# Defining the minimal structural requirements for partial agonism at the type I *myo*-inositol 1,4,5-trisphosphate receptor

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**Abstract The novel synthetic analogues D-3-fluoro-*myo*-inositol 1,5-bisphosphate-4-phosphorothioate, [3F-Ins(1,5)$P_2$-4PS], D-3-fluoro-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate [3F-Ins(1,4)$P_2$-5PS], and D-3-fluoro-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate [3F-Ins(1)P-(4,5)$PS_2$] were utilised to define the structure-activity relationships which could produce partial agonism at the $Ca^{2+}$ mobilising *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$] receptor. Based on prior structure-activity data we hypothesised that the minimal structural requirements for Ins(1,4,5)$P_3$ receptor partial agonism, were phosphorothioate substitution of the crucial vicinal 4,5-bisphosphate pair accompanied by another structural perturbation, such fluorination of 3-position of the *myo*-inositol ring. All the analogues fully displaced [$^3$H]Ins(1,4,5)$P_3$ from a single Ins(1,4,5)$P_3$ binding site in pig cerebellar membranes [3F-Ins(1,5)$P_2$-4PS ($IC_{50}$ = 26 nM), 3F-Ins(1,4)$P_2$-5PS ($IC_{50}$ = 80 nM) and 3F-Ins(1)P-(4,5)$PS_2$ ($IC_{50}$ = 109 nM) cf. Ins(1,4,5)$P_3$ ($IC_{50}$ = 11 nM)]. In contrast, 3F-Ins(1,5)$P_2$-4PS ($IC_{50}$ = 424 nM) and 3F-Ins(1,4)$P_2$-5PS ($IC_{50}$ = 3579 nM) were weak full agonists at the $Ca^{2+}$ mobilising Ins(1,4,5)$P_3$ receptor of permeabilised SH-SY5Y neuroblastoma cells, being respectively 4- and 36-fold less potent than Ins(1,4,5)$P_3$ ($EC_{50}$ = 99 nM). While 3F-Ins(1)P-(4,5)$PS_2$ ($EC_{50}$ = 11345 nM) was a partial agonist releasing only 64.3 ± 1.9% of the Ins(1,4,5)$P_3$-sensitive intracellular $Ca^{2+}$ pools. 3F-Ins(1)P-(4,5)$PS_2$ was unique among the Ins(1,4,5)$P_3$ receptor partial agonists so far identified in having a relatively high affinity for the Ins(1,4,5)$P_3$ binding site, accompanied by a significant loss of intrinsic activity for $Ca^{2+}$ mobilisation. This improved affinity was probably due to the retention of the 1-position phosphate, which enhances interaction with the Ins-(1,4,5)$P_3$ receptor. 3F-Ins(1)P-(4,5)$PS_2$ may be an important lead compound for the development of efficient Ins(1,4,5)$P_3$ receptor antagonists**

*Key words:* Partial agonist; *myo*-Inositol 1,4,5-trisphosphate receptor; Neuroblastoma; Calcium

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*Abbreviations:* Ins(1,4,5)$P_3$, D-*myo*-inositol 1,4,5-trisphosphate; Ins-(1,3,4,6)$P_4$, *myo*-inositol 1,3,4,6-tetrakisphosphate; Ins(1,4,5)$PS_3$, D-*myo*-inositol 1,4,5-trisphosphorothioate; 3F-Ins(1,5)$P_2$-4PS, D-3-fluoro-*myo*-inositol 1,5-bisphosphate-4-phosphorothioate; 3F-Ins(1,4)$P_2$-5PS, D-3-fluoro-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate; 3F-Ins(1)P-(4,5)$PS_2$, D-3-fluoro-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate; Ins(1,4,5)$P_3$R, D-*myo*-inositol 1,4,5-trisphosphate receptor; CLB, cytosolic like buffer; $EC_{50}$, concentration producing 50% of maximal effect; $IC_{50}$, concentration producing 50% of maximal inhibition.

## 1. Introduction

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$] receptors are recognised as a homologous family of tetrameric ligand-gated $Ca^{2+}$ channels, which allow mobilisation of intracellular $Ca^{2+}$ stores in response to activation of cell-surface receptors linked to Ins(1,4,5)$P_3$ generation [1]. The interaction of Ins(1,4,5)$P_3$ with its receptor is highly stereospecific, the vicinal 4,5-bisphosphate and 6-hydroxyl motif being the crucial structural features, and the 1-phosphate of Ins(1,4,5)$P_3$ further contributing to receptor binding specificity (reviewed [2]). Partial or total phosphorothioate substitution of the phosphate groups appears to well tolerated, producing high affinity ligands which are resistant to metabolism catalysed by the specific enzymes Ins(1,4,5)$P_3$-3-kinase and Ins(1,4,5)$P_3$-5-phosphatase [3–5]. Whilst Ins(1,4,5)$PS_3$ has only a slightly lower affinity than Ins(1,4,5)$P_3$ at binding sites characterised with [$^3$H]Ins(1,4,5)$P_3$, inositol 1,4,5-[$^{35}$S(U)]trisphosphorothioate was able to specifically label two specific binding site populations [6]. Thus, Ins(1,4,5)$PS_3$ appears to exhibit a subtly different interaction with the binding pocket of the Ins(1,4,5)$P_3$R [Ins(1,4,5)$P_3$R].

The 2,3,6-hydroxyl groups of Ins(1,4,5)$P_3$ also contribute to the Ins(1,4,5)$P_3$R interaction by either donating or accepting hydrogen bonds with the receptor, or alternatively via fixing the solution conformation of Ins(1,4,5)$P_3$ by intramolecular hydrogen bonding to the neighbouring phosphates [7]. The 6-OH group makes a significant contribution [8,9], whereas the axial 2-hydroxyl (OH) and equatorial 3-OH per se, do not appear to be as critical for Ins(1,4,5)$P_3$R binding and $Ca^{2+}$ release [9,10]. The Ins(1,4,5)$P_3$R has a remarkable tolerance for electronic and steric modification of the 2-position of Ins(1,4,5)$P_3$ for either axial or equatorial substituents, suggesting that the 2-OH is not intimately associated with the receptor binding pocket (reviewed [11]). In contrast, inversion of the 3-OH [12,13] produces loss of activity, while increasing the steric bulk of the 3-position substituent of Ins(1,4,5)$P_3$ analogues correlates with progressively decreasing activity at the Ins(1,4,5)$P_3$R [14]. Notably some modifications of the equatorial 3-position hydroxyl of Ins(1,4,5)$P_3$ affect the intrinsic activity at the Ins(1,4,5)$P_3$R. Indeed, the first partial agonist identified at the Ins(1,4,5)$P_3$R was the naturally occurring *myo*-inositol 1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)$P_4$] [11,15]; significantly Ins(1,3,4,6)$P_4$ adopts a binding conformation which efficiently mimics Ins(1,4,5)$P_3$ with the exception of presenting an axial (pseudo) 3-phosphate [7]. Additionally, recently two low intrinsic activity partial agonists; L-*chiro*-inositol 2,3,5-trisphosphorothioate [L-*ch*-Ins(2,3,5)$PS_3$], D-


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6-deoxy-*myo*-inositol 1,4,5-trisphosphorothioate [6-deoxy-Ins(1,4,5)$PS_3$] [16] and a high intrinsic activity partial agonist; *scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate [11] have been identified. Thus, although Ins(1,4,5)$PS_3$ itself was a full agonist, replacement of the D-1,4,5-trisphosphate motif with phosphorothioates appears to assist in triggering a substantial loss of agonist efficacy, accompanied by a less significant loss of affinity.

We have designed and synthesised three analogues; D-3-fluoro-*myo*-inositol 1,5-bisphosphate-4-phosphorothioate [3F-Ins(1,5)$P_2$-4PS], D-3-fluoro-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate [3F-Ins(1,4)$P_2$-5PS] and D-3-fluoro-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate [3F-Ins(1)P-(4,5)$PS_2$] (Fig. 1), to test whether phosphorothioate substitution at the crucial vicinal 4,5-bisphosphate motif, in combination with a further moderate structural perturbation, could trigger partial agonism. Here we present data to confirm our hypothesis that 4,5-bisphosphorothioate substitution and 3-position fluorination of Ins(1,4,5)$P_3$ can indeed produce partial agonism at the Ins(1,4,5)$P_3$R.

## 2. Materials and methods

### 2.1. Materials

Most reagents used were as previously described [17]. Disodium ATP and EGTA were from Sigma (UK). All other reagents were of the highest purity available. Chemically synthesised Ins(1,4,5)$P_3$ [18] as the hexapotassium salt, was obtained from Cell Signals Inc, Lexington, Kentucky, USA. $^{45}CaCl_2$ (approx. 1000 Ci/mmol, Amersham) and [$^3$H]Ins(1,4,5)$P_3$ (41 Ci/mmol, Amersham, UK) were used throughout. D-3-Fluoro-*myo*-inositol 1,5-bisphosphate-4-phosphorothioate [3F-Ins(1,5)$P_2$-4PS], D-3-fluoro-*myo*-inositol 1,4-bisphosphate-5-phosphorothioate [3F-Ins(1,4)$P_2$-5PS], and D-3-fluoro-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate [3F-Ins(1)P-(4,5)$PS_2$] were used as the tetraethylammonium salts; their full synthesis and a preliminary report on the metabolic stability of 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$ have been published elsewhere [19].

### 2.2. Cell culture

SH-SY5Y human neuroblastoma cell monolayers (passage 70–90), initially a gift from Dr J.L. Biedler (Sloane-Kettering Institute, New York, USA) were subcultured and maintained, as described [17], except that the culture media was serum supplemented with 10% (v/v) new-born calf serum and 1% foetal calf serum (Gibco, UK). Cell monolayers were harvested for experiments using a buffer consisting of 0.02% (w/v) EDTA·$Na_2$ (537 μM), 0.9% (w/v) NaCl (154 mM) and HEPES(free acid) 10 mM, pH 7.2. These neuronally derived cells predominantly express the type 1 (cerebella) Ins(1,4,5)$P_3$R [20,21], thus allowing direct comparison with the binding preparation of cerebellar Ins(1,4,5)$P_3$Rs [22].

### 2.3. Ins(1,4,5)$P_3$ binding studies

Preparation of '$P_2$' membranes from pig cerebellum and the [$^3$H]Ins(1,4,5)$P_3$ binding and displacement assays were performed as described [23]. After incubation for 30 min at 4°C bound and free [$^3$H]Ins(1,4,5)$P_3$ were separated by high-speed centrifugation (28 000 × *g*, 3 min at 4°C) in a refrigerated microfuge (Heraeus Sepatech, Germany). Specifically bound [$^3$H]Ins(1,4,5)$P_3$ was routinely 2500 dpm/assay, while non-specific binding (approx. 150–200 dpm/assay) was defined by addition of 10 μM Ins(1,4,5)$P_3$. Cerebellar membranes were selected for binding studies, because they exhibit an exceptionally high density population of essentially homotetrameric type 1 Ins(1,4,5)$P_3$R [22].

### 2.4. $Ca^{2+}$-mobilisation assay

$^{45}Ca^{2+}$ mobilisation was assessed in saponin-permeabilized SH-SY5Y cells at 20–22°C as previously described for SH-SY5Y cells [17] with each experiment performed in duplicate. Cells were used at a final concentration of 0.5–0.6 mg/ml of protein in a cytosolic-like buffer consisting of KCl, 120 mM; $KH_2PO_4$, 2 mM; $(CH_2COONa)_2 \cdot 6H_2O$, 5 mM; $MgCl_2 \cdot 6H_2O$, 2.4 mM; $Na_2ATP$, 2 mM and HEPES free acid adjusted to pH 7.2 with 20% (w/v) KOH. In each assay ionomycin (5 μM, free acid, Calbiochem, UK) was used to define the total releasable $^{45}Ca^{2+}$ pool and Ins(1,4,5)$P_3$ (20–30 μM) to define the Ins(1,4,5)$P_3$-sensitive $^{45}Ca^{2+}$ pool.

### 2.5. Data analysis

$EC_{50}$ and $IC_{50}$ values (concentrations producing half-maximal stimulation and inhibition, respectively) and slope factors were estimated by computer-assisted curve fitting using Prism version 2.0 (GraphPad Software, USA). Combined data from the independent experiments were expressed as means ± S.E.M., where $n = 4$–8. Statistical comparison of the $\log_{10}[IC_{50}]$ and $\log_{10}[EC_{50}]$ was performed in Excel (Microsoft, UK), using unpaired Student's *t*-tests (assuming unequal variances) at the $p \leq 0.05$ level.

## 3. Results

### 3.1. Recognition by the Ins(1,4,5)$P_3$-receptor

Ins(1,4,5)$P_3$ displaced [$^3$H]Ins(1,4,5)$P_3$ from the Ins(1,4,5)$P_3$-binding sites of pig cerebellar membranes producing a monophasic profile with an $IC_{50}$ of 11 nM (Table 1, Fig. 2). 3F-Ins(1,5)$P_2$-4PS, 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$ also completely displaced specifically bound [$^3$H]Ins(1,4,5)$P_3$ from a single population of binding sites with respective $IC_{50}$ values of 25.8, 80 and 109 nM (Table 1, Fig. 2). 3F-Ins(1,5)$P_2$-4PS was only a 2.5-fold less potent ligand than Ins(1,4,5)$P_3$, and significantly more potent than

Table 1
Interaction of Ins(1,4,5)$P_3$ 3F-Ins(1,5)$P_2$-4PS, 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$ with the pig cerebellar Ins(1,4,5)$P_3$R binding sites and the $Ca^{2+}$ mobilising Ins(1,4,5)$P_3$R of SH-SY5Y neuroblastoma cells

| | (i) Ins(1,4,5)$P_3$ binding | (ii) $^{45}Ca^{2+}$ release: | Ratio $EC_{50}/IC_{50}$ |
|---|---|---|---|
| | Mean $\log(IC_{50}) \pm$ S.E.M.; ($IC_{50}$ nM) | Mean $\log(EC_{50}) \pm$ S.E.M.; ($EC_{50}$ nM) | |
| Ins(1,4,5)$P_3$ | −7.955 ± 0.046 (11) | −7.003 ± 0.019 (99) | 9 |
| 3F-Ins(1,5)$P_2$-4PS | −7.577 ± 0.019 (26) | −6.373 ± 0.075 (424) | 16 |
| 3F-Ins(1,4)$P_2$-5PS | −7.097 ± 0.006 (80) | −5.446 ± 0.041 (3579) | 44.8 |
| 3F-Ins(1)P-(4,5)$PS_2$ | −6.962 ± 0.017 (109) | −4.945 ± 0.002 (11345) | 104 |

(i) Displacement of [$^3$H]Ins(1,4,5)$P_3$ from pig cerebellar Ins(1,4,5)$P_3$ binding sites ($n \geq 3$) by Ins(1,4,5)$P_3$, 3F-Ins(1,5)$P_2$-4PS, 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$. Ins(1,4,5)$P_3$ and all the analogues completely displaced [$^3$H]Ins(1,4,5)$P_3$ from an Ins(1,4,5)$P_3$ binding site. Results are expressed as mean $\log(IC_{50}) \pm$ S.E.M.; the arithmetic means (nM) are indicated in parentheses.
(ii) The $^{45}Ca^{2+}$ mobilisation from SH-SY5Y human neuroblastoma cells ($n \geq 4$) induced by Ins(1,4,5)$P_3$, 3F-Ins(1,5)$P_2$-4PS, 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$. 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1,5)$P_2$-4PS were apparent full agonists mobilising the entire Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool. 3F-Ins(1)P-(4,5)$PS_2$ was a partial agonist with maximally effective concentrations mobilising 64.3 ± 1.9% of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, respectively. The maximal effective concentrations were used for calculation of $\log(EC_{50}) \pm$ S.E.M.; the arithmetic means (nM) are indicated in parentheses. See Section 2 for a complete description of the method.

both 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$, indicating that phosphorothioate modification of the 5-position produces significant perturbation of receptor affinity.

### 3.2. $^{45}Ca^{2+}$ release from saponin-permeabilised SH-SY5Y neuroblastoma cells

Ins(1,4,5)$P_3$ potently mobilised 72.3 ± 0.4% of the total preloaded $^{45}Ca^{2+}$ from SH-SY5Y cells, with an $EC_{50}$ value of 99 nM (Table 1; Fig. 3). 3F-Ins(1,5)$P_2$-4PS ($EC_{50}$ of 424 nM) and 3F-Ins(1,4)$P_2$-5PS ($EC_{50}$ = 3.6 μM) were both relatively weak full agonists for $Ca^{2+}$ release in SH-SY5Y cells releasing $Ca^{2+}$, exhibiting respectively 4- and 36-fold lower potency than Ins(1,4,5)$P_3$, a clear demonstration that the 5-position, compared to 4-position, phosphorothioate substitution produced a more significant perturbation of $Ca^{2+}$ mobilising efficiency. Furthermore, 3F-Ins(1)P-(4,5)$PS_2$ was an Ins(1,4,5)$P_3$R partial agonist releasing only 64.3 ± 1.9% of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, with an $EC_{50}$ of 11.4 μM. Indeed, we have also demonstrated that a maximally effective concentration of 3F-Ins(1)P-(4,5)$PS_2$ (100 μM) was able to partially antagonise $Ca^{2+}$ mobilisation by sub-optimal concentrations of Ins(1,4,5)$P_3$. This inhibition was truly competitive since it could readily br reversed in a concentration-dependent fashion by using increasing doses of Ins(1,4,5)$P_3$ to displace the 3F-Ins(1)P-(4,5)$PS_2$ from the Ins(1,4,5)$P_3$R (Fig. 4).

## 4. Discussion

The interaction of Ins(1,4,5)$P_3$ with its receptor is highly stereospecific, the presence of the vicinal 4,5-bisphosphates and the 6-hydroxyl being the crucial structural motif [2]. However, recent evidence obtained via structure-activity modelling of the very potent Ins(1,4,5)$P_3$R agonists adenophostins A and B [24] and an adenophostin analogue [25] suggests that the 1-phosphate of Ins(1,4,5)$P_3$ significantly enhances receptor binding affinity and efficacy. Phosphorothioate substitution is apparently well tolerated with *myo*-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)$PS_3$] exhibiting only slightly lower affinity than Ins(1,4,5)$P_3$ at binding sites characterised with [$^3$H]Ins(1,4,5)$P_3$. However, while phosphorothioate groups have a molecular volume only slightly greater than that of a phosphate group [14] they exhibit a distinctive chemistry. The replacement of oxygen with sulfur to form the phosphorothioate group results in considerable disturbance in charge distribution with an increased negative charge on the sulfur and reduced negative charge on the remaining oxygens [26, 27]. Indeed, Challiss et al. [6] recently demonstrated that

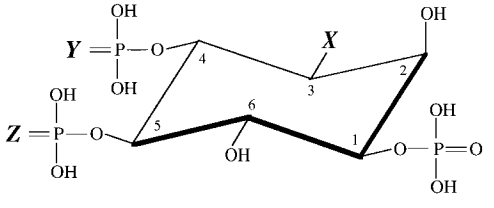


Fig. 1. The structures of Ins(1,4,5)$P_3$, 3F-Ins(1,5)$P_2$-4PS, 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$.

| | | | |
|---|---|---|---|
| Ins(1,4,5)$P_3$: | X = OH | Y = O | Z = O |
| 3F-Ins(1,5)$P_2$-4PS: | X = F | Y = S | Z = O |
| 3F-Ins(1,4)$P_2$-5PS: | X = F | Y = O | Z = S |
| 3F-Ins(1)P-(4,5)$PS_2$: | X = F | Y = S | Z = S. |

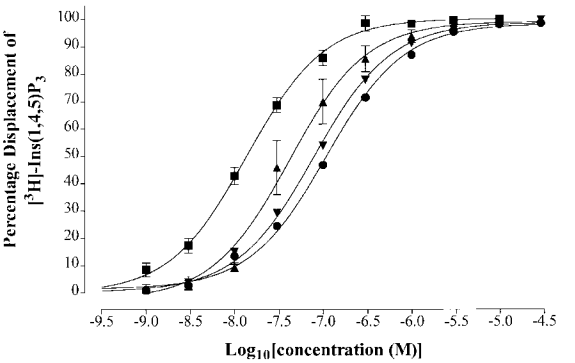


Fig. 2. Percentage displacement of specific [$^3$H]Ins(1,4,5)$P_3$ binding from pig cerebellar membrane Ins(1,4,5)$P_3$ binding sites by Ins(1,4,5)$P_3$ (■), 3F-Ins(1,5)$P_2$-4PS (▲), 3F-Ins(1,4)$P_2$-5PS (▼) and 3F-Ins(1)P-(4,5)$PS_2$ (●). Results are shown as mean ± standard error of $n$ = 4 experiments. Non-specific binding was defined using 10 μM Ins(1,4,5)$P_3$ (100% displacement); see Section 2 for further details.

Ins(1,4,5)$PS_3$ interacts with equal affinity at two sites in rat cerebellar membranes one of which is the Ins(1,4,5)$P_3$R and the other a site which displays low affinity for Ins(1,4,5)$P_3$. These changes in charge distribution appear to subtly affect interactions with the Ins(1,4,5)$P_3$R binding pocket and therefore it is significant that many of the Ins(1,4,5)$P_3$R partial agonists so far identified contain the 1,4,5-trisphosphorothioate motif [11,16]. Indeed, it is significant that the only competitive antagonists of cAMP, that have so far been identified, are derived via phosphorothioate substitution [28,29].

The naturally occurring *myo*-inositol 1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)$P_4$] was the first partial agonist identified at the Ins(1,4,5)$P_3$R [11,15]. Significantly, Ins(1,3,4,6)$P_4$ adopts a binding conformation which efficiently mimics Ins(1,4,5)$P_3$ with the exception of presenting an axial (pseudo) 3-phosphate [7]. However, L-*chiro*-inositol 2,3,5-trisphosphate [L-*ch*-Ins(2,3,5)$P_3$], which binds in a similar orientation to Ins(1,3,4,6)$P_4$, but presents an axial (pseudo) 3-position hydroxyl, was a full agonist [12], and indeed Ins(1,3,4,6)$P_4$ was an apparent full agonist in some cell types [11]. A well recognized approach for probing the interaction of an OH-group with a receptor protein involves replacement with fluorine [30]. The size and electronegativity of a fluorine substituent lies between those of a hydrogen and a hydroxy group, the C-F and C-OH bonds have similar lengths and polarization, but a fluorine substituent can only accept and not donate hydrogen bonds [31]. 3-Fluoro-*myo*-inositol 1,4,5-trisphosphate [3F-Ins(1,4,5)$P_3$] is also among the most potent agonists identified at the Ins(1,4,5)$P_3$ receptor, being only 2–3-fold less potent than Ins(1,4,5)$P_3$, while being intrinsically resistant to Ins(1,4,5)$P_3$-3-kinase activity [12,32,33].

We had previously hypothesised that phosphorothioate substitution of Ins(1,4,5)$P_3$ accompanied by an additional structural perturbation on the inositol ring might produce partial agonism at the Ins(1,4,5)$P_3$R [11]. Here we sought to test this hypothesis by rational design and synthesis of three analogues; 3F-Ins(1,5)$P_2$-4PS, 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$. Using these we hoped to define the importance of phosphorothioate substitution at the crucial vicinal 4,5-bisphosphate motif, and since many of the partial agonists so far identified possessed a structural perturbation at the 3- (or pseudo 3-) position [11,16,34] we decided to substitute the native 3-hydroxyl with a fluorine group in the hope of intro-

ducing a minimal structural perturbation, which could nevertheless induce a loss in intrinsic activity. The native 1-phosphate group was maintained since although not crucial it does appear to significantly enhance the receptor interaction [24,25].

Ins(1,4,5)$P_3$ binding studies revealed that all of the analogues were able to monophasically fully displace [$^3$H]Ins(1,4,5)$P_3$ pig cerebellar Ins(1,4,5)$P_3$R, with the following order of affinity Ins(1,4,5)$P_3$ > 3F-Ins(1,5)$P_2$-4PS ≫ 3F-Ins(1,4)$P_2$-5PS > 3F-Ins(1)P-(4,5)$PS_2$. 3F-Ins(1,4)$P_2$-5PS and 3F-Ins(1)P-(4,5)$PS_2$. Thus, interaction with the Ins(1,4,5)$P_3$R was much more significantly perturbed by 5-phosphorothioate (PS) substitution compared to 4-PS substitution. Significantly this is the first evidence that the 5-position phosphate group is the dominant partner, within the 4,5-bisphosphate moiety that is so crucial for functional interaction with the Ins(1,4,5)$P_3$R.

$Ca^{2+}$-mobilisation studies in permeabilized SH-SY5Y cells revealed a broadly similar trend with respective potencies of Ins(1,4,5)$P_3$ > 3F-Ins(1,5)$P_2$-4PS ≫ 3F-Ins(1,4)$P_2$-5PS ≫ 3F-Ins(1)P-(4,5)$PS_2$. Among the analogues 3F-Ins(1)P-(4,5)$PS_2$ with its vicinal 4,5-bisphosphorothioate substitution was the only apparent partial agonist, mobilising just 64% of the intracellular Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool. We confirmed the dual partial agonist/antagonist nature of 3F-Ins(1)P-(4,5)$PS_2$ by demonstrating that at a maximally effective concentrations it was able to partially inhibit the $Ca^{2+}$ mobilising activity of the full agonist Ins(1,4,5)$P_3$ and that this inhibition could be overcome by competition with increasing concentrations of Ins(1,4,5)$P_3$ (Fig. 4). All the previously identified Ins(1,4,5)$P_3$R partial agonists exhibited binding affinities weaker than Ins(1,4,5)$P_3$ by some 2 orders of magnitude or greater [11,16]. In contrast, the affinity 3F-Ins(1)P-(4,5)$PS_2$ for cerebellar Ins(1,4,5)$P_3$R was only 10-fold weaker than Ins(1,4,5)$P_3$ (Table 1). However, in contrast to the 1,4,5-trisphosphorothioate based partial agonists, 3F-Ins(1)P-(4,5)$PS_2$ retains the 1-position phosphate, which recent evidence suggests significantly stabilizes and enhances interaction with the

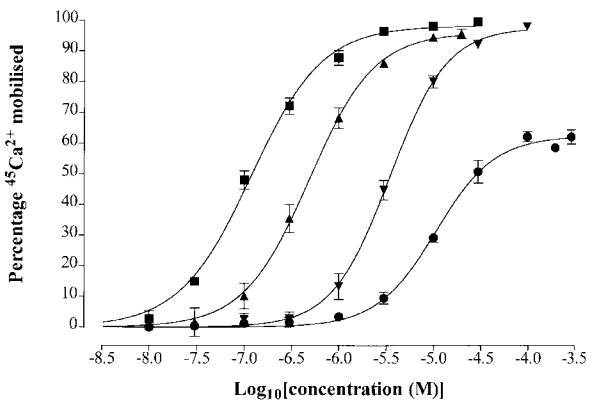


Fig. 3. Intracellular $Ca^{2+}$ mobilisation induced in permeabilized SH-SY5Y cells by Ins(1,4,5)$P_3$ (■), 3F-Ins(1,5)$P_2$-4PS (▲), 3F-Ins(1,4)$P_2$-5PS (▼) and 3F-Ins(1)P-(4,5)$PS_2$ (●). Data indicate the percentage of $^{45}Ca^{2+}$ released at 20–22°C from the intracellular Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores of saponin permeabilised SH-SY5Y neuroblastoma cells in the presence of increasing concentrations of each compound. Results are shown as mean ± standard error of $n$ = 4–8 experiments. Maximal $Ca^{2+}$ release from intracellular Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores was defined using an internal standard of 20–30 μM Ins(1,4,5)$P_3$ (100% release); see Section 2 for a complete description of the method.

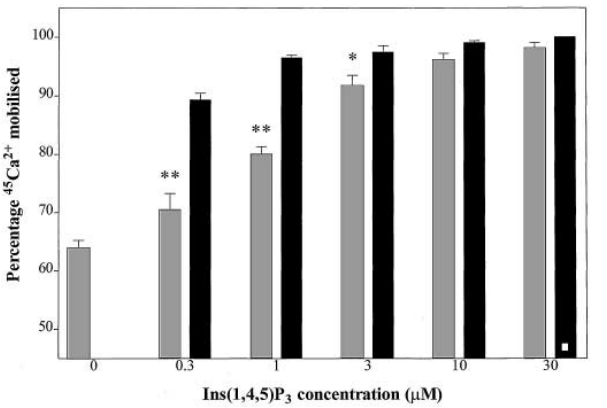


Fig. 4. Effect of increasing concentration of Ins(1,4,5)$P_3$ on maximally effective concentration of the partial agonist 3F-Ins(1)P-(4,5)$PS_2$. A maximally effective concentration of 3F-Ins(1)P-(4,5)$PS_2$ (100 μM), was found to significantly inhibit the ability of sub-maximal concentrations of Ins(1,4,5)$P_3$ (0.3, 1 and 3 μM) to mobilise $^{45}Ca^{2+}$. However, this inhibition could be readily overcome by increasing the concentration of Ins(1,4,5)$P_3$ (10 and 30 μM). Ins(1,4,5)$P_3$ (0.3 to 30 μM) plus control water vehicle are denoted by the black bars, while Ins(1,4,5)$P_3$ (0 to 30 μM) plus maximally effective concentration of 3F-Ins(1)P-(4,5)$PS_2$ (100 μM) are denoted by the grey bars. Statistical comparisons were performed with Excel (Microsoft, UK), using unpaired Student's $t$-tests (assuming unequal variances) statistical significance is indicated by * or ** which represent the $p \leq 0.05$ and $p \leq 0.01$ levels, respectively.

Ins(1,4,5)$P_3$R binding site [24,25]. As for the receptor binding studies the 5-phosphorothioate (PS) substitution produced a much more significant perturbation of $Ca^{2+}$ mobilising efficiency. However, crucially double 4,5-bisphosphothioate substitution was require to trigger partial agonism for $Ca^{2+}$ release at the Ins(1,4,5)$P_3$R.

When ratios of $Ca^{2+}$ releasing potency ($EC_{50}$) to binding affinity ($IC_{50}$) were compared for Ins(1,4,5)$P_3$ and the three analogues, the ratio exhibited a marked increase with the following rank order Ins(1,4,5)$P_3$ > 3F-Ins(1,5)$P_2$-4PS ≫ 3F-Ins(1,4)$P_2$-5PS ≫ 3F-Ins(1)P-(4,5)$PS_2$ (Table 1). In this study since both the SH-SY5Y cells and cerebellar membranes express an essentially pure type 1 Ins(1,4,5)$P_3$R population, this ratio gives an valid estimate of the ability of receptor binding site interaction to subsequently induce the conformation change required to open the type 1 Ins(1,4,5)$P_3$R channel. Clearly Ins(1,4,5)$P_3$ once bound was approx. 10-fold more efficient at producing $Ca^{2+}$ mobilisation than 3F-Ins(1)P-(4,5)$PS_2$. Therefore, although 3F-Ins(1)P-(4,5)$PS_2$ interacts relatively effectively with the ligand binding pocket of the receptor, presumably this interaction must be subtly different from that achieved by Ins(1,4,5)$P_3$, because the interaction of 3F-Ins(1)P-(4,5)$PS_2$ fails to induces full Ins(1,4,5)$P_3$R channel conductance at its maximally effective concentration. Indeed, both full agonists, 3F-Ins(1,5)$P_2$-4PS, and in particular 3F-Ins(1,4)$P_2$-5PS, exhibit progressively higher $EC_{50}/IC_{50}$ ratios compared to Ins(1,4,5)$P_3$. Therefore, perhaps they also induce sub-optimal conformational change of the Ins(1,4,5)$P_3$R, which nevertheless generates a conductance state sufficiently efficient to allow full mobilisation of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores.

Thus, the partial agonist 3F-Ins(1)P-(4,5)$PS_2$ has defined some of the crucial structural features that can lead to loss of intrinsic activity at the Ins(1,4,5)$P_3$R and therefore may be

an important lead compound for the rational design of low intrinsic activity partial agonists and Ins(1,4,5)$P_3$R antagonists.

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