Characterization of inositol 1,4,5-trisphosphate-stimulated calcium release from rat cerebellar microsomal fractions

Comparison with [^{3}H]inositol 1,4,5-trisphosphate binding

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The abilities of D-*myo*-inositol phosphates (Ins*P*s) to promote Ca^{2+} release and to compete for D-*myo*-[^{3}H]-inositol 1,4,5-trisphosphate {[^{3}H]Ins(1,4,5)P_3} binding were examined with microsomal preparations from rat cerebellum. Of the seven Ins*P*s examined, only Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 stimulated the release of Ca^{2+}. Ca^{2+} release was maximal in 4–6 s and was followed by a rapid re-accumulation of Ca^{2+} into the Ins(1,4,5)P_3-sensitive compartment after Ins(1,4,5)P_3, but not after Ins(2,4,5)P_3 or Ins(4,5)P_2. Ca^{2+} re-accumulation after Ins(1,4,5)P_3 was also faster than after pulse additions of Ca^{2+}, and coincided with the metabolism of [^{3}H]Ins(1,4,5)P_3. These data suggest that Ins(1,4,5)P_3-induced Ca^{2+} release and the accompanying decrease in intraluminal Ca^{2+} stimulate the Ca^{2+} pump associated with the Ins(1,4,5)P_3-sensitive compartment. That this effect was observed only after Ins(1,4,5)P_3 may reflect differences in either the metabolic rates of the various Ins*P*s or an effect of the Ins(1,4,5)P_3 metabolite Ins(1,3,4,5)P_4 to stimulate refilling of the Ins(1,4,5)P_3-sensitive store. Ins*P*-induced Ca^{2+} release was concentration-dependent, with EC_{50} values (concn. giving half-maximal release) of 60, 800 and 6500 nM for Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 respectively. Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 also competed for [^{3}H]Ins(1,4,5)P_3 binding, with respective IC_{50} values (concn. giving 50% inhibition) of 100, 850 and 13000 nM. Comparison of the EC_{50} and IC_{50} values yielded a significant correlation ($r = 0.991$). These data provide evidence of an association between the [^{3}H]Ins(1,4,5)P_3-binding site and the receptor mediating Ins(1,4,5)P_3-induced Ca^{2+} release.

INTRODUCTION

In mammalian central-nervous-system (CNS) tissue, a variety of compounds have been shown to stimulate the breakdown of polyphosphoinositides (for a review see Fisher & Agranoff, 1987). Known products of this reaction are 1,2-diacylglycerol, an activator of protein kinase C (Nishizuka, 1984), and D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (Berridge & Irvine, 1984). Ins(1,4,5)P_3 has been shown in peripheral and neuronally derived cells to stimulate Ca^{2+} release from a non-mitochondrial intracellular store, most likely the endoplasmic reticulum (Prentki *et al.*, 1984; Streb *et al.*, 1983, 1984; Ueda *et al.*, 1986; Jean & Klee, 1986). For these reasons, it is now widely believed that Ins(1,4,5)P_3 is a second messenger linking receptor-stimulated breakdown of polyphosphoinositides with the generation of intracellular Ca^{2+} signals.

Ins(1,4,5)P_3 is thought to cause Ca^{2+} release by first binding to specific receptor sites located on the endoplasmic reticulum (Burgess *et al.*, 1984; Irvine *et al.*, 1984; Berridge, 1986). In support of this proposal, specific binding sites for [^{32}P]- and [^{3}H]-Ins(1,4,5)P_3 have been demonstrated to exist on permeabilized guinea-pig hepatocytes and neutrophils (Spät *et al.*, 1986*a*), and on membranes from bovine adrenal cortex and pituitary gland (Baukal *et al.*, 1985; Guillemette *et al.*, 1987; Spät *et al.*, 1987) as well as rat liver and cerebellum (Spät *et al.*, 1986*b*; Worley *et al.*, 1987*a*,*b*; Willcocks *et al.*, 1987). It has also been suggested that the binding site for [^{3}H]Ins(1,4,5)P_3 in brain may have properties different from its peripheral counterpart (Worley *et al.*, 1987*a*). Although an Ins(1,4,5)P_3-sensitive Ca^{2+} gate is clearly present in neuronally derived cells (Ueda *et al.*, 1986; Jean & Klee, 1986), a functional correlate for brain [^{3}H]Ins(1,4,5)P_3-binding sites has not been established. Indeed, a relationship between Ins(1,4,5)P_3-induced Ca^{2+} release and Ins(1,4,5)P_3 binding has only been achieved by using [^{32}P]Ins(1,4,5)P_3 in permeabilized guinea-pig hepatocytes and neutrophils (Spät *et al.*, 1986*a*).

Because the rat cerebellum has a high concentration of [^{3}H]Ins(1,4,5)P_3-binding sites (Worley *et al.*, 1987*a*,*b*; Willcocks *et al.*, 1987; Supattapone *et al.*, 1988), this tissue was chosen to examine (1) if Ins(1,4,5)P_3 functions as a Ca^{2+} mobilizer there, (2) the characteristics of Ins(1,4,5)P_3-stimulated Ca^{2+} release, and (3) whether a relationship exists between [^{3}H]Ins(1,4,5)P_3 binding and Ins(1,4,5)P_3-induced Ca^{2+} release. These experiments were conducted with a microsomal preparation from rat cerebellum.

Abbreviations used: Ins*P*s, D-*myo*-inositol phosphate(s); the positions of phosphate groups for specified inositol phosphates are indicated in parentheses, e.g. Ins(1,4,5)P_3 is D-*myo*-inositol 1,4,5-trisphosphate; EC_{50}, concentration producing half-maximal Ca^{2+} release; IC_{50}, concentration producing 50% inhibition of control [^{3}H]Ins(1,4,5)P_3 binding.

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MATERIALS AND METHODS

Preparation of cerebellar microsomes (microsomal fractions)

Male Sprague–Dawley rats (150–250 g) were killed by decapitation and the cerebella quickly removed and chilled on ice. Subsequent procedures were performed at 4 °C. Four to seven cerebella were homogenized with a glass–Teflon homogenizer in 9 vol. of a microsome-preparation buffer (MPB) containing (mM): sucrose 250, Hepes 5, KCl 10, dithiothreitol 1 and $MgCl_2$ 1, pH 7.05. The homogenate was centrifuged at 1000 $g_{av.}$ for 5 min and the resulting supernatant fraction (S_1) was kept on ice while the pellet was washed once in 10 ml of MPB. The S_1 supernatant fractions were combined and then centrifuged at 8000 $g_{av.}$ for 10 min, after which the supernatant fraction (S_2) was kept and the pellet washed once in 10 ml of MPB. The S_2 supernatant fractions were then combined and centrifuged at 100000 $g_{av.}$ for 40 min to obtain the microsomal pellet (P_3) that was used for all experiments. Electron microscopy of the microsomal pellet revealed that it contained primarily small (< 0.2 μm diam.) smooth vesicles with virtually no rough vesicles, and very little contaminating myelin, mitochondria or synaptosomes.

Ins(1,4,5)P_3-induced Ca^{2+} release

The microsomal pellet was resuspended to a concentration of 200 mg wet wt. of microsomal tissue/ml in a buffer containing (mM): KCl 150, Hepes 5, $MgCl_2$ 2, pH 7.05. Ca^{2+} uptake and release were measured by adding 100 μl of the microsomal suspension (1.3–1.8 mg of protein) into a quartz cuvette containing 1.9 ml of a medium composed of (final concns., mM): KCl 150, Hepes 5, $MgCl_2$ 2, disodium ATP 0.5, phosphocreatine 10, creatine kinase (10 units/ml), oligomycin (1 μg/ml) and NaN_3 1, pH 7.05. The cuvette was then placed into a thermostatically controlled sample compartment of a dual-excitation-wavelength spectrofluorimeter (Photon Technology International, Princeton, NJ, U.S.A.). The tissue mixture was incubated at 37 °C with constant stirring for 15 min before data were recorded. Changes in the extramicrosomal free Ca^{2+} concentration were monitored with the Ca^{2+}-sensitive dye fura-2 (2 μM), which was added 1 min before recording. Fura-2 fluorescence was measured at an emission wavelength of 510 nm with excitation alternating between 340 and 390 nm, so that a 340 nm/390 nm ratio could be obtained 30 times a second. The ratio signal was proportional to the free Ca^{2+} concentration in the cuvette (Grynkiewicz *et al.*, 1985) and was continuously monitored with an IBM-AT computer. To determine the effects of the various Ins*P*s, Ca^{2+}-release responses were measured by the difference between the peak signal after, and the steady-state signal just before, the addition of compound. Responses to the additions of known Ca^{2+} concentrations were used to calibrate the absolute amount of Ca^{2+} released by the Ins*P*s.

Metabolism of [^{3}H]Ins(1,4,5)P_3

These assays were performed under the same conditions as those used to measure Ca^{2+} release, except that fura-2 was omitted. The reaction mixture, containing [^{3}H]Ins(1,4,5)P_3 in addition to the ingredients already listed for Ca^{2+}-release assays, was preincubated for 5 min at 37 °C and was initiated by the addition of microsomes. At various times thereafter, 500 μl samples were removed and added to 500 μl of ice-cold 20 % (w/v) trichloroacetic acid. For the controls (zero time), the tissue was quenched with trichloroacetic acid before addition of [^{3}H]Ins-(1,4,5)P_3. After quenching, the samples were kept on ice for 10–15 min, with intermittent vortex-mixing, and then centrifuged at 6500 $g_{av.}$ for 5 min. The resulting supernatant fraction was extracted with 4 × 4 vol. of water-saturated diethyl ether and then neutralized with NH_4OH. Separation of the various [^{3}H]Ins*P*s was performed by a modification of the h.p.l.c. procedures described by Hansen *et al.* (1986) and Tennes *et al.* (1987), with a Whatman Partisil SAX column (10 μm particle size). The [^{3}H]Ins*P*s were eluted by three successive convex gradients (Waters model 600E gradient controller) with ammonium formate (pH 3.7) concentration steps of 0.5 M, 1.0 M and 1.7 M, each over 15 min intervals. The flow rate was 1.2 ml/min and fractions were collected every 30 s. To each fraction, 1 ml of 50 % methanol (to prevent phase separation) and 9 ml of ACS liquid scintillant (Amersham) were added, and the radioactivity (d.p.m.) was determined by liquid-scintillation spectrophotometry. Identification of h.p.l.c. peaks containing [^{3}H]Ins*P*, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4 was made by comparison with standards.

Binding of [^{3}H]Ins(1,4,5)P_3

The microsomal pellet was resuspended to a concentration of 40 mg wet wt. of microsomal tissue/ml of binding buffer. To mimic intracellular conditions while limiting variabilities caused by [^{3}H]Ins(1,4,5)P_3 metabolism, pH changes and [Ca^{2+}] fluctuations (Worley *et al.*, 1987*b*), we chose a binding buffer similar to that used by Spät *et al.* (1986*b*), which consisted of (mM): NaCl 20, KCl 100, $MgCl_2$ 0.3, NaH_2PO_4 0.96, $NaHCO_3$ 25, EGTA 1, $CaCl_2$ 0.51 (free [Ca^{2+}] 150 nM) and 2,3-bisphosphoglycerate 1, pH 7.1. Use of the same buffer as in the Ca^{2+}-release experiments did not greatly affect binding as long as the pH and free [Ca^{2+}] were carefully controlled. Assays were initiated by the addition of 250 μl of microsomal tissue (about 0.75 mg of protein) to 250 μl of binding buffer containing [^{3}H]Ins(1,4,5)P_3 (20 nM) and various concentrations of competing inositol polyphosphates. The samples were incubated on ice for 10 min, at which time there was no significant degradation of [^{3}H]Ins(1,4,5)P_3. Samples were then centrifuged at 6500 $g_{av.}$ for 5 min, the supernatant fraction was aspirated, and the pellet surface rinsed once with 1 ml of ice-cold 10 mM-phosphate-buffered saline, pH 7.1. The pellet was then solubilized with 100 μl of Protosol, mixed with 6 ml of ACS liquid scintillant (Amersham), and radioactivity determined by liquid-scintillation spectrophotometry. The centrifugation assay was found to be absolutely necessary, because the cerebellar microsomes were too small (see above) to be retained by standard 0.2 or 0.45 μm filters.

Specific binding of [^{3}H]Ins(1,4,5)P_3 was defined as the difference in d.p.m. in the absence (total binding) and the presence (non-specific binding) of 10 μM-Ins(1,4,5)P_3. Typical values at 20 nM-[^{3}H]Ins(1,4,5)P_3 (5.0 Ci/mmol) were 9000–12000 d.p.m. for total binding and 1500–3000 d.p.m. for non-specific binding, which can then be calculated to be about 700–800 fmol of specifically bound [^{3}H]Ins(1,4,5)P_3. The IC_{50} and EC_{50} values were determined graphically from the corresponding con-

centration curves (Figs. 4 and 5). Protein was determined by the method of Lowry *et al.* (1951).

Materials

[^{3}H]Ins(1,4,5)P_3 (3–5 Ci/mmol, > 98% pure) was obtained from Dupont–NEN. Ins(1,4,5)P_3 was purchased from Calbiochem, and all other Ins$P$s (free of isomers) were from Boehringer Mannheim. Fura-2 free acid was from Molecular Probes. All other chemicals were of the highest purity available from Fluka Biochemicals. To remove any contaminating Ca^{2+}, stock solutions of the Ins$P$s were routinely passed through columns containing Chelex resin (Bio-Rad).

RESULTS

In the presence of ATP, rat cerebellar microsomes were able to accumulate Ca^{2+} until the extramicrosomal free Ca^{2+} concentration was decreased to 300–500 nM. Upon addition of Ins(1,4,5)P_3, a rapid release of Ca^{2+} was observed that reached a peak 4–6 s after the onset, followed by a slower rate of Ca^{2+} re-accumulation until steady-state values were re-established (Fig. 1). The maximal initial rate of Ca^{2+} release [at 1 μM-Ins(1,4,5)P_3] was estimated to be 1.5 nmol of Ca^{2+}/s per mg of protein. At maximal concentrations (1 μM or above), Ins(1,4,5)P_3 released approx. 20–40% of the Ca^{2+} released by 2 μM of the Ca^{2+} ionophore ionomycin (results not shown). As suggested in Fig. 1, desensitization to the effects of Ins(1,4,5)P_3 did not occur. Thus, when added at the end of a prior response, the same concentration of Ins(1,4,5)P_3 could be added repeatedly with no evidence of a decrease in the response (results not shown).

The lack of desensitization just mentioned suggested that Ins(1,4,5)P_3 was rapidly metabolized. As shown in Fig. 2(*a*), the time course of [^{3}H]Ins(1,4,5)P_3 metabolism

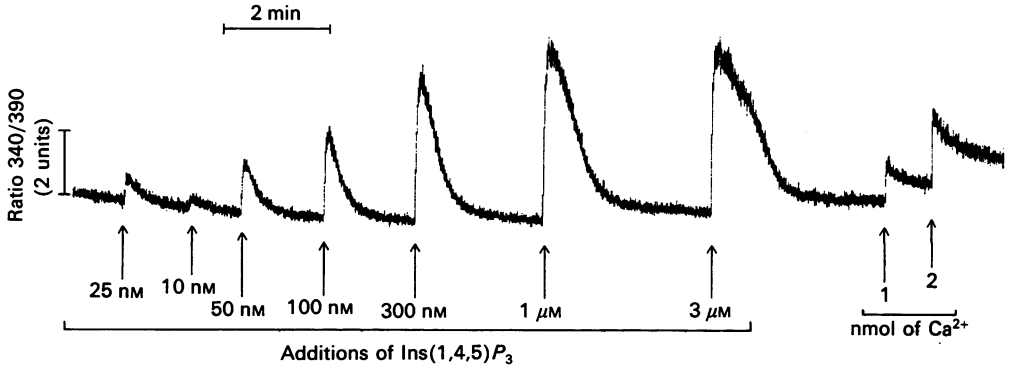

Fig. 1. Ins(1,4,5)P_3-induced Ca^{2+} release from rat cerebellar microsomes

Before data collection, the microsomes were incubated for 15 min at 37 °C in a buffer containing 500 μM-ATP and an ATP-regeneration system (see the Materials and methods section). Then 1 min before recording of data, the Ca^{2+}-sensitive dye fura-2 (2 μM) was added. Fura-2 responses were measured in a dual-excitation-wavelength spectrophotometer with excitation wavelengths alternating between 340 and 390 nm (emission 510 nm). The ratio 340/390 (units are integers) was proportional to the free [Ca^{2+}] outside the microsomes (see the Materials and methods section). The steady-state free [Ca^{2+}] was 300 nM. Shown are responses to various concentrations of Ins(1,4,5)P_3 from a typical experiment. To calibrate the amount of Ca^{2+} released, $CaCl_2$ standards were added in each experiment.

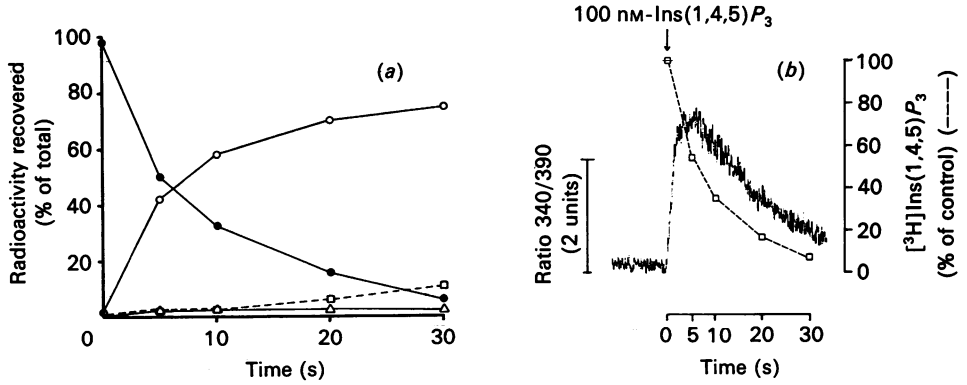

Fig. 2. Comparison of the time course of [^{3}H]Ins(1,4,5)P_3 metabolism (*a*) with Ins(1,4,5)P_3-stimulated Ca^{2+} release (*b*) in rat cerebellar microsomes

The various [^{3}H]Ins$P$s were separated by h.p.l.c. as described in the Materials and methods section. Shown are the results from one experiment that was representative of at least three others. In (*b*), the data for [^{3}H]Ins(1,4,5)P_3 (100 nM) metabolism (*a*) are superimposed over a typical Ca^{2+}-release response to 100 nM-Ins(1,4,5)P_3. The same results were obtained with 25 nM-[^{3}H]Ins(1,4,5)P_3 and Ins(1,4,5)P_3. In (*a*), the data for the individual [^{3}H]Ins$P$s are expressed as a percentage of the total radioactivity (d.p.m.) recovered. The only compounds to appear in significant amounts were [^{3}H]Ins(1,4,5)P_3 (●), [^{3}H]Ins(1,3,4,5)P_4 (△), [^{3}H]Ins(1,3,4)P_3 (□) and [^{3}H]Ins(1,4)P_2 (○).

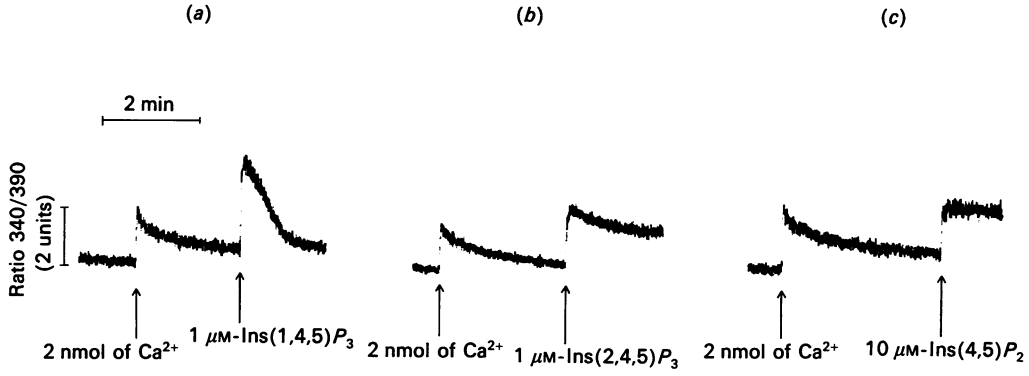

Fig. 3. Comparison of Ca^{2+} release and re-uptake after Ins(1,4,5)P_3 (*a*), Ins(2,4,5)P_3 (*b*) and Ins(4,5)P_2 (*c*)

The procedure for measuring the Ca^{2+} responses was the same as that described in Fig. 1 (see the Materials and methods section). The data were typical of at least four separate experiments. Note the differences in the rate of Ca^{2+} re-uptake after the addition of Ins(1,4,5)P_3 compared with Ins(2,4,5)P_3, Ins(4,5)P_2 or Ca^{2+}.

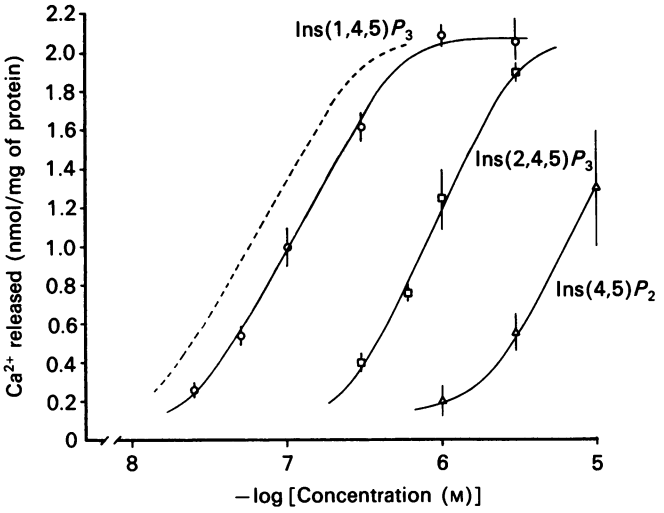

Fig. 4. Concentration–effect curves for Ins(1,4,5)P_3-, Ins(2,4,5)P_3- and Ins(4,5)P_2-induced Ca^{2+} release from rat cerebellar microsomes

Ca^{2+} release was measured as described in the Materials and methods section in response to Ins(1,4,5)P_3 (○), Ins(2,4,5)P_3 (□) and Ins(4,5)P_2 (△). The data points are means (±S.E.M.) from two to five separate experiments. Because of a limited supply of Ins(4,5)P_2, concentrations above 10 μM could not be tested. The broken line represents the predicted concentration–effect curve for Ins(1,4,5)P_3 after taking into account its rapid metabolism during the Ca^{2+}-release assays and assuming that the rate of metabolism is the same at all points on the line.

by the cerebellar microsomes was rapid, with only 54% of [^{3}H]Ins(1,4,5)P_3 remaining at 5 s. The appearance of the [^{3}H]Ins(1,4,5)P_3 metabolites [^{3}H]Ins(1,4)P_2, [^{3}H]-Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,3,4)P_3 indicated that the cerebellar microsomal preparation contained both Ins(1,4,5)P_3 5-phosphomonoesterase and Ins(1,4,5)P_3 kinase activities. When compared with the Ca^{2+}-release/re-uptake response, [^{3}H]Ins(1,4,5)P_3 degradation paralleled the time course of the Ca^{2+} re-accumulation phase after Ins(1,4,5)P_3 (Fig. 2*b*).

In the next experiment, the specificity of Ins*P*-stimulated Ca^{2+} release was examined. No response was observed to additions of Ins(1)*P*, Ins(4)*P* or Ins(1,4)P_2 at 1 μM, and the presence of these compounds did not alter the response to Ins(1,4,5)P_3 (results not shown). Also, 1 μM-D-*myo*-Ins(3,4,5,6)P_4 [i.e. L-*myo*-Ins(1,4,5,6)P_4 recently identified from avian red cells (Stephens *et al.*, 1988)] had no effect. Besides Ins(1,4,5)P_3, release of Ca^{2+} was also elicited by Ins(2,4,5)P_3 and Ins(4,5)P_2 (Fig. 3).

Interestingly, Fig. 3(*a*) (and Fig. 1) shows that the time to return to baseline after a pulse addition of Ca^{2+} was much longer than after Ins(1,4,5)P_3. The steeper slope of the ratio signal during Ca^{2+} re-uptake after Ins(1,4,5)P_3 is an indication that the rate of Ca^{2+} uptake was stimulated relative to the rate of Ca^{2+} uptake after addition of Ca^{2+}. After Ca^{2+} addition, the microsomes were always capable of lowering free [Ca^{2+}] to initial baseline values, showing that the low rate of Ca^{2+} uptake was not because the microsomes were already saturated with Ca^{2+}. Ca^{2+} re-accumulation after Ins(2,4,5)P_3 (Fig. 3*b*) or Ins(4,5)P_2 (Fig. 3*c*) was also slower than after Ins(1,4,5) P_3. Furthermore, repeated additions of Ins(2,4,5)P_3 or Ins(4,5)P_2 led to diminished responses either to themselves or to Ins(1,4,5)P_3 added subsequently (results not shown), in contrast with the lack of desensitization to Ins(1,4,5)P_3 described above.

The release of Ca^{2+} by Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 was concentration-dependent (Fig. 4). The Ins*P* concentrations producing half-maximal Ca^{2+} release (EC_{50}) were 110, 800 and 6500 nM for Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 respectively. However, since the rate of Ins(1,4,5)P_3 degradation was so high (see Fig. 2), during the Ca^{2+}-release assays only about 54% of authentic Ins(1,4,5)P_3 would be expected to remain 5 s after its addition, which was the time when most of the Ca^{2+}-release responses were measured. The true concentration–effect curve for Ins(1,4,5)P_3-induced Ca^{2+} release should then be shifted left to reflect the concentration of Ins(1,4,5)P_3 at the time when measurements were made. With this correction the EC_{50} for Ins(1,4,5)P_3-induced Ca^{2+} release was 60 nM (broken line in Fig. 4) and the Hill coefficient, *h*, was 1.18, consistent with a one-site model. The contribution of metabolism of Ins(2,4,5)P_3

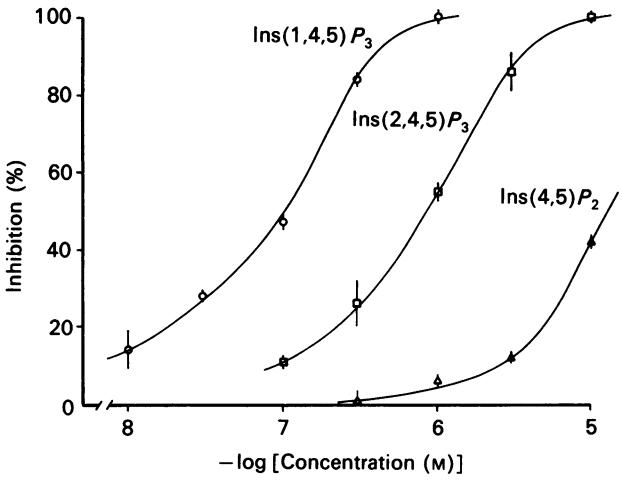

Fig. 5. Competition of [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar microsomes

The concentration of [^{3}H]Ins(1,4,5)P_3 was 20 nM, and specific binding was determined as described in the Materials and methods section. Shown are the competition curves for Ins(1,4,5)P_3 (○), Ins(2,4,5)P_3 (□) and Ins(4,5)P_2 (△). The data are expressed as percentages of control specific binding and are presented as means (±S.E.M.) from three experiments performed in duplicate [except for Ins(4,5)P_2, for which there were only two experiments]. Control specific binding was 1003±74 fmol/mg of protein ($n = 4$). Because of a limited supply of Ins(4,5)P_2, concentrations above 10 μM could not be tested, so the IC_{50} value was estimated by extrapolation.

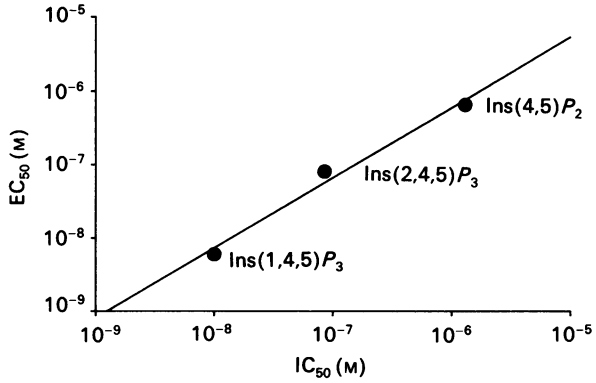

Fig. 6. Correlation of binding (IC_{50}) and Ca^{2+}-release (EC_{50}) properties of Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2

The IC_{50} and EC_{50} values were determined from Figs. 4 and 5. Linear-regression analysis of the points yielded a positive correlation, with a coefficient (r) of 0.991 (slope = 1.09).

and Ins(4,5)P_2 is unknown. Notwithstanding, on the basis of their apparent EC_{50} values, the relative potencies of the compounds tested were Ins(1,4,5)P_3 (1) > Ins(2,4,5)P_3 (13) > Ins(4,5)P_2 (108).

The demonstration of specific binding sites for [^{3}H]Ins(1,4,5)P_3 on rat cerebellar membranes (Worley *et al.*, 1987*a*,*b*; Willcocks *et al.*, 1987) raised a question concerning the functional significance of these sites. To gain more insight into the possible association of [^{3}H]Ins(1,4,5)P_3-binding sites with the receptor mediating Ins(1,4,5)P_3-induced Ca^{2+} release, we next examined the abilities of Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 to compete for [^{3}H]Ins(1,4,5)P_3 binding: the concentrations of Ins$P$s producing 50% inhibition of [^{3}H]Ins(1,4,5)P_3 binding (IC_{50}) were 100, 850 and 13000 (estimated) nM respectively (Fig. 5). Possible isotope-dilution effects in the case of Ins(1,4,5)P_3 would seem unlikely, since the Hill coefficient of its competition curve (0.82) was close to that of Ins(2,4,5)P_3 (1.03), which is consistent with a receptor interaction governed by mass action. Finally, even though only three compounds with agonist activity could be compared, a positive correlation ($r = 0.991$) was found to exist between the binding (IC_{50}) and Ca^{2+}-release (EC_{50}) properties of Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 (Fig. 6).

DISCUSSION

The initial aim of this study was to determine the characteristics of Ins(1,4,5)P_3-induced Ca^{2+} release from rat cerebellar microsomes. Beyond a visual identification, no attempt was made here to identify the components of the microsomal fraction. Although it was assumed that this fraction was enriched with vesicles derived from the endoplasmic reticulum, other membrane components were likely to be present, and therefore a more thorough characterization is needed. Notwithstanding, the microsomes did accumulate Ca^{2+}, and, once steady-state concentrations of extra-microsomal free Ca^{2+} were reached, the addition of Ins(1,4,5)P_3 caused a rapid release of Ca^{2+}, followed by a slower re-accumulation of Ca^{2+}. Consistent with previous findings (Joseph *et al.*, 1984, 1987), the Ca^{2+} re-accumulation phase after the addition of Ins(1,4,5)P_3 coincided with the degradation of Ins(1,4,5)P_3, providing evidence that metabolism was the principal mechanism terminating the effects of Ins(1,4,5)P_3.

That the rate of Ca^{2+} re-uptake after Ins(1,4,5)P_3 was higher than after a pulse addition of Ca^{2+} is noteworthy, because it suggests that the Ca^{2+} pump associated with the Ins(1,4,5)P_3-sensitive Ca^{2+} store in cerebellum can be stimulated by a mechanism other than an increase in cytosolic free [Ca^{2+}]. It is quite possible that either Ca^{2+} depletion of the microsomes, which is known to activate the microsomal Ca^{2+}-transport ATPase in brain (Trotta & de Meis, 1978), or the increased intraluminal K^+ that is thought to accompany Ca^{2+} release by Ins(1,4,5)P_3 (Muallem *et al.*, 1985; Joseph & Williamson, 1986), which can also activate Ca^{2+}-ATPase activity (Robinson, 1976; Kemmer *et al.*, 1987), may be mechanisms involved in enhancing the Ca^{2+}-pump activity. Work is needed to identify the precise nature of this mechanism.

If the addition of Ins(1,4,5)P_3 leads to a stimulation of Ca^{2+} re-uptake after Ca^{2+} release, why was this effect not observed after Ca^{2+} release by Ins(2,4,5)P_3 or Ins(4,5)P_2? One possibility is that Ins(2,4,5)P_3 and Ins(4,5)P_2 are not metabolized as rapidly as Ins(1,4,5)P_3. Unfortunately, limited information is available on the metabolism of Ins(2,4,5)P_3 and Ins(4,5)P_2. The microsomal preparation from cerebellum contained both Ins(1,4,5)P_3 5-phosphomonoesterase and kinase activities, as indicated by the appearance of Ins(1,4)P_2, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 during metabolism of Ins(1,4,5)P_3. Irvine & Moor (1987) and Ryu *et al.* (1987) have reported that Ins(2,4,5)P_3 and Ins(4,5)P_2 are not good substrates for the Ins(1,4,5)P_3

kinase. The activity of the Ins(1,4,5)P_3 5-phosphomonoesterase toward Ins(2,4,5)P_3 and Ins(4,5)P_2 has not been reported. If Ins(2,4,5)P_3 and Ins(4,5)P_2 were slowly metabolized, their continued presence would cause the Ins(1,4,5)P_3-responsive 'channel' to remain open, making it impossible for the Ins(1,4,5)P_3-sensitive compartment to refill rapidly with Ca^{2+}. The released Ca^{2+} would then be slowly accumulated by an Ins(1,4,5)P_3-insensitive compartment (Dawson & Irvine, 1984), and would explain why responses to Ins(2,4,5)P_3 and Ins(4,5)P_2 desensitized, whereas those to Ins(1,4,5)P_3 did not.

A second possibility that must be considered, however, is that formation of Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3, which would only occur from Ins(1,4,5)P_3 via the Ins(1,4,5)P_3 kinase pathway, might participate in the enhanced refilling of the Ins(1,4,5)P_3-sensitive Ca^{2+} compartment. This idea would be consistent with the recently proposed role for Ins(1,3,4,5)P_4 in modulating Ca^{2+} entry into the Ins(1,4,5)P_3-sensitive Ca^{2+} store (Irvine & Moor, 1987; Morris *et al.*, 1987). More work is clearly needed to determine if this mechanism is operative in cerebellar microsomes.

Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 all produced concentration-dependent release of Ca^{2+}. Their relative potencies are comparable with those reported in permeabilized guinea-pig hepatocytes and Swiss-mouse 3T3 cells (Burgess *et al.*, 1984; Irvine *et al.*, 1984). Thus, with regard to the agonist-recognition site, the Ins(1,4,5)P_3 receptors in the cerebellum and periphery appear to be similar.

The first evidence that the receptor mediating Ins(1,4,5)P_3-induced Ca^{2+} release might be associated with [^{3}H]Ins(1,4,5)P_3-binding sites in the cerebellum came from the observation that, after taking rapid metabolism into account, the EC_{50} value for Ins(1,4,5)P_3-induced Ca^{2+} release was 60 nM, which was within the range of K_d values (23–80 nM) previously reported for [^{3}H]-Ins(1,4,5)P_3 binding to rat cerebellar membranes (Worley *et al.*, 1987*a*,*b*; Willcocks *et al.*, 1987; Supattapone *et al.*, 1988). Unfortunately, our limited supply of [^{3}H]Ins(1,4,5)P_3 prevented the determination of a K_d in the present study. Nevertheless, if [^{3}H]Ins(1,4,5)P_3-binding sites represent the receptor mediating Ins(1,4,5)P_3-induced Ca^{2+} release, then the potencies (relative and absolute) of Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 to compete for [^{3}H]Ins(1,4,5)P_3 binding should correspond to their potencies to release Ca^{2+}.

When tested for their ability to compete for [^{3}H]-Ins(1,4,5)P_3 binding, the IC_{50} values for Ins(1,4,5)P_3, Ins(2,4,5)P_3 and Ins(4,5)P_2 were 100, 850 and 13000 nM respectively, which were close to their corresponding EC_{50} values (60, 800 and 6500 nM). Furthermore, although only three compounds could be compared, there was an excellent correlation ($r = 0.991$) between the ability to compete for [^{3}H]Ins(1,4,5)P_3 binding and the ability to cause Ca^{2+} release (Fig. 6). These data provide evidence for an association of the [^{3}H]Ins(1,4,5)P_3-binding site in rat cerebellum with the receptor mediating Ins(1,4,5)P_3-induced Ca^{2+} release. This association supports the idea that Ins(1,4,5)P_3 mobilizes Ca^{2+} by first binding to a specific intracellular receptor.

Given the relationship between binding and Ca^{2+} release by Ins(1,4,5)P_3, it is possible to estimate the ratio of Ca^{2+} ions released for every bound molecule of Ins(1,4,5)P_3 as approx. 400:1, which would be consistent with the proposal that Ins(1,4,5)P_3 binding leads to activation of a Ca^{2+} channel. However, we do not yet know the stoichiometry between the association of Ins(1,4,5)P_3 binding and the opening of Ins(1,4,5)P_3-sensitive Ca^{2+} channels (reported to be 3:1 in rat basophilic leukaemia cells; Meyer *et al.*, 1988). Rat cerebellar microsomes should provide a useful model system for studying the mechanism and kinetics of Ins(1,4,5)P_3-induced Ca^{2+} release.

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