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Inositol Tetrakisphosphate Mobilizes Calcium from Cerebellum Microsomes

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SUMMARY

Ca^{2+} accumulated by rat cerebellum microsomes in the presence of MgATP was released by added inositol tetrakisphosphate [Ins(1,3,4,5)P_4]. The concentrations of D-*myo*-inositol-1,4,5-trisphosphate [D-Ins(1,4,5)P_3], D-Ins(1,3,4,5)P_4, and DL-Ins(1,3,4,5)P_4 required for half-maximal release were 0.15, 4.6, and 7.5 μM, respectively. Maximal concentrations of $InsP_4$ released only 70% of the Ca^{2+} released by maximal concentrations of Ins(1,4,5)P_3. Inositol pentakisphosphate and D-*myo*-inositol-1,3,4-trisphosphate were relatively inactive. Additional Ca^{2+} was released when Ins(1,4,5)P_3 (or a nonhydrolyzable analog) was added after completion of $InsP_4$-mediated Ca^{2+} release but not when this sequence of additions was reversed. This indicates that $InsP_4$ releases Ca^{2+} from part of the $InsP_3$-releasable compartment. No evidence for synergism between $InsP_4$ and $InsP_3$ was obtained and responses to suboptimal concentrations of both inositol phosphates were approximately additive. Heparin was a potent inhibitor of $InsP_4$-mediated Ca^{2+} release. Inhibition by heparin was competitive with respect to $InsP_4$ concentration and the K_i for heparin was 0.6 μg/ml (approximately 100 nM), irrespective of whether $InsP_4$ or $InsP_3$ was used as an agonist. A 3-phosphatase capable of converting [^{3}H]Ins(1,3,4,5)P_3 to [^{3}H]Ins(1,4,5)P_3 could not be detected in cerebellum microsomes. Hence, we conclude that, *in vitro*, receptors capable of recognizing Ins(1,3,4,5)P_4 can also be coupled directly to a Ca^{2+} release system.

Ins(1,4,5)P_3 is the messenger molecule responsible for Ca^{2+} mobilization from intracellular stores during agonist stimulation of many cell types (1, 2). Metabolism of this molecule is known to proceed via two alternative pathways involving either the hydrolysis of the 5-phosphate to generate Ins(1,4)P_2 or phosphorylation of the 3-position to yield Ins(1,3,4,5)P_4 (3). The formation of Ins(1,3,4,5)P_4 in stimulated cells has attracted much interest since the proposal that this molecule could also have a messenger role in stimulating the entry of Ca^{2+} across the plasma membrane. A model has been proposed in which Ins(1,3,4,5)P_4 functions to activate plasma membrane Ca^{2+} flux directly into an Ins(1,4,5)P_3-sensitive store (3). Evidence consistent with this proposal was obtained in experiments performed with mouse lacrimal cells (4) and sea urchin eggs (5). However, data inconsistent with this model have also been reported (6, 7). In voltage-clamped neuronal cells, Ins(1,3,4,5)P_4 microinjection activated an inward monovalent ion current (8). The microinjection of high concentrations of Ins(1,3,4,5)P_4 into *Xenopus* oocytes elicited a Ca^{2+}-dependent Cl^- current, even in the absence of extracellular Ca^{2+} (9, 10). These data, and direct observations using fura-2 in sea urchin eggs (6), suggest that Ins(1,3,4,5)P_4 may be capable of mobilizing intracellular Ca^{2+} stores.

Worley *et al.* (11) have demonstrated that rat cerebellum contains a very high density of [^{3}H]Ins(1,4,5)P_3 binding sites. Further studies revealed that this brain region was also rich in [^{3}H]Ins(1,3,4,5)P_4 binding sites, which could be experimentally distinguished from the [^{3}H]Ins(1,4,5)P_3 binding sites (12). Binding sites for [^{3}H]Ins(1,3,4,5)P_4 have also been studied in membranes derived from HL-60 cells (13) and adrenal cortex (14). Microsomal vesicles prepared from rat cerebellum contain an active Ca^{2+} release system that responds to low concentrations of Ins(1,4,5)P_3 (15, 16). In the present study, we report that this experimental system also releases Ca^{2+} in response to Ins(1,3,4,5)P_4. Our data indicate that an Ins(1,3,4,5)P_4 receptor in these membranes may also be coupled to a Ca^{2+} release mechanism.

Materials and Methods

Microsomes were prepared from cerebellum dissected from rats. The isolation buffer contained 0.32 M sucrose, 10 mM Tris/HEPES (pH

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ABBREVIATIONS: Ins(1,4,5)P_3, D-myo-inositol-1,4,5-trisphosphate; Ins(1,3,4,5)P_4, D-myo-inositol-1-3,4,5-tetrakisphosphate; Ins(1,4)P_2, D-myo-inositol-1,4-bisphosphate; Ins(1,3,4)P_3, D-myo-inositol 1,3,4-trisphosphate; Ins(1,3,4,5,6)P_5, myo-inositol-1,3,4,5,6-pentakisphosphate; GPIP$_2$, glycerophosphoinositol-4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, [ethylenebisoxyethylenenitrilo)]tetracetic acid; HPLC, high pressure liquid chromatography.

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7.6), and 10 μM EGTA. Routinely, cerebellum from four rats were homogenized with a Teflon-glass homogenizer (five strokes) to give a 1 g of wet weight/20 ml suspension. This was centrifuged at 10,000 × *g* for 20 min and the supernatant was centrifuged at 100,000 × *g* for 45 min. The pellet was resuspended in isolation buffer to a final volume of 1 ml/g of starting tissue.

Ca^{2+} fluxes were measured with a Ca^{2+}-sensitive minielectrode prepared with the neutral ionophore resin ETH-1001 (Glasblaserei, Zurich, Switzerland). Measurements were made in a final volume of 0.2 ml, using a medium (30°) containing 120 mM KCl, 20 mM Tris/HEPES (pH 7.2), 0.3 mM $MgCl_2$, 1 mM MgATP, 10 mM phosphocreatine, 10 units/ml creatine kinase, 3.75 μM antimycin A, and 1 mg/ml microsomal protein. The microsomes were allowed to accumulate endogenous Ca^{2+} (1–2 μM) and additions of inositol phosphates were made to the microsomes as they approached a steady state with respect to Ca^{2+} fluxes. The quantity of Ca^{2+} released was calculated from the electrode response to an internal addition of Ca^{2+} that was made at the end of each trace. The free Ca^{2+} concentration of the medium was determined by comparison with standard buffers, prepared according to the method of Tsien and Rink (17). $^{45}Ca^{2+}$ fluxes were studied in the same medium as used for the Ca^{2+} electrode but containing, in addition, 0.5 mM EGTA and 0.2 mM $CaCl_2$ to generate a free calcium concentration of 140–160 nM, as measured directly with a Ca^{2+}-sensitive electrode. This medium was supplemented with $^{45}Ca^{2+}$ (1 μCi/ml; Amersham). Incubations were performed at 30° for 20 min with 0.5 mg of microsomal protein/ml. Intravesicular Ca^{2+} contents before and after inositol phosphate additions were determined with a filtration assay (0.45-μm filter; Millipore) and a wash buffer containing 120 mM KCl and 20 mM Tris/HEPES (pH 7.2) (15).

Metabolism of D-Ins(1,3,4,5)P_4 was followed in a medium containing 120 mM KCl, 20 mM Tris/HEPES (pH 7.2), 0.3 mM $MgCl_2$, 0.5 mM EGTA, 0.2 mM $CaCl_2$, 1 mg/ml microsomal protein, 0.1 μCi/ml [^{3}H]D-Ins(1,3,4,5)P_4, and 1 μM unlabeled D-Ins(1,3,4,5)P_4. The incubation was performed at 30° and quenched at various times with perchloric acid, as described previously (18). Neutralized extracts were analyzed by HPLC using a Partisil SAX column. The mobile phase for elution of the inositol phosphates was ammonium formate (brought to pH 3.7 with H_3PO_4). The protocol used involved an isocratic elution at 1.2 ml/min for 6 min with 0.77 M ammonium formate, a further 12 min with 1.1 M ammonium formate, and 7 min with 1.98 M ammonium formate. Under these conditions, authentic commercially available standards of Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4 eluted at 10, 22, 25, and 34 min, respectively. Fractions of 0.6 ml were mixed with 2 ml of a scintillation cocktail (TruCount; INUS Corporation, Fairfield, NJ) and counted.

D-Ins(1,4,5)P_3 was from Behring Diagnostics. D-Ins(1,3,4,5)P_4 was prepared from D-Ins(1,4,5)P_3 using purified 3-kinase and chromatographic procedures as described in Ref. 19. Chemically synthesized DL-Ins(1,3,4,5)P_4 was a kind gift of Dr. James Meeks, Dupont, Wilmington, DE. Ins(1,3,4,5,6)P_5 was from Calbiochem. The concentrations of all stock solutions of inositol phosphates used in this study were determined by measurement of phosphorus, using an ashing procedure (20).

Results

Dose responses and relationships between Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-sensitive stores. Microsomal vesicles from rat cerebellum actively accumulate Ca^{2+} when incubated in the presence of MgATP. Fig. 1 (*trace A*) shows that the addition of D-Ins(1,3,4,5)P_4 triggers a rapid release of Ca^{2+}. Under identical conditions, a maximal concentration of Ins(1,3,4,5)P_4 released a smaller amount of Ca^{2+} than maximal concentrations of Ins(1,4,5)P_3 (Fig. 1, *trace A* versus *trace B*). Microsomal vesicles prepared from cerebral cortex also responded to Ins(1,3,4,5)P_4 in a similar manner (data not shown). The possibility that contamination of Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3 accounts for the Ca^{2+} mobilization observed with

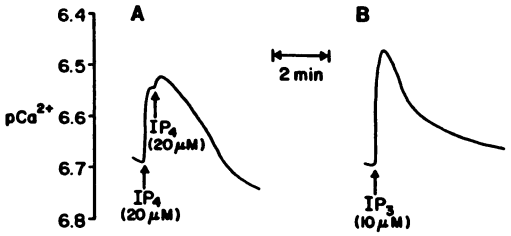

Fig. 1. Mobilization of Ca^{2+} from cerebellum microsomes by Ins(1,3,4,5)P_4. Rat cerebellum microsomes were incubated, in a final volume of 0.2 ml, at a concentration of 1 mg of protein/ml, in the medium described for Ca^{2+} electrode studies (see Materials and Methods). The microsomes were allowed to accumulate endogenous Ca^{2+} in the medium in the presence of MgATP and were then challenged with the indicated concentrations of Ins(1,4,5)P_3 (*IP*$_3$) or D-Ins(1,3,4,5)P_4 (*IP*$_4$). The records are representative of three experiments.

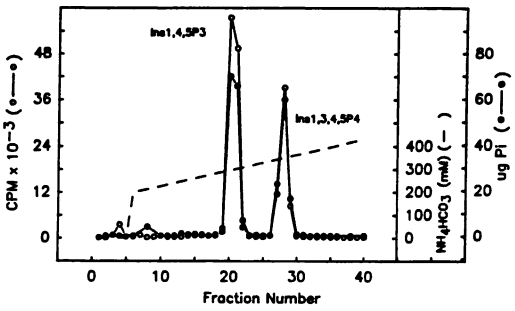

Fig. 2. Separation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 on QAE-Sepharose. A 1.6 × 32 cm column of QAE-Sepharose was washed with 5 column volumes of 1 M ammonium bicarbonate, followed by equilibration with H_2O. A 10-ml sample containing 1 mg of Ins(1,4,5)P_3, 0.5 mg of Ins(1,3,4,5)P_4, 100,000 cpm of [^{3}H]Ins(1,4,5)P_3, and 60,000 cpm of [^{3}H]Ins(1,3,4,5)P_4 was applied to the column and the column was eluted with a 350-ml gradient from 200 to 500 mM ammonium bicarbonate. The flow rate was 1 ml/min and 6.5-ml fractions were collected and assayed for radioactivity and phosphate content.

Ins(1,3,4,5)P_4 can be excluded for the following reasons. 1) Ca^{2+} mobilization was observed with five separate batches of Ins(1,3,4,5)P_4. These were prepared using 3-kinase to convert added Ins(1,3,4,5)P_3 to Ins(1,3,4,5)P_4, followed by chromatographic separation of the Ins(1,3,4,5)P_4 on a QAE-Sepharose column eluted with an ammonium bicarbonate gradient (19). The incubation conditions used result in >95% conversion of added Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 (data not shown). Fig. 2 shows the elution profile resulting from the separation of 1 mg of [^{3}H]Ins(1,4,5)P_3 and 0.5 mg of [^{3}H]Ins(1,3,4,5)P_4, using the same QAE-Sepharose column and gradient conditions employed to prepare the batches of Ins(1,3,4,5)P_4 used in this study. It is clear that the two inositol phosphates are completely resolved from each other, even when the mixture contained Ins(1,4,5)P_3 in excess over Ins(1,3,4,5)P_4. Hence, we conclude that there is no detectable contamination of the enzymically prepared Ins(1,3,4,5)P_4 when purified as described in Fig. 2. 2) One batch of Ins(1,3,4,5)P_4 obtained from the QAE-Sepharose column was rechromatographed on a Hamilton PRP X-100 HPLC column and eluted with an ammonium bicarbonate gradient. The effectiveness of the rechromatographed Ins(1,3,4,5)P_4 in the Ca^{2+} release assay was the same as the original Ins(1,3,4,5)P_4 (data not shown). 3) Ins(1,3,4,5)P_4 obtained from Dr. Peter Bradford (Hahnemann University, Philadelphia, PA) and prepared and purified by Dr. Robin Irvine (AFRC Institute, Cambridge, UK) also mediated Ca^{2+} release. 4) The ability to release Ca^{2+} was also noted with a racemic

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mixture of Ins(1,3,4,5)P_4 (Fig. 3) that had been chemically synthesized and also shown to be free of D-Ins(1,4,5)P_3 (21).

The dose dependency of Ca^{2+} release on inositol phosphate concentration was further explored using cerebellum microsomes (Fig. 3). As documented previously, the Ca^{2+} release system in cerebellum microsomes retains a high sensitivity to Ins(1,4,5)P_3 (EC_{50} = 0.18 μM) and this can be correlated with the high density of Ins(1,4,5)P_3 receptors found in this region of the brain (15, 22). In contrast, half-maximal release required 4.6 μM D-Ins(1,3,4,5)P_4 or 7.5 μM DL-Ins(1,3,4,5)P_4. The lower sensitivity to DL-Ins(1,3,4,5)P_4 would be anticipated if the L-form of Ins(1,3,4,5)P_4 was inactive in releasing Ca^{2+}. Ins(1,3,4,5,6)P_5 and D-Ins(1,3,4)P_3 (data not shown) were much less effective in releasing Ca^{2+} from cerebellum microsomes. In agreement with the data shown in Fig. 1, maximal concentrations of D- or DL-Ins(1,3,4,5)P_4 were able to mobilize only 70% of the amount of Ca^{2+} released by maximal concentrations of Ins(1,4,5)P_3.

The relationship between the Ca^{2+} pools released by Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 was further explored in the experiments shown in Fig. 4. These experiments were performed using $GPIP_2$, a poorly metabolized analog of Ins(1,4,5)P_3 (23, 24). The addition of this compound elicited Ca^{2+} release when added after a maximal dose of Ins(1,3,4,5)P_4 (Fig. 4). The addition of $GPIP_2$ alone released an amount of Ca^{2+} that was approximately equal to that released by the sequential addition of Ins(1,3,4,5)P_4 and $GPIP_2$. Ins(1,3,4,5)P_4 was without effect when added after $GPIP_2$. Similar data were obtained when Ins(1,3,4,5)P_4 was added at the peak of the Ca^{2+} release response obtained with 10 μM Ins(1,4,5)P_3 (data not shown). These data indicate that the Ins(1,3,4,5)P_4-releasable compartment is a part of the same Ca^{2+} pool that is released by Ins(1,4,5)P_3.

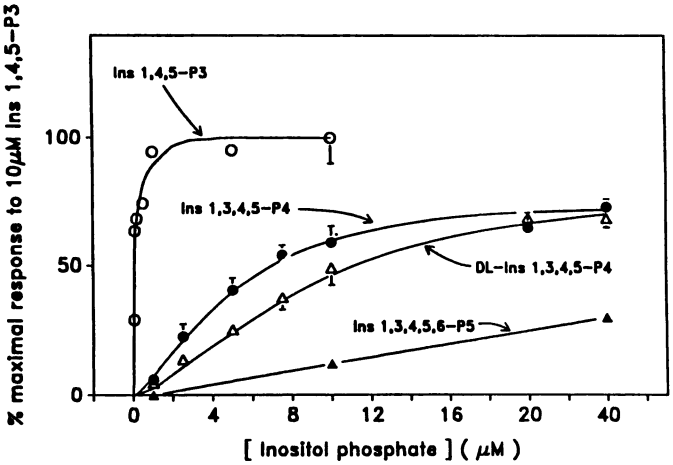

Fig. 3. Inositol phosphate concentration dependence of Ca^{2+} mobilization. Ca^{2+} release in response to increasing concentrations of added inositol phosphates was measured with a Ca^{2+}-sensitive minielectrode, using the procedures described in the text. The amount of Ca^{2+} released by low concentrations of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 was the same, whether the inositol phosphates were added sequentially in one experiment or individually in separate experiments. A 'desensitization' phenomenon was observed when high concentrations were used; therefore, Ca^{2+} release was evaluated from separate incubations. For each set of measurements, Ca^{2+} release was normalized to that obtained with 10 μM Ins(1,4,5)P_3. In seven experiments, this was 1.7 ± 0.1 nmol of Ca^{2+} mg of protein. The data shown for D-Ins(1,3,4,5)P_4 and DL-Ins(1,3,4,5)P_4 are the mean ± standard error of measurements made on three to five separate microsomal preparations.

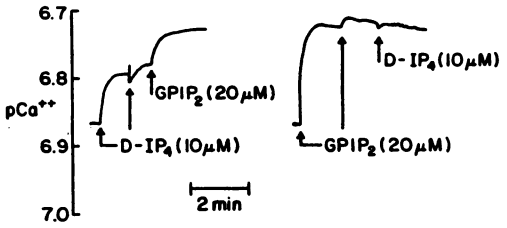

Fig. 4. Additivity of responses to Ins(1,3,4,5)P_4 and $GPIP_2$. Ca^{2+} release was measured with a Ca^{2+}-sensitive electrode, as determined in Fig. 1. In *trace a*, the cerebellum microsomes were challenged with maximal doses of D-Ins(1,3,4,5)P_4 (*D-IP*$_4$), followed by maximal concentrations of $GPIP_2$, a poorly hydrolyzable analog of Ins(1,4,5)P_3. In *trace b*, the sequence of additions were reversed.

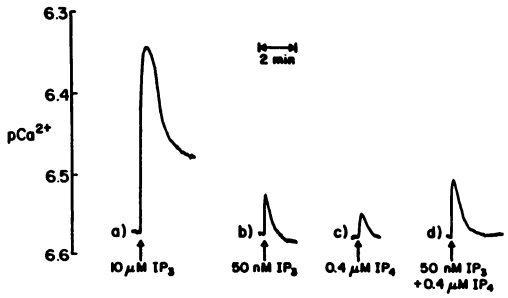

Fig. 5. Additivity of responses to suboptimal concentrations of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. The response of cerebellum microsomes to the indicated concentrations of Ins(1,4,5)P_3 (*IP*$_3$) and Ins(1,3,4,5)$_4$ (*IP*$_4$) was measured with a Ca^{2+}-sensitive electrode, as described in Materials and Methods. Representative traces are shown.

Ins(1,3,4,5)P_4 has been reported to act synergistically with Ins(1,4,5)P_3 in producing effects on ion currents in mouse lacrimal cells (4) and *Xenopus* oocytes (9). In addition, Ins(1,3,4,5)P_4 was reported to stimulate Ins(1,4,5)P_3-mediated Ca^{2+} release from pituitary microsomes while being inactive by itself (25). Experiments were, therefore, performed to measure Ca^{2+} release in response to suboptimal concentrations of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, added separately or together (Fig. 5). The data provided no evidence for synergism in the action of these two inositol phosphates. Indeed, the Ca^{2+} released by addition of suboptimal concentrations of both compounds were approximately additive; Ins(1,4,5)P_3 (0.275 μM), Ins(1,3,4,5)P_4 (5 μM), or both inositol phosphates added together released 1.06 ± 0.16, 0.64 ± 0.08, and 1.38 ± 0.17 nmol of Ca^{2+}/mg of protein, respectively. The maximal response to Ins(1,4,5)P_3 (10 μM) was 1.72 ± 0.19 nmol of Ca^{2+}/mg of protein (mean ± SE of results on three separate preparations).

The nature of the compartment that responds to Ins(1,3,4,5)P_4 in cerebellum microsomes is unknown. Ins(1,4,5)P_3 has been shown to release Ca^{2+} from platelet plasma membrane vesicles that have been loaded with $^{45}Ca^{2+}$ via a Na^+/Ca^{2+} exchange (26). In an attempt to address the issue of whether the Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-sensitive Ca^{2+} stores were functionally separate, we examined the responses to inositol polyphosphates under conditions where plasma membrane vesicles would be expected to be selectively depleted, due to the operation of a Na^+/Ca^{2+} exchange. Vesicles containing a Na^+/Ca^{2+} exchange mechanism would not be expected to accumulate Ca^{2+} in the presence of ATP when incubated in a Na^+-containing medium and would, therefore, not release Ca^{2+} in response to inositol phosphates. Incubation of cerebellum microsomes in a Na^+-containing medium mark-

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edly reduced the amount of Ca^{2+} accumulated in the presence of MgATP (Table 1). A small reduction in the amount of Ca^{2+} released by Ins(1,4,5)P_3 was also noted under these conditions. However, the ratio of Ca^{2+} released by Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 was the same in a K^+ or a Na^+-containing medium. This result indicates that both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 release Ca^{2+} predominantly from a compartment that does not contain a Na^+/Ca^{2+} exchange mechanism.

Metabolism of Ins(1,3,4,5)P_4. The metabolism of Ins(1,3,4,5)P_4 is known to occur by a pathway involving the hydrolysis of the 5-phosphate to produce Ins(1,3,4)P_3. More recently, several studies have documented the conversion of Ins(1,3,4,5)P_4 into Ins(1,4,5)P_3 under particular experimental conditions. This pathway has been observed in membrane fractions of erythrocytes in the absence of Mg^{2+} (27), membrane fractions of rat basophilic leukaemic cells (RBL-2H3) in the presence of ATP (28), and the soluble fraction of bovine brain (29). Removal of the 3-phosphate from Ins(1,3,4,5)P_4 by an enzyme in cerebellum microsomes could potentially explain the Ca^{2+} release observed with this compound. The metabolic products formed from [^{3}H]Ins(1,3,4,5)P_4 were identified using an HPLC method that fully resolves both isomers of inositol trisphosphate (Fig. 6A). Microsomal membranes metabolized [^{3}H]Ins(1,3,4,5)P_4 solely to [^{3}H]Ins(1,3,4)P_3, with no detectable formation of [^{3}H]Ins(1,4,5)P_3 (Fig. 6, C and D). The membrane also did not produce any Ins(1,4)P_2 or inositol-4-monophosphate, excluding the possibility that any [^{3}H]Ins(1,4,5)P_3 formed could have been rapidly dephosphorylated (data not shown). The experiment shown in Fig. 6 was repeated using a range of concentrations of Ins(1,3,4,5)P_4 (0.2–10 μM), in the presence or absence of MgATP (1 mM). Ins(1,4,5)P_3 formation was never observed. The results support the view that Ins(1,3,4,5)P_4 exerts its effects by interacting with a receptor and not as a result of its metabolism to Ins(1,4,5)P_3.

Effect of heparin. Heparin has been shown to be a potent inhibitor of [^{3}H]Ins(1,4,5)P_3 binding to cerebellum microsomes (11) and to block Ins(1,4,5)P_3-mediated Ca^{2+} release in this (15) and other (30–33) experimental systems. Fig. 7 illustrates the typical biphasic pattern of Ca^{2+} release observed upon addition of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 to cerebellum microsomes. The presence of 20 μg/ml porcine heparin completely blocked responses to Ins(1,3,4,5)P_4 and partially inhibited responses to 10 μM Ins(1,4,5)P_3 (Fig. 7). The dependence of this inhibitory effect on the heparin concentration is shown in Fig. 8. In this series of experiments, the concentration of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were held at values of 2 and 40 μM, respectively, which correspond to concentrations that are approximately 10-fold higher than the half-maximally effective concentrations (Fig. 3). Under these conditions, heparin was a more potent inhibitor of responses to Ins(1,3,4,5)P_4 than Ins(1,4,5)P_3, with half-maximal inhibition (IC_{50}) being observed at 4.5 and 8 μg of heparin/ml, respectively. Complete inhibition of Ins(1,4,5)P_3 responses required over 80 μg of heparin/ml, whereas Ins(1,3,4,5)P_4 responses were maximally inhibited at 20 μg of heparin/ml (Fig. 8). When the concentration of Ins(1,3,4,5)P_4 used was decreased to 5 μM, the IC_{50} for heparin decreased to 1.5 μg/ml (data not shown).

In Fig. 9A, the Ca^{2+} release response to increasing concentrations of D-Ins(1,3,4,5)P_4 was measured in the presence or absence of 2 μg/ml heparin. The data show that the inhibitory effect of heparin diminished as the concentration of Ins(1,3,4,5)P_4 was increased. A double-reciprocal plot of these data yields a pattern that is characteristic of competitive inhibition (Fig. 9B). Previous studies using microsomes from smooth-muscle (33) or brain (15) have shown that heparin is also a competitive inhibitor of Ins(1,4,5)P_3-mediated release. An apparent K_i value of 0.59 μg/ml was derived from the intercepts of the x-axis in Fig. 9B. Assuming a M_r of 6000 for porcine heparin, the measured K_i corresponds to a concentration of 100 nM. Values for the K_i were also obtained from the data in Fig. 8, using the relationship $K_i = IC_{50}/(1 + S/EC_{50})$ (Ref. 34) where S is the concentration of inositol phosphate and EC_{50} values were taken from Fig. 3. With Ins(1,3,4,5)P_4 as the agonist, a K_i value of 0.71 μg of heparin/ml (118 nM) was obtained, which is in reasonable agreement with the value obtained from Fig. 9B. A K_i of 0.61 μg/ml (102 nM) was calculated for the heparin inhibition of Ins(1,4,5)P_3 responses, using the data in Fig. 8 and a value of 0.18 μM for the EC_{50}. This is higher then the reported K_i of 23 nM for heparin inhibition of Ins(1,4,5)P_3-mediated Ca^{2+} release from smooth muscle microsomes (33). Our data indicate that the affinity of heparin for its putative site(s) of action is, within experimental error, independent of whether Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 is used to mobilize Ca^{2+}.

TABLE 1

Influence of a Na^+ containing medium on inositol phosphate-induced Ca^{2+} release from brain microsomes

Microsomes (1 mg of protein/ml) were incubated in the medium described in Materials and Methods for studying $^{45}Ca^{2+}$ fluxes, containing either 120 mM KCl or NaCl. Ca^{2+} uptake in either medium reached a steady state in 20 min and the total intravesicular Ca^{2+} was measured after addition of 2 μM A23187. Ca^{2+} release was measured over a 15–30 sec interval, after addition of the inositol phosphates to microsomes that had been incubated for 20 min. The data shown are the mean ± standard error of experiments on three separate microsomal preparations.

		Ca^{2+} flux	
		K^+ Medium	Na^+ Medium
		nmol of $^{45}Ca^{2+}$/mg of protein	
Ca^{2+} uptake		7.99 ± 0.52	3.89 ± 0.31
Ca^{2+} release			
Ins(1,4,5)P_3	(10 μM)	1.57 ± 0.06	1.13 ± 0.05
DL-Ins(1,3,4,5)P_4	(20 μM)	1.23 ± 0.07	0.86 ± 0.06

Discussion

Our results indicate that Ins(1,3,4,5)P_4 can act as a weak partial agonist in mediating Ca^{2+} release from cerebellum microsomes. When compared with Ins(1,4,5)P_3, half-maximal Ca^{2+} release required a 30-fold greater concentration of Ins(1,3,4,5)P_4. The metabolism of Ins(1,3,4,5)P_4 to generate Ins(1,4,5)P_3 or the direct interaction of Ins(1,3,4,5)P_4 with its receptor are two alternative hypotheses that would account for these data. A 3-phosphatase acting on Ins(1,3,4,5)P_4 has been found in membrane fractions of human erythrocytes (27) and RBL-2H3 cells (28). However, in porcine brain (29), rat brain, or NG108-15 cells this activity was soluble and present in extremely low amounts, when compared with the activity of 5-phosphatase (data not shown). Recently, Cullen *et al.* (35) have shown that 2 μM Ins(1,3,4,5)P_4 can mobilize Ca^{2+} from mouse lymphoma cells permeabilized by electroporation but not cells permeabilized by digitonin. Using [^{3}H]Ins(1,3,4,5)P_4, they were able to demonstrate that 3% of the added Ins(1,3,4,5)P_4 was converted to Ins(1,4,5)P_3 within 30 sec by electrophorated cells but not by digitonin-permeabilized cells. We were unable to observe any conversion of Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3 or its

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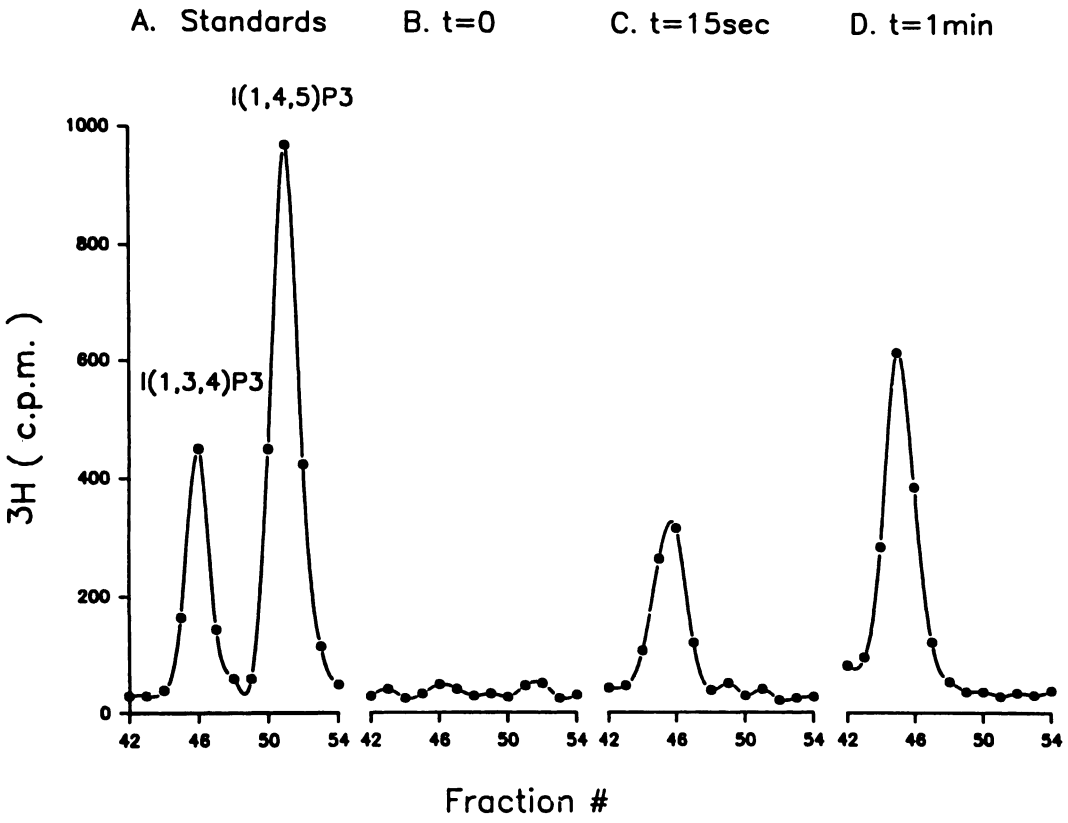

Fig. 6. Products of D-[^{3}H] Ins(1,3,4,5)P_4 metabolism. Rat cerebellum microsomes were incubated in a medium containing 120 mM KCl, 20 mM Tris/HEPES, 0.3 mM $MgCl_2$ 0.5 mM EGTA, and 0.2 mM $CaCl_2$ (free [Ca^{2+}] = 150 nM, as determined with a Ca^{2+}-sensitive electrode). In addition the medium contained 1 μM D-Ins(1,3,4,5)P_4 and 0.1 μCi/ml D-[^{3}H]Ins(1,3,4,5)P_4. Samples were deproteinized at the indicated times and analyzed by HPLC, as described in Materials and Methods. The data are representative of three similar experiments.

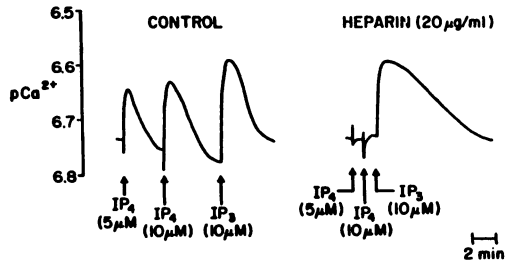

Fig. 7. Inhibition of Ins(1,3,4,5)P_4 responses by heparin. The Ca^{2+} release response to sequential additions of D-Ins(1,3,4,5)P_4 (IP_4) and Ins(1,4,5)P_3 (IP_3) was monitored with a Ca^{2+}-sensitive electrode, as described in Materials and Methods. When present, porcine heparin (20 μg/ml) was added before the initiation of Ca^{2+} uptake. Representative traces are shown.

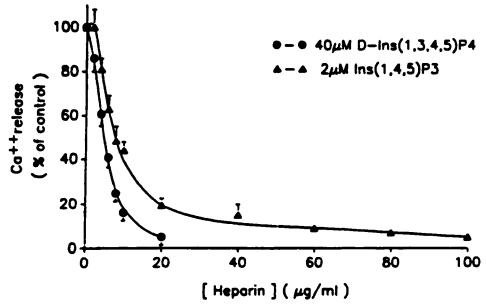

Fig. 8. Comparison of the potency of heparin as an inhibitor of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 responses. The effect of various concentrations of heparin on Ca^{2+} release was measured in experiments of the type shown in Fig. 6. The data are the mean of experiments on two or three different microsomal preparations.

metabolites by cerebellum membranes, under a variety of incubation conditions. A minute conversion of [^{3}H]Ins(1,3,4,5)P_4 to [^{3}H]Ins(1,4,5) P_3, below the detection limit of the HPLC assay, cannot be unequivocally excluded at present.[1] Given the available data, notably the inability of maximal concentrations of Ins(1,3,4,5)P_4 to mobilize the entire Ins(1,4,5)P_3-releasable store and the absence of detectable 3-phosphatase activity in cerebellum microsomes, we favor the hypothesis that Ins(1,3,4,5)P_4 releases Ca^{2+} by direct interaction with a Ca^{2+}-mobilizing receptor.

Membrane receptors that show some selectivity for Ins(1,3,4,5)P_4 over Ins(1,4,5)P_3 have been described in HL-60 cells (13) and adrenal cortex (14). In the latter case, both high and low affinity binding sites were observed. A proportion of the low affinity binding sites were also able to bind Ins(1,4,5)P_3. In cerebellum, the purified Ins(1,4,5)P_3 receptor does not bind Ins(1,3,4,5)P_4 (36). However, [^{3}H]Ins(1,3,4,5)P_4 bound to cerebellum membranes can be displaced by both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (12). The evidence, therefore, suggests that cerebellum membranes may contain both a specific Ins(1,4,5)P_3 receptor and a nonselective Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 receptor. The coupling of both classes of receptor to Ca^{2+} release in different vesicle populations would account for the experimental data, i.e., partial release with Ins(1,3,4,5)P_4 and full release with Ins(1,4,5)P_3. However, Scatchard analysis of [^{3}H] Ins(1,4,5)P_3 binding data indicates only a single class of binding sites in cerebellum membranes (11, 15). An alternative model, which is also compatible with the data, is that all the responsive

[1] The minimum size of a peak that could be resolved above baseline levels was judged to be 75 cpm. In these experiments, the total counts applied to the HPLC column was on average 8000 cpm. Hence, the detection limit of hydrolysis can be estimated to be 0.9%.

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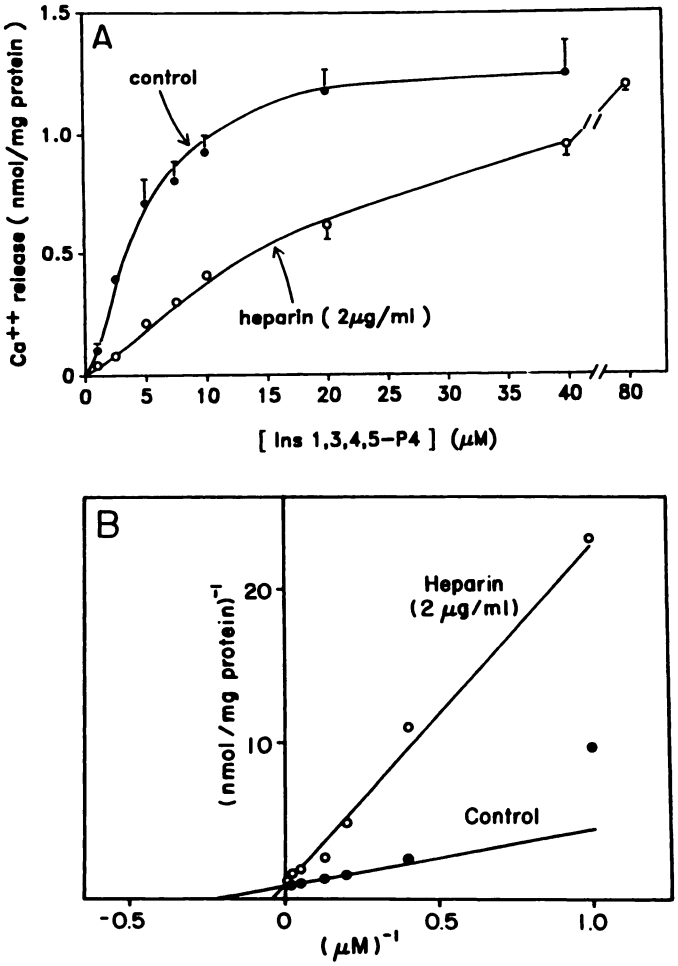

Fig. 9. Competitive inhibition of the Ins(1,3,4,5)P_4 Ca^{2+} release system by heparin. A, Ca^{2+} release in response to increasing concentrations of D-Ins(1,3,4,5)P_4 was measured as described in Fig. 2, in the presence and absence of 2 μg/ml heparin. The data were pooled from experiments on three different microsomal preparations. B, A double-reciprocal plot of the data shown in A. The *lines* shown were obtained from the linear regression analysis of the data. The data point corresponding to the lowest concentration of D-Ins(1,3,4,5)P_4 in the absence of heparin was omitted from the analysis.

vesicles contain a receptor that is specific for Ins(1,4,5)P_3, with some vesicles containing, in addition, a receptor that is specific for Ins(1,3,4,5)P_4. Distinguishing between these alternatives in cerebellum membranes will require further studies on the pharmacology of inositol phosphate receptors and their distribution within the vesicle population.

Heparin was found to competitively inhibit Ins(1,3,4,5)P_4 responses (Fig. 9). Competitive inhibition of Ins(1,4,5)P_3-mediated Ca^{2+} release has been previously noted (15, 33). At equivalent concentrations of both inositol phosphates, heparin was a more potent inhibitor of Ins(1,3,4,5)P_4 responses. However, the K_i values for heparin inhibition were similar, whether Ins(1,3,4,5)P_4 or Ins(1,4,5)P_3 was used as agonist. Hence, the difference in inhibitory potency of heparin can be entirely ascribed to the competitive nature of heparin action and the differences in the effective dose responses of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4.

Several reports in the literature also suggest that high concentrations of Ins(1,3,4,5)P_4 can mobilize internal stores of Ca^{2+}. Microinjection of Ins(1,3,4,5)P_4 into sea urchin eggs that were loaded with fura-2 initiated a Ca^{2+} transient that was indistinguishable from that produced by Ins(1,4,5)P_3 (6). Microinjection of Ins(1,3,4,5)P_4 into *Xenopus* oocytes, incubated in the absence of extracellular Ca^{2+}, activated a Ca^{2+}-dependent Cl^- conductance. In this case, there were qualitative and quantitative differences between the membrane current responses elicited by Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 (9, 10). These differences become particularly important with regard to the possibility that Ins(1,3,4,5)P_4 may be metabolized to Ins(1,4,5)P_3 under certain conditions. It should also be pointed out that the literature contains reports where the addition of Ins(1,3,4,5)P_4 to permeabilized cells or subcellular fractions does not induce Ca^{2+} release (24, 37). Recently, experiments on a rat liver epithelial cell line have shown that Ins(1,3,4,5)P_4 can stimulate the uptake of added Ca^{2+} or the reuptake of Ca^{2+} released by Ins(2,4,5)P_3 (a nonmetabolizable analog) (38). These effects of Ins(1,3,4,5)P_4 were heparin insensitive. However, in our experiments, Ins(1,3,4,5)P_4 did not stimulate the reuptake of Ca^{2+} released by $GPIP_2$ (Fig. 4). The underlying reason for these differences between experimental systems has not been established. The cerebellar membranes used in the present study possess an extremely high density of Ins(1,4,5)P_3 receptors, which have been immunohistochemically localized predominantly to the Purkinje neuron (39). The receptor appears to be distributed in several intracellular membranes, including those present in the perinuclear region (39).

Oscillations of Ca^{2+} associated with spontaneous electrical activity have been recorded from the soma and dendrites of the Purkinje neuron, using fura-2 (40). Recent experiments on the exocrine pancreas suggest that formation of Ins(1,3,4,5)P_4 from Ins(1,4,5)P_3 is unlikely to play a major role in the generation of agonist-mediated oscillations in this tissue (41). Relatively high concentrations of Ins(1,3,4,5)P_4 are required to observe effects on Ca^{2+} release *in vitro*. At present, no information is available on the actual concentrations of inositol phosphates in the cerebellum. The role of the inositol-lipid signal transduction pathway in modulating the functioning of the Purkinje neuron is also unknown. Hence, the physiological significance of the Ca^{2+}-mobilizing effect of Ins(1,3,4,5)P_4 must remain an open question.

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