myo-Inositol 1,4,6-Trisphosphorothioate and myo-Inositol 1,3,6-Trisphosphorothioate: Partial Agonists with Very Low Intrinsic Activity at the Platelet myo-Inositol 1,4,5-Trisphosphate Receptor

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ABSTRACT

Racemic mixtures and enantiomerically pure d-isomers of both myo-inositol 1,3,6-trisphosphorothioate \([\text{Ins}(1,3,6)\text{PS}_3]\) and myo-inositol 1,4,6-trisphosphorothioate \([\text{Ins}(1,4,6)\text{PS}_3]\), prepared by total synthesis, were examined in \(\text{Ca}^{2+}\) flux and binding assays. Both \(\alpha\)-Ins(1,3,6)PS\(_3\) and \(\alpha\)-Ins(1,4,6)PS\(_3\) were shown to be low intrinsic activity partial agonists at the platelet \(\alpha\)-Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) store. \(\alpha\)-Ins(1,4,6)PS\(_3\) displaced specifically bound \([3H]\)Ins(1,4,5)P\(_3\) from rat cerebellar membranes, although displacement was some 34-fold weaker than by \(\alpha\)-Ins(1,4,5)P\(_3\). \(\alpha\)-Ins(1,4,6)PS\(_3\) displaced \([3H]\)Ins(1,4,5)P\(_3\) from cerebellar membranes with roughly twice the affinity of \(\alpha\)-Ins(1,4,5)P\(_3\) \((IC_{50} \text{ value} = 1.4 \pm 0.35 \text{ mM} \text{ compared with} 2.15 \pm 0.13 \text{ mM})\), whereas \(\alpha\)-Ins(1,3,6)PS\(_3\) displaced \([3H]\)Ins(1,4,5)P\(_3\) with roughly twice the affinity of \(\alpha\)-Ins(1,3,6)PS\(_3\) \((IC_{50} \text{ value} = 17.5 \pm 5.8 \text{ mM})\), confirming that the activity of both these phosphorothioates resides in their d-enantiomers. Increasing concentrations of either \(\alpha\)-Ins(1,3,6)PS\(_3\) or \(\alpha\)-Ins(1,4,6)PS\(_3\) were able to partially antagonize Ca\(^{2+}\) release induced by submaximal concentrations of Ins(1,4,5)P\(_3\), an inhibition that could be overcome by increasing the concentration of Ins(1,4,5)P\(_3\), suggesting competition for binding at the Ins(1,4,5)P\(_3\)-R. The only low-efficacy partial agonists at the Ins(1,4,5)P\(_3\)-R discovered to date have been phosphorothioates; the novel \(\alpha\)-Ins(1,3,6)PS\(_3\) and \(\alpha\)-Ins(1,4,6)PS\(_3\) can now be added to this small group of analogs. However, \(\alpha\)-Ins(1,4,6)PS\(_3\) has a relatively high affinity for the Ins(1,4,5)P\(_3\)-R but maintains the lowest efficacy of all the partial agonists thus far identified. As such, it may be a useful tool for pharmacological intervention in the polyphosphoinositide pathway and an important lead compound for the development of further Ins(1,4,5)P\(_3\)-R antagonists.

An elevated level of cytosolic \(\text{Ca}^{2+}\) is known to be a principle mediator of activation-response coupling in numerous cell types in response to a wide range of extracellular stimuli. In non–voltage-excitable cells, \(\text{Ca}^{2+}\) is elevated via two pathways: mobilization from the intracellular stores and influx across the plasma membrane (Berridge, 1993; Putney and Bird, 1993; Clapham, 1995). Agonist-receptor coupling activates the hydrolysis of phosphatidylinositol 4,5-bisphosphate, producing the signal molecule inositol 1,4,5-trisphosphate, which, via ligation of specific receptors on Ins(1,4,5)P\(_3\)-sensitive intracellular \(\text{Ca}^{2+}\) stores, induces \(\text{Ca}^{2+}\) mobilization into the cytoplasm, (for review, see Patel et al., 1999). Three Ins(1,4,5)P\(_3\)-receptor \([\text{Ins}(1,4,5)\text{P}_3]\) subtypes, together with splice variants of each of these, have now been identified and the genes cloned (Furuichi et al., 1989; Sudhof et al., 1991; Blondel et al., 1993). The Ins(1,4,5)P\(_3\)-R is now known to exist as a heterotetrameric complex that forms the Ins(1,4,5)P\(_3\)-gated \(\text{Ca}^{2+}\) channel (Joseph et al., 1995; Monkawa et al., 1995; Wójcikiewicz and He, 1995). Expression of the Ins(1,4,5)P\(_3\)-R was found to enhance both Ins(1,4,5)P\(_3\) binding and \(\text{Ca}^{2+}\)-releasing activities in transfected cell lines, indicating expression of protein with both binding sites for Ins(1,4,5)P\(_3\) and \(\text{Ca}^{2+}\) channel activity (Miyawaki et al., 1990).

To investigate the relative importance of Ins(1,4,5)P\(_3\)-induced \(\text{Ca}^{2+}\) release in mediating the physiological processes within cells, a specific, high-affinity Ins(1,4,5)P\(_3\)-R antagonist is required. In the rational design of an Ins(1,4,5)P\(_3\)-R antagonist or a low intrinsic activity partial agonist, extensive knowledge of the structure-activity relationships of...
Ins(1,4,5)P₃ is required (for review, see Wilcox et al., 1998). At present, structure-activity studies using analogs of Ins(1,4,5)P₃ have not identified any distinct structural motifs of Ins(1,4,5)P₃ that are responsible solely for either its receptor binding capability or its Ca²⁺-releasing activity (Potter and Lampe, 1995), although the pivotal role of the vicinal 4,5-bisphosphate system augmented by other auxiliary motifs has long been recognized.

The first inositol phosphate demonstrated to be a partial agonist at the Ins(1,4,5)P₃-R was the naturally occurring D-Ins(1,4,5)PS₃. Although the partial agonist properties of this compound were confirmed (Murphy et al., 1996). Both of these active enantiomers possess one of the features found in the majority of partial agonists: a modification at either the C-3 or C-6 groups. The other characteristic feature found in common in the partial agonists is the replacement of the vicinal 4,5-bisphosphate with phosphorothioate groups, with no other perturbation of the molecular structure of Ins(1,4,5)P₃, however, had no effect on efficacy and only a small decrease in affinity at the Ins(1,4,5)P₃-R in numerous cell types (for review, see Potter and Nahorski, 1992). Most recently, D-3-fluoro-myo-inositol 1-phosphate-4,5-bisphosphorothioate [3F-Ins(1)P(4,5)PS₂] (Fig. 1a) was found to be only 10-fold less potent than Ins(1,4,5)P₃ at displacing [³H]Ins(1,4,5)P₃ from its receptor on pig cerebellum and to mobilize up to 60% of total Ca²⁺ in permeabilized SH-SY5Y cells (Wilcox et al., 1997). Therefore, all of the partial agonists thus far described at the Ins(1,4,5)P₃-R are Ins(1,4,5)P₃ analogs and, with the exception of scyllo-inositol 1,2,4,5-tetakisphosphorothioate and Ins(1,3,4,6)P₄, have combined modifications at C-3 or C-6 with phosphorothioate substitutions.

The Ca²⁺-mobilizing activity of Ins(1,3,4,6)P₄ was rationalized by envisaging two alternative receptor-binding orientations in which the 1,6-vicinal bisphosphate of Ins(1,3,4,6)P₄ mimics the normal 4,5-bisphosphate in the Ins(1,4,5)P₃ binding orientation (although this did not explain its partial agonist properties). This model predicted that two Ins(1,4,5)P₃ regiosomers (i.e., D-myo-inositol 1,4,6-trisphosphate [D-Ins(1,4,6)P₃] and D-myo-inositol 1,3,6-trisphosphate [D-Ins(1,3,6)P₃] (L-Ins(1,3,4)P₃)]) should be able to mobilize Ca²⁺ and indeed this was confirmed (Murphy et al., 1996). Both of these active enantiomers possess one of the features found in the majority of partial agonists: a modification at either the C-3 or C-6 groups. The other characteristic feature found in common in the partial agonists is the replacement of the vicinal 4,5-bisphosphate group with phosphorothioate groups. To determine whether adoption of these minimal criteria, found in common with other partial agonists, was adequate in the rational design of a partial agonist, we replaced the phosphatase groups of both Ins(1,3,6)P₃ and Ins(1,4,6)P₃ with phosphorothioates in the synthesis of Ins(1,3,6)PS₃ and Ins(1,4,6)PS₃. Preliminary data suggested that the racemic mixtures of the phosphorothioates Ins(1,4,6)PS₃ and Ins(1,3,6)PS₃, and Ins(1,4,6)PS₃, Preliminary data suggested that the racemic mixtures of the phosphorothioates Ins(1,4,6)PS₃ and Ins(1,3,6)PS₃ (Fig. 1a) were partial agonists at the Ins(1,4,5)P₃-R in permeabilized rabbit platelets (Al-Hafidh et al., 1994; Mills et al., 1995). Using the same rationalization for the Ca²⁺-mobilizing activity of the partial agonist Ins(1,3,4,6)P₄, we predicted that the two chiral phosphorothioate analogs, D-Ins(1,3,6)PS₃ and D-Ins(1,4,6)PS₃, were responsible for the observed partial agonist properties of their racemic mixtures. In this study, we demonstrate clearly that both of these phosphorothioate analogs are low-intrinsic-activity partial agonists at the Ins(1,4,5)P₃-R and that one of them [D-Ins(1,4,6)PS₃] possesses particularly promising potency coupled with very low intrinsic activity.

**Experimental Procedures**

Chemically synthesized Ins(1,4,5)P₃ was purchased from the Rhode Island Chemical Group (Kingston, RI). [³H]Ins(1,4,5)P₃ (20–60 Ci/mmol, 10 μCi/ml) and ⁴⁰Ca²⁺ (5–50 mCi/mg Ca²⁺, 2 mCi/ml) were purchased from Amersham International (Buckingham-

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1 Alternative nomenclature for D-Ins(1,3,6)P₃ is L-Ins(1,3,4)P₃.
Methods

Preparation of Platelets. Washed rabbit platelets were prepared as described previously (Murphy et al., 1991). The resulting platelet pellet from this preparation was resuspended in HEPES-buffered Tyrode’s solution (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.5 mM Na₂HPO₄, 5.5 mM glucose and 0.25% BSA, pH 7.4) before performing the following procedures.

45Ca²⁺ Release from Intracellular Stores. Platelets were washed in high-K⁺ buffer A [120 mM KCl, 2 mM KH₂PO₄, 5 mM (CH₃COONa)₂, 6 mM MgCl₂, 20 mM HEPES, in MilliQ water; 5 mM ATP was added, pH adjusted to 6.9 and free Ca²⁺ concentration adjusted below 150 nM] and then suspended to 3 × 10⁹/ml. The platelets were then permeabilized with 40 μg/ml saponin A, which was removed by further washing in buffer A. The intracellular Ca²⁺ stores were loaded with 45Ca²⁺ (2 μCi/ml) for 1 h in the presence of 10 μg/ml oligomycin. Total release of 45Ca²⁺ from the stores was determined by a 3-min incubation with 75 μM ionomycin. Release of 45Ca²⁺ from the intracellular stores at 4°C was determined 3 min after the addition of the inositol phosphate by separation of free and retained 45Ca²⁺ by filtration of cells using Whatman FP100 filters (Murphy and Westwick, 1994).

Displacement of [³H]Ins(1,4,5)P₃ Binding to Specific Ins(1,4,5)P₃ Receptors on Rat Cerebellar Membranes. The preparation of rat cerebellar membranes and displacement of [³H]Ins(1,4,5)P₃ bound to the Ins(1,4,5)P₃ receptors on the membranes was performed as described previously (Challis et al., 1991). Briefly, cerebella were removed from 6 rats (200–250 g) and homogenized (2 × 10 s, 4°C) in buffer C (20 mM Tris·HCl, 20 mM NaCl, 100 mM KCl, 1 mM EDTA, 1 mg/ml BSA, pH 7.7) containing the protease inhibitors 10 μM leupeptin and 10 μM pepstatin after centrifugation (50,000 × g for 10 min). The supernatant was removed by further washing in buffer A. The intracellular Ca²⁺ and Westwick, 1994).

Results

Ca²⁺ Release from Permeabilized Platelets. Rabbit platelets permeabilized with saponin and in the presence of oligomycin displayed ATP-dependent 45Ca²⁺ uptake into their nonmitochondrial stores. Uptake reached a steady state by 45 min and was monitored throughout the time course of the experiment and found to remain essentially unchanged. The ionomycin releasable component of accumulated 45Ca²⁺ was found to be >92%; again, this was not found to change significantly throughout the time course of any of the 45Ca²⁺ release experiments undertaken.

Treatment of permeabilized platelets with d-Ins(1,4,5)P₃ (0.01–30 μM) for 3 min (4°C) caused a dose-dependent release of 45Ca²⁺ from preloaded intracellular stores (Fig. 2). dl-Ins(1,3,6)PS₃ (1–3000 μM) alone caused a dose-dependent release of 45Ca²⁺ from the stores of permeabilized platelets. Maximal release, however, was only around 20% of the Ins(1,4,5)P₃-sensitive Ca²⁺ pool, even at concentrations above 1 mM (some of which may have been caused by nonspecific release), demonstrating a very low efficacy for d-Ins(1,3,6)PS₃ at the Ins(1,4,5)P₃-R of rabbit platelets (Fig. 2a). Treatment of permeabilized platelets with 1 μM Ins(1,4,5)P₃, together with increasing concentrations of dl-Ins(1,3,6)PS₃, caused an inhibition of Ins(1,4,5)P₃-induced Ca²⁺ release (Fig. 2a). Ca²⁺ release induced by Ins(1,4,5)P₃ was reduced as the concentration of dl-Ins(1,3,6)PS₃ increased, until release approached a level near the intrinsic efficacy of Ins(1,3,6)PS₃ itself. The concentration of dl-Ins(1,3,6)PS₃ required to inhibit release of Ca²⁺ induced with 1 μM Ins(1,4,5)P₃ by 50% (IC₅₀) was >100 μM. However, increasing the concentration of dl-Ins(1,3,6)PS₃ to 5 mg/ml. The cerebellar membranes were either used immediately or frozen (−80°C) until use. The binding assay mixture in a total volume of 250 μl contained 1 nM [³H]Ins(1,4,5)P₃, and synthetic ligand dilute in buffer C at appropriate concentrations. Binding was initiated by the addition of 250 μg of the cerebellar membrane preparation. The assay tubes were incubated (4°C) for 10 min before termination of the reaction by centrifugation (10,000 g, 4 min, 4°C). Nonspecific binding of [³H]Ins(1,4,5)P₃ was assessed as the counts remaining upon inclusion of 10 μM cold Ins(1,4,5)P₃ in the assay mixture. After centrifugation, the supernatant was carefully removed, the pellet resuspended, and radioactivity bound to the cerebellar membrane was determined by liquid scintillation counting.
more than 100 μM caused no further inhibition of Ca^{2+}-release, suggesting that maximal inhibition of Ca^{2+}-release induced with 1 μM Ins(1,4,5)P₃ is reached by 100 μM (Fig. 2a).

DL-Ins(1,4,6)PS₃ (0.03–300 μM) was also found to have a very low efficacy at the Ins(1,4,5)P₃-R, releasing only ~15% of the Ins(1,4,5)P₃-sensitive Ca²⁺ store at the highest concentration used (300 μM) (Fig. 2b). However, Ca²⁺ release induced by 1 μM Ins(1,4,5)P₃ could be inhibited with increasing concentrations of DL-Ins(1,4,6)PS₃ (Fig. 2b). As described previously for DL-Ins(1,3,6)PS₃, Ca²⁺ release induced by Ins(1,4,5)P₃ was further inhibited as the concentration of DL-Ins(1,4,6)PS₃ increased, until release declined to a level near the intrinsic efficacy of DL-Ins(1,4,6)PS₃ itself. The concentration of DL-Ins(1,4,6)PS₃ required to inhibit release of Ca²⁺ induced with 1 μM Ins(1,4,5)P₃ by 50% (IC₅₀) was found to be 56 ± 3.6 μM. Maximal inhibition of Ca²⁺-release induced by 1 μM Ins(1,4,5)P₃ seems to have been achieved with 100 μM DL-Ins(1,4,6)PS₃ as an increased Ca²⁺ release was observed with 300 μM (Fig. 2b).

**Displacement of Specific [³H]Ins(1,4,5)P₃ Binding to Rat Cerebellar Membranes.** [³H]Ins(1,4,5)P₃ was readily displaced from specific binding sites on rat cerebellar membranes by cold D-Ins(1,4,5)P₃ with an IC₅₀ of 0.043 ± 0.01 μM (Fig. 3). The ability of DL- and D-Ins(1,3,6)PS₃ and of DL- and D-Ins(1,4,6)PS₃ to displace [³H]Ins(1,4,5)P₃ from rat cerebellar membranes was also examined (Fig. 3). D-Ins(1,3,6)PS₃ displaced specifically bound [³H]Ins(1,4,5)P₃ from rat cerebellar membranes, although displacement by D-Ins(1,3,6)PS₃ was 500 fold weaker than by D-Ins(1,4,5)P₃ (Fig. 3a). However, comparing the IC₅₀ value for D-Ins(1,3,6)PS₃ (17.5 ± 5.8 μM) with that of the racemic mixture DL-Ins(1,3,6)PS₃ (34 ± 10 μM), demonstrated that D-Ins(1,3,6)PS₃ was able to displace ([³H]Ins(1,4,5)P₃ with roughly twice the affinity of the racemic mixture (IC₅₀ value of 1.4 ± 0.35 μM compared with an IC₅₀ value of 2.15 ± 0.13 μM for the racemic mixture), indicating that the activity resides in the D-enantiomer (Fig. 3b).

Therefore, the ability of D-Ins(1,3,6)PS₃ and D-Ins(1,4,6)PS₃ to displace [³H]Ins(1,4,5)P₃ from specific binding sites on rat cerebellar membranes contrasted with their relative inability to release ⁴²Ca²⁺ from the intracellular stores of permeabilized platelets.

**Effect of Trisphosphorothioates on Ins(1,4,5)P₃-Induced Ca²⁺ Release.** From the binding studies, it seems that D-Ins(1,3,6)PS₃ is the active component of the racemic mixture DL-Ins(1,3,6)PS₃, whereas D-Ins(1,4,6)PS₃ is the active component of the racemic mixture of DL-Ins(1,4,6)PS₃. As for the racemic mixtures, both D-Ins(1,3,6)PS₃ and D-Ins(1,4,6)PS₃ were also found to have a very low efficacy at the Ins(1,4,5)P₃-receptor of platelets, releasing only a small percentage (<15%) of the Ins(1,4,5)P₃-sensitive Ca²⁺ store even at 300 μM (Fig. 4a). It is possible that the release of Ca²⁺ by high concentrations of both D-Ins(1,3,6)PS₃ and D-Ins(1,4,6)PS₃ may be non-specific.

Increasing concentrations of D-Ins(1,3,6)PS₃ (30, 100, and 300 μM) were able to partially antagonize Ca²⁺ release induced by submaximal concentrations of Ins(1,4,5)P₃ (0.1–3 μM); however, by increasing the concentration of Ins(1,4,5)P₃ (10 and 30 μM), this inhibition was no longer observed, suggesting competition for binding at the Ins(1,4,5)P₃-R (Fig. 4b). The IC₅₀ value for the inhibition of Ca²⁺ elevation induced by 1 μM Ins(1,4,5)P₃ was >100 μM for D-Ins(1,3,6)PS₃. Competitive partial antagonism of Ins(1,4,5)P₃-induced Ca²⁺ release was also observed with D-Ins(1,4,6)PS₃ (10, 30, and 100 μM) (Fig. 4c). The IC₅₀ value of D-Ins(1,4,6)PS₃ for Ca²⁺ release induced by 1 μM Ins(1,4,5)P₃ was 27 ± 8.1 μM, approximately half that required of the racemic mixture (56 ± 3.6 μM).

**Discussion**

Structure-activity studies performed to date using Ins(1,4,5)P₃ analogs have concluded that the vicinal 4,5-bisphosphate configuration plays the key role in receptor recognition and mediation of Ca²⁺ release from intracellular stores (for reviews, see Potter and Nahorski, 1992; Potter and Lampe, 1995; Wilcox et al., 1998). Other structural require-

![Fig. 3. Displacement of specific [³H]Ins(1,4,5)P₃ binding from Ins(1,4,5)P₃ receptors on rat cerebellar membranes. Percentage displacement of specific [³H]Ins(1,4,5)P₃ binding to rat cerebellar membranes by (a) Ins(1,4,5)P₃ and DL- and (b) Ins(1,3,6)PS₃ and DL- and (b) Ins(1,4,6)PS₃. The values shown are the mean ± S.E. of three to four experiments, each performed in duplicate. (a) ■, Ins(1,4,5)P₃; ▲, DL-Ins(1,3,6)PS₃; ●, D-Ins(1,3,6)PS₃; (b) ■, Ins(1,4,5)P₃; ●, DL-Ins(1,4,6)PS₃; ▲, D-Ins(1,4,6)PS₃; ●, D-Ins(1,4,6)PS₃.
ments for Ca\textsuperscript{2+} release include an additional phosphate group at the 1-position (but it can be tolerated at the 2-position), which increases affinity at the Ins(1,4,5)P\textsubscript{3}-R (Potter and Lampe, 1995). The importance of the hydroxyl groups of Ins(1,4,5)P\textsubscript{3} is well characterized, with modification of the three hydroxyl groups at either the 2-, 3- or 6-position of Ins(1,4,5)P\textsubscript{3} varying the impact on Ca\textsuperscript{2+} release and the binding of Ins(1,4,5)P\textsubscript{3} to its receptor (for review, see Potter and Lampe, 1995).

At present very few partial agonists at the Ins(1,4,5)P\textsubscript{3}-R have been reported; these include Ins(1,3,4,6)P\textsubscript{4} (Gawler et al., 1991), \textit{L-} and \textit{D-}ins(1,3,6)PS\textsubscript{3}, and 6-deoxy-Ins(1,4,5)P\textsubscript{3} (Safirany et al., 1993). \textit{scylo}-inositol 1,2,4,5-tetraakisphosphorothioate (Wilcox et al., 1994), and 3F-Ins(1)P-(4,5)PS\textsubscript{2} (Wilcox et al., 1997). By using rapid kinetic measurements of \textit{45}Ca\textsuperscript{2+} mobilization, Ins(1,4,5)P\textsubscript{3} has also been demonstrated to be a partial agonist at hepatic Ins(1,4,5)P\textsubscript{3}-Rs (Marchant et al., 1997). Because of the quantal mechanism of Ca\textsuperscript{2+} release, whereby even partial agonists may completely empty the Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores, albeit at slower rates than Ins(1,4,5)P\textsubscript{3}, it is possible that the partial agonist properties of other inositol phosphates may be distinguished under high temporal resolution (Menza and Michelangeli, 1998). D-3-Amino-3-deoxy-Ins(1,4,5)P\textsubscript{3} has also been described as a partial agonist in SH-SY5Y neuroblastoma cells, but increasing pH from 6.8 to 7.2 negates the partial agonist properties (Kozikowski et al., 1994).

In a previous study (Murphy et al., 1996), we rationalized how Ins(1,3,4,6)P\textsubscript{4} elicits Ca\textsuperscript{2+} release by envisaging two alternative receptor binding orientations, where the 1,6-vicinal diphosphate is presumed to mimic the normal 4,5-bisphosphate of Ins(1,4,5)P\textsubscript{3}. As either the 4-phosphate or the 3-phosphate of Ins(1,3,4,6)P\textsubscript{4} could mimic the 1-phosphate of Ins(1,4,5)P\textsubscript{3}, it is likely that Ins(1,3,4,6)P\textsubscript{4} evokes Ca\textsuperscript{2+} release by a similar binding mechanism to Ins(1,4,5)P\textsubscript{3}. We went on to show that two related trisphosphates [D-Ins(1,3,6)PS\textsubscript{3} and D-Ins(1,3,6)PS\textsubscript{3}] were also able to displace \textit{[3H]}Ins(1,4,5)P\textsubscript{3} from the Ins(1,4,5)P\textsubscript{3}-R and to possess Ca\textsuperscript{2+} mobilization ability, whereas their enantiomers were inactive (Murphy et al., 1996).

Noting that Ins(1,4,6)P\textsubscript{3} and Ins(1,3,6)P\textsubscript{3} possessed one of the features common to the known partial agonist, namely modification at some of the positions corresponding to C-2, C-3, or C-6 of Ins(1,4,5)P\textsubscript{3}, we went on to replace their phosphate groups with phosphorothioates, giving Ins(1,4,6)PS\textsubscript{3} and Ins(1,3,6)PS\textsubscript{3}. From the preceding structure-activity arguments, we predicted that D-Ins(1,4,6)PS\textsubscript{3} and D-Ins(1,3,6)PS\textsubscript{3} would show partial agonist properties, whereas their enantiomers would be inactive. Both the racemic trisphosphorothioates DL-Ins(1,3,6)PS\textsubscript{3} and DL-Ins(1,4,6)PS\textsubscript{3} were found to have very low efficacy at the Ins(1,4,5)P\textsubscript{3}-R of rabbit platelets. Taken in isolation, this result does not show that either of these compounds is a partial agonist; an extremely-low-potency full agonist could give similar results. However, when platelets were treated with 1 \textmu M Ins(1,4,5)P\textsubscript{3}, together with increasing concentrations of either DL-Ins(1,3,6)PS\textsubscript{3} or DL-Ins(1,4,6)PS\textsubscript{3}, a definite inhibition of Ins(1,4,5)P\textsubscript{3}-stimulated Ca\textsuperscript{2+} release was observed, demonstrating that both DL-Ins(1,3,6)PS\textsubscript{3} and DL-Ins(1,4,6)PS\textsubscript{3} were acting as true partial agonists.

It was assumed that the partial agonist activity of racemic DL-Ins(1,4,6)PS\textsubscript{3} and DL-Ins(1,3,6)PS\textsubscript{3} resided in the D-enantiomers. These isomers have in common the possession of a “pseudo” vicinal d-4,5-bisphosphate motif of the same absolute stereochemistry as that found in Ins(1,4,5)P\textsubscript{3}. On the other hand, neither the L-enantiomer of Ins(1,4,6)PS\textsubscript{3} nor the L-enantiomer of Ins(1,3,6)PS\textsubscript{3} possesses this motif; rather, they are similar to L-Ins(1,4,5)P\textsubscript{3}. To examine this theory, the ability of both DL-Ins(1,4,6)PS\textsubscript{3} and DL-Ins(1,3,6)PS\textsubscript{3} to displace \textit{[3H]}Ins(1,4,5)P\textsubscript{3} from its binding site on rat cerebellar membranes was compared with their respective racemic mixtures. Both the D-isomer of Ins(1,4,6)PS\textsubscript{3} and the D-isomer of Ins(1,3,6)PS\textsubscript{3} were found to have roughly twice the affinity.

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**Fig. 4.** Effect of increasing concentrations of D-Ins(1,3,6)PS\textsubscript{3} and of D-Ins(1,4,6)PS\textsubscript{3} on release of Ca\textsuperscript{2+} induced by both submaximal and maximal concentrations of Ins(1,4,5)P\textsubscript{3}. (a) Permeabilized platelets, preloaded with \textit{45}Ca\textsuperscript{2+}, were treated with either Ins(1,4,5)P\textsubscript{3} ( ), or D-Ins(1,4,6)PS\textsubscript{3} alone ( ). (b) Platelets were treated with Ins(1,4,5)P\textsubscript{3} either alone ( ) or in the presence of increasing concentrations of D-Ins(1,3,6)PS\textsubscript{3}, 30 \textmu M ( ), 100 \textmu M ( ), or 300 \textmu M ( ). (c) Platelets were treated with Ins(1,4,5)P\textsubscript{3} either alone ( ) or in the presence of increasing concentrations of D-Ins(1,4,6)PS\textsubscript{3}, 10 \textmu M ( ), 30 \textmu M ( ), or 100 \textmu M ( ). Release of \textit{45}Ca\textsuperscript{2+} was terminated by rapid filtration and is given as a percentage of maximal \textit{45}Ca\textsuperscript{2+} releasable upon treatment of platelets with 75 \textmu M ionomycin. The values are the mean ± S.E. of three to four separate experiments, each performed in triplicate.
for the Ins(1,4,5)P₃-R of their racemic mixtures. This confirms that the activity of the racemic mixtures resides with the enantiomers possessing a vicinal bisphosphate of the correct absolute stereochemistry.

Compared with d-Ins(1,3,6)PS₃, d-Ins(1,4,6)PS₃ showed a higher affinity for the Ins(1,4,5)P₃-R in binding studies. In d-Ins(1,4,6)PS₃, the orientation of the 5-OH [which mimics the 6-ΟH of Ins(1,4,5)P₃] is equatorial [as in d-Ins(1,4,5)P₃], whereas the OH-group corresponding to the 3-ΟH is axial rather than equatorial (Fig. 1a). The 2-ΟH [which mimics the 6-ΟH of Ins(1,4,5)P₃] is reoriented to axial in d-Ins(1,3,6)PS₃ and is therefore different from that in d-Ins(1,4,5)P₃, whereas the OH group corresponding to the 3-ΟH of Ins(1,4,5)P₃ remains equatorial. From structure-activity studies, the 3-ΟH group of Ins(1,4,5)P₃ seems to have only a minor role in receptor recognition (Hirata et al., 1989; Seewald et al., 1990); thus, reorientation of the OH-group on the “pseudo” 3-position (actually the 2-position) of the inositol ring [as in Ins(1,4,6)PS₃] might not be expected to have a significant effect on Ins(1,4,5)P₃ binding. However, modification at the 6-ΟH group [as in Ins(1,3,6)PS₃] would be expected to reduce binding and activity (Polokoff et al., 1988; Safrany et al., 1991). The finding that d-Ins(1,4,6)PS₃ is more potent than d-Ins(1,3,6)PS₃ at displacing [3H]Ins(1,4,5)P₃ conforms with these structural requirements and confirms the conclusion that the 6-ΟH group of Ins(1,4,5)P₃ is more important for binding than the 3-ΟH group (Hirata et al., 1993; Murphy et al., 1996).

Increasing concentrations of either d-Ins(1,3,6)PS₃ or d-Ins(1,4,6)PS₃ were able to partially antagonize Ca²⁺ release induced by submaximal concentrations of Ins(1,4,5)P₃. However, by increasing the concentration of Ins(1,4,5)P₃, this inhibition was no longer observed, suggesting competition for binding at the Ins(1,4,5)P₃-R (Fig. 4b). The IC₅₀ value for the inhibition of Ca²⁺ elevation induced by 1 μM Ins(1,4,5)P₃ was >100 μM for both racemic and d-Ins(1,3,6)PS₃ and was 27 ± 8.1 μM for d-Ins(1,4,6)PS₃, approximately half that of the racemic mixture.

The only two low-intrinsic-activity partial agonists described previously are l-chr-Ins(2,3,5)PS₃ and 6-deoxy-Ins(1,4,5)P₃, which were found to release 34 and 42% of Ca²⁺ respectively in SH-SYSY cells (Safrany et al., 1993). It is interesting to note that the only structural difference between d-Ins(1,4,6)PS₃ and l-chr-Ins(2,3,5)PS₃ is that the hydroxyl group that mimics the 2-ΟH of Ins(1,4,5)P₃ is reoriented from axial to equatorial in Ins(1,4,6)PS₃ relative to l-chr-Ins(2,3,5)PS₃ (see Fig. 1b). In structure-activity studies, the 2-ΟH group has been shown to have the least importance in receptor recognition (Hirata et al., 1989; Wilcox et al., 1994), yet this reorientation seems to contribute both to lower efficacy of d-Ins(1,4,6)PS₃ and an increase in its affinity for the receptor [l-chr-Ins(2,3,5)PS₃ was found to have some 100-fold lower affinity for the Ins(1,4,5)P₃-R in bovine adrenal cortical membranes (Safrany et al., 1993), whereas the affinity of Ins(1,4,6)PS₃ was only 34-fold lower than Ins(1,4,5)P₃ in rat cerebellar membranes]. There are two differences between 6-deoxy-Ins(1,4,5)P₃ and d-Ins(1,3,6)PS₃: first, the hydroxyl group is deleted in 6-deoxy-Ins(1,4,5)P₃ whereas the “pseudo” 6-ΟH is axial in Ins(1,3,6)PS₃; second, the 2-ΟH group is axial in 6-deoxy-Ins(1,4,5)P₃ [as in Ins(1,4,5)P₃], whereas the “pseudo” 2-ΟH is equatorial in Ins(1,3,6)PS₃. These differences cause a 2-fold increase in the affinity for the receptor and reduce the efficacy from 42% to less than 20% (Safrany et al., 1993).

Wilcox et al. (1997) investigated the three compounds d-3-fluoro-3-deoxy-myo-inositol 1,5-bisphosphate-4-phosphorothioate, d-3-fluoro-3-deoxy-myo-inositol 1,4-bisphosphate-5-phosphorothioate, and 3F-Ins(1)P-(4,5)PS₂ for partial agonist activity (Wilcox et al., 1997). Similarly to d-Ins(1,4,6)PS₃, these compounds possessed a structural perturbation at the hydroxyl group that mimics the 3-ΟH of Ins(1,4,5)P₃. This was achieved by the replacement of the native 3-ΟH with a fluorine group. Again, like d-Ins(1,4,6)PS₃, these compounds had phosphorothioate substitutions, although only one, 3F-Ins(1)P-(4,5)PS₂, had phosphorothioate substitutions at both members of the crucial vicinal 4,5-bisphosphate motif. Of these compounds, 3F-Ins(1P)-(4,5)PS₂ was the only one identified as a partial agonist, able to inhibit Ca²⁺ mobilization induced by submaximal concentrations of Ins(1,4,5)P₃ (Wilcox et al., 1997). It was also demonstrated to be an antagonist of receptor-mediated Ca²⁺ signaling (Davis et al., 1998). Compared with 3F-Ins(1P)-(4,5)PS₂, d-Ins(1,4,6)PS₃ had a lower affinity for the Ins(1,4,5)P₃-R [it is ~34-fold weaker than Ins(1,4,5)P₃ at displacing [3H]Ins(1,4,5)P₃], whereas 3F-Ins(1)P-(4,5)PS₂ was only 10-fold weaker (Wilcox et al., 1997). Of all the phosphorothioate-containing partial agonists, 3-F-Ins(1P)-(4,5)PS₂ is the only one that has not substituted the “pseudo”-1-phosphate with a phosphorothioate; this may account for its increased affinity above the other partial agonists described so far. However, compared with d-Ins(1,4,6)PS₃, 3F-Ins(1P)-(4,5)PS₂ has a relatively high efficacy, causing over 60% of Ca²⁺ to be released. Therefore, although 3F-Ins(1P)-(4,5)PS₂ has a high affinity, its potential as a partial antagonist and a lead compound is reduced by its lower efficacy. It is possible, therefore, that the lower affinity of d-Ins(1,4,6)PS₃ at the Ins(1,4,5)P₃-R compared with 3F-Ins(1P)-(4,5)PS₂ is because it has three phosphorothioates, one of which is at the 1-position. Thus a version of d-Ins(1,4,6)PS₃ with a vicinal bisphosphoanote and a pseudo-1-phosphate [namely d-Ins(1P)-(4,5)PS₂] may combine the high affinity of 3F-Ins(1P)-(4,5)PS₂ and the low efficacy of d-Ins(1,4,6)PS₃.

The only low-efficacy partial agonists at the Ins(1,4,5)P₃-R discovered to date have been phosphorothioates; d-Ins(1,3,6)PS₃ and d-Ins(1,4,6)PS₃ now expand this small group of such analogs. However, d-Ins(1,4,6)PS₃ in particular has a relatively high affinity for the Ins(1,4,5)P₃ receptor and yet maintains very low efficacy. Thus, d-Ins(1,4,6)PS₃ may be a useful tool for pharmacological intervention in the polyphosphoinositide pathway and an important lead compound for the development of Ins(1,4,5)P₃ receptor antagonists. Indeed, it has recently been successfully employed via microinjection to inhibit Ins(1,4,5)P₃-induced Ca²⁺ mobilization in intact Jurkat T-lymphocytes (Guse et al., 1997, 1999).

References
myo-Inositol 1,4,5-Trisphosphate Receptor Partial Agonists


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