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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

CHRISTINE T. MURPHY, ANDREW M. RILEY, STEVE J. MILLS, CATHERINE J. LINDLEY, BARRY V. L. POTTER, and JOHN WESTWICK

Department of Pharmacy and Pharmacology, University of Bath, Bath, UK

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ABSTRACT

Racemic mixtures and enantiomerically pure D-isomers of both *myo*-inositol 1,3,6-trisphosphorothioate [Ins(1,3,6)PS_3] and *myo*-inositol 1,4,6-trisphosphorothioate [Ins(1,4,6)PS_3], prepared by total synthesis, were examined in Ca^{2+} flux and binding assays. Both D-Ins(1,3,6)PS_3 and D-Ins(1,4,6)PS_3 were shown to be low intrinsic activity partial agonists at the platelet *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] receptor, releasing less than 20% of the Ins(1,4,5)P_3-sensitive Ca^{2+} store. D-Ins(1,4,6)PS_3 displaced specifically bound [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes, although displacement was some 34-fold weaker than by D-Ins(1,4,5)P_3. D-Ins(1,4,6)PS_3 displaced [^{3}H]Ins(1,4,5)P_3 from cerebellar membranes with roughly twice the affinity of DL-Ins(1,4,6)PS_3 (IC_{50} value = 1.4 ± 0.35 μM compared with 2.15 ± 0.13 μM), whereas D-Ins(1,3,6)PS_3 displaced [^{3}H]Ins(1,4,5)P_3 with roughly twice the affinity of DL-Ins(1,3,6)PS_3 (IC_{50} value = 17.5 ± 5.8 μM compared with 34 ± 10 μM), confirming that the activity of both these phosphorothioates resides in their D-enantiomers. Increasing concentrations of either D-Ins(1,3,6)PS_3 or D-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 D-Ins(1,3,6)PS_3 and D-Ins(1,4,6)PS_3 can now be added to this small group of analogs. However, D-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 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, 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 [Ins(1,4,5)P_3], which, via ligation of specific receptors on Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores, induces Ca^{2+} mobilization into the cytoplasm, (for review, see Patel et al., 1999). Three Ins(1,4,5)P_3-receptor [Ins(1,4,5)P_3-R] 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 Ca^{2+} channel (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz 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 Ca^{2+}-releasing activities in transfected cell lines, indicating expression of protein with both binding sites for Ins(1,4,5)P_3 and Ca^{2+} channel activity (Miyawaki et al., 1990).

To investigate the relative importance of Ins(1,4,5)P_3-induced 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

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ABBREVIATIONS: Ins(1,4,5)P_3, *myo*-inositol 1,4,5-trisphosphate; Ins(1,3,6)PS_3, *myo*-inositol 1,3,6-trisphosphorothioate; Ins(1,4,6)PS_3, *myo*-inositol 1,4,6-trisphosphorothioate; Ins(1,4,6)P_3, *myo*-inositol 1,4,6-trisphosphate; Ins(1,3,6)P_3, *myo*-inositol 1,3,6-trisphosphate; Ins(1,4,5)P_3-R, *myo*-Ins(1,4,5)P_3-receptor; Ins(1,3,4,6)P_4, *myo*-inositol 1,3,4,6-tetrakisphosphate; L-*chr*-Ins(2,3,5)PS_3, L-*chiro*-inositol 2,3,5-trisphosphorothioate; 3F-Ins(1)P-(4,5)PS_2, D-3-fluoro-3-deoxy-*myo*-inositol 1-phosphate-4,5-bisphosphorothioate.

$Ins(1,4,5)P_3$ is required (for review, see Wilcox et al., 1998). At present, structure-activity studies using analogs of $Ins(1,4,5)P_3$ have not identified any distinct structural motifs of $Ins(1,4,5)P_3$ that are responsible solely for either its receptor binding capability or its Ca^{2+}-releasing activity (Potter and Lampe, 1995), although the pivotal role of the vicinal 4,5-trisphosphate system augmented by other auxiliary motifs has long been recognized.

The first inositol phosphate demonstrated to be a partial agonist at the $Ins(1,4,5)P_3$-R was the naturally occurring higher polyphosphate *myo*-inositol 1,3,4,6-tetrakisphosphate [$Ins(1,3,4,6)P_4$] in SH-SY5Y cells (Gawler et al., 1991), although this compound was a full agonist in rabbit platelets (Murphy et al., 1996). L-*chiro*-Inositol 2,3,5-trisphosphorothioate [L-*chr*-$Ins(2,3,5)PS_3$] and D-6-deoxy-*myo*-inositol 1,4,5-trisphosphorothioate [6-deoxy-$Ins(1,4,5)PS_3$] (Fig. 1a) were found to be low-efficacy partial agonists at the $Ins(1,4,5)P_3$-R (Safrany et al., 1993; Liu et al., 1994). L-*chr*-$Ins(2,3,5)PS_3$ and 6-deoxy-$Ins(1,4,5)PS_3$ are the C-3- and C-6-modified analogs of $Ins(1,4,5)P_3$, respectively, in addition to carrying phosphorothioate groups rather than phosphates at the 1-, 4- and 5-positions. A high intrinsic activity partial agonist *scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate also combined phosphorothioate substitutions at the key vicinal 4,5-bisphosphate motif with further structural modifications (Wilcox et al., 1994). Replacement of all the phosphate groups on $Ins(1,4,5)P_3$ with phosphorothioate groups, with no other perturbation of the molecular structure of $Ins(1,4,5)P_3$, however, had no effect on efficacy and only a small decrease in affinity at the $Ins(1,4,5)P_3$-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-$Ins(1)P$-$(4,5)PS_2$] (Fig. 1a) was found to be only 10-fold less potent than $Ins(1,4,5)P_3$ at displacing [^{3}H]$Ins(1,4,5)P_3$ from its receptor on pig cerebellum and to mobilize up to 60% of total Ca^{2+} in permeabilized SH-SY5Y cells (Wilcox et al., 1997). Therefore, all of the partial agonists thus far described at the $Ins(1,4,5)P_3$-R are $Ins(1,4,5)P_3$ analogs and, with the exception of *scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate and $Ins(1,3,4,6)P_4$, have combined modifications at C-3 or C-6 with phosphorothioate substitutions.

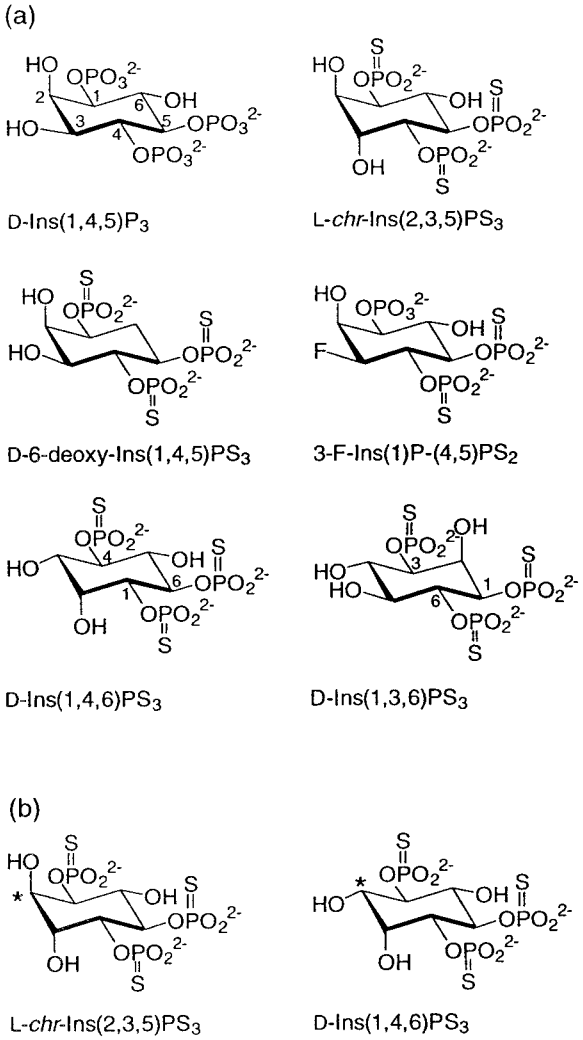

Fig. 1. (a) Structures of D-$Ins(1,4,5)P_3$, D-$Ins(1,4,6)PS_3$, and D-$Ins(1,3,6)PS_3$ compared with other partial agonists: L-*chr*-$Ins(2,3,5)PS_3$, D-6-deoxy-$Ins(1,4,5)PS_3$, and 3F-$Ins(1)P$-$(4,5)PS_2$ (Safrany et al., 1993). (b) Close structural relationship of D-$Ins(1,4,5)P_3$ with L-*chr*-$Ins(2,3,5)PS_3$ showing the difference at one chiral center (asterisk).

The Ca^{2+}-mobilizing activity of $Ins(1,3,4,6)P_4$ was rationalized by envisaging two alternative receptor-binding orientations in which the 1,6-vicinal bisphosphate of $Ins(1,3,4,6)P_4$ mimics the normal 4,5-bisphosphate in the $Ins(1,4,5)P_3$ binding orientation (although this did not explain its partial agonist properties). This model predicted that two $Ins(1,4,5)P_3$ regioisomers (i.e., D-*myo*-inositol 1,4,6-trisphosphate [D-$Ins(1,4,6)P_3$] and D-*myo*-inositol 1,3,6-trisphosphate [D-$Ins(1,3,6)P_3$ {L-$Ins(1,3,4)P_3$[1]}]) should be able to mobilize Ca^{2+} 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 phosphate groups of both $Ins(1,3,6)P_3$ and $Ins(1,4,6)P_3$ with phosphorothioates in the synthesis of $Ins(1,3,6)PS_3$ and $Ins(1,4,6)PS_3$. Preliminary data suggested that the racemic mixtures of the phosphorothioates $Ins(1,4,6)PS_3$ and $Ins(1,3,6)PS_3$ (Fig. 1a) were partial agonists at the $Ins(1,4,5)P_3$-R in permeabilized rabbit platelets (Al-Hafidh et al., 1994; Mills et al., 1995). Using the same rationalization for the Ca^{2+}-mobilizing activity of the partial agonist $Ins(1,3,4,6)P_3$, we predicted that the two chiral phosphorothioate analogs, D-$Ins(1,3,6)PS_3$ and D-$Ins(1,4,6)PS_3$, 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 $Ins(1,4,5)P_3$-R and that one of them [D-$Ins(1,4,6)PS_3$] possesses particularly promising potency coupled with very low intrinsic activity.

Experimental Procedures

Materials

Chemically synthesized $Ins(1,4,5)P_3$ was purchased from the Rhode Island Chemical Group (Kingston, RI). [^{3}H]$Ins(1,4,5)P_3$ (20–60 Ci/mmol, 10 μCi/ml) and $^{45}Ca^{2+}$ (5–50 mCi/mg Ca^{2+}, 2 mCi/ml) were purchased from Amersham International (Buckingham-

[1] Alternative nomenclature for D-$Ins(1,3,6)P_3$ is L-$Ins(1,3,4)P_3$.

shire, UK). FP100 filters were purchased from Whatman (Clifton, NJ). Saponin A, oligomycin, leupeptin, pepstatin, and ATP were obtained from Sigma (St. Louis, MO), ionomycin was purchased from Calbiochem (San Diego, CA). DL-Ins(1,3,6)PS_3 and DL-Ins(1,4,6)PS_3 were synthesized as described by Mills et al. (1995). D-Ins(1,3,6)PS_3 was synthesized from 1D-2,4,5-tri-*O*-benzyl-*myo*-inositol (Riley et al., 1994) and D-Ins(1,4,6)PS_3 was synthesized from 1D-2,3,5-tri-*O*-benzyl *myo*-inositol (Mills and Potter, 1996) using methods similar to those described for the racemic mixture (Mills et al., 1995). All synthetic compounds were homogenous by ^1H and ^{31}P NMR spectroscopy and mass spectroscopy after purification by ion-exchange chromatography. The compounds were quantified by total phosphate assay and then used as their triethylammonium salts.

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 $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose and 0.25% BSA, pH 7.4) before performing the following procedures.

$^{45}Ca^{2+}$ Release from Intracellular Stores. Platelets were washed in high-K^+ buffer A [120 mM KCl, 2 mM KH_2PO_4, 5 mM $(CH_2COONa)_2.6H_2O$, 6 mM $MgCl_2$, 20 mM HEPES, in MilliQ water; 5 mM ATP was added, pH adjusted to 6.9 and free Ca^{2+} concentration adjusted below 150 nM] and then suspended to 3×10^9/ml. The platelets were then permeabilized with 40 μg/ml saponin A, which was removed by further washing in buffer A. The intracellular Ca^{2+} stores were loaded with $^{45}Ca^{2+}$ (2 μCi/ml) for 1 h in the presence of 10 μg/ml oligomycin. Total release of $^{45}Ca^{2+}$ from the stores was determined by a 3-min incubation with 75 μM ionomycin. Release of $^{45}Ca^{2+}$ from the intracellular stores at 4°C was determined 3 min after the addition of the inositol phosphate by separation of free and retained $^{45}Ca^{2+}$ by filtration of cells using Whatman FP100 filters. $^{45}Ca^{2+}$ release was determined by liquid-scintillation counting (Murphy and Westwick, 1994).

Displacement of [^{3}H]Ins(1,4,5)P_3 Binding to Specific Ins(1,4,5)P_3 Receptors on Rat Cerebellar Membranes. The preparation of rat cerebellar membranes and displacement of [^{3}H]Ins(1,4,5)P_3 bound to the Ins(1,4,5)P_3 receptors on the membranes was performed as described previously (Challiss 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*, 13 min, 4°C), the pellet was resuspended in buffer C, homogenized as above, and the protein content adjusted 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 [^{3}H]Ins(1,4,5)P_3, and synthetic ligand diluted 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 [^{3}H]Ins(1,4,5)P_3 was assessed as the counts remaining upon inclusion of 10 μM cold Ins(1,4,5)P_3 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.

Results

Ca^{2+} Release from Permeabilized Platelets. Rabbit platelets permeabilized with saponin and in the presence of oligomycin displayed ATP-dependent $^{45}Ca^{2+}$ 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 $^{45}Ca^{2+}$ was found to be >92%; again, this was not found to change significantly throughout the time course of any of the $^{45}Ca^{2+}$ release experiments undertaken.

Treatment of permeabilized platelets with D-Ins(1,4,5)P_3 (0.01–30 μM) for 3 min (4°C) caused a dose-dependent release of $^{45}Ca^{2+}$ from preloaded intracellular stores (Fig. 2). DL-Ins(1,3,6)PS_3 (1–3000 μM) alone caused a dose-dependent release of $^{45}Ca^{2+}$ from the stores of permeabilized platelets. Maximal release, however, was only around 20% of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool, even at concentrations above 1 mM (some of which may have been caused by nonspecific release), demonstrating a very low efficacy for DL-Ins(1,3,6)PS_3 at the Ins(1,4,5)P_3-R of rabbit platelets (Fig. 2a). Treatment of permeabilized platelets with 1 μM Ins(1,4,5)P_3, together with increasing concentrations of DL-Ins(1,3,6)PS_3, caused an inhibition of Ins(1,4,5)P_3-induced Ca^{2+}-release (Fig. 2a). Ca^{2+} release induced by Ins(1,4,5)P_3 was reduced as the concentration of DL-Ins(1,3,6)PS_3 increased, until release approached a level near the intrinsic efficacy of DL-Ins(1,3,6)PS_3 itself. The concentration of DL-Ins(1,3,6)PS_3 required to inhibit release of Ca^{2+} induced with 1 μM Ins(1,4,5)P_3 by 50% (IC_{50}) was >100 μM. However, increasing the concentration of DL-Ins(1,3,6)PS_3 to

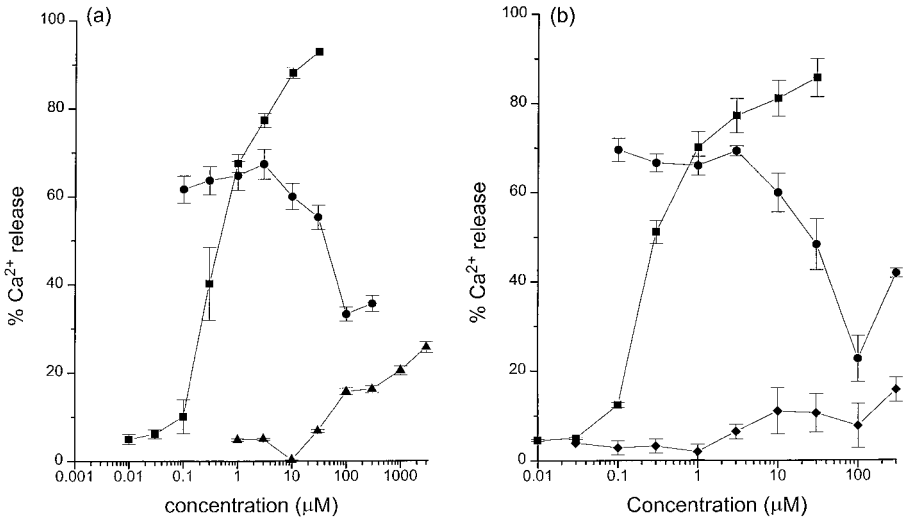

Fig. 2. Ins(1,4,5)P_3, DL-Ins(1,3,6)PS_3, and DL-Ins(1,4,6)PS_3-induced $^{45}Ca^{2+}$ release from permeabilized platelets. (a) Permeabilized platelets, preloaded with $^{45}Ca^{2+}$, were treated with either Ins(1,4,5)P_3 (■), DL-Ins(1,3,6)PS_3 alone (▲), or increasing concentrations of DL-Ins(1,3,6)PS_3 and 1 μM Ins(1,4,5)P_3 (●) for 3 min (4°C). (b) As in (a), except treated with either Ins(1,4,5)P_3 (■), DL-Ins(1,4,6)PS_3 alone (♦) or increasing concentrations of Ins(1,4,6)PS_3 and 1 μM Ins(1,4,5)P_3 (●). Release of $^{45}Ca^{2+}$ was terminated by rapid filtration and is given as a percentage of maximal $^{45}Ca^{2+}$ releasable upon treatment of platelets with 75 μM ionomycin. The values are the mean ± S.E. of three to six separate experiments, each performed in triplicate.

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)P_3 is reached by 100 μM (Fig. 2a).

DL-Ins(1,4,6)PS_3 (0.03–300 μM) was also found to have a very low efficacy at the Ins(1,4,5)P_3-R, releasing only ~15% of the Ins(1,4,5)P_3-sensitive Ca^{2+} store at the highest concentration used (300 μM) (Fig. 2b). However, Ca^{2+} release induced by 1 μM Ins(1,4,5)P_3 could be inhibited with increasing concentrations of DL-Ins(1,4,6)PS_3 (Fig. 2b). As described previously for DL-Ins(1,3,6)PS_3, Ca^{2+} release induced by Ins(1,4,5)P_3 was further inhibited as the concentration of DL-Ins(1,4,6)PS_3 increased, until release declined to a level near the intrinsic efficacy of DL-Ins(1,4,6)PS_3 itself. The concentration of DL-Ins(1,4,6)PS_3 required to inhibit release of Ca^{2+} induced with 1 μM Ins(1,4,5)P_3 by 50% (IC_{50}) was found to be 56 ± 3.6 μM. Maximal inhibition of Ca^{2+}-release induced by 1 μM Ins(1,4,5)P_3 seems to have been achieved with 100 μM DL-Ins(1,4,6)PS_3 as an increased Ca^{2+} release was observed with 300 μM (Fig. 2b).

Displacement of Specific [^{3}H]Ins(1,4,5)P_3 Binding to Rat Cerebellar Membranes. [^{3}H]Ins(1,4,5)P_3 was readily displaced from specific binding sites on rat cerebellar membranes by cold D-Ins(1,4,5)P_3 with an IC_{50} of 0.043 ± 0.01 μM (Fig. 3). The ability of DL- and D-Ins(1,3,6)PS_3 and of DL- and D-Ins(1,4,6)PS_3 to displace [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes was also examined (Fig. 3). D-Ins(1,3,6)PS_3 displaced specifically bound [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes, although displacement by D-Ins(1,3,6)PS_3 was 500 fold weaker than by D-Ins(1,4,5)P_3 (Fig. 3a). However, comparing the IC_{50} value for D-Ins(1,3,6)PS_3 (17.5 ± 5.8 μM) with that of the racemic mixture DL-Ins(1,3,6)PS_3 (34 ± 10 μM), demonstrated that D-Ins(1,3,6)PS_3 was able to displace [^{3}H]Ins(1,4,5)P_3 with roughly twice the affinity of the racemic mixture, confirming that activity resides in the D-enantiomer (Fig. 3a). D-Ins(1,4,6)PS_3 displaced specifically bound [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes, although displacement by D-Ins(1,4,6)PS_3 was some 34-fold weaker than by D-Ins(1,4,5)P_3 (Fig. 3a). D-Ins(1,4,6)PS_3 was able to displace [^{3}H]Ins(1,4,5)P_3 from cerebellar membranes with roughly twice the affinity of its racemic mixture (IC_{50} value of 1.4 ± 0.35 μM compared with an IC_{50} 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)PS_3 and D-Ins(1,4,6)PS_3 to displace [^{3}H]Ins(1,4,5)P_3 from specific binding sites on rat cerebellar membranes contrasted with their relative inability to release $^{45}Ca^{2+}$ from the intracellular stores of permeabilized platelets.

Effect of Trisphosphorothioates on Ins(1,4,5)P_3-Induced Ca^{2+} Release. From the binding studies, it seems that D-Ins(1,3,6)PS_3 is the active component of the racemic mixture DL-Ins(1,3,6)PS_3, whereas D-Ins(1,4,6)PS_3 is the active component of the racemic mixture of DL-Ins(1,4,6)PS_3. As for the racemic mixtures, both D-Ins(1,3,6)PS_3 and D-Ins(1,4,6)PS_3 were also found to have a very low efficacy at the Ins(1,4,5)P_3–receptor of platelets, releasing only a small percentage (<15%) of the Ins(1,4,5)P_3-sensitive Ca^{2+} store even at 300 μM (Fig. 4a). It is possible that the release of Ca^{2+} by high concentrations of both D-Ins(1,3,6)PS_3 and D-Ins(1,4,6)PS_3 may be nonspecific.

Increasing concentrations of D-Ins(1,3,6)PS_3 (30, 100, and 300 μM) were able to partially antagonize Ca^{2+} release induced by submaximal concentrations of Ins(1,4,5)P_3 (0.1–3 μM); however, by increasing the concentration of Ins(1,4,5)P_3 (10 and 30 μM), this inhibition was no longer observed, suggesting competition for binding at the Ins(1,4,5)P_3-R (Fig. 4b). The IC_{50} value for the inhibition of Ca^{2+} elevation induced by 1 μM Ins(1,4,5)P_3 was >100 μM for D-Ins(1,3,6)PS_3. Competitive partial antagonism of Ins(1,4,5)P_3-induced Ca^{2+} release was also observed with D-Ins(1,4,6)PS_3 (10, 30, and 100 μM) (Fig. 4c). The IC_{50} value of D-Ins(1,4,6)PS_3 for Ca^{2+} release induced by 1 μM Ins(1,4,5)P_3 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)P_3 analogs have concluded that the vicinal 4,5-bisphosphate configuration plays the key role in receptor recognition and mediation of Ca^{2+} release from intracellular stores (for reviews, see Potter and Nahorski, 1992; Potter and Lampe, 1995; Wilcox et al., 1998). Other structural require-

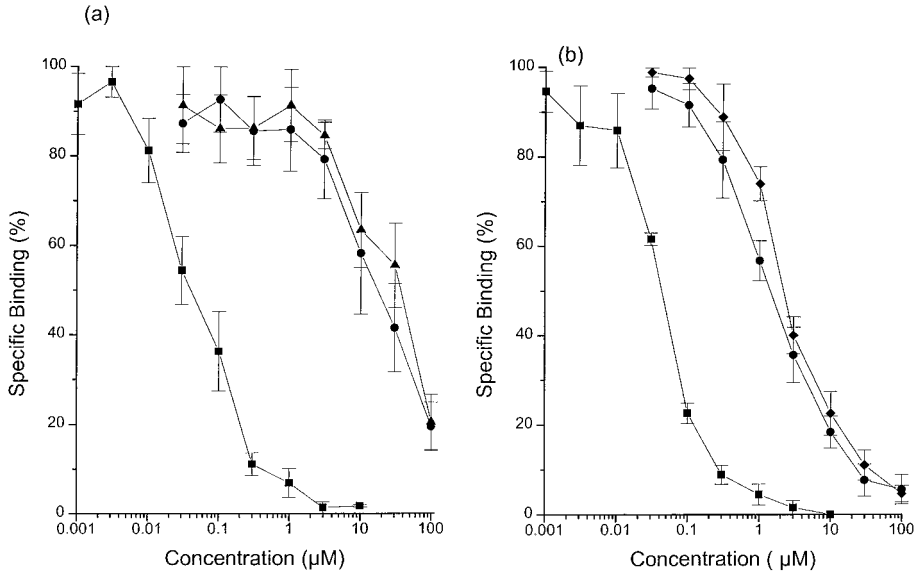

Fig. 3. Displacement of specific [^{3}H]Ins(1,4,5)P_3 binding from Ins(1,4,5)P_3 receptors on rat cerebellar membranes. Percentage displacement of specific [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar membranes by (a) Ins(1,4,5)P_3 and DL- and D-Ins(1,3,6)PS_3 and (b) Ins(1,4,5)P_3, DL- and D-Ins(1,4,6)PS_3. The values shown are the mean ± S.E. of three to four experiments, each performed in duplicate. (a) ■, Ins(1,4,5)P_3; ▲, DL-Ins(1,3,6)PS_3; ●, D-Ins(1,3,6)PS_3. (b) ■, Ins(1,4,5)P_3; ◆, DL-Ins(1,4,6)PS_3; ●, D-Ins (1,4,6)PS_3.

ments for Ca^{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_3-R (Potter and Lampe, 1995). The importance of the hydroxyl groups of Ins(1,4,5)P_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_3 varying the impact on Ca^{2+} release and the binding of Ins(1,4,5)P_3 to its receptor (for review, see Potter and Lampe, 1995).

At present very few partial agonists at the Ins(1,4,5)P_3-R have been reported; these include Ins(1,3,4,6)P_4 (Gawler et al., 1991), L-*chr*-Ins(2,3,5)PS_3, and 6-deoxy-Ins1,4,5)PS_3 (Safrany et al., 1993), *scyllo*-inositol 1,2,4,5-tetrakisphosphorothioate (Wilcox et al., 1994), and 3F-Ins(1)P-(4,5)PS_2 (Wilcox et al., 1997). By using rapid kinetic measurements of $^{45}Ca^{2+}$ mobilization, Ins(2,4,5)P_3 has also been demonstrated to be a partial agonist at hepatic Ins(1,4,5)P_3-Rs (Marchant et al., 1997). Because of the quantal mechanism of Ca^{2+} release, whereby even partial agonists may completely empty the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, albeit at slower rates than Ins(1,4,5)P_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_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_4 elicits Ca^{2+} release by envisaging two alternative receptor binding orientations, where the 1,6-vicinal bisphosphate is presumed to mimic the normal 4,5-bisphosphate of Ins(1,4,5)P_3. As either the 4-phosphate or the 3-phosphate of Ins(1,3,4,6)P_4 could mimic the 1-phosphate of Ins(1,4,5)P_3, it is likely that Ins(1,3,4,6)P_4 evokes Ca^{2+} release by a similar binding mechanism to Ins(1,4,5)P_3. We went on to show that two related trisphosphates [D-Ins(1,4,6)P_3 and D-Ins(1,3,6)P_3] were also able to displace [^{3}H]Ins(1,4,5)P_3 from the Ins(1,4,5)P_3-R and to possess Ca^{2+} mobilization ability, whereas their enantiomers were inactive (Murphy et al., 1996). Noting that Ins(1,4,6)P_3 and Ins(1,3,6)P_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_3, we went on to replace their phosphate groups with phosphorothioates, giving Ins(1,4,6)PS_3 and Ins(1,3,6)PS_3. From the preceding structure-activity arguments, we predicted that D-Ins(1,4,6)PS_3 and D-Ins(1,3,6)PS_3 would show partial agonist properties, whereas their enantiomers would be inactive.

Both the racemic trisphosphorothioates DL-Ins(1,3,6)PS_3 and DL-Ins(1,4,6)PS_3 were found to have very low efficacy at the Ins(1,4,5)P_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 μM Ins(1,4,5)P_3, together with increasing concentrations of either DL-Ins(1,3,6)PS_3 or DL-Ins(1,4,6)PS_3, a definite inhibition of Ins(1,4,5)P_3-stimulated Ca^{2+} release was observed, demonstrating that both DL-Ins(1,3,6)PS_3 and DL-Ins(1,4,6)PS_3 were acting as true partial agonists.

It was assumed that the partial agonist activity of racemic DL-Ins(1,4,6)PS_3 and DL-Ins(1,3,6)PS_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_3. On the other hand, neither the L-enantiomer of Ins(1,4,6)PS_3 nor the L-enantiomer of Ins(1,3,6)PS_3 possesses this motif; rather, they are similar to L-Ins(1,4,5)P_3. To examine this theory, the ability of both D-Ins(1,4,6)PS_3 and D-Ins(1,3,6)PS_3 to displace [^{3}H]Ins(1,4,5)P_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_3 and the D-isomer of Ins(1,3,6)PS_3 were found to have roughly twice the affinity

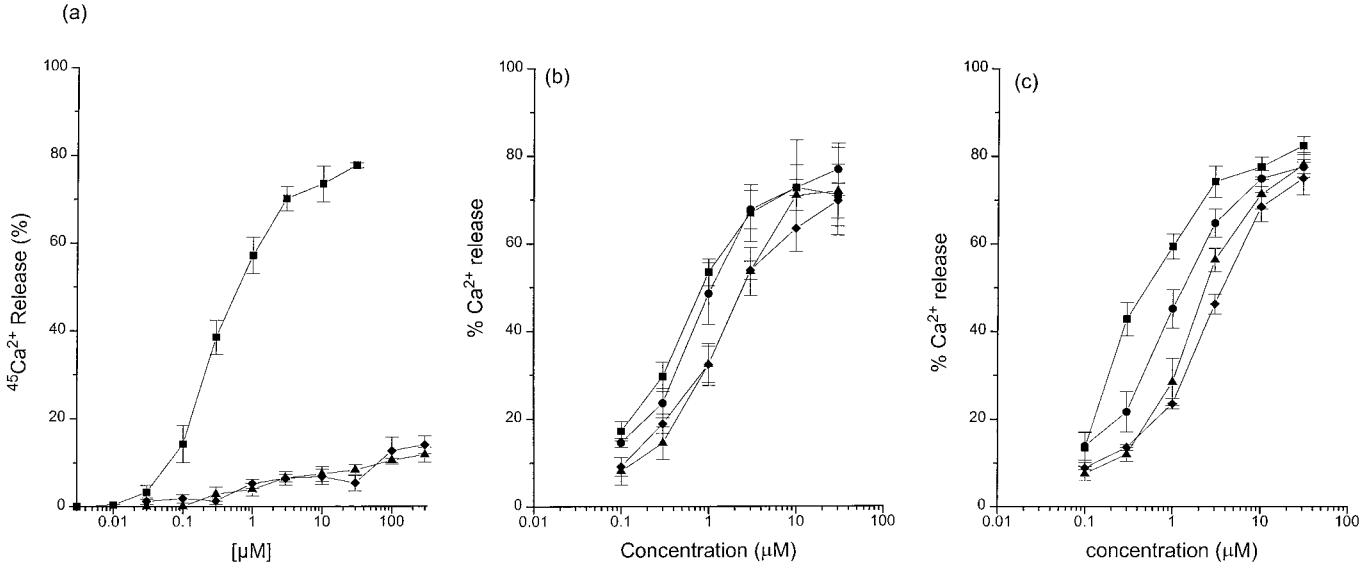

Fig. 4. Effect of increasing concentrations of D-Ins(1,3,6)PS_3 and of D-Ins(1,4,6)PS_3 on release of Ca^{2+} induced by both submaximal and maximal concentrations of Ins(1,4,5)P_3. (a) Permeabilized platelets, preloaded with $^{45}Ca^{2+}$, were treated with either Ins(1,4,5)P_3 (■), D-Ins(1,3,6)PS_3 alone (▲), or D-Ins(1,4,6)PS_3 alone (♦) for 3 min (4°C). (b) Platelets were treated with Ins(1,4,5)P_3 either alone (■) or in the presence of increasing concentrations of D-Ins(1,3,6)PS_3: 30 μM (●), 100 μM (▲), or 300 μM (♦). (c) Platelets were treated with Ins(1,4,5)P_3 either alone (■) or in the presence of increasing concentrations of D- Ins(1,4,6)PS_3: 10 μM (●), 30 μM (▲), or 100 μM (♦). Release of $^{45}Ca^{2+}$ was terminated by rapid filtration and is given as a percentage of maximal $^{45}Ca^{2+}$ releasable upon treatment of platelets with 75 μM ionomycin. The values are the mean ± S.E. of three to four separate experiments, each performed in triplicate

for the Ins(1,4,5)P_3-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)PS_3, D-Ins(1,4,6)PS_3 showed a higher affinity for the Ins(1,4,5)P_3-R in binding studies. In D-Ins(1,4,6)PS_3, the orientation of the 5-OH [which mimics the 6-OH of Ins(1,4,5)P_3] is equatorial [as in D-Ins(1,4,5)P_3], whereas the OH-group corresponding to the 3-OH is axial rather than equatorial (Fig. 1a). The 2-OH [which mimics the 6-OH of Ins(1,4,5)P_3] is reoriented to axial in D-Ins(1,3,6)PS_3 and is therefore different from that in D-Ins(1,4,5)P_3, whereas the OH group corresponding to the 3-OH of Ins(1,4,5)P_3 remains equatorial. From structure-activity studies, the 3-OH group of Ins(1,4,5)P_3 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)PS_3] might not be expected to have a significant effect on Ins(1,4,5)P_3 binding. However, modification at the 6-OH group [as in Ins(1,3,6)PS_3] would be expected to reduce binding and activity (Polokoff et al., 1988; Safrany et al., 1991). The finding that D-Ins(1,4,6)PS_3 is more potent than D-Ins(1,3,6)PS_3 at displacing [^{3}H]Ins(1,4,5)P_3 conforms with these structural requirements and confirms the conclusion that the 6-OH group of Ins(1,4,5)P_3 is more important for binding than the 3-OH group (Hirata et al., 1993; Murphy et al., 1996).

Increasing concentrations of either D-Ins(1,3,6)PS_3 or D-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. However, by increasing the concentration of Ins(1,4,5)P_3, this inhibition was no longer observed, suggesting competition for binding at the Ins(1,4,5)P_3-R (Fig. 4b). The IC_{50} value for the inhibition of Ca^{2+} elevation induced by 1 μM Ins(1,4,5)P_3 was >100 μM for both racemic and D-Ins(1,3,6)PS_3 and was 27 $\pm$ 8.1 μM for D-Ins(1,4,6)PS_3, approximately half that of the racemic mixture.

The only two low-intrinsic-activity partial agonists described previously are L-*chr*-Ins(2,3,5)PS_3 and 6-deoxy-Ins(1,4,5)PS_3, which were found to release 34 and 42% of Ca^{2+} respectively in SH-SY5Y cells (Safrany et al., 1993). It is interesting to note that the only structural difference between D-Ins(1,4,6)PS_3 and L-*chr*-Ins(2,3,5)PS_3 is that the hydroxyl group that mimics the 2-OH of Ins(1,4,5)P_3, is reoriented from axial to equatorial in Ins(1,4,6)PS_3 relative to L-*chr*-Ins(2,3,5)PS_3 (see Fig. 1b). In structure-activity studies, the 2-OH 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)PS_3 and an increase in its affinity for the receptor [L-*chr*-Ins(2,3,5)PS_3 was found to have some 100-fold lower affinity for the Ins(1,4,5)P_3-R in bovine adrenal cortical membranes (Safrany et al., 1993), whereas the affinity of Ins(1,4,6)PS_3 was only 34-fold lower than Ins(1,4,5)P_3 in rat cerebellar membranes]. There are two differences between 6-deoxy-Ins(1,4,5)PS_3 and D-Ins(1,3,6)PS_3: first, the 6-hydroxyl group is deleted in 6-deoxy-Ins(1,4,5)PS_3, whereas the "pseudo" 6-OH is axial in Ins(1,3,6)PS_3; second, the 2-OH group is axial in 6-deoxy-Ins(1,4,5)PS_3 [as in Ins(1,4,5)P_3], whereas the "pseudo" 2-OH is equatorial in Ins(1,3,6)PS_3. 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)PS_2 for partial agonist activity (Wilcox et al., 1997). Similarly to D-Ins(1,4,6)PS_3, these compounds possessed a structural perturbation at the hydroxyl group that mimics the 3-OH of Ins(1,4,5)P_3. This was achieved by the replacement of the native 3-OH with a fluorine group. Again, like D-Ins(1,4,6)PS_3, these compounds had phosphorothioate substitutions, although only one, 3F-Ins(1)P-(4,5)PS_2, had phosphorothioate substitutions at both members of the crucial vicinal 4,5-bisphosphate motif. Of these compounds, 3F-Ins(1)P-(4,5)PS_2 was the only one identified as a partial agonist, able to inhibit Ca^{2+} mobilization induced by submaximal concentrations of Ins(1,4,5)P_3 (Wilcox et al., 1997). It was also demonstrated to be an antagonist of receptor-mediated Ca^{2+} signaling (Davis et al., 1998). Compared with 3F-Ins(1)P-(4,5)PS_2, D-Ins(1,4,6)PS_3 has a lower affinity for the Ins(1,4,5)P_3-R [it is ~34-fold weaker than Ins(1,4,5)P_3 at displacing [^{3}H]Ins(1,4,5)P_3], whereas 3F-Ins(1)P-(4,5)PS_2 was only 10-fold weaker (Wilcox et al., 1997). Of all the phosphorothioate-containing partial agonists, 3-F-Ins(1)P-(4,5)P_2 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)PS_3, 3F-Ins(1)P-(4,5)PS_2, has a relatively high efficacy, causing over 60% of Ca^{2+} to be released. Therefore, although 3F-Ins(1)P-(4,5)PS_2 has a high affinity, its potential as a partial antagonist and a lead compound is reduced by its higher efficacy. It is possible, therefore, that the lower affinity of D-Ins(1,4,6)PS_3 at the Ins(1,4,5)P_3-R compared with 3F-Ins(1)P-(4,5)PS_2 is because it has three phosphorothioates, one of which is at the 1-position. Thus a version of D-Ins(1,4,6)PS_3 with a vicinal bisphosphorothioate and a pseudo-1-phosphate [namely D-Ins(4)P-(1,6)PS_2] may combine the high affinity of 3F-Ins(1)P-(4,5)PS_2 and the low efficacy of D-Ins(1,4,6)PS_3.

The only low-efficacy partial agonists at the Ins(1,4,5)P_3-R discovered to date have been phosphorothioates; D-Ins(1,3,6)PS_3 and D-Ins(1,4,6)PS_3 now expand this small group of such analogs. However, D-Ins(1,4,6)PS_3 in particular has a relatively high affinity for the Ins(1,4,5)P_3 receptor and yet maintains very low efficacy. Thus D-Ins(1,4,6)PS_3 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)P_3 receptor antagonists. Indeed, it has recently been successfully employed via microinjection to inhibit Ins(1,4,5)P_3-induced Ca^{2+} mobilization in intact Jurkat T-lymphocytes (Guse et al., 1997, 1999).

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Send reprint requests to: Dr. C. T. Murphy, Department of Pharmacy & Pharmacology, University of Bath, Bath, BA2 7AY UK. E-mail: c.t.murphy@bath.ac.uk