Ins(1,4,5)P_3, but was found to noncompetitively inhibit 5-phosphate cleavage from Ins(1,3,4,5)P_4, with apparent K_i values of 0.67 and 0.43 μM, respectively (Figures 3a and 3b). L-*chr* Ins(1,4,6)PS_3 is the first phosphorothioate analogue of Ins(1,4,5)P_3 to display noncompetitive kinetics for inhibition of inositol polyphosphate metabolism. This result suggests that L-*chr* Ins(1,4,6)PS_3 may inhibit Ins(1,3,4,5)P_4 dephosphorylation by binding to a different region on the Ins(1,(3,)4,5)PP 5-phosphatase to the Ins(1,3,4,5)P_4 binding site. However, the noncompetitive inhibition may indicate enzyme inactivation by the L-*chr* Ins(1,4,6)PS_3, or alternatively, the presence of isoforms of soluble type I Ins(1,(3,)4,5)PP 5-phosphatase in muscle (17). The K_i values for L-*chr* Ins(1,4,6)P_3 inhibition of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 metabolism are not equivalent, which further suggests the presence of isoforms. The increased effectiveness of L-*chr* Ins(1,4,6)PS_3 on Ins(1,(3,)4,5)PP 5-phosphatase, in comparison to L-*chr* Ins(1,4,6)P_3, directly reflects the effects of phosphorothioate substitution, as inorganic phosphate or phosphorothioate are not released from the respective analogue on incubation with membrane

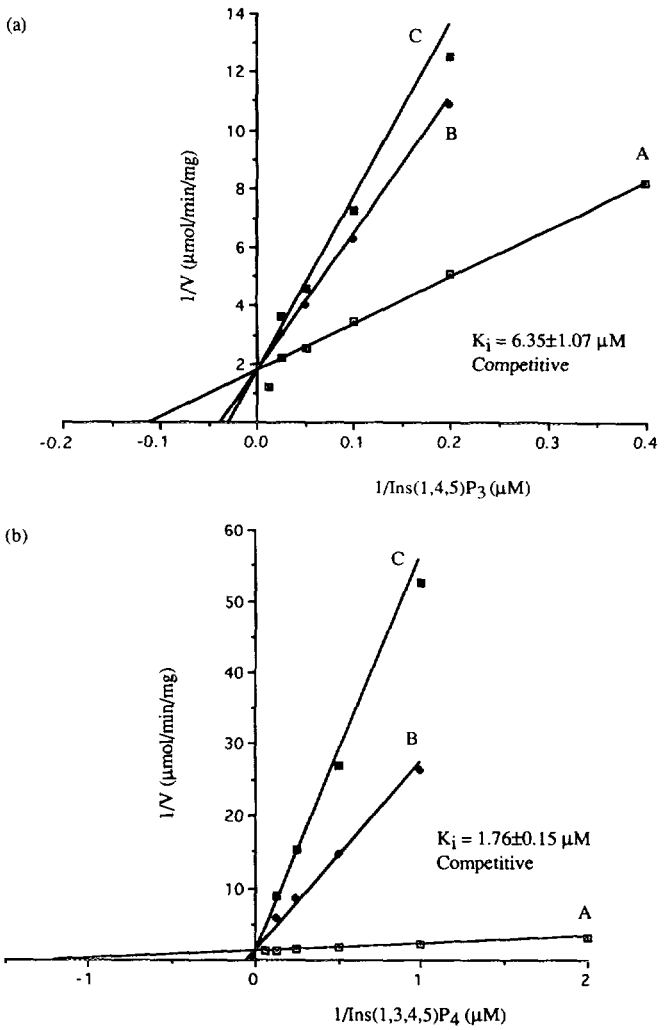

FIGURE 2. The inhibition of Ins(1,(3,)4,5)PP 5-phosphatase soluble type I by L-*chr* Ins(1,4,6)P_3.

Competitive inhibition by L-*chr* Ins(1,4,6)P_3 of both (a) Ins(1,4,5)P_3 and (b) Ins(1,3,4,5)P_4 metabolism is shown. K_i values of 6.35±1.07 and 1.76±0.15 μM, respectively, were obtained. Kinetic assays were carried out in the presence of; (A) 0, (B) 40 and (C) 80 μM of L-*chr* Ins(1,4,6)P_3 and 2.5-80 (A) or 5-40 (B,C) μM Ins(1,4,5)P_3 or 0.5-16 (A) or 1-8 (B,C) μM Ins(1,3,4,5)P_4. The results shown are means±SE of duplicate experiments from three preparations of purified enzyme.

Ins(1,4,5)P_3 5-phosphatase (20). Similar results have been observed with other phosphorothioate substituted analogues (24). L-*chr* Ins(1,4,6)P_3 (6 μM) inhibited the phosphorylation of Ins(1,4,5)P_3 by purified soluble Ins(1,4,5)P_3 3-kinase from skeletal muscle by only 10.6% (apparent K_m for Ins(1,4,5)P_3 phosphorylation 0.46 μM [18]). However, L-*chr* Ins(1,4,6)PS_3 (2 μM) did not affect activity (results not shown). In the

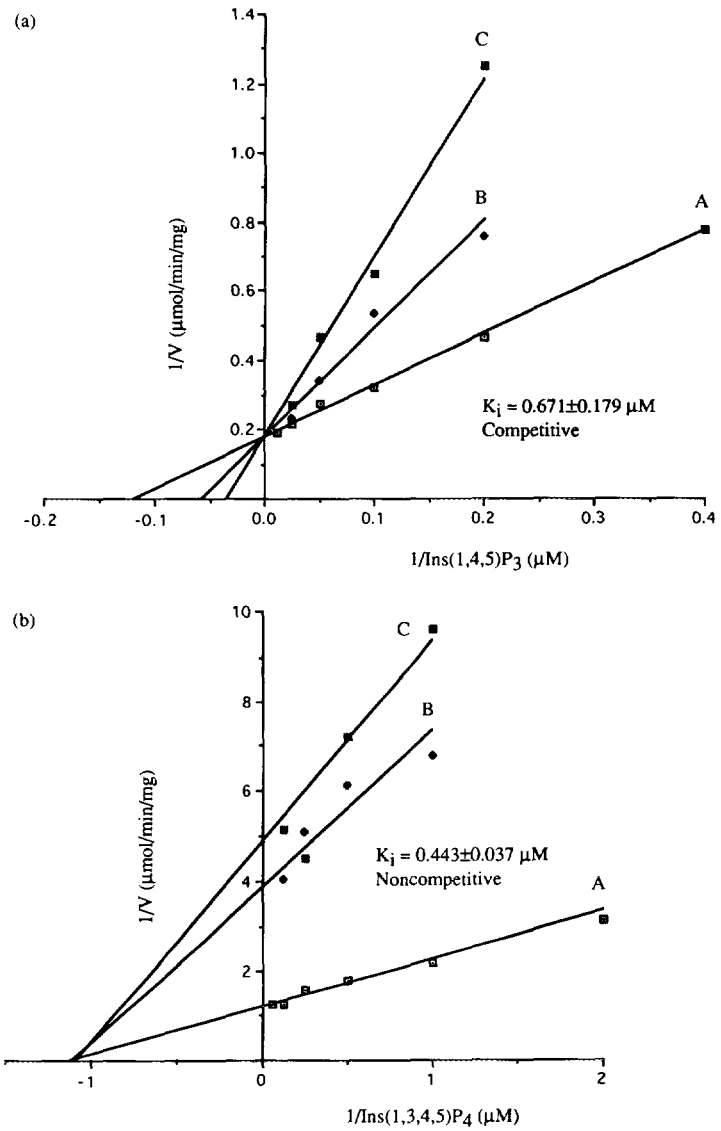

FIGURE 3. The inhibition of Ins(1,(3,)4,5)PP 5-phosphatase soluble type I by L-*chr* Ins(1,4,6)PS_3.

Competitive inhibition of (a) Ins(1,4,5)P_3 metabolism and noncompetitive inhibition of (b) Ins(1,3,4,5)P_4 metabolism by L-*chr* Ins(1,4,6)PS_3 is shown. K_i values of 0.671±0.179 and 0.443±0.037 µM, respectively, were obtained. Kinetic assays were carried out with (A) 0, (B) 1 and (C) 2 µM of inhibitor and 2.5-80 (A) or 5-40 (B,C) µM Ins(1,4,5)P_3 or 0.5-16 (A) or 1-8 (B,C) µM Ins(1,3,4,5)P_4. The results shown are means±SE of duplicate experiments from three preparations of purified enzyme.

present investigation L-*chr* Ins(1,4,6)P_3 and particularily L-*chr* Ins(1,4,6)PS_3 have been shown to be the most potent and selective inhibitors of inositol polyphosphate metabolism yet described.

In investigations with electrically permeabilised human neuroblastoma cells L-*chr* Ins(1,4,6)P_3 and L-*chr* Ins(1,4,6)PS_3 did not elicit Ca^{2+} transients from intracellular stores, while Ins(1,4,5)P_3 released Ca^{2+} potently (EC_{50} 0.12 μM) (20). Furthermore, neither analogue inhibited Ins(1,4,5)P_3-induced Ca^{2+} mobilisation at concentrations as high as 30 μM. Thus, L-*chr* Ins(1,4,6)P_3 and L-*chr* Ins(1,4,6)PS_3 potently inhibit inositol polyphosphate metabolism without affecting Ca^{2+} stores, Ins(1,4,5)P_3 receptor occupancy or 3-hydroxyl specific phosphorylation of Ins(1,4,5)P_3. The structure-activity relationships between these analogues and others described in the literature highlight the differences in the inositol polyphosphate recognition sites of the above proteins and that of the Ins(1,(3,)4,5)PP 5-phosphatases.

L-*chr* Ins(1,4,6)P_3 and L-*chr* Ins(1,4,6)PS_3 should prove to be important pharmacological agents for use in understanding the roles of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and their metabolites in the regulation of intracellular Ca^{2+}.

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