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Interaction of synthetic D-6-deoxy-*myo*-inositol 1,4,5-trisphosphate with the Ca^{2+}-releasing D-*myo*-inositol 1,4,5-trisphosphate receptor, and the metabolic enzymes 5-phosphatase and 3-kinase

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The ability of D-6-deoxy-*myo*-inositol 1,4,5-trisphosphate [6-deoxy-Ins(1,4,5)P_3], a synthetic analogue of the second messenger D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], to mobilise intracellular Ca^{2+} stores in permeabilised SH-SY5Y neuroblastoma cells was investigated. 6-Deoxy-Ins(1,4,5)P_3 was a full agonist (EC_{50} = 6.4 μM), but was some 70-fold less potent than Ins(1,4,5)P_3 (EC_{50} = 0.09 μM), indicating that the 6-hydroxyl group of Ins(1,4,5)P_3 is important for receptor binding and stimulation of Ca^{2+} release, but is not an essential structural feature. 6-Deoxy-Ins(1,4,5)P_3 was not a substrate for Ins(1,4,5)P_3 5-phosphatase, but inhibited both the hydrolysis of 5-[^{3}H]+Ins(1,4,5)P_3 (K_i 76 μM) and the phosphorylation of [^{3}H]Ins(1,4,5)P_3 (apparent K_i 5.7 μM). 6-Deoxy-Ins(1,4,5)P_3 mobilized Ca^{2+} with different kinetics to Ins(1,4,5)P_3, indicating that it is probably a substrate for Ins(1,4,5)P_3 3-kinase

Second messenger, Inositol phosphate analogue, Ca^{2+} mobilisation

1. INTRODUCTION

D-*myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3, (1), Fig. 1) is recognised as a second messenger which mediates the release of sequestered Ca^{2+} from intracellular stores [1,2]. Recent studies have led to the purification [3], cloning and sequencing of the Ins(1,4,5)P_3 receptor [4,5], which when incorporated into liposomes can mediate Ca^{2+} release in response to Ins(1,4,5)P_3 [6]. A major challenge is now the elucidation of molecular aspects of the interaction of Ins(1,4,5)P_3 with its receptor and with the enzymes involved in its metabolism, i.e. Ins(1,4,5)P_3 3-kinase and 5-phosphatase, and the chemical design of novel Ins(1,4,5)P_3 agonists, antagonists and enzyme inhibitors. Recent progress in inositol phosphate chemistry [7,8] and the molecular recognition of Ins(1,4,5)P_3 by these three proteins has been reviewed [9].

In the absence of structural information on the Ins(1,4,5)P_3 receptor, chemical modification of the second messenger molecule is an approach to probe molecular interactions. Several inositol ring-modified and phosphate-modified analogues have already been synthesized [7–17] and some progress has been made in understanding the role of the three phosphate and hydroxyl groups of Ins(1,4,5)P_3 in determining receptor binding specificity and stimulation of Ca^{2+} release. As yet, however, little structure–activity data have emerged, apart from recognition of the fundamental importance of the vicinal D-4,5-bisphosphate moiety [8,9]. We report here a study on the interaction of the synthetic analogue D-6-deoxy-*myo* inositol 1,4,5-trisphosphate (6-deoxy-Ins(1,4,5)P_3, (2), Fig. 1) with the Ca^{2+} releasing Ins(1,4,5)P_3 receptor of permeabilised SH-SY5Y neuroblastoma cells and with rat brain Ins(1,4,5)P_3 3-kinase and human erythrocyte membrane Ins(1,4,5)P_3 5-phosphatase.

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2. MATERIALS AND METHODS

D-6-deoxy-Ins(1,4,5)P_3, D-6-deoxy-Ins(1,2-cyclic-4,5)P_3 and D-6-deoxy-Ins(1,5)P_2 were prepared by total synthesis from D-galactose (D. Dubreuil, J. Cleophax, B.V.L. Potter and S.D. Gero, manuscript in preparation). Full details of the synthetic procedures will be reported elsewhere. D-*myo*-inositol 1,4,5-trisphosphorothioate (D-Ins(1,4,5)PS_3) was synthesized and purified similarly to the procedure described for racemic material [10]. D-Ins(1,4,5)P_3 was from Calbiochem. All cell culture reagents were from Gibco. $^{45}CaCl_2$ (approx. 1000 Ci/mmol) was from Amersham International. EC_{50} values were derived using ALLFIT computer-assisted curve fitting [18]. Combined data from a number of independent experiments (n) are expressed as mean ± SEM, where $n \geq 3$.

2.1. *Ca^{2+} release*

$^{45}Ca^{2+}$ release experiments were carried out essentially as described [19], but using saponin-permeabilised SH-SY5Y neuroblastoma cells [17]. Temporal characteristics of Ca^{2+} mobilisation were monitored using a Ca^{2+}-specific electrode [20].

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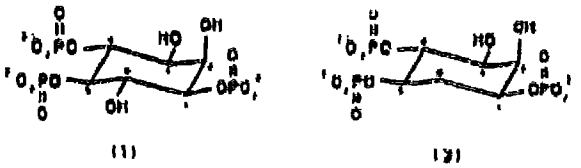

(1) (2)

Fig. 1. Structures of (1) D-Ins(1,4,5)P_3 and (2) D-6-deoxy-Ins(1,4,5)P_3

2.2. *Ins(1,4,5)P_3 5-phosphatase*

Inhibition by 6-deoxy-Ins(1,4,5)P_3 of human erythrocyte ghost 5-phosphatase-catalysed breakdown of 5-[^{32}P]-Ins(1,4,5)P_3 was examined essentially as described for *myo*-inositol 1,4,5-trisphosphorothioate [21]. Erythrocyte ghosts (1 mg protein/ml) were incubated at 37°C for 15 min in the presence of 30 μM Ins(1,4,5)P_3 (approx 5000 dpm 5-[^{32}P]-Ins(1,4,5)P_3) and 0–1 mM 6-deoxy-Ins(1,4,5)P_3. Under these conditions no more than 20% of Ins(1,4,5)P_3 was consumed. To ascertain whether 6-deoxy-Ins(1,4,5)P_3 was a substrate for 5-phosphatase, Ins(1,4,5)P_3 (40 μM), 6-deoxy-Ins(1,4,5)P_3 (80 μM) or KH_2PO_4 (0–50 μM) were incubated with erythrocyte ghosts (1 mg/ml) at 37°C for 30 min. Inorganic phosphate assay to determine phosphate released was carried out as described [21].

2.3. *Ins(1,4,5)P_3 3-kinase*

Inhibition of [^{3}H]-Ins(1,4,5)P_3 phosphorylation by 6-deoxy-Ins(1,4,5)P_3 was examined by incubating a crude 3-kinase preparation [17] (0.1% w/v) at 37°C in the presence of 3, 10 or 30 μM Ins(1,4,5)P_3, ca. 10000 dpm [^{3}H]-Ins(1,4,5)P_3 and increasing amounts of 6-deoxy-Ins(1,4,5)P_3 under conditions where no more than 20% of 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 [22]. Rates of Ins(1,3,4,5)P_4 formation were then calculated.

3. RESULTS AND DISCUSSION

Only 4 studies concerned with biological activity of ring-modified analogues of Ins(1,4,5)P_3 have been reported [13–16]. An interesting aspect concerns the potential role of the 6-hydroxyl group of Ins(1,4,5)P_3, adjacent to the crucial vicinal 4,5-bisphosphate, in determining the affinity and specificity of Ins(1,4,5)P_3 for its intracellular receptor and its interaction with the metabolic enzymes Ins(1,4,5)P_3 3-kinase and 5-phosphatase. We have examined this by deleting the 6-hydroxyl group of Ins(1,4,5)P_3. 6-deoxy-Ins(1,4,5)P_3 has previously been chemically synthesized by a different route to our procedure [13] and other 6-modified analogues which have been synthesized include 6-methoxy-Ins(1,4,5)P_3 [13,14], 6-methyl-Ins(1,4,5)P_3 [13] and 6-fluoro-Ins(1,4,5)P_3 [13]. However, only 6-methoxy-Ins(1,4,5)P_3 has been biologically evaluated and found to be a weak agonist [14]. Apart from a study of the effects of multiple hydroxyl group deletion of Ins(1,4,5)P_3 on biological activity [14], the only other similar investigations already reported concern hydroxyl group deletion in *myo*-inositol 1-phosphate (Ins(1)P), a substrate for inositol 1-phosphatase. 6-Deoxy-*myo*-inositol 1-phosphate is a competitive inhibitor of *myo*-inositol 1-phosphatase [23] and 3,5,6-trideoxy-*myo*-inositol 1-phosphate is the most potent inhibitor yet identified [24].

The EC_{50} for Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilised SH-SY5Y human neuroblastoma cells was 0.09 ± 0.02 μM (Fig. 2). For D-6-deoxy-Ins(1,4,5)P_3 (2) the EC_{50} was 6.4 ± 1.7 μM (Fig. 2) and the analogue was a full agonist for Ca^{2+} release. Thus, deletion of the 6-hydroxyl group makes the analogue approximately 70-fold less potent than Ins(1,4,5)P_3. These data suggest that 6-deoxy-Ins(1,4,5)P_3 has a lower affinity than Ins(1,4,5)P_3 for the Ca^{2+}-releasing receptor. This has been confirmed by radioligand binding to rat cerebellar receptors (A.L. Willcocks, B.V.L. Potter and S.R. Nahorski, data not shown), where the IC_{50} for 6-deoxy-Ins(1,4,5)P_3 in displacing bound [^{3}H]-Ins(1,4,5)P_3 was 6 μM (for comparison, the IC_{50} for Ins(1,4,5)P_3 was 60 nM). The related synthetic compound, D-6-deoxy (1,2-cyclic)-*myo*-inositol 4,5-trisphosphate, had an IC_{50} of 40 μM (for comparison, the IC_{50} for D-Ins(1,2-cyclic-4,5)P_3 was 2.5 μM). Synthetic D-6-deoxy-*myo*-inositol 1,5-bisphosphate showed no detectable displacement up to 100 μM.

In contrast, only a small decrease in Ca^{2+}-releasing potency was observed upon deletion of the 2-hydroxyl group of Ins(1,4,5)P_3. The EC_{50} for DL-2-deoxy-Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilised macrophages was 0.5 μM, whereas that for Ins(1,4,5)P_3 was 0.2 μM [15]. Multiple deletion of hydroxyl groups to produce 1,2,4-cyclohexane trisphosphate was shown to lead to substantial loss of potency (ca 130-fold for racemic material) [14]. 3-Deoxy-Ins(1,4,5)P_3 has not yet been synthesized. DL-6-Methoxy-Ins(1,4,5)P_3 was ca 200-fold weaker than Ins(1,4,5)P_3 in mobilising Ca^{2+} [14].

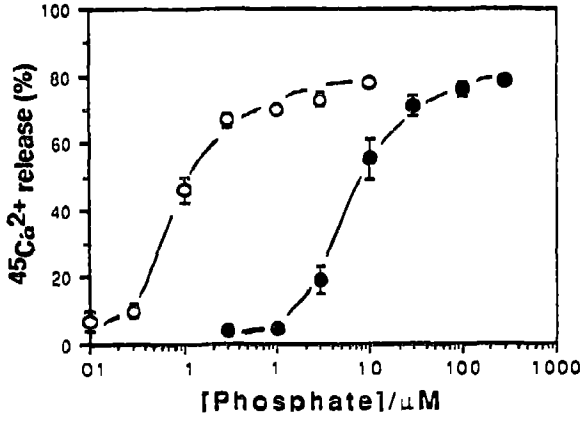

Fig. 2. Dose-dependence of $^{45}Ca^{2+}$ releasing effects of Ins(1,4,5)P_3 (○) and D-6-deoxy-Ins(1,4,5)P_3 (●) in permeabilised SH-SY5Y cells. Saponin-permeabilised SH-SY5Y cells were loaded with $^{45}Ca^{2+}$ and then challenged with agonist. Incubations at 20°C were terminated after 2 min, at which point the amount of $^{45}Ca^{2+}$ released was assessed as described in Section 2 (Ins(1,4,5)P_3, n = 6, 6-deoxy-Ins(1,4,5)P_3, n = 3).

The three negatively charged phosphate groups of Ins(1,4,5)P_3 will most likely make ionic interactions with appropriate positive centres of the Ins(1,4,5)P_3 receptor and make the major contribution to binding energy. The three hydroxyl groups at the 2, 3 and 6 positions may be either hydrogen bond donors to, or acceptors from the protein and, additionally, they may be involved in fixing the solution conformation of Ins(1,4,5)P_3 by intramolecular hydrogen bonding to neighbouring phosphate groups. The vicinal D-4,5-bisphosphate system is known to be essential for Ca^{2+}-releasing activity, while the 1-phosphate enhances receptor binding [9]. Therefore, removal of the 6-hydroxyl group, a neighbour to both the 1- and the 5-phosphate groups, may affect the conformation of these phosphates and the population of appropriate conformer for receptor binding. No data are, however, yet available concerning the conformation of Ins(1,4,5)P_3 bound to its receptor.

Ins(1,4,5)P_3 is metabolised by two major routes that involve, as the first step, dephosphorylation by a 5-phosphatase and phosphorylation by a 3-kinase [9]. The metabolism of 6-deoxy-Ins(1,4,5)P_3 was examined initially using electrically-permeabilised SH-SY5Y cells and continuous monitoring of Ca^{2+} release. SH-SY5Y cells exhibit both Ins(1,4,5)P_3 5-phosphatase and 3-kinase activities [25]. The Ca^{2+} release profile for 6-deoxy-Ins(1,4,5)P_3 differed from that exhibited by Ins(1,4,5)P_3 (Fig. 3a) insofar as Ca^{2+} re-uptake, and therefore inositol phosphate metabolism [20], appeared to be significantly retarded. This re-uptake was, however, faster than that for the non-hydrolysable analogue Ins(1,4,5)PS_3 [9] (Fig. 3b), which is resistant to metabolism by both 5-phosphatase and 3-kinase [9,21,25]. Thus, 6-deoxy-Ins(1,4,5)P_3 may be metabolised slowly by one or both of the enzymes.

We therefore investigated the interaction of D-6-deoxy-Ins(1,4,5)P_3 with Ins(1,4,5)P_3 5-phosphatase from human erythrocyte ghosts. D-6-deoxy-Ins(1,4,5)P_3 inhibited [^{32}P]Ins(1,4,5)P_3 dephosphorylation with a K_i of 76.0 ± 5.2 μM (Fig. 4). Since the K_m for D-Ins(1,4,5)P_3 is ca 40 μM [21], D-6-deoxy-Ins(1,4,5)P_3 clearly binds with relatively high affinity to the 5-phosphatase. The approximately 2-fold weaker affinity of 6-deoxy-Ins(1,4,5)P_3 for erythrocyte 5-phosphatase underlines the marked non-selectivity of this enzyme for inositol phosphates, as previously noted [4,9], and is comparable with the approximately 4-fold lower affinity of DL-6-methoxy-Ins(1,4,5)P_3 for the aortic smooth muscle enzyme [14]. D-6-deoxy-Ins(1,4,5)P_3 was, however, resistant to dephosphorylation by Ins(1,4,5)P_3 5-phosphatase, since inorganic phosphate was not liberated after a 30 min incubation with the enzyme under conditions where 40% of Ins(1,4,5)P_3 was hydrolysed (data not shown). This is in agreement with the conclusion of Polokoff et al. [14] that the minimal structural requirements for substrate hydrolysis by 5-phosphatase include phosphate groups at the D-4,5-positions and a free D-6-hydroxyl group

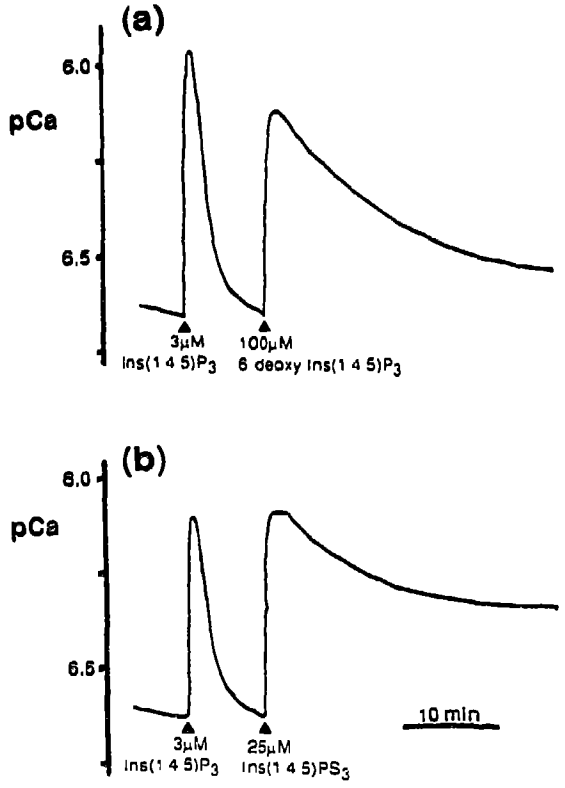

Fig 3 Kinetics of Ca^{2+} release induced by D-Ins(1,4,5)P_3, 6-deoxy Ins(1,4,5)P_3 and Ins(1,4,5)PS_3 monitored in SH-SY5Y neuroblastoma cells using a Ca^{2+}-specific electrode Suspensions of electrically-permeabilised SH-SY5Y cells (3 6–3.9 mg protein/ml) were challenged with (a) 3 μM Ins(1,4,5)P_3 followed by 100 μM 6-deoxy-Ins(1,4,5)P_3, or (b) 3 μM Ins(1,4,5)P_3 followed by 25 μM Ins(1,4,5)PS_3 Data shown are representative of 3 independent experiments

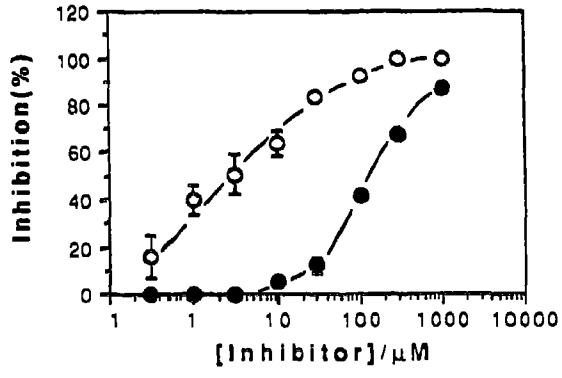

Fig 4. Inhibition of human erythrocyte Ins(1,4,5)P_3 5-phosphatase-catalysed dephosphorylation of [^{32}P]Ins(1,4,5)P_3 by D 6-deoxy-Ins(1,4,5)P_3 (●) and DL-Ins(1,4,5)PS_3 (○) Ins(1,4,5)P_3 (30 μM) containing ca 10000 dpm 5-[^{32}P]Ins(1,4,5)P_3 was incubated at 37°C for 15 min with 5-phosphatase and 0 1 μM–1 mM Ins(1,4,5)PS_3, or 6-deoxy-Ins(1,4,5)P_3 (n = 3) The rate of liberation of inorganic [^{32}P]phosphate was monitored as described [21] K_i values were calculated according to Cheng and Prusoff [26].

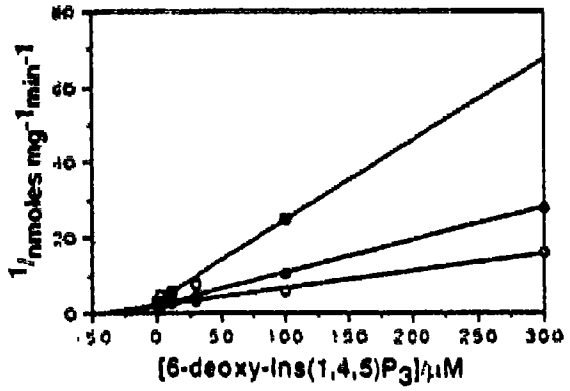

Fig. 5. Dixon plot showing competitive inhibition of Ins(1,4,5)P_3 3-kinase-catalysed phosphorylation of D-Ins(1,4,5)P_3 by D-6-deoxy-Ins(1,4,5)P_3. Various concentrations of Ins(1,4,5)P_3 (3 μM (□), 10 μM (●) and 30 μM (○)) were incubated at 37°C for 15 min with the Ins(1,4,5)P_3 3-kinase preparation (0.1% w/v) and 0–300 μM 6-deoxy-Ins(1,4,5)P_3 (n = 5–6). The rate of formation of Ins(1,3,4,5)P_4 was monitored as described in Section 2

[14], D-6-deoxy-Ins(1,4,5)P_3 is therefore a relatively potent inhibitor of 5-phosphatase.

The interaction of D-6-deoxy-Ins(1,4,5)P_3 with Ins(1,4,5)P_3 3-kinase was also investigated. The phosphorylation of Ins(1,4,5)P_3 by crude rat brain 3-kinase was inhibited by 6-deoxy-Ins(1,4,5)P_3 in a competitive fashion with an apparent K_i of 5.7 ± 2.7 μM (Fig. 5). As the K_m for Ins(1,4,5)P_3 in the present study was 3.2 μM (data not shown), it is clear that 6-deoxy-Ins(1,4,5)P_3 binds with relatively high affinity to the 3-kinase. The 3-kinase is known to exhibit a very high selectivity for binding of inositol phosphates and 6-deoxy-Ins(1,4,5)P_3 is one of a small number of compounds which are recognised by the enzyme with high affinity [9,14–17] This small drop in affinity of 6-deoxy-Ins(1,4,5)P_3 for the 3-kinase, relative to Ins(1,4,5)P_3, should be contrasted with the marked (ca 120-fold) drop in affinity noted for DL-6-methoxy-Ins(1,4,5)P_3 [14]. Since H-bonding potential to neighbouring phosphates is removed both by hydroxyl deletion or methylation, it seems likely that the reason for the low 3-kinase affinity for the 6-methoxy analogue lies with a low tolerance of this enzyme for increased steric bulk at the 6-position.

Direct studies on the substrate properties of 6-deoxy-Ins(1,4,5)P_3 like those reported for fluoro-analogues of Ins(1,4,5)P_3 [17], were not possible due to the low potency of 6-deoxy-Ins(1,4,5)P_3 as a mobiliser of Ca^{2+}. Pretreatment of the analogue at sub-millimolar concentrations with preparations high in 3-kinase activity, prior to Ca^{2+} release studies, was not successful as only limited dilutions of the buffers used were possible. This buffer interfered with $^{45}Ca^{2+}$ mobilisation from a permeabilised cell preparation. It remains possible, however, that 6-deoxy-Ins(1,4,5)P_3 is a substrate for the 3-kinase and this explains the slow re-uptake of Ca^{2+} as seen in Fig. 3a.

We conclude that the 6-hydroxyl group of Ins(1,4,5)P_3, while not absolutely required for stimulation of Ca^{2+} release, is probably important in receptor binding or fixing the conformation of neighbouring phosphate groups, to a significantly greater extent than the 2-hydroxyl group. Clearly, hydroxyl group deletion remote from the 3-hydroxyl site does not markedly affect affinity for the 3-kinase. Indeed, 6-deoxy-Ins(1,4,5)P_3 may be a weak substrate for this enzyme. Hydroxyl deletion adjacent to the 5-phosphate group, however, makes 6-deoxy-Ins(1,4,5)P_3 a relatively potent 5-phosphatase inhibitor.

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