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Binding of inositol phosphates and induction of Ca^{2+} release from pituitary microsomal fractions

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Bovine anterior-pituitary microsomal fractions exhibit high-affinity, saturable and reversible binding of inositol 1,4,5-[^{32}P]trisphosphate; 50% of the labelled ligand is displaced by 3.5 nM-inositol 1,4,5-trisphosphate, 0.5 μM-inositol 1,4-bisphosphate and 10 μM-ATP. Inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from the microsomal vesicles (half-maximal effect at 290 nM), and its action is potentiated by inositol tetrakisphosphate (half-maximal effect at 4 μM).

INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$), formed through the phosphodiesteratic hydrolysis of phosphatidylinositol 4,5-bisphosphate (Berridge, 1983; Berridge & Irvine, 1984) induces the release of Ca^{2+} from the endoplasmic reticulum (as reviewed by Abdel-Latif, 1986; Berridge, 1986; Irvine, 1986; Williamson, 1986) and thus triggers the biological response to 'calcium-mobilizing' hormones and neurotransmitters. In order to explore the mode of the Ca^{2+}-releasing action of $InsP_3$, we characterized its binding to specific intracellular binding sites. The high affinity, saturable and reversible binding of $InsP_3$ to non-mitochondrial intracellular sites in permeabilized guinea-pig hepatocytes and rabbit peritoneal polymorphonuclear cells showed a strong correlation with Ca^{2+} release in terms of concentration-dependence as well as specificity (Spät *et al.*, 1986*a*). We localized the binding site in the microsomal fraction of rat liver (Spät *et al.*, 1986*b*) and bovine adrenal cortex (Baukal *et al.*, 1985). In order to generalize and extend these observations, in the present experiments we studied the binding characteristics and effect of $InsP_3$ in a hitherto non-examined type of tissue, the peptide-hormone-secreting anterior pituitary. $InsP_3$ is formed in the cells of the anterior pituitary in response to gonadotropin-releasing hormone (Kiesel *et al.*, 1986), thyrotropin-releasing hormone (Martin, 1983; Rebecchi & Gershengorn, 1983; Drummond & Raeburn, 1984) or angiotensin II (Canonico & Macleod, 1986; Enjalbert *et al.*, 1986). $InsP_3$ induces Ca^{2+} release in permeabilized pituitary-tumour cells (Gershengorn *et al.*, 1984; Biden *et al.*, 1986). Here we provide direct evidence for the first time in favour of the microsomal Ca^{2+}-releasing action of $InsP_3$ in non-tumorous anterior pituitary. The results suggest that similar or identical binding sites are responsible for the control of Ca^{2+} efflux from the endoplasmic reticulum in different mammalian cell types. Moreover, we observed that ATP competes with $InsP_3$ for its binding site. This effect may account for the difference between K_d and EC_{50} of $InsP_3$. We also report on a synergistic action of $InsP_3$ and $InsP_4$ on intracellular Ca^{2+} metabolism.

MATERIALS AND METHODS

[^{32}P]$InsP_3$, prepared from [γ-^{32}P]ATP-labelled human red-blood-cell ghosts (Spät *et al.*, 1986*a*), was obtained from du Pont–New England Nuclear. Its specific radioactivity at the time of the experiments, as estimated by self-displacement analysis (Catt *et al.*, 1976), was 7–10 Ci/mmol. $InsP_3$ and $InsP_4$ were given by Dr. R. F. Irvine (Cambridge, U.K.). $InsP_2$ was obtained from Amersham International. All other chemicals were purchased from Sigma or Serva.

Fresh bovine pituitaries were collected in liquid N_2 and stored at −80 °C until homogenization. The separated anterior pituitary was homogenized first in a Polytron homogenizer and then with a glass–Teflon Potter homogenizer at 4 °C. Microsomal fraction (microsomes) was prepared as described by Dawson & Irvine (1984), with the modification that the pellet obtained after the first 35000 *g* centrifugation was resuspended in 250 mM-sucrose/5 mM-Hepes/10 mM-KCl/1 mM-dithiothreitol/0.15 mM-EGTA (pH 7.0) and re-centrifuged at 35000 *g* for 30 min.

For binding studies, microsomal vesicles (250 μg of protein) were incubated in a cytoplasmic-type medium (pH 7.0), containing 10 mM-NaCl, 100 mM-KCl, 0.513 mM-$MgCl_2$, 1 mM-NaH_2PO_4, 10 mM-Hepes, 1 mM-EGTA, 0.330 mM-$CaCl_2$ and 1 mg of bovine serum albumin/ml. The calculated (Fabiato & Fabiato, 1979) free Ca^{2+} and Mg^{2+} concentrations were about 235 nM and 0.5 mM respectively. The medium was completed with 8–13 nCi of [^{32}P]$InsP_3$/ml. Except for kinetic studies, the volume of the incubation medium was 500 μl. Non-specific binding was determined in the presence of 2–10 μM-$InsP_3$. Incubations were carried out in a shaking ice bath, for 5 min (except for kinetic studies) and terminated by filtration through GF/C filters as described by Spät *et al.* (1986*b*). All samples were counted for radioactivity at least twice for 20 min by liquid-scintillation spectrometry. Binding parameters were estimated by graphical analysis of the limiting slopes of the Scatchard curve (Thakur *et al.*, 1980).

For Ca^{2+}-transport measurements, microsomes (about 1 mg/ml) were incubated at 30 °C in the fol-

Abbreviations used: $InsP_2$, inositol 1,4-bisphosphate; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; EC_{50} and IC_{50}, concentrations causing half-maximal effect or inhibition respectively.

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lowing medium: 100 mM-KCl, 10 mM-NaCl, 1 mM-$MgCl_2$, 10 mM-Hepes, 1.5 mM-ATP, 4 mM-phosphocreatine, 20 μg of creatine kinase/ml, 0.5 mM-dithiothreitol and 1 μg of oligomycin/ml, pH 7.0. The free Ca^{2+} concentration was monitored with a Ca^{2+}-selective electrode (Lukács & Fonyó, 1986), calibrated with calcium buffers (Tsien & Rink, 1980). Ca^{2+} release was quantified on the basis of the electrode response to known amounts of $CaCl_2$ added after microsomes reached the steady-state Ca^{2+} concentration.

RESULTS AND DISCUSSION

Pituitary microsomal binding sites for InsP_3 obey all the criteria for receptor function: high affinity, saturability, reversibility, specificity, and also the ability to trigger the biological response. Binding of [^{32}P]InsP_3 (equivalent to 1.3 nM) by pituitary microsomes was complete within 10 s ($n = 3$; results not shown). Addition of excess InsP_3 induced the dissociation of the ligand–receptor complex, and in two experiments half-times of 3 and 5 min were observed (Fig. 1). The binding was saturable, and half-maximal displacement of the tracer was attained by 3.5 nM unlabelled ligand on average (Fig. 2). This value is comparable with that observed in rat liver (8 nM) (Spät *et al.*, 1986*b*) and bovine adrenocortical microsomes (5 nM) (Baukal *et al.*, 1985) as well as in a bovine pituitary particulate fraction (0.9 nM) (Guillemette *et al.*, 1987) obtained by a homogenization and isolation method different from that used in the present work. Accordingly, it may be assumed that similar or identical binding sites are to be found in different mammalian tissues. Scatchard analysis of the

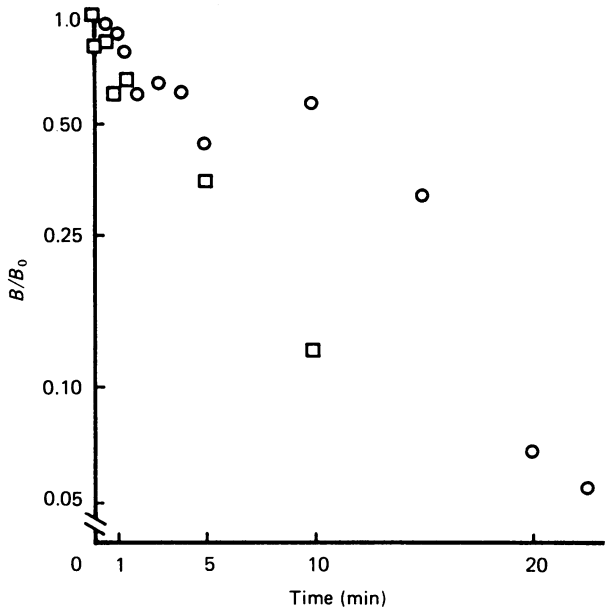

Fig. 1. Dissociation of [^{32}P]InsP_3 from pituitary microsomal binding sites

After incubation with labelled ligand for 5 min (as detailed in the Materials and methods section), unlabelled InsP_3 was added to give a final concentration of 20 μM. Samples (0.5 ml) were filtered at various time intervals. The two sets of symbols represent data derived from two separate preparations. Zero-time specific binding before dissociation, in the presence of 6.6 and 4.2 nCi/ml, was 105 and 74, and non-specific binding was 55 and 48 c.p.m./mg of protein respectively.

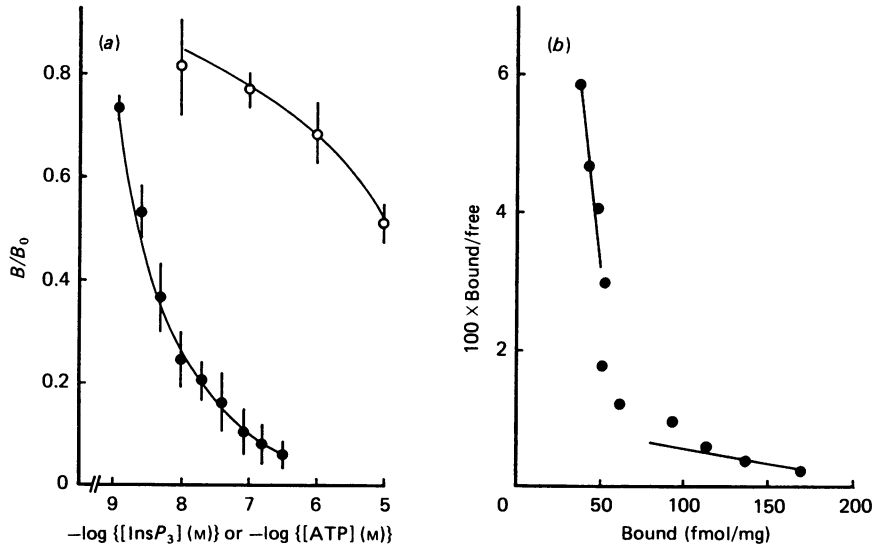

Fig. 2. Displacement of [^{32}P]InsP_3 by unlabelled InsP_3 (●) or ATP (○)

Pituitary microsomes were incubated in an ice bath in a cytosol-like medium containing 1.3 nM-labelled ligand, in the absence (B_0) or the presence (B) of various concentrations of unlabelled ligand. Incubation was terminated after 5 min by filtration. Binding values, corrected for non-specific binding (81 ± 15 c.p.m./mg of protein) and expressed relative to specific binding observed in the absence of unlabelled ligand (B_0, 100 ± 22 c.p.m./mg of protein) are shown in (*a*). Values are means ± S.E.M. for two or three separate preparations, each done in duplicate. In (*b*) a Scatchard plot of the specific-binding data (together with its limiting slopes) is shown for one of three similar experiments.

data (Fig. 2) demonstrates a curvilinear plot, which can be resolved into two components. The mean value for the apparent K_d, calculated from slope of the high-affinity component, is 1.38 ± 0.27 nM and that for the intercept on the abscissa, representative of receptor concentration, is 38 ± 12 fmol/mg of protein (both $\pm$S.E.M.; $n = 3$). The apparent K_d of the low-affinity compartment is 50.8 ± 13.1 nM, with an apparent receptor concentration of 197 ± 93 fmol/mg. Whether the upward concavity of the Scatchard plot is indicative of two separate receptor population, or of a complex receptor–ligand interaction, requires further studies.

In the presence of ATP, the microsomes accumulated Ca^{2+} from the medium until the steady-state extravesicular Ca^{2+} concentration (0.2 μM) was attained. A mitochondrial uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone; 2 μM) and an inhibitor of the mitochondrial Ca^{2+} uptake (Ruthenium Red; 2 μM) both failed to alter the steady-state Ca^{2+} concentration (results not shown). InsP_3 induced the release of Ca^{2+} from the vesicles. The maximal extent of release was 0.81 ± 0.15 nmol/mg, which is 7% of the ionophore-A-23189-releasable Ca^{2+} pool. A second pulse of maximally effective InsP_3, added within 3 min after the first pulse, failed to induce further Ca^{2+} release. Half-maximal release was attained with 0.29 ± 0.10 μM-InsP_3 (mean $\pm$S.E.M., $n = 4$), a concentration comparable with that described in other cell types (cf. Berridge & Irvine, 1984) and with estimated intracellular concentrations of InsP_3 (Bradford & Rubin, 1986; Wollheim & Biden, 1986). This concentration is, however, two orders of magnitude higher than that required for half-maximal occupancy of the InsP_3 receptor. This discrepancy may be due to the affinity of ATP (present at 1.5 mM in the Ca^{2+}-release studies) for InsP_3 receptors. As shown in Fig. 2, ATP displaced [^{32}P]InsP_3 with an IC_{50} of 10 μM.

Binding and effect of InsP_3 were compared with that of InsP_2. This metabolic product of InsP_3 does not evoke Ca^{2+} release in permeabilized pancreatic acinar cells (Streb *et al.*, 1983), hepatocytes (Burgess *et al.*, 1984) or Swiss-mouse 3T3 cells (Irvine *et al.*, 1984). In the present experiments a 50% displacement of [^{32}P]InsP_3 was achieved by 0.5 μM-InsP_2 (of unknown purity) ($n = 2$). It did not induce Ca^{2+} release when applied in concentrations up to 12 μM (results not shown). [In evaluation of the Ca^{2+} curves, correction has been made for the Ca^{2+}-like contamination of Ins(1,4)P_2.]

The present study was the first to examine the effect of the recently described inositol metabolite InsP_4 (Batty *et al.*, 1985; Heslop *et al.*, 1985) on the binding of InsP_3. In accordance with previous observations (Hansen *et al.*, 1986; Irvine *et al.*, 1986; Wollheim & Biden, 1987), InsP_4, in concentrations up to 8 μM, failed to induce Ca^{2+} release. We observed, however, that it potentiated the Ca^{2+}-releasing effect of InsP_3. In two separate experiments, where InsP_3 was applied at 0.22 and 0.55 μM respectively, half-maximal potentiation was attained by 4μM-InsP_4 (Fig. 3*a*). The extent of maximal Ca^{2+} release increased by 27%, and the EC_{50} of InsP_3 decreased by 30% on average (Fig. 3*b*). The fact that InsP_4 had no effect in itself, but augmented the InsP_3-induced Ca^{2+} release to supramaximal values, indicates that its effect may not be attributed to contamination with InsP_3. The possibility arose that InsP_4 increases the actual concentration of InsP_3 and enhances Ca^{2+} release by competing with InsP_3 for InsP_3 5-phosphomonoesterase (Connolly

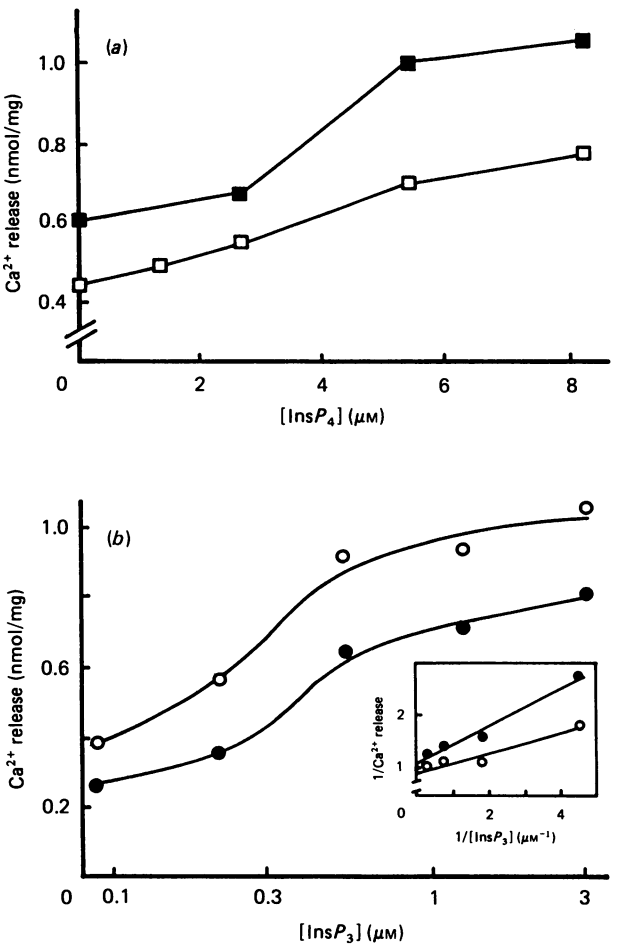

Fig. 3. Effect of InsP_4 on InsP_3-induced Ca^{2+} release from pituitary microsomes

The microsomal suspension was incubated and Ca^{2+} concentration monitored with a Ca^{2+}-selective electrode as described in the Materials and methods section. After the steady-state Ca^{2+} concentration (0.2 μM) was attained, inositol phosphates were added. (*a*) InsP_3 (■, 0.55 μM; □, 0.22 μM)-induced Ca^{2+} release as a function of InsP_4 concentration in two separate experiments. (*b*) InsP_3-induced Ca^{2+} release in the absence (●) or presence (○) of 5.4 μM-InsP_4. Averaged data from two separate experiments are shown. Inset: double-reciprocal plot of the same data.

et al., 1987). But this was not the case, since 2,3-bisphosphoglycerate, a competitive inhibitor of the enzyme, did not potentiate the effect of InsP_3 in our system. Moreover, the effect of InsP_4 to increase InsP_3-induced maximal Ca^{2+} release suggests that a Ca^{2+} pool other than InsP_3-sensitive endoplasmic-reticular vesicles may represent the site of action of InsP_4. This assumption is compatible with the observation that a single pulse of maximally effective InsP_3 apparently depletes the responsive vesicles of Ca^{2+}. The results of Irvine & Moor (1986) suggest that InsP_4, in the presence of any Ca^{2+}-releasing inositol trisphosphate isomer, elicits Ca^{2+} influx through the plasma membrane in sea-urchin eggs. In view of this phenomenon, our finding may be accounted for by a release of Ca^{2+} from plasma-membrane vesicles in response to the two inositol phosphate species. Such a mechanism would also

explain the failure of $InsP_4$ to influence $InsP_3$-induced Ca^{2+} release in permeabilized cells (Irvine *et al.*, 1986; Hansen *et al.*, 1986), where the plasma membrane is freely diffusible to Ca^{2+}. Experiments are now needed to localize the site of action of $InsP_4$, a potential new second messenger.

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