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Enantiomers of *myo*-Inositol-1,3,4-trisphosphate and *myo*-Inositol-1,4,6-trisphosphate: Stereospecific Recognition by Cerebellar and Platelet *myo*-Inositol-1,4,5-trisphosphate Receptors

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SUMMARY

The naturally occurring tetrakisphosphate *myo*-inositol-1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)P_4] was able to release Ca^{2+} from the intracellular stores of permeabilized rabbit platelets but was 40-fold less potent than D-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]. The Ca^{2+} releasing activity of Ins(1,3,4,6)P_4 was rationalized by envisaging two alternative receptor binding orientations in which the vicinal D-1,6-bisphosphate of Ins(1,3,4,6)P_4 mimics the D-4,5-bisphosphate in the Ins(1,4,5)P_3 binding conformation. This rationalization predicted that 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]] should also possess Ca^{2+}-releasing activity. The unambiguous total synthesis of the enantiomers of Ins(1,4,6)P_3 [i.e., D-Ins(1,4,6)P_3 and D-Ins(3,4,6)P_3] and the enantiomers of Ins(1,3,4)P_3 [i.e., D-Ins(1,3,6)P_3 and D-Ins(1,3,4)P_3] allowed an examination of this prediction. D-Ins(1,4,6)P_3 released Ca^{2+} from the intracellular stores of permeabilized platelets and was only 2–3-fold less potent than Ins(1,4,5)P_3. D-Ins(1,3,6)P_3 [alternative nomenclature, L-Ins(1,3,4)P_3] also released Ca^{2+} but was 12-fold less potent than Ins(1,4,5)P_3. Both D-Ins(1,4,6)P_3 and D-Ins(1,3,6)P_3 displaced specifically bound [^{3}H]Ins(1,4,5)P_3 from the Ins(1,4,5)P_3 receptor on rat cerebellar membranes. In contrast, however, D-Ins(3,4,6)P_3 [alternative nomenclature, L-Ins(1,4,6)P_3] and D-Ins(1,3,4)P_3 neither possessed Ca^{2+}-releasing activity nor displaced [^{3}H]Ins(1,4,5)P_3. The ability of D-Ins(1,3,6)P_3 to release Ca^{2+} in permeabilized platelets is in contrast to its apparent lack of Ca^{2+}-mobilizing activity previously reported in rat basophilic leukemic cells. The possibility that this is a reflection of the different Ins(1,4,5)P_3 receptor subtypes possessed by these two cell types is discussed.

Elevated cytosolic Ca^{2+} is known to be a principal mediator of activation/response coupling in numerous cell types in response to a wide range of extracellular stimuli. Agonist/receptor coupling activates the hydrolysis of phosphatidyl inositol-4,5,-bisphosphate, producing the signal molecule Ins(1,4,5)P_3 (Fig. 1a), which induces Ca^{2+} mobilization into the cytoplasm via ligation of specific receptors on Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores (1). Three Ins(1,4,5)P_3-R subtypes, together with splice variants of each of these, have been identified, and the genes have been cloned (2, 3). 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 (4). Transfection of the Ins(1,4,5)P_3-R cDNA 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 binding sites for both Ins(1,4,5)P_3 and Ca^{2+} channel activity (5).

Two enzymes are mainly responsible for the metabolism of Ins(1,4,5)P_3 [Ins(1,4,5)P_3-5-phosphatase, which dephosphorylates Ins(1,4,5)P_3 to Ins(1,4)P_2, and Ins(1,4,5)P_3-3-kinase, which phosphorylates Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4]. Ins(1,4)P_2 is inactive, and its formation is therefore a mechanism for switching-off the action of Ins(1,4,5)P_3. The role of Ins(1,3,4,5)P_4 remains controversial, but the recent finding that an Ins(1,3,4,5)P_4 binding protein is located in the

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ABBREVIATIONS: Ins(1,4,5)P_3, *myo*-inositol-1,4,5-trisphosphate; Ins(1,3,4,6)P_4, *myo*-inositol-1,3,4,6-tetrakisphosphate; Ins(1,3,4)P_3, *myo*-inositol-1,3,4-trisphosphate; Ins(1,3,6)P_3, *myo*-inositol-1,3,6-trisphosphate; Ins(1,4,6)P_3, *myo*-inositol-1,4,6-trisphosphate; Ins(3,4,6)P_3, *myo*-inositol-3,4,6-trisphosphate; Ins(1,4,5)P_3-R, D-*myo*-inositol-1,4,5-trisphosphate receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; RBL, rat basophilic leukemic.

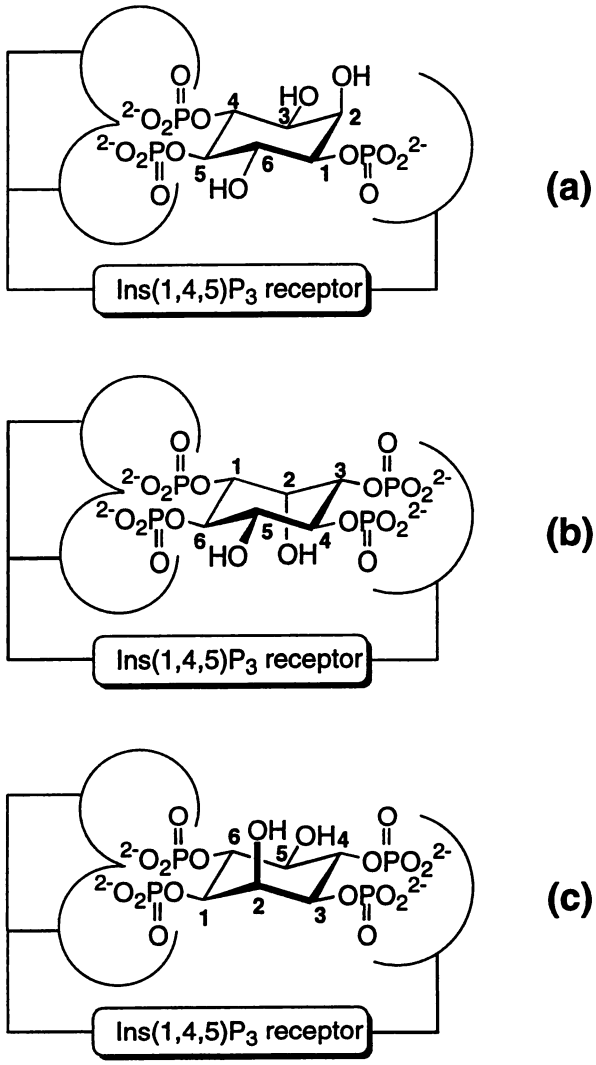

Fig. 1. a, Representation of D-Ins(1,4,5)P_3 at the Ins(1,4,5)P_3-binding site. b and c, The two binding orientations of Ins(1,3,4,6)P_4 at the Ins(1,4,5)P_3-binding site.

plasma membrane of platelets and has been identified as a member of the GTPase activating protein-1 family suggests a potential signaling role for Ins(1,3,4,5)P_4 (6).

Structure-activity studies using analogs of Ins(1,4,5)P_3 have demonstrated that distinct structural motifs in Ins(1,4,5)P_3 are responsible for its receptor-binding capability and Ca^{2+}-releasing activity (7). An important feature of the structure-activity studies that have been performed with Ins(1,4,5)P_3 analogs (7, 8) is the key role of the vicinal D-4,5-bisphosphate system in mediating intracellular Ca^{2+} release (9), whereas a 1-phosphate group and an equatorial 6-OH are thought to be responsible for enhanced binding (Fig. 1a) (8, 10). Surprisingly, the higher polyphosphate Ins(1,3,4,6)P_4 also possessed moderate Ca^{2+}-mobilizing activity and was shown to be a partial agonist in SH-SY5Y cells (11) despite the apparent absence of a D-4,5-bisphosphate motif (8, 12).

In this study, the Ca^{2+}-releasing activity of Ins(1,3,4,6)P_4 was investigated in platelets. The activity of this compound has been rationalized by envisaging two alternative receptor binding orientations in which the vicinal D-1,6-bisphosphate of Ins(1,3,4,6)P_4 mimics the normal D-4,5-bisphosphate in the Ins(1,4,5)P_3 binding orientation (Fig. 1). This rationalization predicts that two Ins(1,4,5)P_3 regioisomers [D-Ins(1,4,6)P_3 and D-Ins(1,3,6)P_3][1] should mobilize Ca^{2+}, with the vicinal D-1,6-bisphosphate motif in each case mimicking the 4,5-bisphosphate of Ins(1,4,5)P_3 (Figs. 1 and 2). Total and unambiguous syntheses of both the enantiomers of Ins(1,3,4)P_3 (13) and of Ins(1,4,6)P_3 (14, 15) allowed us to investigate this proposal and to explain potential features underlying the partial agonism of Ins(1,3,4,6)P_4. In this study, we report the Ca^{2+}-releasing activity of both enantiomers of Ins(1,4,6)P_3 and Ins(1,3,4)P_3 in permeabilized rabbit platelets together with their ability to displace [^{3}H]Ins(1,4,5)P_3 from its specific binding site in rat cerebellum.

Experimental Procedures

Materials. Chemically synthesized Ins(1,4,5)P_3 was purchased from the Rhode Island Chemical Group (Kingston, RI). Fura-2 (pentapotassium salt) was from Molecular Probes (Eugene, OR). [^{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 (Buckinghamshire, UK). FP100 filters were purchased from Whatman. Heparin, oligomycin, creatine phosphokinase, phosphocreatine, saponin A, leupeptin, pepstatin, and ATP were obtained from Sigma Chemical (St. Louis, MO). Ins(1,3,4,6)P_4 and ionomycin were purchased from Calbiochem (San Diego, CA). Enantiomers of Ins(1,3,4)P_3 were synthesized according to Riley *et al.* (13), and those of Ins(1,4,6)P_3 were synthesized according to Mills *et al.* (14, 15).

Preparation of platelets. Washed rabbit platelets were prepared as described previously (16). 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% bovine serum albumin, pH 7.4) before the following procedures were performed.

$^{45}Ca^{2+}$ release from intracellular stores. Platelets were washed in high-K^+ buffer A [120 mM KCl, 2 mM KH_2PO_4, 5 mM $(CH_2COONa)_2{\cdot}6H_2O$, 6 mM $MgCl_2$, 20 mM HEPES, in MilliQ water (Millipore, Watford, UK); 5 mM ATP was added, pH adjusted to 6.9, and free Ca^{2+} concentration was 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 hr in the presence of 10 μg/ml oligomycin. Total release of $^{45}Ca^{2+}$ from the stores was determined at 20° by a 3-min incubation with 75 μM ionomycin. Release of $^{45}Ca^{2+}$ from the intracellular stores was determined 3 min after the addition of the inositol phosphate by separation of free and retained $^{45}Ca^{2+}$ through filtration of cells using Whatman FP100 filters. $^{45}Ca^{2+}$ release was determined by liquid scintillation spectroscopy (17).

Ins(1,4,5)P_3-induced Ca^{2+} release from permeabilized platelets monitored by spectrophotofluorimetry. Platelets were isolated and washed as above and then resuspended in high-K^+ buffer B (100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES, 2 mM EGTA, pH 7.2) at a concentration of 3×10^9/ml. After permeabilization with 40 μg/ml saponin A (1 min, 20°), the platelets were washed again in buffer B in the absence of EGTA but in the presence of 20 units/ml creatine phosphokinase and 10 μg/ml oligomycin following a modification of a previously described method (18). Ca^{2+} uptake into stores was initiated by the addition of 3 mM ATP and 50 mM phosphocreatine. Ca^{2+} release from the stores was monitored using Fura-2 (free acid, 0.5 μM) in the extracellular buffer. Changes in fluorescence were measured using a PTI dual-wavelength spectrophotofluorimeter (excitation, 340 and 380 nm; emission, 510 nm; slit width, 4 nm) (Photon Technology, Surbiton, UK). Experiments

[1] For the sake of clarity, L-Ins(1,3,4)P_3 = D-Ins(1,3,6)P_3, and L-Ins(1,4,6)P_3 = D-Ins(3,4,6)P_3. All compounds are listed in their D-configuration throughout the article.

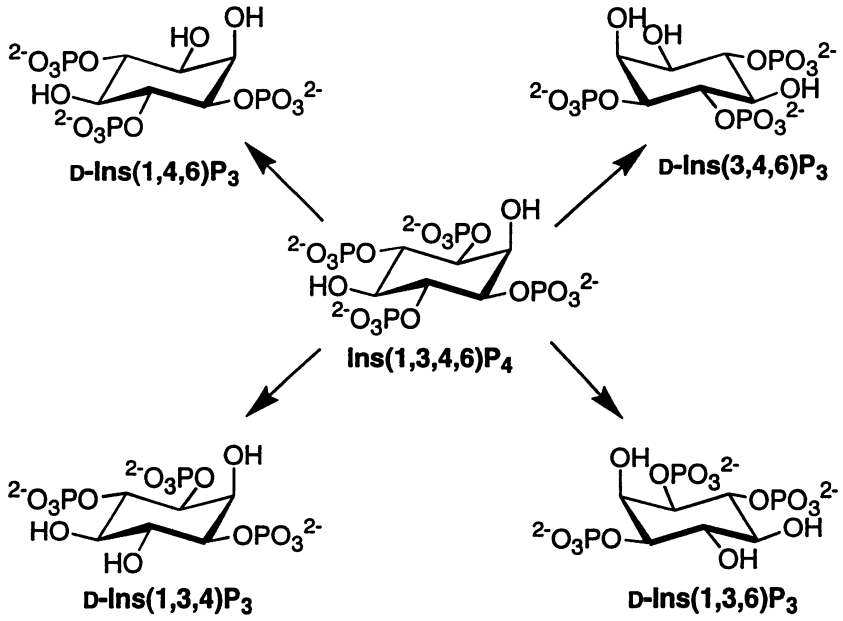

Fig. 2. Structural relationship of Ins(1,3,4,6)P_4 to D-Ins(1,3,4)P_3, D-Ins(1,3,6)P_3, D-Ins(1,4,6)P_3, and D-Ins(3,4,6)P_3 formed through sequential removal of phosphate groups.

were performed at 20°. The traces shown in the figures represent an increase in fluorescence of Fura-2, which is due to the transient release of Ca^{2+} from the intracellular stores, followed by a decrease in fluorescence, which is due to Ca^{2+} resequestration; this technique therefore provides an indirect measurement of the metabolism of Ca^{2+}-releasing agents. The Ca^{2+}/Fura-2-fluorescence was calibrated as described previously (19).

Displacement of [^{3}H]Ins(1,4,5)P_3 binding to specific Ins(1,4,5)P_3-Rs 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-Rs on the membranes were performed as described previously (20). Briefly, the cerebella were removed from six rats (200–250 g) and homogenized (2 × 10 sec, 4°) in buffer C (20 mM Tris·HCl, 20 mM NaCl, 100 mM KCl, 1 mM EDTA, 1 mg/ml bovine serum albumin, pH 7.7) containing the protease inhibitors 10 μM leupeptin and 10 μM pepstatin. After centrifugation (50,000 × *g*, 13 min, 4°), the pellet was resuspended in buffer C and homogenized as described above, and the protein content was adjusted to 5 mg/ml. The cerebellar membranes were either used immediately or frozen (−80°) 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°) for 10 min before termination of the reaction by centrifugation (10,000 × *g*, 4 min, 4°). Nonspecific binding of [^{3}H]Ins(1,4,5)P_3 was assessed as the counts remaining on inclusion of 10 μM cold Ins(1,4,5)P_3 in the assay mixture. After centrifugation, the supernatant was carefully removed, the pellet was resuspended, and radioactivity bound to the cerebellar membrane was determined by liquid scintillation counting.

Results

Ca^{2+} release from permeabilized platelets. In the presence of oligomycin, rabbit platelets permeabilized with saponin A displayed ATP-dependent $^{45}Ca^{2+}$ uptake into their nonmitochondrial stores. Uptake reached a steady state by 45 min; it was monitored throughout the time course of the experiment and was 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 that we conducted.

Treatment of permeabilized platelets with D-Ins(1,4,5)P_3 (0.01–30 μM) for 3 min (20°) caused a dose-dependent release of $^{45}Ca^{2+}$ from preloaded intracellular stores (Fig. 3). A time of 3 min was chosen because at this point, $^{45}Ca^{2+}$ release had reached a maximal plateau. Ins(1,3,4,6)P_4 (0.1–100 μM) also caused a dose-dependent release of $^{45}Ca^{2+}$ from the stores of permeabilized platelets (Fig. 3, *top*). However, it displayed a ~40-fold higher EC_{40} value than D-Ins(1,4,5)P_3 (Table 1). Synthetic Ins(1,3,4,6)P_4 from an alternative source, which had previously been shown to exhibit partial agonistic properties in SH-SY5Y cells (11), was also examined. This Ins(1,3,4,6)P_4 was found to give essentially the same results as described above. At the highest concentration of Ins(1,3,4,6)P_4 (100 μM), the release of Ca^{2+} had not reached a plateau, thus providing no evidence that Ins(1,3,4,6)P_4 was a partial agonist in permeabilized platelets. Using higher concentrations, we demonstrated that Ins(1,3,4,6)P_4 is as efficacious as Ins(1,4,5)P_3 in releasing Ca^{2+} from permeabilized platelets (results not shown).

Two synthetic chiral regioisomers of D-Ins(1,4,5)P_3 [i.e., D-Ins(1,3,4)P_3 and D-Ins(1,3,6)P_3] were also examined for their ability to release $^{45}Ca^{2+}$ from permeabilized platelets (Fig. 3, *center*). D-Ins(1,3,6)P_3 was a full agonist at the Ins(1,4,5)P_3-R and was found to be ~11-fold weaker at releasing $^{45}Ca^{2+}$ than D-Ins(1,4,5)P_3 (Fig. 3, *center*; Table 1). However, D-Ins(1,3,4)P_3 possessed very weak ability to release Ca^{2+}, with only 16.7 ± 4.1% of $^{45}Ca^{2+}$ released at the highest concentration of D-Ins(1,3,4)P_3 examined (100 μM) (Fig. 3, *center*; Table 1).

Another enantiomeric pair of Ins(1,4,5)P_3 regioisomers [i.e., D-Ins(3,4,6)P_3 and D-Ins(1,4,6)P_3] was also examined for ability to release $^{45}Ca^{2+}$ from the stores (Fig. 3, *bottom*). D-Ins(1,4,6)P_3 was a potent and full agonist at the Ins(1,4,5)P_3-R and found to be ~2-3-fold weaker than Ins(1,4,5)P_3 in releasing $^{45}Ca^{2+}$ from the intracellular stores (Fig. 3, *bottom*; Table 1). However, its enantiomer, D-Ins(3,4,6)P_3, displayed very weak Ca^{2+}-mobilizing activity, inducing only 28.8 ± 2.9% $^{45}Ca^{2+}$ release from permeabilized platelets at 100 μM (Fig. 3, *bottom*; Table 1).

Therefore, Ins(1,3,4,6)P_4, D-Ins(1,3,6)P_3, and D-Ins(1,4,6)P_3

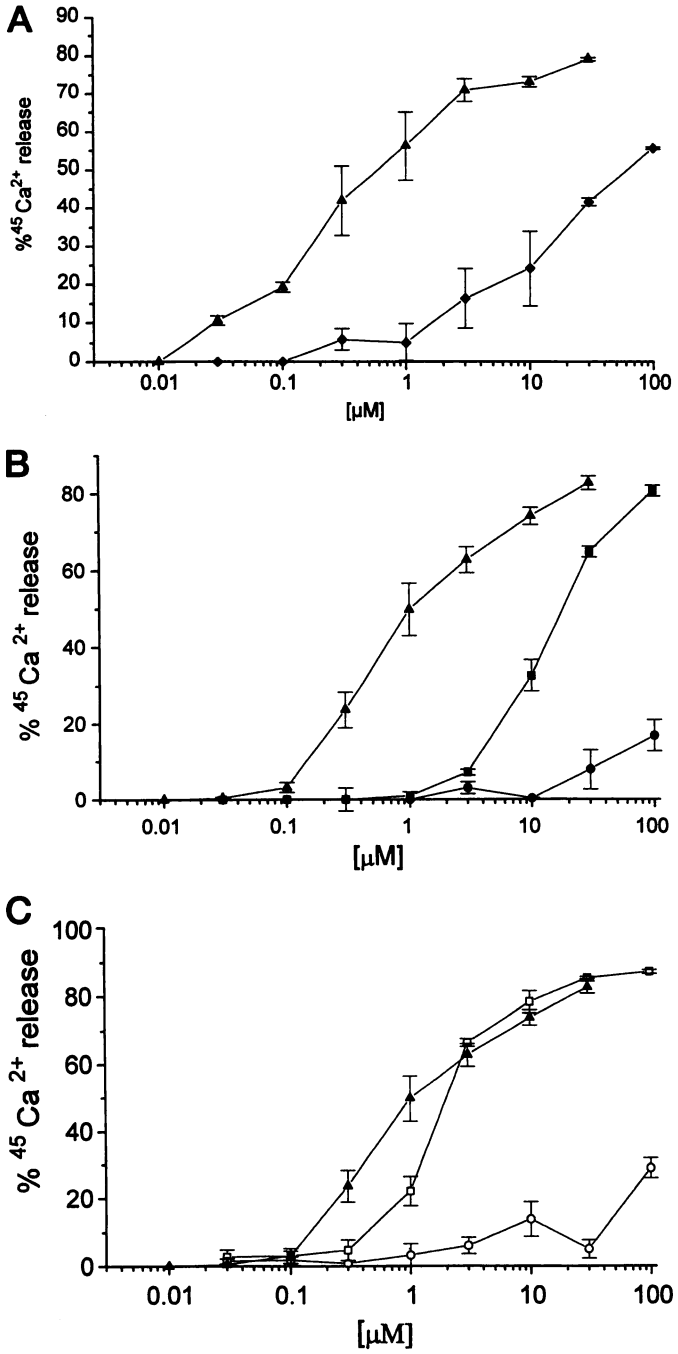

Fig. 3. $^{45}Ca^{2+}$ release from permeabilized platelets induced by Ins(1,4,5)P_3, Ins(1,3,4,6)P_4, D-Ins(1,3,4)P_3, D-Ins(1,3,6)P_3, D-Ins(1,4,6)P_3, and D-Ins(3,4,6)P_3. Permeabilized platelets preloaded with $^{45}Ca^{2+}$ were treated with (A) Ins(1,4,5)P_3 and Ins(1,3,4,6)P_4; (B) Ins(1,4,5)P_3, D-Ins(1,3,4)P_3, and D-Ins(1,3,6)P_3; or (C) Ins(1,4,5)P_3, D-Ins(1,4,6)P_3, and D-Ins(3,4,6)P_3 at 20° for 3 min. Release of $^{45}Ca^{2+}$ was terminated by rapid filtration and is given as a percentage of maximal $^{45}Ca^{2+}$ releasable on treatment of platelets with 75 μM ionomycin. Values are mean ± standard error of five separate experiments, each performed in triplicate. ▲, Ins(1,4,5)P_3; ♦, Ins(1,3,4,6)P_4; ■, D-Ins(1,3,6)P_3; ●, D-Ins(1,3,4)P_3; ○, D-Ins(3,4,6)P_3; and □, D-Ins(1,4,6)P_3.

were all able to release $^{45}Ca^{2+}$ from the intracellular stores of permeabilized platelets. In contrast, D-Ins(1,3,4)P_3 and D-Ins(3,4,6)P_3 displayed extremely weak Ca^{2+} release activity, even at high micromolar levels. This almost negligible Ca^{2+}-releasing activity displayed by D-Ins(1,3,4)P_3 and

TABLE 1

Comparison of inositol phosphates on $^{45}Ca^{2+}$ release and displacement of [^{3}H]Ins(1,4,5)P_3

Values are the mean ± standard error of 3 to 10 experiments.

Compound	$^{45}Ca^{2+}$ release (concentration causing 40% release)	Relative potency	Displacement of [^{3}H]Ins(1,4,5)P_3 binding (IC_{50})	Relative potency
	μM		μM	
Ins(1,4,5)P_3	0.69 ± 0.24	1	0.045 ± 0.01	1
Ins(1,3,4,6)P_4	28.50 ± 1.06	0.024	1.62 ± 0.39	0.028
D-Ins(1,3,6)P_3	8.05 ± 0.98	0.083	4.42 ± 1.29	0.010
D-Ins(1,3,4)P_3	>100		>30	
D-Ins(3,4,6)P_3	>100		>10	
D-Ins(1,4,6)P_3	1.56 ± 0.34	0.5	1.42 ± 0.34	0.031

D-Ins(3,4,6)P_3 could be due to contamination with <1% of their respective enantiomer from the optical resolution procedure used during the synthetic procedure.

The above findings were extended through examination of the kinetics of Ca^{2+} release of the chiral regioisomers of D-Ins(1,4,5)P_3; Ca^{2+} release was monitored in the presence of the fluorescent dye Fura-2 (free acid) by spectrophotofluorimetry. The addition of 1 μM D-Ins(1,4,5)P_3 caused release of Ca^{2+} from the intracellular stores of permeabilized platelets, detected as a rapid increase in the fluorescence of Fura-2-free acid (Fig. 4). The increase in fluorescence was transient, presumably due to metabolism of Ins(1,4,5)P_3 to give inactive products, resulting in resequestration of Ca^{2+} back into the intracellular stores by Ca^{2+}/ATPase activity. The addition of D-Ins(1,3,6)P_3 caused a dose-dependent release of Ca^{2+} from the intracellular stores. However, unlike the Ca^{2+} release of Ins(1,4,5)P_3, which was transient, Ca^{2+} release by D-Ins(1,3,6)P_3 reached a maximal level and was then maintained at a plateau phase over the time course of the experiment (Fig. 4A). In agreement with the findings for $^{45}Ca^{2+}$ release, D-Ins(1,3,4)P_3 seemed to be inactive, with no increase in fluorescence monitored at 30 μM D-Ins(1,3,4)P_3 (Fig. 4B).

The ability of both enantiomers of Ins(1,4,6)P_3 to release Ca^{2+} from permeabilized platelets was also monitored by spectrophotofluorimetry. D-Ins(1,4,6)P_3 caused a dose-dependent increase in Fura-2 fluorescence, indicating release of Ca^{2+} from the intracellular stores of permeabilized platelets. However, similar to D-Ins(1,3,6)P_3, the fluorescence signal was maintained at a plateau once it had reached a maximal level, indicating poor metabolism of D-Ins(1,4,6)P_3 and, therefore, sustained release of Ca^{2+} from the intracellular stores. In agreement with the $^{45}Ca^{2+}$-release studies, D-Ins(3,4,6)P_3 seemed to be inactive with no increase in fluorescence, and therefore no Ca^{2+} release was detected on the addition of 30 μM D-Ins(3,4,6)P_3 to the platelets (Fig. 4C).

The addition of 1 μM Ins(1,4,5)P_3 and 10 μM D-Ins(1,3,6)P_3 together demonstrated an additive effect, with an increased Ca^{2+} release compared with the use of either D-Ins(1,4,5)P_3 or D-Ins(1,3,6)P_3 alone. Importantly, the increase in fluorescence was more transient than that with the addition of D-Ins(1,3,6)P_3 alone but was maintained for a longer period than that with the addition of D-Ins(1,4,5)P_3 alone (Fig. 4D). This finding indicates that the maintained plateau on the addition of D-Ins(1,3,6)P_3 is the result of slower metabolism compared with D-Ins(1,4,5)P_3, as opposed to inhibition by

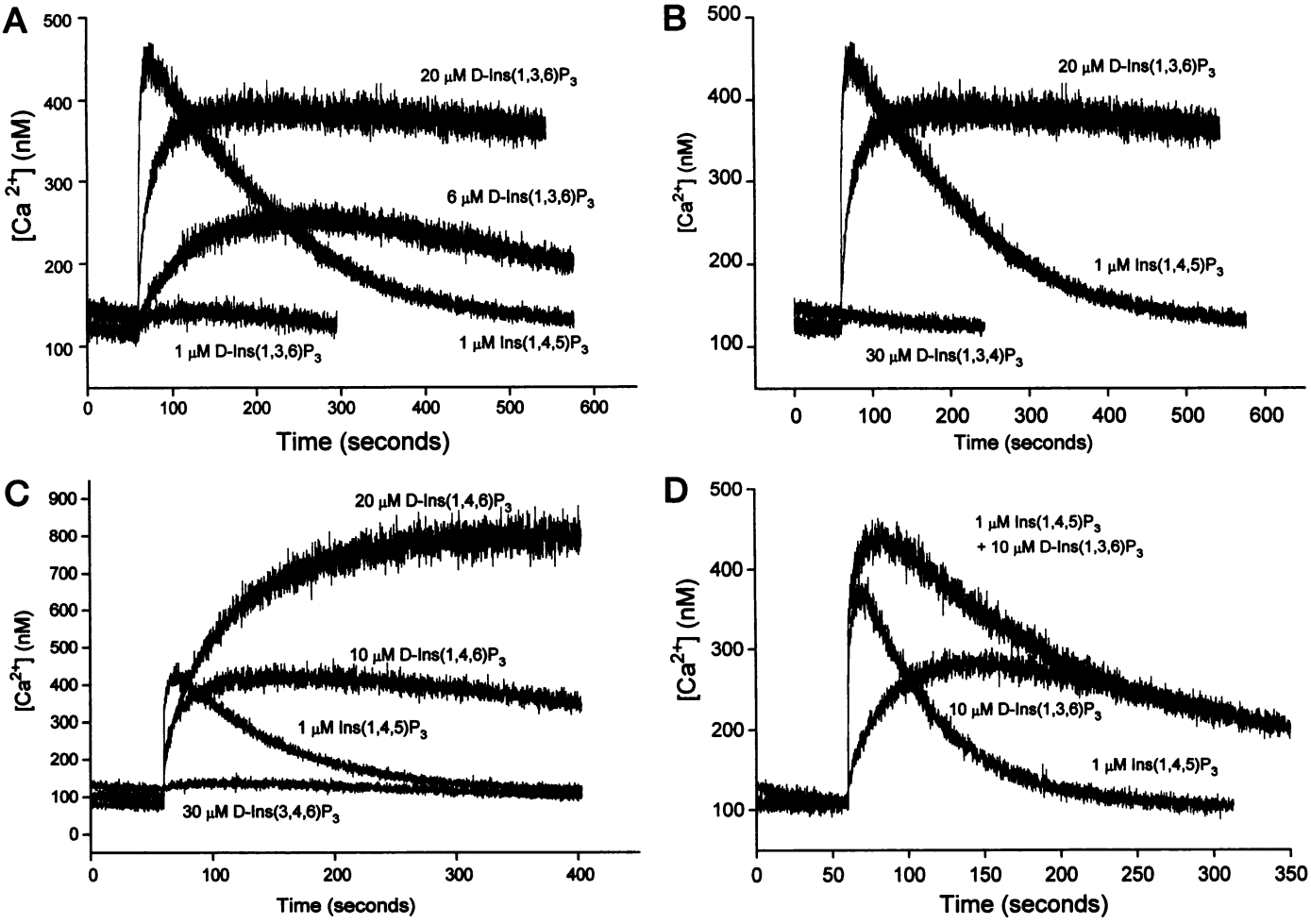

Fig. 4. Ca^{2+} mobilization induced by $Ins(1,4,5)P_3$, D-$Ins(1,3,4)P_3$, D-$Ins(1,3,6)P_3$, D-$Ins(1,4,6)P_3$, and D-$Ins(3,4,6)P_3$, as monitored by spectrophotofluorimetry. Release of Ca^{2+} from the intracellular stores of permeabilized platelets in the presence of Fura-2 (free acid) was monitored by spectrophotofluorimetry in platelets treated with (A) D-$Ins(1,3,6)P_3$, (B) D-$Ins(1,3,4)P_3$, (C) D-$Ins(1,4,6)P_3$, and D-$Ins(3,4,6)P_3$ and (D) $Ins(1,4,5)P_3$ added with D-$Ins(1,3,6)P_3$. *Traces*, fluorescent output from Fura-2 after conversion of fluorescence to Ca^{2+} [nM], taken from a single experiment but representative of four individual experiments.

D-$Ins(1,3,6)P_3$ of Ca^{2+}/ATPase activity, which is responsible for resequestration of Ca^{2+} into the stores.

Heparin has previously been demonstrated to bind at the $Ins(1,4,5)P_3$-R, competitively inhibiting the binding of D-$Ins(1,4,5)P_3$ and thus its ability to release Ca^{2+} from the intracellular stores. Heparin was found to inhibit D-$Ins(1,3,6)P_3$-induced Ca^{2+} release from permeabilized platelets, indicating that the effects of D-$Ins(1,3,6)P_3$ are also mediated by its binding to the $Ins(1,4,5)P_3$-R (results not shown).

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} value of 0.045 ± 0.01 μM (Fig. 5 and Table 1). $Ins(1,3,4,6)P_4$ also displaced specifically bound [^{3}H]$Ins(1,4,5)P_3$ from rat cerebellar membranes, although displacement by $Ins(1,3,4,6)P_4$ was 36-fold weaker than that by D-$Ins(1,4,5)P_3$ (Fig. 5, *top*).

Displacement of [^{3}H]$Ins(1,4,5)P_3$ from the binding sites on rat cerebellar membranes by D-$Ins(1,3,6)P_3$ was ~100-fold weaker than displacement by D-$Ins(1,4,5)P_3$. However, D-$Ins(1,3,4)P_3$ was able to only weakly displace [^{3}H]$Ins(1,4,5)P_3$, even at the highest concentration examined (30 μM) and with an IC_{50} value of >30 μM (Fig. 5, *center*). D-$Ins(1,4,6)P_3$ displaced [^{3}H]$Ins(1,4,5)P_3$ from its receptor, although it was ~30-fold weaker than D-$Ins(1,4,5)P_3$. D-$Ins(3,4,6)P_3$, however, was almost inactive at displacing [^{3}H]$Ins(1,4,5)P_3$, with an IC_{50} value that was greater than the highest concentration examined (10 μM) (Fig. 5, *bottom*).

Therefore, the ability of both enantiomers of $Ins(1,3,4)P_3$ and $Ins(1,4,6)P_3$ to displace [^{3}H]$Ins(1,4,5)P_3$ from specific binding sites on rat cerebellar membranes paralleled their ability (or lack of ability) to release $^{45}Ca^{2+}$ from the intracellular stores of permeabilized platelets.

Discussion

Structure-activity studies performed using $Ins(1,4,5)P_3$ analogs have indicated that the vicinal 4,5-bisphosphate configuration plays a key role in mediating Ca^{2+} release from intracellular stores (9, 21). The naturally occurring tetrakisphosphate, $Ins(1,3,4,6)P_4$, formed by the phosphorylation of D-$Ins(1,3,4)P_3$ by D-$Ins(1,3,4)P_3$-6-kinase and by the phosphorylation of D-$Ins(1,4,6)P_3$ by D-$Ins(1,4,6)P_3$-3-kinase has been identified in several cell types, including adrenal glomerulosa cells, macrophages, and platelets (22–24). The findings that $Ins(1,3,4,6)P_4$ could mobilize Ca^{2+} in permeabilized

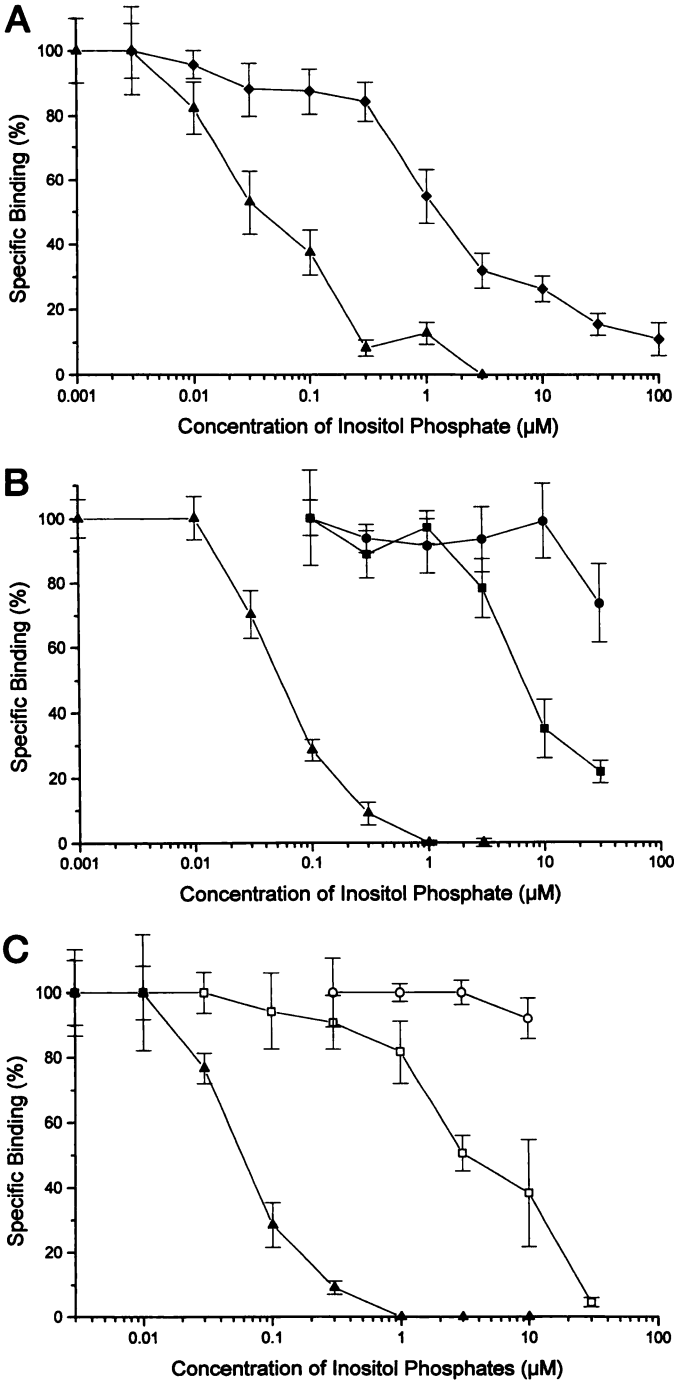

Fig. 5. Displacement of specific [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar membranes by (A) Ins(1,4,5)P_3 and Ins(1,3,4,6)P_4; (B) Ins(1,4,5)P_3, D-Ins(1,3,4)P_3, and D-Ins(1,3,6)P_3; and (C) Ins(1,4,5)P_3, D-Ins(1,4,6)P_3, and D-Ins(3,4,6)P_3. Values are mean ± standard error of three experiments, each performed in duplicate. ▲, Ins(1,4,5)P_3; ♦, Ins(1,3,4,6)P_4; ■, D-Ins(1,3,6)P_3; ●, D-Ins(1,3,4)P_3; ○, D-Ins(3,4,6)P_3; and □, D-Ins(1,4,6)P_3.

SH-SY5Y human neuroblastoma cells (11), *Xenopus laevis* oocytes (25), and rat brain microsomes (26) and displace [^{3}H]Ins(1,4,5)P_3 from specific binding sites on rat cerebellar membranes (11), despite the lack of a 4,5-bisphosphate binding motif, seemed initially to be at odds with the established basic structural requirements considered necessary for Ca^{2+} release at the Ins(1,4,5)P_3-R. The Ca^{2+}-releasing activity of Ins(1,3,4,6)P_4 was found to be ~50-fold less than that of Ins(1,4,5)P_3 in permeabilized SH-SY5Y human neuroblastoma cells, in which it was described as a partial agonist (11). In permeabilized rabbit platelets, however, we found Ins(1,3,4,6)P_4 to be ~40-fold less potent than Ins(1,4,5)P_3, although equally as efficacious. The reason that Ins(1,3,4,6)P_4 is a full agonist in rabbit platelets but only a partial agonist in SH-SY5Y cells is at present unclear, but it may be connected to differences in the relative abundance of the Ins(1,4,5)P_3-R subtypes possessed by the two cell types (27, 28) or to a difference in the affinity of the type I Ins(1,4,5)P_3-R to D-Ins(1,4,5)P_3 in neuronal compared with peripheral tissue (for a review, see Ref. 29).

How Ins(1,3,4,6)P_4 elicits Ca^{2+} release was rationalized by envisaging two alternative receptor binding orientations for Ins(1,3,4,6)P_4, in which the vicinal D-1,6-bisphosphate is presumed to mimic the normal D-4,5-bisphosphate of Ins(1,4,5)P_3 (Fig. 1, b and c). Furthermore, because 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. If these alternative binding orientations derived from Ins(1,3,4,6)P_4 are responsible for its Ca^{2+}-releasing activity, then it can be predicted that both D-Ins(1,4,6)P_3 and D-Ins(1,3,6)P_3 enantiomers, which possess a vicinal bisphosphate of the correct relative stereochemistry ("pseudo"-vicinal bisphosphate), should be able to bind to the Ins(1,4,5)P_3-R and possess Ca^{2+}-mobilizing activity (Fig. 1). It was also of interest to examine whether the enantiomers D-Ins(1,3,4)P_3 and D-Ins(3,4,6)P_3, which do not possess this motif but rather are similar to D-Ins(3,5,6)P_3 (alternative nomenclature, L-Ins(1,4,5)P_3), exhibit biological activity. As predicted, only D-Ins(1,4,6)P_3 and D-Ins(1,3,6)P_3 possessed the ability to release a significant amount of Ca^{2+} from permeabilized platelets and to displace [^{3}H]Ins(1,4,5)P_3 from its specific rat cerebellar binding site, whereas their enantiomers were inactive. This study, which is the first to examine a role for D-Ins(3,4,6)P_3, has therefore established the inability of this enantiomer to release Ca^{2+}.

Other structural requirements for Ca^{2+} release include an additional phosphate group, preferably 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 (7, 9). 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 a review, see Ref. 7). Neither the 2-OH nor the 3-OH seems to be particularly important for receptor recognition; however, modification at the 6-OH by *O*-methylation and dehydroxylation significantly reduced Ca^{2+}-releasing activity and receptor binding (10, 30). It could therefore be predicted that modification of the orientation of the OH groups at the 2 and 3 positions on the inositol ring might not have a significant effect on Ins(1,4,5)P_3 binding and activity, whereas modification at 6-OH would reduce Ins(1,4,5)P_3 binding and activity. The finding that D-Ins(1,4,6)P_3 is more potent at releasing Ca^{2+} and displacing [^{3}H]Ins(1,4,5)P_3 than D-Ins(1,3,6)P_3 conforms with these structural requirements. In D-Ins(1,4,6)P_3, the orientation of the 5-OH [which mimics the 6-OH of Ins(1,4,5)P_3] is equatorial, the same as in D-Ins(1,4,5)P_3; however, in D-Ins(1,3,6)P_3, the 2-OH [which

mimics the equatorial 6-OH of Ins(1,4,5)P_3] is axial and therefore different from that in D-Ins(1,4,5)P_3.

In an earlier study, which examined the ability of D-Ins(1,3,6)P_3 to release Ca^{2+} from permeabilized RBL cells, D-Ins(1,3,6)P_3 was found to be ~3000-fold weaker than Ins(1,4,5)P_3, but it was only 90-fold weaker at displacing [^{3}H]Ins(1,4,5)P_3 from specific binding sites on rat cerebellar membranes (31). Similar to that described above, we demonstrated displacement of [^{3}H]Ins(1,4,5)P_3 with D-Ins(1,3,6)P_3 in rat cerebellar membranes to be ~98-fold less efficient than Ins(1,4,5)P_3; however, in contrast, the ability of D-Ins(1,3,6)P_3 to release Ca^{2+} from the intracellular stores of permeabilized platelets was only 12-fold less efficient than Ins(1,4,5)P_3. There are several potential reasons for the anomaly in the potency of D-Ins(1,3,6)P_3 to release Ca^{2+} in RBL cells compared with platelets; one is that different preparations of D-Ins(1,3,6)P_3 were used in the two studies. However, the fact that D-Ins(1,3,6)P_3 is able to displace [^{3}H]Ins(1,4,5)P_3 from rat cerebellar membranes with an equal affinity in the two studies makes this unlikely. Alternatively, the Ca^{2+}-mobilizing effect of D-Ins(1,3,6)P_3 may be unique to platelets. However, again, this is unlikely because D-Ins(1,3,6)P_3 was able to mobilize Ca^{2+} when injected into *Limulus* photoreceptors (13). Another possibility is that the disparity is due to a difference between the Ins(1,4,5)P_3-R subtypes possessed by the two cell types. Platelets possess the type I Ins(1,4,5)P_3-R but not the type III (28). In contrast, RBL cells are known to possess predominantly type IV and some type V Ins(1,4,5)P_3-R (both of which are members of the subtype II family) while possessing very low amounts of Ins(1,4,5)P_3-R type I (32). The significance of Ins(1,4,5)P_3-R diversity has not been established. Clearly, however, selective expression of Ins(1,4,5)P_3-R subtypes will influence the action of an agonist at the receptor if the receptors (subtype I, II, or III) exhibit regulatory and/or functional differences. Indeed, phosphorylation of the Ins(1,4,5)P_3-R subtype I (but not II or III) by cAMP-dependent kinase that results in a modification in sensitivity is one well-described regulatory difference between the receptors (33, 34). Whether there are functional differences between the receptor subtypes is more contentious, with some studies describing differences in Ins(1,4,5)P_3 affinity among receptor subtypes (35, 36) and other studies reporting that type I, II, and III receptors bind Ins(1,4,5)P_3 with very similar characteristics (3, 37). A greater understanding of the functional and regulatory characteristics that distinguish the Ins(1,4,5)P_3-R subtypes is therefore required before differences in Ca^{2+} homeostasis among cell types can be categorically attributed to their Ins(1,4,5)P_3-R subtype diversity.

Because of the central position of D-Ins(1,3,4)P_3 in inositol phosphate metabolism (just two metabolic steps removed from Ins(1,4,5)P_3), interest has surrounded a possible role for this inositol phosphate in the regulation of Ca^{2+} homeostasis; many conflicting reports have surrounded the potential biological role of D-Ins(1,3,4)P_3 (30, 38–40). The unambiguous total synthesis of enantiomers of Ins(1,3,4)P_3 allowed us to demonstrate unequivocally that D-Ins(1,3,6)P_3 and not D-Ins(1,3,4)P_3 is able to bind to the Ins(1,4,5)P_3-R and release Ca^{2+}. This is in agreement with preliminary findings in which a comparison was made of the effects of these two enantiomers microinjected into *Limulus* ventral photoreceptors. D-Ins(1,3,6)P_3 induced a rapid burst of depolarization due to Ca^{2+} release that was not observed on injection with D-Ins(1,3,4)P_3 (13). D-Ins(1,3,6)P_3 caused a sustained release of Ca^{2+} from the intracellular stores of platelets, which correlates with the repetitive bursts of depolarization observed on injection of D-Ins(1,3,6)P_3 into *Limulus* ventral photoreceptors and is in contrast to the single burst observed with D-Ins(1,4,5)P_3, thus indicating poor metabolism of D-Ins(1,3,6)P_3. It is possible that previously conflicting results regarding the role of D-Ins(1,3,4)P_3 were the result of contamination of D-Ins(1,3,4)P_3 with either D-Ins(1,4,5)P_3 or D-Ins(1,3,6)P_3.

Whether D-Ins(1,3,6)P_3 or D-Ins(1,4,6)P_3 has any physiological significance remains unknown, although both have been identified in extracts of avian erythrocytes, albeit as minor constituents of the inositol trisphosphate pool (41). Certainly, Ins(1,3,4,6)P_4, which may be a precursor of these inositol phosphates (24), does accumulate in some cells after agonist stimulation (22, 23, 42), although the cellular concentration of Ins(1,3,4,6)P_4 seems to be relatively small even when levels of D-Ins(1,3,4)P_3 are significantly elevated (23, 42). A physiological role for Ins(1,3,4,6)P_4 is nevertheless a possibility. Indeed, it was suggested that because Ins(1,3,4,6)P_4 remains elevated for several hours during prolonged stimulation of adrenal glomerulosa cells with angiotensin II, it may have a role in keeping Ca^{2+} stores at a certain capacity (22). The finding that D-Ins(1,3,4)P_3-6-kinase [responsible for Ins(1,3,4,6)P_4 formation from Ins(1,3,4)P_3] is regulated by Ca^{2+}-, cAMP-, and phosphokinase C-dependent mechanisms (22, 23) suggests tight regulation of the formation of higher inositol phosphates, and Ins(1,3,4,6)P_4 may simply be an intermediate in their formation (22).

In conclusion, our findings that both D-Ins(1,3,6)P_3 and D-Ins(1,4,6)P_3, but not their enantiomers, were able to release Ca^{2+} from permeabilized platelets and displace [^{3}H]Ins(1,4,5)P_3 from specific binding sites on rat cerebellar membranes confirmed that the Ca^{2+}-release activity of Ins(1,3,4,6)P_4 is due to the mimicking by vicinal D-1,6-bisphosphate of the D-4,5-bisphosphate of Ins(1,4,5)P_3. The findings of this study also demonstrate that D-Ins(1,4,6)P_3 is a surprisingly potent agonist at the Ins(1,4,5)P_3-R. The unambiguous total synthesis of enantiomers of Ins(1,3,4)P_3 allowed us to demonstrate unequivocally that D-Ins(1,3,4)P_3 is unable to release Ca^{2+} from the intracellular Ca^{2+} storage sites and cannot displace [^{3}H]Ins(1,4,5)P_3 from its receptor. The results from this work are in contrast to the findings of Hirata *et al.* (31), who reported that an almost negligible release of Ca^{2+} was observed from RBL cells treated with D-Ins(1,3,6)P_3. This disparity in findings between platelets and RBL cells opens up the possibility that differences in the subtypes of Ins(1,4,5)P_3-R possessed by the two cell types may in some way, because of functional or regulatory differences, be contributing to the observed differences in Ca^{2+} release.

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