Inositol 1,3,4,5-tetrakisphosphate stimulates calcium release from bovine adrenal microsomes by a mechanism independent of the inositol 1,4,5-trisphosphate receptor

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In bovine adrenal microsomes, $\text{Ins}(1,4,5)\text{P}_3$ binds to a specific high-affinity receptor site ($K_d = 11 \text{ nM}$) with low affinity for two other InsP_3 isomers, $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$. In the same subcellular fractions $\text{Ins}(1,4,5)\text{P}_3$ was also the most potent stimulus of Ca^{2+} release of all the inositol phosphates tested. Of the many inositol phosphates recently identified in angiotensin-II-stimulated adrenal glomerulosa and other cells, $\text{Ins}(1,3,4,5)\text{P}_4$ has been implicated as an additional second messenger that may act in conjunction with $\text{Ins}(1,4,5)\text{P}_3$ to elicit Ca^{2+} mobilization. In the present study, an independent action of $\text{Ins}(1,3,4,5)\text{P}_4$ was observed in bovine adrenal microsomes. Heparin, a sulphated polysaccharide which binds to $\text{Ins}(1,4,5)\text{P}_3$ receptors in several tissues, inhibited both the binding of radiolabelled $\text{Ins}(1,4,5)\text{P}_3$ and its Ca^{2+} -releasing activity in adrenal microsomes. In contrast, heparin did not inhibit the mobilization of Ca^{2+} by $\text{Ins}(1,3,4,5)\text{P}_4$, even at doses that abolished the $\text{Ins}(1,4,5)\text{P}_3$ response. Such differential inhibition of the $\text{Ins}(1,4,5)\text{P}_3$ - and $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} responses by heparin indicates that $\text{Ins}(1,3,4,5)\text{P}_4$ stimulates the release of Ca^{2+} from a discrete intracellular store, and exerts this action via a specific receptor site that is distinct from the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

INTRODUCTION

The hydrolysis of inositol phospholipids during receptor activation by Ca^{2+} -mobilizing agonists results in the production of $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (Berridge & Irvine, 1984). In angiotensin-stimulated bovine adrenal-glomerulosa cells, rapid formation of $\text{Ins}(1,4,5)\text{P}_3$ is accompanied by mobilization of Ca^{2+} from vesicular intracellular stores that co-purify with the plasma-membrane fraction during centrifugal fractionation of adrenal homogenates (Baukal *et al.*, 1985; Guillemette *et al.*, 1987a; Rossier *et al.*, 1989). Several other inositol phosphates are formed in angiotensin-stimulated glomerulosa cells, including $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Guillemette *et al.*, 1987b), but the physiological role of these compounds is unclear. Specific binding sites for $\text{Ins}(1,3,4,5)\text{P}_4$ have been identified in several cell types (Theibert *et al.*, 1987; Bradford & Irvine, 1987; Enyedi & Williams, 1988; Enyedi *et al.*, 1989), suggesting that there is a receptor through which $\text{Ins}(1,3,4,5)\text{P}_4$ may act on intracellular processes.

Recent studies have suggested that $\text{Ins}(1,3,4,5)\text{P}_4$ may play a role in the mobilization of Ca^{2+} from intracellular stores, possibly by acting in conjunction with $\text{Ins}(1,4,5)\text{P}_3$ (see review by Irvine *et al.*, 1988). However, to date there have been no reports of a direct and independent action of $\text{Ins}(1,3,4,5)\text{P}_4$ on Ca^{2+} mobilization in either whole- or broken-cell preparations. In fact, one study has suggested that $\text{Ins}(1,3,4,5)\text{P}_4$ may even induce Ca^{2+} sequestration under certain conditions (Hill *et al.*, 1988).

Heparin sulphate has been shown to act as a potent inhibitor of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release in a variety of cell types (Hill *et al.*, 1987; Nilsson *et al.*, 1988; Ghosh *et al.*, 1988) by preventing the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor (Worley *et al.*, 1987; Guillemette *et al.*, 1989). The results of the present study confirm that heparin inhibits $\text{Ins}(1,4,5)\text{P}_3$ binding in a dose-dependent manner and also selectively inhibits the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} response. We also report the finding of an $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} mobilization in bovine adrenal microsomes that

was not inhibited by heparin. In addition, evidence is presented to show that the Ca^{2+} -mobilizing action of $\text{Ins}(1,3,4,5)\text{P}_4$ is not the result of its rapid metabolism to $\text{Ins}(1,4,5)\text{P}_3$.

These results provide evidence that $\text{Ins}(1,3,4,5)\text{P}_4$ may act through a specific receptor site, distinct from the $\text{Ins}(1,4,5)\text{P}_3$ receptor, to effect a change in intracellular Ca^{2+} levels.

MATERIALS AND METHODS

Materials

$[\text{32P}]\text{Ins}(1,4,5)\text{P}_3$ (1000 Ci/mmol) and $[\text{3H}]\text{Ins}(1,4,5)\text{P}_3$ (20 Ci/mmol) were obtained from Amersham and $[\text{3H}]\text{Ins}(1,3,4,5)\text{P}_4$ (20 Ci/mmol) was from New England Nuclear. Fura-2 free acid was from Calbiochem, Chelex resin was from Bio-Rad, heparin sulphate was from General Biochemicals and Sigma Chemical Co., and all other reagents were from either Calbiochem or Boehringer Mannheim. *D-myo*- $\text{Ins}(1,3,4)\text{P}_3$ was generously given by Dr. G. D. Prestwich (Department of Chemistry, SUNY at Stony Brook, NY, U.S.A.).

Preparation of particulate cell fraction for binding studies

A particulate subcellular fraction containing microsomal membranes was prepared from bovine adrenal cortices, which were homogenized first with a Polytron (setting 10, 15 s) and then with five strokes of a Dounce homogenizer (tight pestle) in 20 mM- NaHCO_3 . The homogenate was then filtered through nylon mesh and centrifuged at 500 *g* for 15 min. The supernatant was centrifuged at 35000 *g* for 20 min, and the pellet was washed and centrifuged again, resuspended in 20 mM-Tris/HCl, pH 7.4, at a concentration of 15–20 mg of protein/ml and stored at -70°C until utilized.

Binding studies

Portions of the adrenal particulate fraction (200–400 μg of protein per sample) were incubated in a buffer containing 25 mM-

Abbreviations used: IC_{50} , concn. producing 50% inhibition of control $[\text{32P}]\text{Ins}(1,4,5)\text{P}_3$ binding.

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Hepes, 20 mM-NaCl, 100 mM-KCl, 1 mM-EDTA and 1 mg of BSA/ml. Incubations were performed in a final volume of 200 μ l with [32 P]Ins(1,4,5) P_3 (25000 d.p.m. $\approx$ 10 fmol $\approx$ 0.05 nM) and the appropriate concentrations of unlabelled inositol phosphates. Non-specific binding was determined by the addition of 10 μ M-Ins(1,4,5) P_3 . After 20 min at 0 $^{\circ}$ C, the incubation was terminated by dilution of samples with 3 ml of ice-cold phosphate-buffered saline (138 mM-NaCl/2.8 mM-KCl/0.8 mM- Na_2HPO_4 /1.45 mM- KH_2PO_4), pH 7.0, and rapid filtration through GF/C filters (Whatman), followed by 3 $\times$ 3 ml washes with the same solution. The bound radioactivity on the filters was measured by β -spectrometry in a liquid-scintillation counter.

Ca $^{2+}$ -release studies

Bovine adrenocortical tissue was dissected from fresh adrenal glands and homogenized in Buffer A (20 mM-Hepes/KOH, pH 7.2, 110 mM-KCl, 10 mM-NaCl, 2 mM- MgCl_2 , 5 mM- KH_2PO_4 , 2 mM-EGTA and 1 mM-dithiothreitol) by using a Polytron (setting 10, 15 s) and then a Dounce homogenizer (five strokes, tight pestle). The homogenate was filtered through nylon mesh before centrifugation at 500 g for 15 min. The supernatant was then centrifuged at 35000 g for 20 min, and the subsequent pellet was resuspended in Buffer B (Buffer A, without EGTA) and re-centrifuged. The pellet was washed once again, and the final pellet was resuspended in an intracellular-type buffer composed of 110 mM-KCl, 10 mM-NaCl, 2 mM- MgCl_2 , 5 mM- KH_2PO_4 and 20 mM-Hepes/KOH, pH 7.2. The volume was adjusted to give a final concentration of 30 mg of protein/ml.

A 400 μ l portion of the particulate suspension (5–6 mg of protein) was added to a quartz cuvette containing the intracellular medium to give a final volume of 2 ml. An ATP-regenerating system composed of 20 units of creatine kinase/ml and 20 mM-phosphocreatine was added to the medium and 2.5 μ g of oligomycin/ml to inhibit mitochondrial ATPase activity.

The changes in free Ca $^{2+}$ concentration of the incubation medium containing the adrenal particles were monitored by using the fluorescent calcium indicator Fura-2 free acid (1 μ M) in a Perkin-Elmer LS-5 fluorescence spectrophotometer. The data were collected by using an excitation wavelength of 340 nm (slit 5 nm) and emission wavelength of 500 nm (slit 10 nm).

In order to detect a Ca $^{2+}$ response from the test compounds, the microsomes were first re-loaded with Ca $^{2+}$ by addition of ATP (1 mM) to decrease free Ca $^{2+}$ to ambient intracellular levels. All responses were measured as the peak fluorescence induced by the addition of the test inositol phosphate; this change in free Ca $^{2+}$ was then expressed as nmol of Ca $^{2+}$ released. In some experiments we used a Chelex resin to remove any possible contaminating Ca $^{2+}$ from our Ins(1,3,4,5) P_4 stock solution. The Chelex treatment caused no detectable difference in the amplitude or kinetics of the Ins(1,3,4,5) P_4 -induced Ca $^{2+}$ release.

The inositol phosphates and heparin to be tested for Ca $^{2+}$ -releasing activity were added in small volumes to the cuvette, and each experiment was calibrated by the addition of a known amount of Ca $^{2+}$. The free Ca $^{2+}$ concentration of the medium was determined from the F_{max} and F_{min} (Grynkiewicz *et al.*, 1985). The F_{max} value was obtained by addition of excess Ca $^{2+}$ after addition of 1 μ M-ionomycin, and F_{min} was determined by addition of 1 mM-EGTA/Tris, pH 8.3.

Metabolism of inositol phosphates

Metabolism of [^3H]Ins(1,3,4,5) P_4 was measured simultaneously in a cuvette while the Ca $^{2+}$ -releasing activity of the unlabelled Ins(1,3,4,5) P_4 was monitored with Fura-2; recordings were made with an on-line printer. The particles were incubated in an intracellular medium under the conditions described for the Ca $^{2+}$ -release protocol. [^3H]Ins(1,3,4,5) P_4 was added

(100000 c.p.m.) in the presence of 2 μ M unlabelled Ins(1,3,4,5) P_4 ; at various times 100 μ l samples were removed from the cuvette, and the incubation was stopped by addition to 100 μ l of ice-cold 10% (w/v) HClO_4 .

The samples were centrifuged (2000 g, 20 min), and the supernatant containing the solubilized inositol phosphates was extracted with trifluoroethane/trioctylamine (1:1, v/v) (Downes *et al.*, 1986). Samples were neutralized and then analysed by h.p.l.c. on an Absorbosphere SAX column (5 μ m) (Alltech Associates, Deerfield, IL, U.S.A.) and eluted with a linear gradient of ammonium phosphate as previously described (Balla *et al.*, 1986). The radioactivity in the effluent was continuously

Fig. 1. Competition for [32 P]Ins(1,4,5) P_3 binding by unlabelled inositol phosphates in a bovine adrenal particulate fraction

Data are expressed as percentages of the total binding in the absence of unlabelled ligand, and are corrected for non-specific binding. (a) Displacement curve for Ins(1,4,5) P_3 binding. Scatchard analysis of the data (see inset) revealed a single class of high-affinity binding sites. This experiment was performed in triplicate, and is representative of 14 different experiments. (b) Competitive displacement of [32 P]Ins(1,4,5) P_3 by increasing concentration of unlabelled inositol phosphates in bovine adrenal microsomes: $\bullet$, Ins(1,4,5) P_3 ; $\circ$, Ins(1,3,4,5) P_4 ; $\blacktriangle$, Ins(2,4,5) P_3 ; $\triangle$, Ins(1,3,4,5) P_4 . Data are means of three different experiments, each performed in triplicate [for Ins(1,4,5) P_3 , $n = 14$ experiments]; s.d. values (less than 15%) have been omitted for clarity.

monitored with an on-line radioactive flow detector (Flo-one, Beta IC; Radiomatic Instruments, Tampa, FL, U.S.A.).

RESULTS

Bovine adrenal membranes contain high-affinity low-capacity binding sites for $\text{Ins}(1,4,5)\text{P}_3$. $^{32}\text{P}\text{Ins}(1,4,5)\text{P}_3$ binding to particulate receptor sites was inhibited by increasing amounts of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ and was completely abolished by addition of $1\text{ }\mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 1a). Scatchard analysis (Fig. 1, inset) of the displacement data indicates that $\text{Ins}(1,4,5)\text{P}_3$ binds to a single, specific, high-affinity receptor site in bovine adrenal microsomes, with a K_d of $11.2 \pm 2.9\text{ nM}$ and a binding capacity of $388 \pm 60.2\text{ fmol/mg}$ of protein (means $\pm$ S.E.M.; $n = 14$).

The competition by other inositol phosphates for binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor site is shown in Fig. 1(b). $\text{Ins}(1,3,4,5)\text{P}_4$ was a less potent inhibitor of $^{32}\text{P}\text{Ins}(1,4,5)\text{P}_3$ binding, with $\text{IC}_{50} \approx 40\text{ nM}$. Evaluation of the activities of other InsP_3 isomers revealed that $\text{Ins}(1,3,4)\text{P}_3$ was ineffective at doses up to

500 nM , and then displaced only 20% of the specific binding at $1\text{ }\mu\text{M}$. In contrast, $\text{Ins}(2,4,5)\text{P}_3$ displaced $^{32}\text{P}\text{Ins}(1,4,5)\text{P}_3$ with $\text{IC}_{50} \approx 300\text{ nM}$.

The order of potencies of these inositol phosphates was similar to that observed by Willcocks *et al.* (1987) and by Palmer *et al.* (1989) in bovine adrenal cortex, where $\text{Ins}(1,3,4)\text{P}_3$ appeared to be ineffectual in displacing radiolabelled $\text{Ins}(1,4,5)\text{P}_3$ from the receptor, and $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(2,4,5)\text{P}_3$ were about one order of magnitude less potent than $\text{Ins}(1,4,5)\text{P}_3$, with IC_{50} values of about 110 nM and 120 nM respectively. Several studies have indicated that $\text{Ins}(2,4,5)\text{P}_3$ can displace radiolabelled $\text{Ins}(1,4,5)\text{P}_3$ from its binding sites with IC_{50} values within the range reported here (Spät *et al.*, 1986; Stauderman *et al.*, 1988; Palmer *et al.*, 1989). The relatively greater potency of $\text{Ins}(1,3,4,5)\text{P}_4$ in competing for $^{32}\text{P}\text{Ins}(1,4,5)\text{P}_3$ binding ($\text{IC}_{50} \approx 40\text{ nM}$) suggests that $\text{Ins}(1,3,4,5)\text{P}_4$ may have some activity on the InsP_3 -receptor site. However, previous studies in bovine adrenal-glomerulosa cells indicate that under physiological conditions $\text{Ins}(1,4,5)\text{P}_3$ levels are at least 2–5 times those of $\text{Ins}(1,3,4,5)\text{P}_4$ (Balla *et al.*, 1988).

Fig. 2. Effects of maximal doses of $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(2,4,5)\text{P}_3$ on Ca^{2+} release in a bovine adrenal particulate fraction

Fura-2 was used to measure Ca^{2+} release induced by different inositol phosphates in bovine adrenal microsomes. Before addition of inositol phosphates, the microsome-containing fraction was incubated with 1 mM -ATP to decrease free $[\text{Ca}^{2+}]$ to baseline levels. Ca^{2+} release was induced by: (1) $10\text{ }\mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$; (2) $20\text{ }\mu\text{M}$ - $\text{Ins}(1,3,4,5)\text{P}_4$; (3) $40\text{ }\mu\text{M}$ - $\text{Ins}(2,4,5)\text{P}_3$. Panel (a): (1) followed by (2). Panel (b): (2) followed by (1). Panel (c): (1) followed within 10 s by (2). Panel (d): (2) followed within 10 s by (1). Panel (e): (3) followed within 15 s by (2). Panel (f): (2) followed after 2.5 min by (3). The $\text{Ins}(1,3,4,5)\text{P}_4$ response typically exhibited different kinetics from those of the InsP_3 -induced Ca^{2+} response, showing a secondary rise or plateau after the initial spike in the free Ca^{2+} level.

Considering that $\text{Ins}(1,3,4,5)P_4$ has about one-seventh the affinity of $\text{Ins}(1,4,5)P_3$ for its receptor, it is unlikely that $\text{Ins}(1,3,4,5)P_4$ exerts its physiological activity through the $\text{Ins}(1,4,5)P_3$ receptor.

The Ca^{2+} signals generated by the addition of $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ to adrenal microsomes exhibited different response kinetics. Typically, the response to $\text{Ins}(1,4,5)P_3$ was a prompt rise to a peak Ca^{2+} level followed by a relatively rapid exponential decay back to baseline levels. The Ca^{2+} response to $\text{Ins}(1,3,4,5)P_4$, particularly at higher doses, usually exhibited a peak, followed by a prolonged phase or plateau, frequently with a secondary rise after the initial peak and a slow decay toward baseline levels, which were not usually attained during the 10 min observation period (Fig. 2a). The order of addition of inositol phosphates did not affect the kinetics of either the $\text{Ins}(1,4,5)P_3$ - or the $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} response. $\text{Ins}(1,4,5)P_3$ elicited its characteristic 'spike' response when added after $\text{Ins}(1,3,4,5)P_4$, although the magnitude of the response was often diminished as compared with responses where $\text{Ins}(1,4,5)P_3$ was first added to the membrane preparation (Fig. 2b).

The independent effect of a maximal dose of $\text{Ins}(1,3,4,5)P_4$ was superimposable on the $\text{Ins}(1,4,5)P_3$ -induced Ca^{2+} release. When $\text{Ins}(1,3,4,5)P_4$ was added shortly after a maximal dose of $\text{Ins}(1,4,5)P_3$ (10 s), there was an additional small increase in the amount of Ca^{2+} released (Fig. 2c); however, the rapid decay in the Ca^{2+} response seen with $\text{Ins}(1,4,5)P_3$ alone was slowed by the addition of $\text{Ins}(1,3,4,5)P_4$, and pre-stimulated baseline levels were not attained. The additive effect of the $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} release was more evident when the order of addition was reversed. When $\text{Ins}(1,4,5)P_3$ was added after $\text{Ins}(1,3,4,5)P_4$, a marked additional increase in Ca^{2+} release was observed and the characteristic $\text{Ins}(1,4,5)P_3$ -stimulated Ca^{2+} -response kinetics were superimposed on top of the $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} response (Fig. 2d). This independent effect of $\text{Ins}(1,3,4,5)P_4$ on Ca^{2+} release could also be clearly seen when $\text{Ins}(2,4,5)P_3$, a non-metabolized analogue of $\text{Ins}(1,4,5)P_3$, was utilized. $\text{Ins}(2,4,5)P_3$

alone elicited a smaller response than did maximal doses of $\text{Ins}(1,4,5)P_3$ (trace not shown); the $\text{Ins}(2,4,5)P_3$ -induced Ca^{2+} signal remained elevated [but to a lesser extent than that elicited by $\text{Ins}(1,3,4,5)P_4$], presumably owing to the inability of $\text{Ins}(2,4,5)P_3$ to be metabolized. When a maximal dose of $\text{Ins}(1,3,4,5)P_4$ was added after $\text{Ins}(2,4,5)P_3$ there was a discernible increase in Ca^{2+} release (Fig. 2e). This additivity was more apparent when $\text{Ins}(1,3,4,5)P_4$ was added before $\text{Ins}(2,4,5)P_3$ (Fig. 2f); in this trace the additive effect of $\text{Ins}(2,4,5)P_3$ above the elevated Ca^{2+} -response plateau induced by $\text{Ins}(1,3,4,5)P_4$ was clearly evident.

When Ca^{2+} -mobilization dose/response curves for $\text{Ins}(1,4,5)P_3$, $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(2,4,5)P_3$ were constructed, the $\text{Ins}(1,4,5)P_3$ showed half-maximal activity of $\sim 1.5 \mu\text{M}$, whereas $\text{Ins}(1,3,4,5)P_4$ had an estimated half-maximal activity of $2 \mu\text{M}$. $\text{Ins}(1,3,4,5)P_4$ was less active than $\text{Ins}(1,4,5)P_3$ at all doses tested; a $2 \mu\text{M}$ dose of $\text{Ins}(1,4,5)P_3$ stimulated the release of $0.205 \pm 0.022 \text{ nmol}$ of Ca^{2+} /mg of protein, as compared with $0.100 \pm 0.013 \text{ nmol}$ of Ca^{2+} /mg of protein for $\text{Ins}(1,3,4,5)P_4$ (means $\pm$ S.E.M.; $n = 42$; Fig. 3). At lower doses $\text{Ins}(2,4,5)P_3$ appeared to be a partial agonist: comparing $2 \mu\text{M}$ doses, $\text{Ins}(2,4,5)P_3$ was less than one-seventh as potent as $\text{Ins}(1,4,5)P_3$ and one-third as potent as $\text{Ins}(1,3,4,5)P_4$, in Ca^{2+} -releasing activity. However, at very high doses ($\geq 20 \mu\text{M}$) the Ca^{2+} response induced by $\text{Ins}(2,4,5)P_3$ surpassed the Ca^{2+} -releasing potency of $\text{Ins}(1,3,4,5)P_4$ (Fig. 3).

Several reports have shown that heparin is a potent inhibitor of both $\text{Ins}(1,4,5)P_3$ binding and $\text{Ins}(1,4,5)P_3$ -stimulated Ca^{2+} release (Worley *et al.*, 1987; Ghosh *et al.*, 1988; Nilsson *et al.*, 1988; Guillemette *et al.*, 1989). Addition of heparin ($200 \mu\text{g}/\text{ml}$) to the microsomal preparation, after establishing the Ca^{2+} responses to $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$, abolished or substantially decreased the subsequent $\text{Ins}(1,4,5)P_3$ -induced Ca^{2+} response; in contrast, the Ca^{2+} response to $\text{Ins}(1,3,4,5)P_4$ remained unaffected (Fig. 4). A few of the post-heparin $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} responses showed a small decrease, whereas the majority were unchanged or showed slight enhancement of Ca^{2+} release (traces not shown).

When the quantitative effects of heparin ($200 \mu\text{g}/\text{ml}$) on the $\text{Ins}P_3$ - and $\text{Ins}P_4$ -induced Ca^{2+} responses were compared, the $\text{Ins}(1,4,5)P_3$ response was decreased to $33 \pm 5.7\%$ (S.E.M., $n = 18$) of the control value, whereas the $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} release was almost unchanged ($94 \pm 12\%$, $n = 17$). The same concentration of heparin inhibited the binding of radiolabelled $\text{Ins}(1,4,5)P_3$ to the bovine adrenocortical preparation by about 90% (results not shown). Displacement curves for four doses of

Fig. 3. Dose/response curves for inositol phosphate-induced Ca^{2+} release in bovine adrenal particles

Ca^{2+} -release data were obtained as described in the Materials and methods section. ●, $\text{Ins}(1,4,5)P_3$; ▲, $\text{Ins}(1,3,4,5)P_4$; ○, $\text{Ins}(2,4,5)P_3$. Data points are means of at least three determinations in different experiments. Comparing $2 \mu\text{M}$ doses, $\text{Ins}(1,4,5)P_3$ is 2-fold more active than $\text{Ins}(1,3,4,5)P_4$ and 7-fold more active than $\text{Ins}(2,4,5)P_3$. The $40 \mu\text{M}$ -dose for $\text{Ins}(2,4,5)P_3$ is depicted with an arrow.

Fig. 4. Effect of heparin on $\text{Ins}(1,4,5)P_3$ - and $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} release in a bovine adrenal particulate fraction

Fura-2 was used to measure Ca^{2+} release induced by $2 \mu\text{M}$ - $\text{Ins}(1,4,5)P_3$ and $-\text{Ins}(1,3,4,5)P_4$ before and after treatment with $200 \mu\text{g}$ of heparin/ml. The subsequent $\text{Ins}(1,4,5)P_3$ response after heparin addition was markedly decreased, whereas the $\text{Ins}P_4$ response remained unchanged.

heparin tested (Fig. 5) indicate that a dose of $50 \mu\text{g/ml}$ was sufficient to inhibit binding of $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ by more than 50%; $500 \mu\text{g}$ of heparin/ml completely inhibited binding of $\text{Ins}(1,4,5)\text{P}_3$. The inhibition of $\text{Ins}(1,4,5)\text{P}_3$ binding by heparin has been reported to have an IC_{50} of $5\text{--}10 \mu\text{g/ml}$, similar to the IC_{50} values reported for heparin inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release (Hill *et al.*, 1987; Worley *et al.*, 1987; Ghosh *et al.*, 1988; Nilsson *et al.*, 1988; Guillemette *et al.*, 1989).

The Ca^{2+} -mobilizing effect of $\text{Ins}(1,3,4,5)\text{P}_4$ in adrenal microsomes appears to be due to the intrinsic activity of the tetra-kisphosphate itself. However, a recent report has shown that an apparent Ca^{2+} -releasing action of $\text{Ins}(1,3,4,5)\text{P}_4$ was due to its rapid conversion into $\text{Ins}(1,4,5)\text{P}_3$ (Cullen *et al.*, 1989). To determine whether $\text{Ins}(1,4,5)\text{P}_3$ formation contributed to the $\text{Ins}(1,3,4,5)\text{P}_4$ -stimulated Ca^{2+} response, we analysed the metabolism of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ while simultaneously measuring the Ca^{2+} response in Fura-2-loaded adrenal microsomes. The addition of $2 \mu\text{M}$ unlabelled $\text{Ins}(1,3,4,5)\text{P}_4$ mimicked the conditions employed for Ca^{2+} -release studies and ensured that an adequate Ca^{2+} response could be monitored while following the metabolism of the radioactive compound. The data shown in Fig. 6 are representative of four $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ -metabolism experiments, in which $\text{Ins}(1,3,4,5)\text{P}_4$ was rapidly metabolized to $\text{Ins}(1,3,4)\text{P}_3$ and was almost completely dephosphorylated after 5 min. A concomitant slow rise in the formation of $\text{Ins}(1,4)\text{P}_2$ was attributable to the dephosphorylation of $\text{Ins}(1,3,4)\text{P}_3$. There was also a small degree of dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$ to $\text{Ins}(1,4,5)\text{P}_3$, as determined by h.p.l.c. analysis, with a gradual increase from 10 s to 5 min. The extent of this conversion, which was at most 5% of the added radioactivity, corresponds to approx. $100 \text{ nM-Ins}(1,4,5)\text{P}_3$ (Fig. 6, inset). The Ca^{2+} response of our membrane preparation to this concentration of $\text{Ins}(1,4,5)\text{P}_3$ was barely detectable above baseline. The finding that $\text{Ins}(1,4,5)\text{P}_3$ was a very minor product of $\text{Ins}(1,3,4,5)\text{P}_4$ metabolism is in agreement with previous studies in permeabilized adrenal cells, which suggest that $\text{Ins}(1,3,4,5)\text{P}_4$ is predominantly dephosphorylated to $\text{Ins}(1,3,4)\text{P}_3$ (Balla *et al.*, 1988).

DISCUSSION

$\text{Ins}(1,4,5)\text{P}_3$ stimulated a rapid release of Ca^{2+} from bovine adrenal microsomal particles, followed by rapid re-uptake and

Fig. 5. Effects of increasing doses of heparin on binding of $\text{Ins}(1,4,5)\text{P}_3$ in a bovine adrenal particulate preparation

The membrane fraction was incubated with $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ in the presence of increasing concentrations of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ and increasing doses of heparin sulphate: $\circ$, none; $\square$, $10 \mu\text{g/ml}$; $\blacksquare$, $50 \mu\text{g/ml}$; $\triangle$, $100 \mu\text{g/ml}$; $\blacktriangle$, $500 \mu\text{g/ml}$. Each data point was performed in triplicate from data collected in two different experiments.

Fig. 6. Kinetics of metabolism of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ in bovine adrenal particles

The metabolism of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was monitored while Ca^{2+} release was simultaneously measured in a microsomal preparation containing $2 \mu\text{M}$ unlabelled $\text{Ins}(1,3,4,5)\text{P}_4$ (see the Materials and methods section for details). The Ca^{2+} response typically reached its new baseline 10 min after addition of $\text{Ins}(1,3,4,5)\text{P}_4$, within the time frame of metabolite measurement. Metabolites detected by h.p.l.c. were: $\bullet$, $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$; $\circ$, $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$; $\blacktriangle$, $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$; $\triangle$, $[^3\text{H}]\text{InsP}_2$. Inset: $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ formation during $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ metabolism; $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ appeared at 10 s and was completely dephosphorylated by 5 min. At its maximum, $\text{Ins}(1,4,5)\text{P}_3$ comprised only 5% of the total radioactivity in the sample ($\sim 100 \text{ nM}$). These results are representative of four individual experiments.

return of the free Ca^{2+} level to baseline. $\text{Ins}(2,4,5)\text{P}_3$ induced a much smaller release of Ca^{2+} at lower doses than did $\text{Ins}(1,4,5)\text{P}_3$, and also exhibited different kinetics, with a prolonged plateau phase. More remarkably, $\text{Ins}(1,3,4,5)\text{P}_4$ induced rapid and prominent Ca^{2+} mobilization when added to adrenal microsomes. The Ca^{2+} -response kinetics for $\text{Ins}(1,3,4,5)\text{P}_4$ differed from the response to $\text{Ins}(1,4,5)\text{P}_3$, and usually exhibited a rapid transient spike followed by a prolonged plateau phase. $\text{Ins}(1,3,4,5)\text{P}_4$ was approximately half as active as $\text{Ins}(1,4,5)\text{P}_3$ and 3-fold more active than $\text{Ins}(2,4,5)\text{P}_3$, when responses to $2 \mu\text{M}$ concentrations of each agonist are compared.

An independent and additive effect of $\text{Ins}(1,3,4,5)\text{P}_4$ was clearly evident when maximal doses of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were added in rapid succession. The additive effect was enhanced when $\text{Ins}(1,4,5)\text{P}_3$ was applied after $\text{Ins}(1,3,4,5)\text{P}_4$. Heparin sulphate consistently inhibited $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release, but did not decrease the Ca^{2+} response to $\text{Ins}(1,3,4,5)\text{P}_4$. The dose of heparin ($200 \mu\text{g/ml}$) which maximally inhibited $\text{Ins}(1,4,5)\text{P}_3$ binding also inhibited the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. This suggested that the $\text{Ins}(1,3,4,5)\text{P}_4$ -stimulated Ca^{2+} mobilization was due to the action of $\text{Ins}(1,3,4,5)\text{P}_4$ at a site that was independent of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Heparin appears to be a selective inhibitor at the adrenal InsP_3 -receptor site in terms of binding and Ca^{2+} responses. In the bovine adrenal microsomal preparation (J. A. Ely, unpublished work) and in studies on a rat liver cell line (Hill *et al.*, 1987), $\text{Ins}(2,4,5)\text{P}_3$ -induced Ca^{2+} release was also inhibited by heparin, indicating that its Ca^{2+} -mobilizing action is mediated by the $\text{Ins}(1,4,5)\text{P}_3$ -receptor site. A selective action of heparin on the InsP_3 -binding site has also been noted in the Ca^{2+} -sequestration response induced in this same rat liver cell line, in which $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} sequestration [after addition of either $\text{Ins}(2,4,5)\text{P}_3$ or Ca^{2+}] was not inhibited by the addition of heparin. This finding indicates that the Ca^{2+} -regulating action of $\text{Ins}(1,3,4,5)\text{P}_4$, albeit different in this cell type, is

also not blocked by heparin. Thus it appears that $\text{Ins}(1,3,4,5)P_4$ can exert its action via a receptor site that is pharmacologically distinct from the high-affinity $\text{Ins}(1,4,5)P_3$ receptor. Specific binding sites for $\text{Ins}(1,3,4,5)P_4$ have been identified in at least three different cell types (Bradford & Irvine, 1987; Theibert *et al.*, 1987; Enyedi & Williams, 1988), providing a locus through which $\text{Ins}(1,3,4,5)P_4$ may act. Recently, mobilization of Ca^{2+} by $\text{Ins}(1,3,4,5)P_4$ has also been reported in cerebellar microsomes (Joseph *et al.*, 1989), but here the process was inhibited by heparin, in contrast with the responses that we observed in the adrenal microsomal fraction.

Our results are compatible with the proposal that under physiological conditions $\text{Ins}(1,3,4,5)P_4$ may participate in the transfer of Ca^{2+} between various intracellular pools, some of which are sensitive to $\text{Ins}(1,4,5)P_3$ -induced Ca^{2+} release (Irvine *et al.*, 1988). Although an independent effect of $\text{Ins}(1,3,4,5)P_4$ has not been observed in several permeabilized or whole-cell preparations (Irvine *et al.*, 1986; Irvine & Moor, 1986; Hansen *et al.*, 1986; Morris *et al.*, 1987; Nilsson *et al.*, 1988), its presence in our particulate fraction may reflect the physical or functional separation of such Ca^{2+} pools during homogenization, with formation of vesicles that are individually sensitive to $\text{Ins}(1,4,5)P_3$ or $\text{Ins}(1,3,4,5)P_4$. Furthermore, the kinetics of the Ca^{2+} response induced by $\text{Ins}(1,3,4,5)P_4$ differ from those of the response to $\text{Ins}(1,4,5)P_3$, suggesting a different mode of action of $\text{Ins}P_4$. Although the Ca^{2+} -releasing activity of $\text{Ins}(1,3,4,5)P_4$ is quantitatively less than that of $\text{Ins}(1,4,5)P_3$, its action is independent from that of $\text{Ins}(1,4,5)P_3$, raising the possibility that $\text{Ins}(1,3,4,5)P_4$ has a specific messenger function during hormone-induced Ca^{2+} signalling.

The specific action of $\text{Ins}(1,3,4,5)P_4$ on the release of stored Ca^{2+} was further evaluated by analysis of the metabolism of radiolabelled $\text{Ins}(1,3,4,5)P_4$ in adrenal microsomes. Recent studies have described the formation of $\text{Ins}(1,4,5)P_3$ from $\text{Ins}(1,3,4,5)P_4$ via its dephosphorylation by a specific 3-phosphatase, in addition to its dephosphorylation by a 5-phosphatase to form $\text{Ins}(1,3,4)P_3$ (Doughney *et al.*, 1988; Hoer *et al.*, 1988; Cullen *et al.*, 1989). It has been suggested that the observed Ca^{2+} -mobilizing action of $\text{Ins}(1,3,4,5)P_4$ may be due, in part, to the action of $\text{Ins}(1,4,5)P_3$, which was rapidly converted from $\text{Ins}(1,3,4,5)P_4$. However, our metabolism studies reveal very little conversion of $[\text{H}]\text{Ins}(1,3,4,5)P_4$ into $[\text{H}]\text{Ins}(1,4,5)P_3$ in bovine adrenal microsomes, where the major pathway is that of dephosphorylation to $\text{Ins}(1,3,4)P_3$ (Balla *et al.*, 1988). This finding indicates that the $\text{Ins}(1,3,4,5)P_4$ -induced Ca^{2+} responses seen in our adrenal preparation are due to the intrinsic action of $\text{Ins}(1,3,4,5)P_4$ and not to formation of $\text{Ins}(1,4,5)P_3$, which in any case would be ineffective in the presence of heparin. It is not likely that the major metabolic product of $\text{Ins}(1,3,4,5)P_4$ dephosphorylation, $\text{Ins}(1,3,4)P_3$, could stimulate Ca^{2+} release, since it has much lower potency than $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ for Ca^{2+} release in adrenal microsomes (results not shown) and in other studies (Polokoff *et al.*, 1988; Strupish *et al.*, 1988).

In conclusion, the individual effects of $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ on Ca^{2+} release, the different kinetics of the Ca^{2+} response induced by these two compounds, and the differential inhibitory effect of heparin on the two responses, suggest that $\text{Ins}(1,3,4,5)P_4$ acts on a specific receptor to exert an independent effect on the release or transfer of intracellular Ca^{2+} in adrenocortical cells.

We thank Dr. Tamás Balla for helpful suggestions and discussion of the data, and Dr. Kathryn Sandberg for a critical review of the

manuscript. We are grateful to Dr. Glenn D. Prestwich (SUNY, Stony Brook, NY) for generously providing us with $\text{Ins}(1,3,4)P_3$. J.A.E. was supported by a fellowship from Sigma-Tau, S.p.A., Rome, Italy.

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