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

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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 (Spát et al., 1986a). We localized the binding site in the microsomal fraction of rat liver (Spát et al., 1986b) 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 (Canonicco & 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

[32P]InsP$_3$, prepared from [γ-32P]ATP-labelled human red-blood-cell ghosts (Spát et al., 1986a), 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$_4$ 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 Poltron 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/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 cytoplasmatic-type medium (pH 7.0), containing 10 mM-NaCl, 100 mM-KCl, 0.513 mM-MgCl$_2$, 1 mM-Na$_2$PO$_4$, 10 mM-Hepes, 1 mM-EGTA, 0.330 mM-CaCl$_2$ and 1 mg of bovine serum albumin/ml. The calculated (Fabiano & 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 [32P]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 Spát et al. (1986b). 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$_3$, inositol 1,4-bisphosphate; InsP$_4$, inositol 1,4,5-trisphosphate; InsP$_5$, 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-
MgCl₂, 10 mM-Hepes, 1.5 mM-ATP, 4 mM-phospho-
creatine, 20 μg of creatine kinase/ml, 0.5 mM-dithiothre-
tol and 1 μg of oligomycin/ml, pH 7.0. The free Ca²⁺
concentration was monitored with a Ca²⁺-selective
electrode (Lukács & Fonyó, 1986), calibrated with
calcium buffers (Tsien & Rink, 1980). Ca²⁺ release was
quantified on the basis of the electrode response to known
amounts of CaCl₂ added after microsomes reached the
steady-state Ca²⁺ concentration.

RESULTS AND DISCUSSION

Pituitary microsomal binding sites for InsP₃ obey all
the criteria for receptor function: high affinity, saturat-
bility, reversibility, specificity, and also the ability to
trigger the biological response. Binding of [³²P]InsP₃
(equivalent to 1.3 nM) by pituitary microsomes was
complete within 10 s (n = 3; results not shown).
Addition of excess InsP₃ 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 displace-
ment 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., 1986b) 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 [³²P]InsP₃ from pituitary microsomal
binding sites](image)

After incubation with labelled ligand for 5 min (as
detailed in the Materials and methods section), unlabelled InsP₃
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 dissocia-
tion, 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 [³²P]InsP₃ by unlabelled InsP₃ (●) or ATP (○)](image)

Pituitary microsomes were incubated in an ice bath in a cytosol-like medium containing 1.3 nM-labelled ligand, in the absence
(Bₒ) 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 (Bₒ, 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.

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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 $1.38 \pm 0.27 \text{ nm}$ and that for the intercept on the abscissa, representative of receptor concentration, is $38 \pm 12 \text{ fmol/mg of protein (both} \pm \text{S.E.M.; } n = 3)$. The apparent $K_d$ of the low-affinity compartment is $50.8 \pm 13.1 \text{ nm}$, with an apparent receptor concentration of $197 \pm 93 \text{ 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 $0.81 \pm 0.15 \text{ nmol/mg}, which is 7\% of the ionophore-A-23187-releasable Ca$^{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 $0.29 \pm 0.10 \mu$M-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$_4$. This metabolic product of InsP$_3$ does not evoke Ca$^{2+}$ release in permeabilized pancreatic acinar cells (Streeter 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$_4$ (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$_3$ (Fig. 3a). The extent of maximal Ca$^{2+}$ release increased by 27%, and the EC$_{50}$ of InsP$_3$ decreased by 30% on average (Fig. 3b). 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 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$_3$. 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 InsP₄ to influence InsP₃-induced Ca²⁺ release in permeabilized cells (Irvine et al., 1986; Hansen et al., 1986), where the plasma membrane is freely diffusible to Ca²⁺. Experiments are now needed to localize the site of action of InsP₄, a potential new second messenger.

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REFERENCES


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