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myo-Inositol 1,4,5-trisphosphorothioate is a potent competitive inhibitor of human erythrocyte 5-phosphatase

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The effect of the *myo*-inositol 1,4,5-trisphosphate (IP_3) analogue, *myo*-inositol 1,4,5-trisphosphorothioate (IPS_3) on the dephosphorylation of D-5-[^{32}P]IP_3 by the 5-phosphatase from human erythrocyte membranes has been investigated. DL-IPS_3 was found to act as a competitive inhibitor with a K_i of 6 μM, making it the most potent inhibitor currently available for this enzyme. L-IP_3 inhibited the enzyme with a K_i of 124 μM and was more potent than D-2,3-diphosphoglycerate (K_i 978 μM).

Second messenger; Inositol phosphate; Inhibitor; Phosphatase

1. INTRODUCTION

D-*myo*-Inositol 1,4,5-trisphosphate (D-IP_3) (**1**) is now established as the intracellular second messenger mediating calcium release from intracellular stores in stimulated cells [1]. D-IP_3 is metabolized via two pathways: an ATP-dependent phosphorylation to D-1,3,4,5-IP_4 [2] and a 5-phosphatase-catalyzed dephosphorylation to D-1,4-IP_2 [3]. 5-Phosphatase was originally identified in human erythrocyte membranes [4] and has subsequently been found in many different types of cell, including liver [5] and platelets, from which this enzyme has been purified to homogeneity [6]. Despite the obvious importance of this enzyme in deactivation of D-IP_3 and termination of the second messenger response, no potent inhibitors of the 5-phosphatase are known, although D-2,3-diphosphoglycerate (2,3-DPG) was found to inhibit the enzyme relatively weakly with a K_i of 350 μM [4].

We have recently introduced the novel IP_3 analogue DL-*myo*-inositol 1,4,5-trisphosphoro-

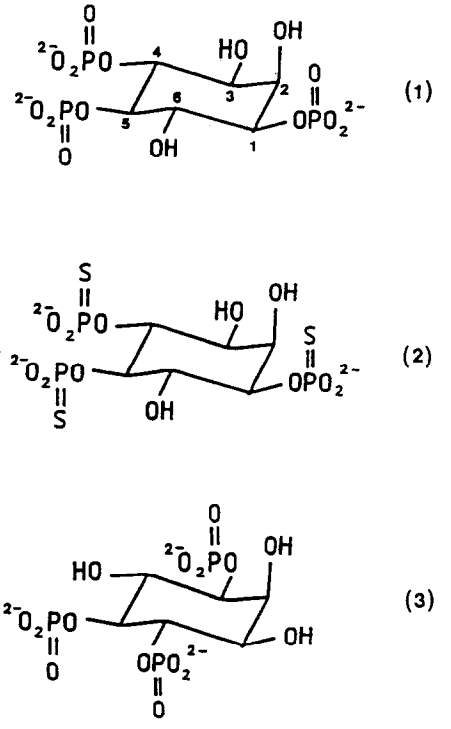

Fig.1. Inositol derivatives studied (single enantiomers shown): (1) D-*myo*-inositol 1,4,5-trisphosphate; (2) D-*myo*-inositol 1,4,5-trisphosphorothioate; (3) L-*myo*-inositol 1,4,5-trisphosphate.

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thioate (IPS_3) (2) [7], wherein the three phosphate groups have been replaced by phosphorothioate groups (fig.1) and which is a potent mobiliser of calcium [8,9] but is resistant to 5-phosphatase [10]. We reasoned that, since D-IPS_3 is a good structural analogue of D-IP_3, it might be expected to be an effective inhibitor of the 5-phosphatase. We report here an investigation of this possibility.

2. MATERIALS AND METHODS

DL-IP_3 [11] and DL-IPS_3 [7] were synthesized as described. L-IP_3 was synthesized as for DL-IP_3 using the resolved precursor D-1,2,4-tri-*O*-benzyl-*myo*-inositol [12]. D-IP_3 and D-5-[^{32}P]IP_3 (100 μCi/ml; 1000 Ci/mmol) were purchased from Amersham, England.

Human erythrocyte ghosts were prepared according to Downes and Michell [13] and were stored at −40°C and 2.6 mg protein/ml. The activity of 5-phosphatase was determined either by colorimetric monitoring of phosphate release from D-IP_3 using an inorganic phosphate assay or by using the specifically labelled substrate D-5-[^{32}P]IP_3 and monitoring release of radioactivity in a similar fashion to Downes et al. [4]. Assay mixtures (50 μl), prepared in duplicate, in buffer (30 mM Hepes, 2 mM Mg^{2+}, pH 7.2), contained D-5-[^{32}P]IP_3 (0.33 nM, chosen to give ~10000 dpm), D-IP_3 (30, 60 or 120 μM) and human erythrocyte ghost preparation (0.026 mg protein). Incubations were performed at 37°C for 30 min, under which conditions no more than 20% of the substrate was consumed at a linear rate. For the competition experiments appropriate concentrations of inhibitor were included in the mixtures. The release of ^{32}P-inorganic phosphate was monitored as follows: mixtures were quickly diluted with 200 μl ice-cold buffer, quenched with 250 μl ice-cold $HClO_4$ (2 M) and centrifuged at 16000 × *g* for 5 min. 450 μl of the supernatant were taken and 50 μl ammonium molybdate (100 mg/ml) were added. The complexed inorganic phosphate was separated from inositol phosphates by addition of isobutyl alcohol (400 μl) and toluene (400 μl). After vigorous mixing and phase separation by centrifugation, 500 μl of the upper layer were taken, added to 5 ml of scintillation fluid (Optiphase X, LKB) and counted for radioactivity. The data were analysed by the method of Dixon (see Cornish-Bowden [15]). Kinetic data were computer fitted using standard linear regression techniques.

Inorganic phosphate assays were carried out according to Moslen et al. [14]. Working colour reagent was freshly prepared daily. Thus, 10 ml ammonium molybdate (99.99%, Aldrich; 4.2%, w/v, solution in 4.2 M HCl) and 30 ml malachite green hydrochloride (Sigma, 0.045%, w/v) were stirred together for 20 min and filtered through Whatman 42 filter paper. Within 1 h of assay 1.2 ml Tween 20 (Sigma, 1.5%, w/v) was added. Inorganic phosphate release from D-IP_3 was determined in incubations containing 200 μl D-IP_3 (50 μM, in 30 mM Hepes/2 mM Mg^{2+} buffer, pH 7.2) and 50 μl membrane preparation (0.13 mg protein) at 37°C. Reactions were terminated by boiling for 10 min. The samples were centrifuged at 16000 × *g* for 5 min, 200 μl of the supernatant were taken and 1.2 ml of the colour reagent was added. Samples were then in-

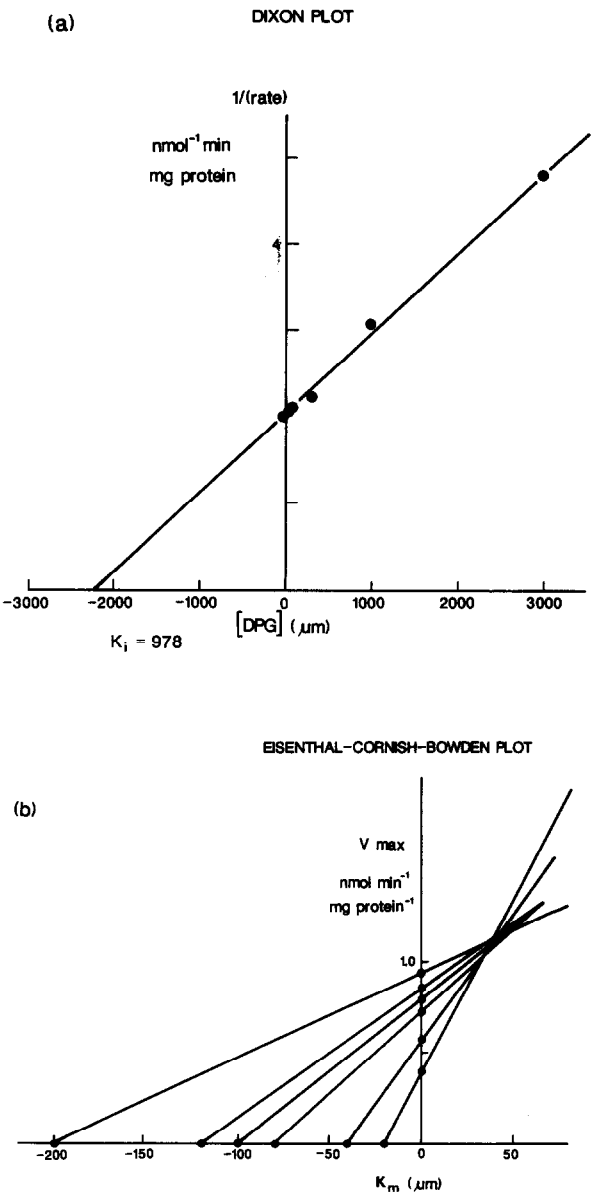

Fig.2. (a) Dixon plot for the determination of the K_i for phosphatase inhibition by D-2,3-diphosphoglycerate: Diphosphoglycerate was added in various concentrations to 50 μM D-IP_3 spiked with 5-[^{32}P]IP_3 (see section 2). The mixtures were then incubated with membrane preparation for 30 min at 37°C. The reaction was quenched and the initial rate determined by counting the released radioactivity. (b) Eisenthal-Cornish-Bowden plot for the determination of the K_m of IP_3 for 5-phosphatase: various concentrations of D-IP_3 spiked with 5-[^{32}P]IP_3 were incubated with membrane preparation for 30 min at 37°C. Dephosphorylation of IP_3 was monitored by counting the released ^{32}P-inorganic phosphate.

cubated at 37°C for 40 min and on cooling to ambient temperature the absorbance at 630 nm was measured within 1 h. A standard curve was prepared for each assay by running blanks and a series of standards containing 0.5–10 nmol KH_2PO_4 through the entire procedure. The assay is sensitive down to 0.3 nmol inorganic phosphate.

3. RESULTS

In our hands the K_m and V_{max} parameters for the human erythrocyte membrane 5-phosphatase were 40 μM and 1.1 nmol/min per mg protein, respectively (fig.2b; cf. 25 μM and 2.8 nmol/min per mg membrane protein, respectively [4]), and the K_i for D-2,3-DPG, the most potent inhibitor presently known, 978 μM (fig.2a; cf. 350 μM [4]). In contrast, *myo*-inositol 1,4,5-trisphosphorothioate (DL-IPS$_3$), a non-hydrolysable analogue of the second messenger D-*myo*-inositol 1,4,5-trisphosphate (1), was a potent and competitive inhibitor of the enzyme with a K_i of 6 μM (fig.3).

L-IP$_3$ was a much weaker inhibitor of the 5-phosphatase (K_i 124 μM: fig.4) and in our hands is not a substrate for the enzyme. Incubation of L-IP$_3$ with 5 times as much 5-phosphatase as used for the inhibition studies for long periods (up to 5 h) caused no detectable release of inorganic

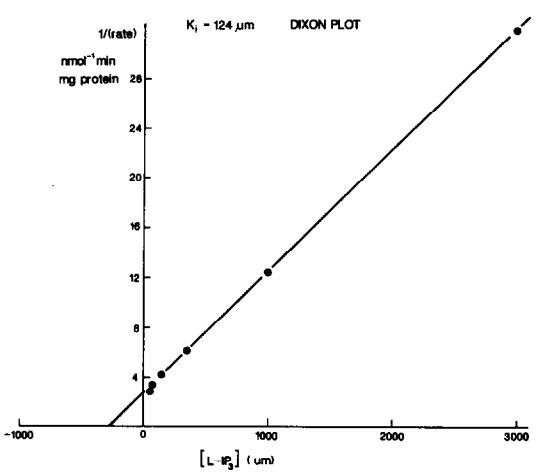

Fig.3. Dixon plot for the determination of the K_i for phosphatase inhibition by DL-IPS$_3$. The effect of various concentrations of DL-IPS$_3$ on the rate of release of radioactive inorganic phosphate in mixtures containing various concentrations of D-IP$_3$ spiked with [^{32}P]IP$_3$ after 30 min incubations at 37°C with 5-phosphatase was measured as described in section 2.

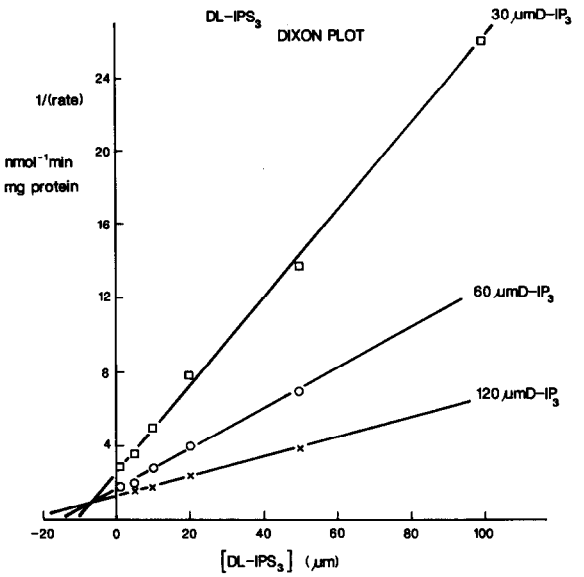

Fig.4. Dixon plot for the determination of the K_i for phosphatase inhibition by L-IP$_3$: L-IP$_3$ was added in various concentrations to 50 μM D-IP$_3$ spiked with 5-[^{32}P]IP$_3$. The mixtures were incubated with 5-phosphatase for 30 min at 37°C. The reaction was quenched and the initial rate determined by measuring release of radioactive inorganic phosphate.

phosphate as measured by molybdate assay. Under identical conditions D-IP$_3$ released its maximal amount of inorganic phosphate in approx. 1 h (not shown).

4. DISCUSSION

Myo-Inositol 1,4,5-trisphosphorothioate (2) was the first structural analogue of IP$_3$ to be synthesized and is resistant to 5-phosphatase-catalyzed degradation. Although sulphur is somewhat larger than oxygen and the pK_a values of phosphorothioates are slightly different from phosphates, phosphorothioates are generally expected to be well bound to biomolecules which recognize the natural substrate, and such analogues, especially those of nucleotides, have found considerable use as substrate analogues in mechanistic investigations [16]. Indeed, phosphorothioate analogues of another second messenger, cAMP, have recently been of great interest [16] and have been demonstrated to be both agonists and antagonists [17]. DL-IPS$_3$ is recognized with high affinity by

intracellular sites mediating calcium release [8,9] and a specific D-IP_3 binding site in cerebellum [10]. In both cases this analogue displayed only a slightly reduced affinity compared to the natural messenger. Thus, D-IPS_3 might be expected to bind strongly to metabolic enzymes, such as 5-phosphatase or 3-kinase, but as a potentially poor substrate should act as a potent inhibitor.

In our hands the membrane-bound 5-phosphatase from human erythrocytes exhibited broadly similar values for the K_m for D-IP_3 and the K_i for D-2,3-DPG as previously reported [4]. DL-IPS_3 was found to be a potent competitive inhibitor of this enzyme possessing a K_i of 6 μM. It is of interest that this value is some 4-fold lower than the previously reported substrate K_m for D-IP_3 [4]. Despite the larger size of sulphur compared to oxygen, which might be expected to reduce the binding affinity, the binding is obviously very tight. It is possible that at the pH used for these experiments (7.2) D-IPS_3 is slightly more charged than D-IP_3, due to the lower pK_a of the phosphorothioate group [18,19] and therefore more favourable interactions may be possible with the groups normally involved in phosphate binding at the active site. No pK_a data are, however, yet available for either compound. Alternatively, the higher hydrophobicity of sulphur over oxygen may have a role to play in enhancing binding to the active site of 5-phosphatase.

Since the DL-IPS_3 used was a racemic mixture it was necessary to establish whether the L-enantiomer (3) possessed inhibitory properties, or indeed was a substrate for the enzyme. The L-enantiomer of IP_3 has already been shown to be very weak at binding to the D-IP_3-specific cerebellum site [10] and is very poor at releasing calcium [9]. In the present experiments, no breakdown of L-IP_3 by 5-phosphatase was discernable, but this enantiomer does inhibit the enzyme. The K_i of 124 μM found for the L-isomer is clearly significantly lower than that for 2,3-DPG, where inhibition is most likely a function of the weak binding of a conformationally mobile vicinal bisphosphate moiety to a bisphosphate-binding site on the enzyme. L-IP_3 (alternative name D-3,5,6-IP_3) also possesses a vicinal bisphosphate but the presence of the inositol ring constrains the conformational mobility of the phosphate groups and they are fixed in an equatorial-equatorial relationship to each other in a similar fashion to the 4,5-bisphosphate of D-IP_3. It is therefore most likely that this extra rigidity is the factor primarily responsible for the approx. 8-fold lower K_i of L-IP_3 compared to D-2,3-DPG. It does not seem likely that the L-1-phosphate group should have any appreciable role to play in binding interactions, although the possibility of an inverse binding mode to the D-IP_3 site, where the three phosphates of an inverted L-IP_3 occupy the phosphate binding sites for D-IP_3 should not be excluded.

In a Dixon plot for inhibition of an enzyme by a 50:50 racemic mixture where each enantiomer has different inhibitory properties (inhibition constants K_i^1 and K_i^2) the plots for different substrate concentrations intersect at $-2K_i^1K_i^2/(K_i^1 + K_i^2)$. Thus, using 124 μM, the measured K_i of L-IP_3, as an upper limit for the putative K_i of L-IPS_3, it is reasonable to presume that the contribution of the L-enantiomer to the overall inhibition measured will be small and a rough estimate of 3.1 μM for the K_i of D-IPS_3 can be made. Although it has not been possible to estimate an accurate K_i for the D-enantiomer of IPS_3, it is clear that even with an upper limit for this value of 6 μM, D-IPS_3 will find use not only as a non-hydrolysable calcium mobilising analogue of D-IP_3, but also as a potent inhibitor of 5-phosphatase. In view of the enhanced metabolic stability of D-IPS_3 its use when bound to an affinity chromatography support should open up new ways for purification of 5-phosphatases and perhaps receptor sites linked to calcium release.

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