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D-6-Deoxy-*myo*-inositol 1,3,4,5-tetrakisphosphate, a mimic of D-*myo*-inositol 1,3,4,5-tetrakisphosphate: biological activity and pH-dependent conformational properties

Graeme Horne,[a] Clarisse Maechling,[b] Andrea Fleig,[c] Masato Hirata,[d] Reinhold Penner,[c] Bernard Spiess,[b] and Barry V.L. Potter[a,*]

[a] *Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK*
[b] *Laboratoire de Pharmacochimie de la Communication Cellulaire, UMR 7081 du CNRS-ULP, Faculté de Pharmacie 74, Route du Rhin, B.P. 24, 67401 Illkirch Cedex, France*
[c] *Laboratory of Cell and Molecular Biology, Center for Biomedical Research, The Queen's Medical Center and Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI, USA*
[d] *Department of Biochemistry, Kyushu University and Kyushu University Station for Collaborative Research, Fukuoka, Japan*

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Abstract

D-6-Deoxy-*myo*-inositol 1,3,4,5-tetrakisphosphate [D-6-deoxy-Ins(1,3,4,5)P_4] **3** is a novel deoxygenated analogue of D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] **2**, a central and enigmatic molecule in the polyphosphoinositide pathway of cellular signalling. D-6-Deoxy-Ins(1,3,4,5)P_4 is a moderate inhibitor of Ins(1,4,5)P_3 5-phosphatase [1.8 μM] compared to Ins(1,3,4,5)P_4 [0.15 μM] and similar to that of L-Ins(1,3,4,5)P_4 [1.8 μM]. In displacement of [^{3}H] Ins(1,4,5)P_3 from the rat cerebellar Ins(1,4,5)P_3 receptor, while slightly weaker [$IC_{50} = 800$ nM] than that of D-Ins(1,3,4,5)P_4 [$IC_{50} = 220$ nM], **3** is less markedly different and again similar to that of L-Ins(1,3,4,5)P_4 [$IC_{50} = 660$ nM]. **3** is an activator of I_{CRAC} when inward currents are measured in RBL-2H3-M1 cells using patch-clamp electrophysiological techniques with a facilitation curve different to that of Ins(1,3,4,5)P_4. Physicochemical properties were studied by potentiometric ^{31}P and ^{1}H NMR titrations and were similar to those of Ins(1,3,4,5)P_4 apart from the observation of a biphasic titration curve for the P1 phosphate group. A novel vicinal phosphate charge-induced conformational change of the inositol ring above pH 10 was observed for D-6-deoxy-Ins(1,3,4,5)P_4 that would normally be hindered because of the central stabilising role played by the 6-OH group in Ins(1,3,4,5)P_4. We conclude that the 6-OH group in Ins(1,3,4,5)P_4 is crucial for its physicochemical behaviour and biological properties of this key inositol phosphate.

Keywords: Signal transduction; Inositol phosphates; ^{31}P NMR; Potentiometry; Electrophysiology; Structure–activity relationships; IP_3-kinase; IP_3 5-phosphatase

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] **1** (Fig. 1), produced in response to stimulation of a wide variety of cellular membrane receptors, exhibits well-known second messenger properties [1] mediating a large number of calcium-regulated signal transduction events [2]. Ins(1,4,5)P_3 releases Ca^{2+} from intracellular stores and activates store-operated Ca^{2+} influx through plasma membrane channels collectively known as store-operated channels (SOCs) [3]. The best characterised SOCs are Ca^{2+} release activated Ca^{2+} channels (CRAC) which pass the inward Ca^{2+} current, I_{CRAC}. After this Ca^{2+} release the signal must be removed and this is accomplished by one or more metabolic pathways: Ins(1,4,5)P_3 5-phosphatase [yielding Ins(1,4)P_2] and Ins(1,4,5)P_3 3-kinase which generates D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4, **2**, Fig. 1]. While the role of Ins(1,4,5)P_3 and its calcium mobilising ability is well defined in many tissues, exact reasons for the rapid production of Ins(1,3,4,5)P_4 following receptor

* Corresponding author. Fax: +44-0-1225-386114.
E-mail address: B.V.L.Potter@bath.ac.uk (B.V.L. Potter).

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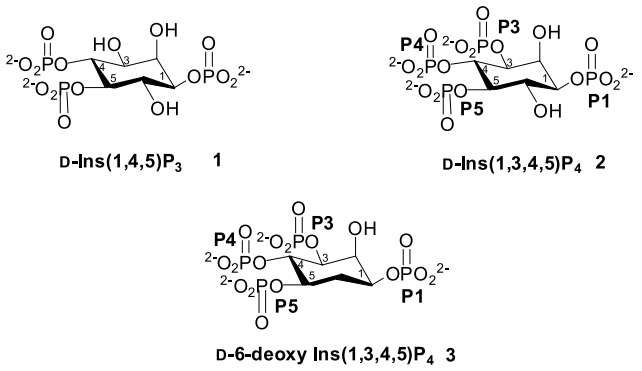

Fig. 1. Structures of Ins(1,4,5)P_3 **1**, Ins(1,3,4,5)P_4 **2**, and 6-deoxy-Ins(1,3,4,5)P_4 **3**.

stimulation are still less well defined. Essentially, there are three broad possibilities: (1) the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 is considered to be an off-switch terminating the release of calcium by Ins(1,4,5)P_3; (2) Ins(1,3,4,5)P_4 is a metabolic intermediate with no other function; and (3) Ins(1,3,4,5)P_4 has a second messenger function itself, initiated through binding to its own specific intracellular receptor.

A role mediating Ca^{2+} entry through plasma membrane channels has been proposed. An Ins(1,3,4,5)P_4-sensitive Ca^{2+}-permeable channel has been characterised from endothelial cells [4] and Ins(1,3,4,5)P_4 binding proteins have been identified from pig and rat cerebellum [5] and porcine platelets [6]. The latter example has been characterised and identified as a putative Ins(1,3,4,5)P_4 receptor designated as GAP^{IP4BP} [7]. GAP^{IP4BP} is a Ras GTPase-activating protein that in vitro is regulated by Ins(1,3,4,5)P_4 and contains C-terminal Ins(1,3,4,5)P_4-binding pleckstrin homology (PH) domains [8]. The interaction of Ins(1,3,4,5)P_4 with the PH domain of Bruton's tyrosine kinase (Btk) may be involved in B-cell activation and development [9]; mutations in the Btk PH domain causing human X-linked agammaglobulinaemia (XLA) and murine X-linked immunodeficiency (Xid) are associated with dramatically reduced Ins(1,3,4,5)P_4-binding activity [10]. We reported an X-ray crystal structure of synthetic Ins(1,3,4,5)P_4 [11] bound to the PH domain of Btk [12], that has been used to define a model describing the Ins(1,3,4,5)P_4-binding site within the PH domain of GAP^{IP4BP} [13].

Recently, we suggested a role for Ins(1,3,4,5)P_4 in facilitating store-operated Ca^{2+} influx in cells by inhibition of Ins(1,4,5)P_3 5-phosphatase [14]. It is known that Ins(1,3,4,5)P_4 is hydrolysed by the same 5-phosphatase that hydrolyses Ins(1,4,5)P_3, but the enzyme has a 10-fold higher affinity and 100-fold lower V_{max} for Ins(1,3,4,5)P_4 than it does for Ins(1,4,5)P_3 [15], implying that Ins(1,3,4,5)P_4 can protect Ins(1,4,5)P_3 against hydrolysis and therefore increase its overall effectiveness. Ins(1,3,4,5)P_4 sensitises Ins(1,4,5)P_3-mediated activation of the store-operated Ca^{2+} current I_{CRAC} through inhibition of type I Ins(1,4,5)P_3 5-phosphatase. Consequently, after cell stimulation has ceased Ins(1,3,4,5)P_4 will have a longer half-life than Ins(1,4,5)P_3 and will protect any new Ins(1,4,5)P_3 that is formed by subsequent stimulation. This inhibition of metabolism results in increased levels of available Ins(1,4,5)P_3. The antagonistic action of Ins(1,3,4,5)P_4 was also revealed with higher concentrations of Ins(1,3,4,5)P_4 acting to inhibit Ins(1,4,5)P_3 receptors. Through these regulatory effects on Ins(1,4,5)P_3 metabolism, Ins(1,3,4,5)P_4 is suggested to behave as a potent bi-modal regulator of cellular sensitivity to Ins(1,4,5)P_3 that provides both facilitatory and inhibitory feedback on Ca^{2+} influx.

In endothelial cells [4] and neurons [16,17] Ins(1,3,4,5)P_4 has been shown to activate Ca^{2+} channels in the plasma membrane. At high concentrations Ins(1,3,4,5)P_4 can also interact with Ins(1,4,5)P_3 receptors on the endoplasmic membrane with differing effects. In some cell types it has been shown to inhibit the actions of Ins(1,4,5)P_3 [18], whereas in others it apparently mimics its action by causing the mobilisation of Ca^{2+} [19]. This apparent contradictory nature of Ins(1,3,4,5)P_4 could be accounted for through the presence of at least three isoforms of the Ins(1,4,5)P_3 receptor in mammals with each possibly responding differently to Ins(1,3,4,5)P_4. To date, three types of Ins(1,4,5)P_3 receptors have been cloned [20] and are expressed in varying abundance in different tissues [21]. Recently, first three-dimensional structures of the type I Ins(1,4,5)P_3 receptor have been reported [22a,22b] and also an X-ray crystal structure of the ligand-binding domain in complex with Ins(1,4,5)P_3 [22c].

In order to further investigate the biological function of Ins(1,3,4,5)P_4 at several potential biological targets the synthesis of structural analogues of this molecule is of continuing interest. We recently reported the synthesis of 6-deoxy-Ins(1,3,4,5)P_4 [23] in racemic and chiral form, as well as the synthesis of D-2-deoxy-Ins(1,3,4,5)P_4 from D-glucose [24]. We now report the protonation constants and an overview of the protonation process of the individual phosphate groups for D-6-deoxy-Ins(1,3,4,5)P_4 (**3**, Fig. 1) and the results of the interaction of D-6-deoxy-Ins(1,3,4,5)P_4 with Ins(1,4,5)P_3 5-phosphatase and Ins(1,4,5)P_3 receptors. The results are compared to the biological and acid–base behaviour of Ins(1,3,4,5)P_4 to aid in the understanding of the enigmatic role of Ins(1,3,4,5)P_4.

Experimental

Materials. Full synthetic details for the preparation of D-Ins-(1,3,4,5)P_4 [11] and D-6-deoxy-Ins(1,3,4,5)P_4 [23] have previously been reported. All materials used for titrations and biological evaluations

were spectroscopically homogeneous and were used as their tri-ethylammonium salts.

5-Phosphatase inhibition and Ins(1,4,5)P_3 receptor-binding assays [14]. Assays of Ins(1,4,5)P_3 5-phosphatase activity were performed at pH 7.2 using purified recombinant enzyme expressed in *Escherichia coli*. Ins(1,4,5)P_3 receptor-binding assays were performed using microsomal fractions of rat cerebellum prepared as described. The assay mixture (0.5 ml) contained 50 mM Hepes/NaOH buffer, pH 7.2, 1 mM EDTA, 6 nM [^{3}H]Ins(1,4,5)P_3, and ~30 μg microsomal fraction, in the presence of various concentrations of compounds of interest. Incubation was performed on ice for 10 min, followed by the separation of bound radioactivity from free form by centrifugation (15,000 rpm for 10 min). Non-specific binding (150–200 dpm) was determined in the presence of 10 μM Ins(1,4,5)P_3 and was subtracted from that in its absence to determine the specific binding (4000–5000 dpm).

Electrophysiology. For patch-clamp experiments, RBL-2H3-M1 cells grown on glass coverslips were transferred to a recording chamber and kept in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, $CaCl_2$ 10, $MgCl_2$ 2, glucose 10, and Hepes–NaOH 10, pH 7.2. The standard intracellular pipette-filling solution contained (in mM): Cs-glutamate 145, NaCl 8, $MgCl_2$ 1, ATP 0.5, and GTP 0.3, pH 7.2, adjusted with CsOH. The internal solution was supplemented with a mixture of 10 mM Cs-BAPTA and 4.3–5.3 mM $CaCl_2$ to buffer $[Ca^{2+}]_i$ to resting levels of 100–150 nM and avoid spontaneous activation of I_{CRAC}. Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21–25 °C. High-resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA, Lambrecht, Germany). Patch pipettes had resistances between 2 and 4 MΩ after filling with the standard intracellular solution. Immediately following establishment of the whole-cell configuration, voltage ramps of 50 ms duration spanning the voltage range of −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 300–800 s. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.3 kHz and digitised at 100 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. For analysis, the very first ramps before activation of I_{CRAC} (usually 1–3) were digitally filtered at 2 kHz, pooled, and used for leak-subtraction of all subsequent current records. The low-resolution temporal development of inward currents was extracted from the leak-corrected individual ramp current records by measuring the current amplitude at −80 mV.

Potentiometric studies and NMR determinations. Potentiometric and NMR determinations were carried out as previously reported [25,26]. The experiments were performed in two steps in which 0.50 cm^3 of the same initial solution of 6-deoxy-Ins(1,3,5,6)P_4, 3.0×10^{-3} mol dm^{-3} in ^2H_2O was successively subjected to potentiometric and NMR titrations. It should be noted that the glass electrode was calibrated in a concentration scale and the measurements were done in ^2H_2O, so that pH here means the co-logarithm of the concentration of $^2H^+$. The processing of the pH measurements allowed the total concentration of the ligand and the acid, as well as the macroscopic protonation constants, to be determined. One-dimensional ^{31}P NMR spectra were recorded at 121.50 MHz on a Bruker DPX-300 Fourier transform spectrometer. ^{31}P chemical shift values were referenced to an external 85% H_3PO_4 signal at 0.00 ppm with downfield shifts represented by positive values. Spectra were acquired over a spectral width of 10 ppm using a 0.1 s relaxation delay and a π/2 pulse. Typically 1K data points were sampled with a corresponding 0.4 s acquisition time. Data were zero-filled and a 1 Hz exponential line broadening function was applied prior to Fourier transformation. The spectra had a digital resolution of 1.19 Hz per point. The HypNMR program was used to check the potentiometrically determined protonation constants. The ^{1}H NMR titration was performed on the same equipment as before operating at 300.13 MHz. Spectra were acquired with water presaturation over a spectral width of 6 ppm using a 3 s relaxation delay and a π/2 pulse. 4K data points were sampled with a corresponding 1.14 s acquisition time. The spectra had a digital resolution of 0.44 Hz per point. The temperature was controlled at 310 ± 0.5 K. The proton and phosphorus resonances were assigned by performing proton–proton and phosphorus–proton 2D thus allowing the titration curves to be characterised.

Results and discussion

Macroscopic and microscopic protonation constants

Determination of structural and thermodynamic parameters at an inframolecular level (i.e., at each functional group) is one of the key issues in the comprehension of molecular interactions. In addition to structure–activity studies, NMR and potentiometric investigations may also contribute to delineate the electronic and conformational demands for optimal binding in the receptor cleft. This is especially true if the parameters that are considered are related to the individual functional groups of the molecules. We have previously determined the acid–base properties of Ins(1,3,4,5)P_4 at an inframolecular level [27]. It is therefore interesting to compare such properties with those of the 6-deoxy analogue. Using both ^{31}P and ^{1}H NMR titration curve analysis provides valuable information on the conformational dependence of the inositol ring with respect to the ionisation state of the individual phosphate groups and in particular the dynamics of the complex intramolecular interactions which govern the conformation of the phosphate groups and of the inositol ring [28–35]. Thus, this physicochemical part first describes the protonation pattern of the 6-deoxy-Ins(1,3,4,5)P_4 and then analyses the conformational consequences of the phosphate protonation process.

In the 12–2 pH range, 6-deoxy-Ins(1,3,4,5)P_4 is able to bind four protons which are involved in the following macroscopic stepwise protonation equilibria:

$$H_{y-1}L^{(9-y)-} + H^+ \overset{K_y}{\rightleftharpoons} H_yL^{(8-y)-} \quad (1)$$

characterised by the related K_y constants, where y takes the values 1–4. It can be noted that $\log K_y$ correspond to the usual pK_y values.

Derivation of the microprotonation schemes is straightforward for difunctional ligands, becomes more complicated for trifunctional ligands such as inositol-trisphosphates [25], but appears as a very intricate task for tetrafunctional molecules, since the number of microspecies to consider is, respectively, 4, 8, and 16. Fortunately, ^{31}P NMR spectroscopy is well suited to monitor the ionisation state of individual phosphates, the chemical shift variations of the phosphorus nuclei with pH being mainly the result of the protonation state variations of the phosphate groups. Thus, even for tetrafunctional ligands, the relative protonation degree of

each phosphate group can be calculated at a given pH from the protonation fraction curves. These protonation fractions can be easily obtained according to the following equation:

$$f_{i,\mathrm{p}} = \frac{\delta_i^{\mathrm{obs}} - \delta_{i,\mathrm{d}}}{\delta_{i,\mathrm{p}} - \delta_{i,\mathrm{d}}}, \quad (2)$$

where δ_i^{obs}, $\delta_{i,\mathrm{p}}$, and $\delta_{i,\mathrm{d}}$ correspond, respectively, to the observed chemical shift and the chemical shifts of protonated and deprotonated fractions of the phosphates in position i.

The ^{31}P NMR titration curves of 6-deoxy-Ins(1,3,4,5)P_4 from which the protonation fraction curves are calculated are displayed in Fig. 2. The general shape of these curves differs only slightly from those of Ins(1,3,4,5)P_4 (curves not shown). As for the latter, P3 and P5 behave similarly and P4 shows the usual features of an equatorial phosphate centrally located between two other equatorial phosphates. Only the P1 curves look different in the 8–12 pH range: the curve for Ins(1,3,4,5)P_4 is monophasic with a slight deshielding upon protonation at the beginning of the curve, whereas that of 6-deoxy-Ins(1,3,4,5)P_4 is biphasic.

Differences also appear on the macroprotonation constants of both ligands. These constants determined by NMR titration [36] in the same experimental conditions (0.2 M KCl in 100% ^2H_2O at 37 °C) are listed in Table 1. It can be observed that there is no unique tendency in the variation of the acid–base character of both compounds. If $\log K_1$ and $\log K_2$ are higher for Ins(1,3,4,5)P_4 than for its 6-deoxy analogue, the inverse occurs for $\log K_3$ and $\log K_4$. This is in line with the observations that can be made on the protonation fractions ($f_{i,\mathrm{p}}$) vs pH curves (Fig. 3).

On these curves it can be seen that the P3, P5, and especially P1 curves for 6-deoxy-Ins(1,3,4,5)P_4 are shifted to higher pH with respect to those of Ins(1,3,4,5)P_4, thereby indicating a more basic character

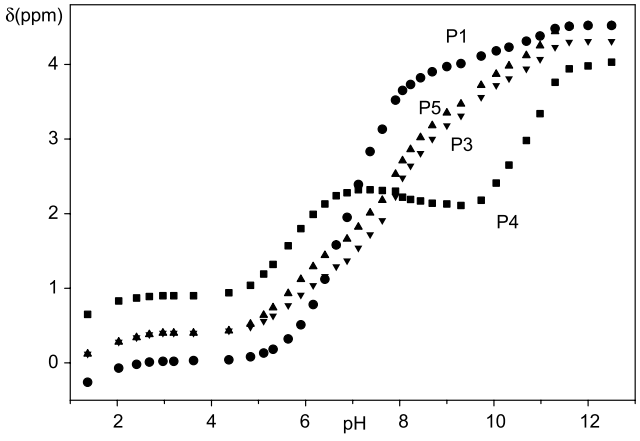

Fig. 2. Chemical shifts (δ) from ^{31}P NMR titrations for 6-deoxy-Ins(1,3,4,5)P_4 as a function of pH in KCl 0.2 M at 37 °C (^2H_2O).

Table 1
Logarithms of the stepwise macroprotonation constants ($\log K_y$) for 6-deoxy-Ins(1,3,4,5)P_4 and Ins(1,3,4,5)P_4

y, ligand	$\log K_y$, 6-deoxy-Ins(1,3,4,5)P_4	$\log K_y$, Ins(1,3,4,5)P_4
1	10.44 (0.04)	10.79 (0.03)
2	7.08 (0.02)	8.00 (0.02)
3	6.87 (0.03)	6.56 (0.03)
4	5.68 (0.02)	5.33 (0.02)

The uncertainties are estimates of the standard deviation as calculated by HypNMR [36]. It can be noted that $\log K_y$ also correspond to the classical pK_a values which refer to a proton dissociation process.

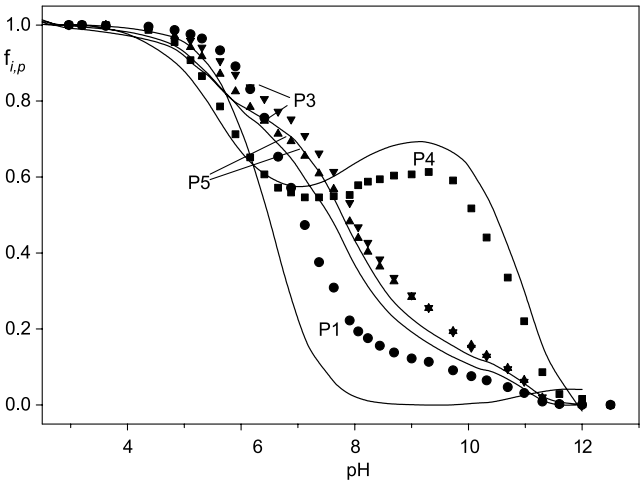

Fig. 3. Protonation fraction curves $f_{i,\mathrm{p}}$ as a function of pH in KCl 0.2 M at 37 °C (^2H_2O) for 6-deoxy-Ins(1,3,4,5)P_4 (points) and Ins(1,3,4,5)P_4 (solid line).

for these phosphates of 6-deoxy-Ins(1,3,4,5)P_4. However, the shift is inverted for P4 showing that P4 is more basic for Ins(1,3,4,5)P_4.

To shed light on these observations, ^{1}H NMR titration experiments of both compounds were performed. The curves for Ins(1,3,4,5)P_4 (Fig. 4A) are fully in line with those previously obtained for Ins(1,4,5)P_3 and related compounds [33]. Upon protonation of a neighbouring phosphate, the ^{1}H resonances generally move downfield due to the decreased electron density occurring at this phosphate. The opposite trend earlier called "wrongway shift" [37,38] has recently been attributed to the establishment of a C–H···O hydrogen bond between a phosphate oxygen constrained to closely approach a neighbouring equatorial hydrogen atom [33]. This effect is particularly obvious for H2, undergoing a highfield shift of 0.17 ppm between pH 10.8 and 7.0, which primarily results from the protonation of P4 but also P3 and P5 phosphates. Partial neutralisation of their negative charges releases the strong constraints due to both P3–P4 and P4–P5 repulsions. A similar behaviour, although much less marked, is shown for the axial H6 which slightly experiences the P4–P5 repulsion. From Fig. 4B, it can be seen that the ^{1}H NMR titration curves

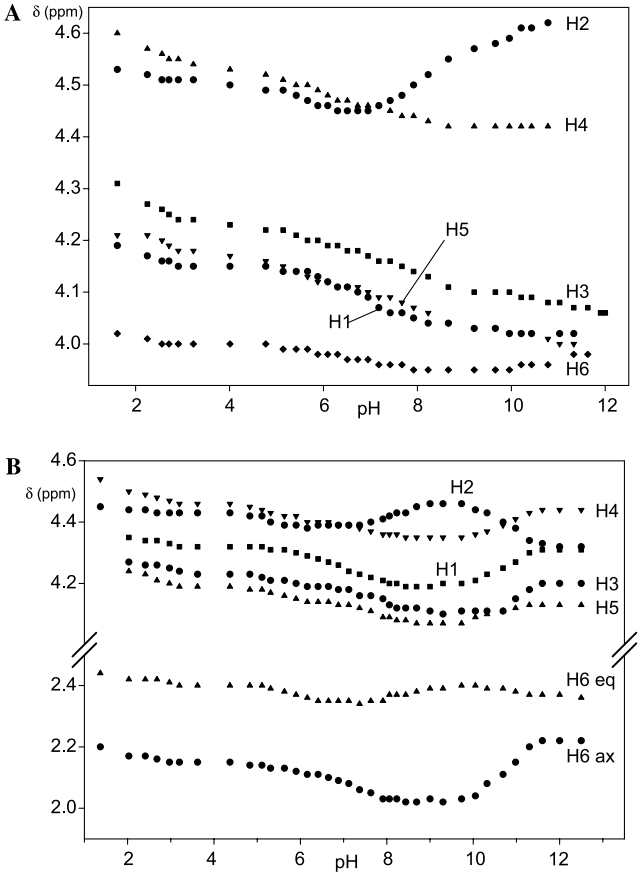

Fig. 4. Chemical shifts δ from ^{1}H NMR titrations for Ins(1,3,4,5)P_4 (A) and 6-deoxy-Ins(1,3,4,5)P_4 (B) as a function of pH in 0.2 M KCl at 37 °C (^2H_2O).

for 6-deoxy-Ins(1,3,4,5)P_4 appear much more complicated. From pH 12.3 to 10 the curves of all the protons take exactly the opposite direction than those of Ins(1,3,4,5)P_4, then below pH 10 they behave again as expected. This is, according to previous observations [34], clearly the sign of a conformational change in the inositol ring. A closer look at its $^3J_{H,H}$ coupling constants shows that at pH 12.3 $^3J_{4,5} \approx {}^3J_{3,4} \approx 6.6$ Hz, whereas the remaining $^3J_{H,H}$ axial–axial coupling constants are nearly 9.2 Hz, both values accounting for dihedral angles which correspond to a pronounced distorted chair conformation (Fig. 5). In between pH 11 and pH 10, $^3J_{4,5}$ and $^3J_{3,4}$ suddenly reach 9.2 Hz, i.e., coupling constants expected for a classical chair inositol

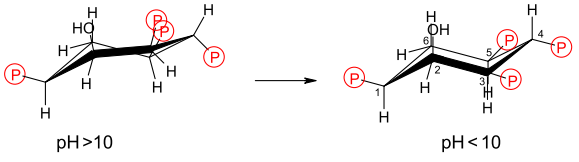

Fig. 5. Inositol ring distortion occurring at pH 10 for 6-deoxy-Ins(1,3,4,5)P_4.

ring. The transition from a distorted to an undistorted conformation accompanies the protonation of the P4 phosphate which again relieves the strong repulsions between the three vicinal phosphate groups. Even though the phosphates are exactly at the same position for Ins(1,3,4,5)P_4 as for its 6-deoxy analogue, such a distortion does not take place due to strong hydrogen bonds established between OH6 and the P1 and P5 phosphates. Nevertheless, for 6-deoxy-Ins(1,3,4,5)P_4, the electrostatic repulsions are not sufficient to provoke the flipping of the inositol ring as recently described for the Ins(1,4,5,6)P_4 carrying four vicinal phosphate groups [34]. Inspection of a simple molecular model shows that, due to the ring distortion for 6-deoxy-Ins(1,3,4,5)P_4 in comparison to Ins(1,3,4,5)P_4, the interactions between the vicinal phosphates are weakened and correlatively the P1–P3, P1–P4, and P1–P5 interactions are strengthened. As a consequence, P4 is more basic for Ins(1,3,4,5)P_4 than for its 6-deoxy analogue. However, the higher basicity of P1 for the latter may rather result from the deletion of the polar equatorial OH6 producing a less hydrophilic pocket in between P1 and P5 which decreases the effective dielectric constant and therefore increases the basicity of the phosphates [35].

The differences observed in the acid–base behaviour of both the studied compounds, although notable, should not solely account for any potential differences in their biological activity. As for Ins(1,4,5)P_3, OH6 seems to play a central role by engaging in strong hydrogen bonds with its neighbouring phosphates. By doing so it participates in the transfer of an electrostatic effect between P1 and P5 [21], but also prevents the inositol ring distortion that would result from the repulsion of vicinal phosphate groups.

Ins(1,4,5)P_3 5-phosphatase inhibition and Ins(1,4,5)P_3 receptor binding

Ins(1,4,5)P_3 releases Ca^{2+} from stores and activates store-operated Ca^{2+} influx through plasma membrane channels collectively known as store-operated channels (SOCs). The best characterised SOCs are Ca^{2+} release activated Ca^{2+} channels (CRAC) which pass the inward Ca^{2+} current, I_{CRAC}. The relationship between Ins(1,4,5)P_3 and I_{CRAC} activation has been reported to be non-linear, exhibiting a steep dose–response curve [39,40]. This non-linearity is thought to be due to Ins(1,4,5)P_3 metabolism, because the non-metabolisable analogue Ins(2,4,5)P_3 has been shown to cause a more graded I_{CRAC} activation. Recent evidence suggests that a functionally distinct store is involved in activating I_{CRAC} and that depletion of this store requires fairly high Ins(1,4,5)P_3 levels [39,41]. It seems likely that the lower sensitivity is due to significant Ins(1,4,5)P_3 metabolism which effectively lowers Ins(1,4,5)P_3 available to gate the

Ins(1,4,5)P_3 receptors in these stores. Ins(1,4,5)P_3 5-phosphatase has been implicated as a major contributing factor to the non-linearity of I_{CRAC} activation. We recently reported the Ins(1,4,5)P_3-mediated activation of I_{CRAC} in living cells by Ins(1,3,4,5)P_4 and other pharmacological 5-phosphatase inhibitors [14]. Based on structural considerations 6-deoxy-Ins(1,3,4,5)P_4 was also believed to be inhibitory to Ins(1,4,5)P_3 5-phosphatase—6-deoxy-Ins(1,4,5)P_3 was shown to be a moderately potent inhibitor of erythrocyte 5-phosphatase and we reported that Ins(1,3,4,5)P_4 (IC_{50} 0.15 μM) is a more potent 5-phosphatase inhibitor than previously described synthetic inhibitors L-*chiro*-Ins(2,3,6)P_3 (IC_{50} 0.23 μM)and L-*chiro*-Ins(1,4,6)PS_3 (IC_{50} 0.30 μM) [14]. We found that 6-deoxy-Ins(1,3,4,5)P_4 inhibits 5-phosphatase at least one order of magnitude less potently than Ins(1,3,4,5)P_4 [Fig. 6A, Ins(1,3,4,5)P_4 IC_{50} 0.15 μM, 6-deoxy-Ins(1,3,4,5)P_4 IC_{50} 1.8 μM]. This value is the same as that for L-Ins(1,3,4,5)P_4 that we previously reported [14]. Looked at in terms of binding orientation in the active site of 5-phosphatase we can assume it to be most likely that 6-deoxy-Ins(1,3,4,5)P_4 (Fig. 7C) binds in the same way as Ins(1,3,4,5)P_4 (Fig. 7A) and that L-Ins(1,3,4,5)P_4 binds probably in the relative orientation shown in Fig. 7B with the polyphosphate spatial configuration maintained, but the axial 2-position and equatorial 6-position hydroxyls binding in the sites normally occupied by the equatorial

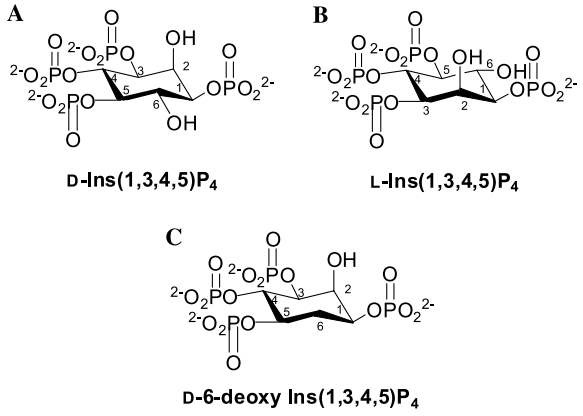

Fig. 7. Proposed relative ligand-binding orientations for Ins(1,4,5)P_3 receptor and 5-phosphatase interactions: (A) Ins(1,3,4,5)P_4; (B) L-Ins(1,3,4,5)P_4 is shown in a binding orientation in which it can mimic its enantiomer Ins(1,3,4,5)P_4; and (C) D-6-deoxy-Ins(1,3,4,5)P_4 is shown in a binding orientation in which it can mimic Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4. Note the relative differences at C-2 and C-6.

6-OH of (A), the CH_2 of (B), and the axial 2-OH of (A) and of (B), respectively.

As 6-deoxy-Ins(1,3,4,5)P_4 behaves as a 5-phosphatase inhibitor we then looked at the potential interaction of this molecule with Ins(1,4,5)P_3 receptors (Fig. 6B). The Ins(1,4,5)P_3 receptor binding assays show that 6-deoxy-Ins(1,3,4,5)P_4 inhibits Ins(1,4,5)P_3 binding to the Ins(1,4,5)P_3-receptor only slightly less effectively

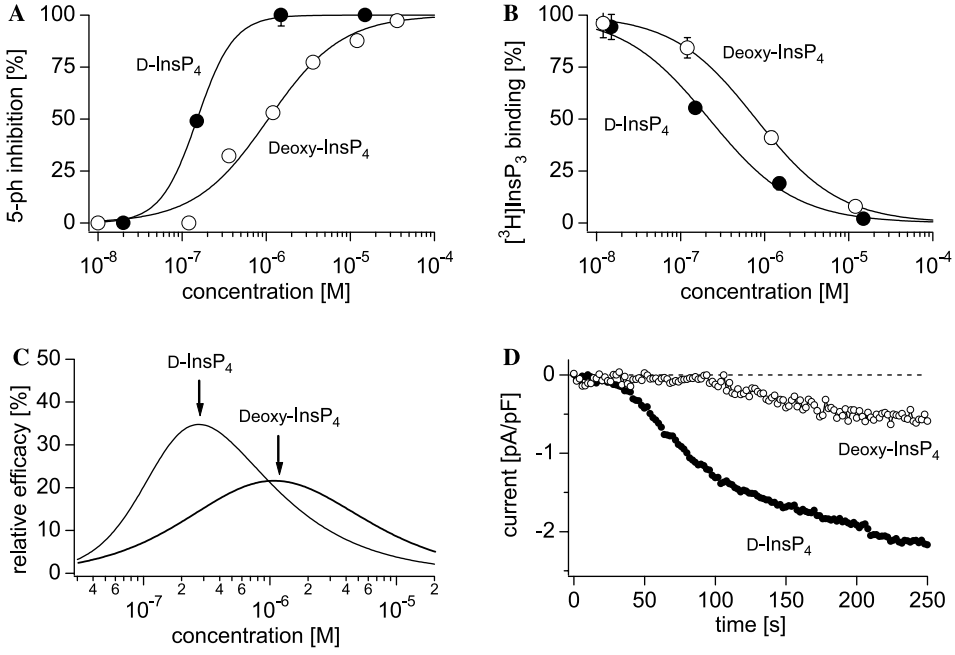

Fig. 6. Ins(1,3,4,5)P_4 and 6-deoxy-Ins(1,3,4,5)P_4 inhibit 5-phosphatase and Ins(1,4,5)P_3 receptor binding. (A) Inhibition profile of Type I 5-phosphatase activity by ligands (see Experimental). Data points are averages ± SEM ($n = 3$). (B) Inhibition profile of [^{3}H]Ins(1,4,5)P_3 binding to InsP_3 receptors for ligands (see Experimental). Data points are averages ± SEM ($n = 3$). (C) Facilitation profiles of the two ligands. Net facilitation curves reflect the product of inhibition profiles of 5-phosphatase activity and Ins(1,4,5)P_3 binding of InsP_3 receptors from (A) and (B). (D) Average inward currents carried by I_{CRAC} at −80 mV. Cells were co-perfused with either Ins(1,3,4,5)P_4 or 6-deoxy-Ins(1,3,4,5)P_4 and sub-threshold levels of Ins(1,4,5)P_3 (1 μM). Traces are averages of experiments in which D-Ins(1,3,4,5)P_4 ($n = 14$) and D-6-deoxy-Ins(1,3,4,5)P_4 ($n = 8$) were used at 20 μM.

[$IC_{50} = 800$ nM] than Ins(1,3,4,5)P_4 [$IC_{50} = 220$ nM]. Ins(1,4,5)P_3-receptor binding and 5-phosphatase inhibition would have opposing effects on Ins(1,4,5)P_3-mediated activation of I_{CRAC}, with the first contributing a positive and the second, a negative effect. The resulting net facilitation can be calculated as the product of these two factors. This analysis is illustrated in Fig. 6C for both Ins(1,3,4,5)P_4 and 6-deoxy-Ins(1,3,4,5)P_4, where the product of the superimposed 5-phosphatase inhibition and Ins(1,4,5)P_3-receptor binding curves yields in each case a bell-shaped net facilitation curve. The amplitude of the latter (*Y*-axis) roughly reflects efficacy, whereas potency is reflected by the location of the curve on the *X*-axis. Compared to Ins(1,3,4,5)P_4, 6-deoxy-Ins(1,3,4,5)P_4 is, as predicted, an effective facilitator of I_{CRAC}, but less potent than the natural inositol phosphate. Qualitatively, these predictions are borne out by patch-clamp experiments which clearly show that Ins(1,3,4,5)P_4 is a more effective facilitator of I_{CRAC} than 6-deoxy-Ins(1,3,4,5)P_4 (Fig. 6D). 6-Deoxy-Ins(1,3,4,5)P_4 is, however, a slightly more effective I_{CRAC} facilitator than the Ins(1,3,4,5)P_4 enantiomer L-Ins(1,3,4,5)P_4[14], which had an IC_{50} for [^{3}H]Ins(1,4,5)P_3 displacement of 660 nM.

If we assume that 6-deoxy-Ins(1,3,4,5)P_4 and L-Ins(1,3,4,5)P_4 most likely also bind to the Ins(1,4,5)P_3 receptor in the relative modes as shown (Fig. 7) then, not surprisingly, placing the axial 2-OH group of L-Ins(1,3,4,5)P_4 in the position normally occupied by the equatorial 6-OH group of D-Ins(1,3,4,5)P_4 [or Ins(1,4,5)P_3] is presumably in SAR terms equivalent to deoxygenation of D-Ins(1,3,4,5)P_4 at the 6-position. Since the relative IC_{50} values for L-Ins(1,3,4,5)P_4 and D-6-deoxy-Ins(1,3,4,5)P_4 are very similar we can assume that the equatorial 6-OH group of L-Ins(1,3,4,5)P_4, binding at a site normally occupied by the axial 2-OH group of D-Ins(1,3,4,5)P_4 [or Ins(1,4,5)P_3] does not have a significant effect on binding. This observation is perfectly in line with the established SAR for Ins(1,4,5)P_3 binding to its receptor where the 2-position modifications found in *scyllo*-Ins(1,4,5)P_3 [equatorial 2-OH] and 2-deoxy-Ins(1,4,5)P_3 [no 2-OH group] have a minimal effect on potency [1b].

Structural considerations indicated that 6-deoxy-Ins(1,3,4,5)P_4 should most likely have an inhibitory effect on Ins(1,4,5)P_3 5-phosphatase and this has been demonstrated with 6-deoxy-Ins(1,3,4,5)P_4 behaving as a relatively potent 5-phosphatase inhibitor. Through this inhibition 6-deoxy-Ins(1,3,4,5)P_4 like Ins(1,3,4,5)P_4 facilitates Ca^{2+} influx by sensitising Ins(1,4,5)P_3-mediated activation of I_{CRAC}. This action is shared by other 5-phosphatase inhibitors and therefore appears to be a general mechanism involved in regulating Ins(1,4,5)P_3-mediated Ca^{2+} signalling [14]. However, 6-deoxy-Ins(1,3,4,5)P_4 has a more varying and reduced potency with respect to the natural ligand Ins(1,3,4,5)P_4 in all biological indicators examined. This can be rationalised to some extent through consideration of the inframolecular data presented here.

It is particularly revealing here that phosphates 1, 3, and 5 of 6-deoxy-Ins(1,3,4,5)P_4 are more basic than the corresponding groups of the natural polyphosphate and that the inverse is true for the 4-phosphate. This alteration in the physicochemical status of 6-deoxy-Ins(1,3,4,5)P_4 with respect to Ins(1,3,4,5)P_4 could explain the observed differences in the biological activity of these structurally similar inositol phosphates. Thus, changes in charge distribution could account for the reduced potency of 6-deoxy-Ins(1,3,4,5)P_4—any interaction between these phosphate groups and an Ins(1,3,4,5)P_4 receptor that are key to activity will undoubtedly be affected by reduced/increased basicity of the phosphate moieties. Furthermore, it has been shown that OH-6 plays a central role in the conformational preference of the inositol ring. The OH-6 motif participates in the electrostatic effect between phosphates 1 and 5, as well as preventing inositol ring distortion. Consequently, removal of this stabilising effect could have important consequences for biological activity. Indeed, we show here that deletion of OH-6 has dramatic effects upon the relative basicity of the individual phosphate groups—a consequence that has been shown to influence the conformation of 6-deoxy-Ins(1,3,4,5)P_4. The ^{1}H NMR titration studies revealed that at relatively high pH a distorted chair conformation is formed in which the three vicinal phosphates are strongly repulsed by each other. Upon protonation of phosphate 4, as the pH is lowered, the inositol ring of 6-deoxy-Ins(1,3,4,5)P_4 returns to an undistorted conformation. This transition from distorted to undistorted conformations does not occur in Ins(1,3,4,5)P_4 where such conformational changes are hindered by the formation of a hydrogen bond between phosphate 5 and OH 6, presumably making the inositol ring more rigid. Due to the resultant ring distortion that occurs in 6-deoxy-Ins(1,3,4,5)P_4 interactions between vicinal phosphates are weakened and P1–P3, P1–P4, and P1–P5 interactions are strengthened. The biological consequences of such pH-dependent conformational changes have been implicated in the binding mechanisms and regulation processes of Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4 [34].

The protonation constants of inositol polyphosphates containing several vicinal phosphate groups are known to vary by several orders of magnitude and it is known that the conformational equilibrium of such phosphates is governed by the ionisation state of the phosphate groups concerned. This, in itself, strongly depends upon the immediate ionic environment in which the molecule in question finds itself. Consequently, the observed difference in the relative potencies of Ins(1,3,4,5)P_4 and its 6-deoxy congener could potentially be attributed to such

a phenomenon occurring in the microenvironment of the ligand–receptor interaction.

Thus, in summary, we have demonstrated that D-6-deoxy-Ins(1,3,4,5)P_4 is a moderate inhibitor of Ins(1,4,5)P_3 5-phosphatase compared to natural Ins(1,3,4,5)P_4, thus illustrating a role for the 6-hydroxyl group in the binding of Ins(1,3,4,5)P_4 to this enzyme. 6-Deoxy-Ins(1,3,4,5)P_4, however, inhibits the binding of [^{3}H]Ins(1,4,5)P_3 to its receptor only slightly less effectively than Ins(1,3,4,5)P_4. The resulting bell-shaped facilitation curve for 6-deoxy-Ins(1,3,4,5)P_4 reveals a compound of overall lower efficacy and potency and this molecule is an effective facilitator of I_{CRAC}, but less effective than Ins(1,3,4,5)P_4. The pH-dependent protonation characteristics of 6-deoxy-Ins(1,3,4,5)P_4 were determined and found to be broadly similar to those of Ins(1,3,4,5)P_4, apart from the data for P1. ^{1}H NMR titrations uncovered evidence for a novel conformational charge of the inositol ring above pH 10 for 6-deoxy-Ins(1,3,4,5)P_4, something that is normally prevented in Ins(1,3,4,5)P_4 because of the stabilising effect of the 6-OH group.

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