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Binding of inositol trisphosphate by a liver microsomal fraction

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Accumulating evidence suggests that the increase in cytosolic Ca^{2+} induced by receptor agonists is mediated by inositol 1,4,5-trisphosphate, a product of phospholipase C-mediated breakdown of phosphatidylinositol 4,5-bisphosphate. The present study employs inositol tris[^{32}P]phosphate to demonstrate a specific receptor binding site in a microsomal fraction of rat liver.

INTRODUCTION

Calcium-mobilizing hormones and neurotransmitters evoke rapid and characteristic changes in the metabolism of phosphoinositides, primarily PtdIns(4,5)P_2 (Michell *et al.*, 1981; Berridge & Irvine, 1984). Ins(1,4,5)P_3 is formed in response to hormonal stimulation by phospholipase C mediated hydrolysis of PtdIns(4,5)P_2, and is thought to be responsible for the release of calcium ions from cellular stores (Berridge, 1983). The time course of Ins(1,4,5)P_3 formation as well as its effect on calcium metabolism in various types of permeabilized cells supports this postulate (Berridge, 1984; Berridge & Irvine, 1984; Williamson *et al.*, 1985). Moreover, it was found that Ins(1,4,5)P_3 is capable of releasing calcium from various microsomal preparations (Prentki *et al.*, 1984; Streb *et al.*, 1984; O'Rourke *et al.*, 1985), including a rat liver microsomal fraction (Dawson & Irvine, 1984; Muallem *et al.*, 1985). In this report we present evidence for the specific, saturable, and reversible binding of radiolabelled Ins(1,4,5)P_3 to a crude microsomal fraction of rat liver, which demonstrates a specific receptor for this putative second messenger.

MATERIALS AND METHODS

Materials

Ins(1,4)P_2, Ins(4,5)P_2 and Ins(1,4,5)P_3 were prepared as previously described and generously provided by Dr. R. F. Irvine (Irvine *et al.*, 1984). All other materials were from Sigma Chemical Co. and Boehringer Mannheim Biochemicals. [γ-^{32}P]ATP (at least 3000 Ci/mmol) was obtained from New England Nuclear, and [2-^{3}H]mannose (22 Ci/mmol) was from ICN Pharmaceuticals.

Preparation of [^{32}P]Ins(1,4,5)P_3

Human erythrocyte ghosts (1.5 mg of protein) were incubated for 20 min at room temperature in 200 μl of a solution containing 5 mM-$MgCl_2$, 1 mM-EGTA, 25 mM-imidazole/HCl (pH 7.0) and 4 mCi of [^{32}P]ATP (modified after Quist, 1985). The incubation was terminated by adding 8 vol. of 1 mM-EDTA in 10 mM-Tris/HCl buffer (pH 7.5). The membranes were then repeatedly washed with buffer. Hydrolysis of labelled polyphosphoinositides was induced by incubating the membranes in 25 mM-imidazole buffer (pH 7.0) containing 0.18 mM-$CaCl_2$ for 10 min at room temperature. The reaction was stopped by adding $HClO_4$. The acid-soluble supernatant was neutralized with KOH, and the inositol phosphates were separated on Dowex-1 anion-exchange resin (Berridge *et al.*, 1983). Ammonium ions were removed by passing the sample through a Dowex-50 (H^+ form) column. The eluate was lyophilized and stored (in dry state or dissolved in water) at −76 °C. The radiochemical purity of the product, as examined by paper chromatography (Desjobert & Petek, 1956) and/or h.p.l.c. (Irvine *et al.*, 1984), was greater than 90%. Specific radioactivity, determined by self-displacement analysis in the binding system (Catt *et al.*, 1976), was approx. 40 Ci/mmol.

Preparation of subcellular fractions

Subcellular fractions were prepared from livers of male Sprague–Dawley rats (starved overnight), as described by Dawson & Irvine (1984) and then stored at −76 °C prior to use. In order to assess the relative purity of the subcellular fractions, activities of rotenone-insensitive NADH–cytochrome *c* reductase [a marker for endoplasmic reticulum (Fleischer & Fleischer, 1970)] and succinate–cytochrome *c* reductase [a marker for mitochondria (Fleischer & Fleischer, 1967)] were determined, as well as total protein (Miller, 1959). Rotenone-insensitive NADH–cytochrome *c* reductase activity in the mitochondrial and cytosolic fractions amounted to 38 ± 6 and 16 ± 7% respectively of that in the microsomal fraction (per unit mass of protein). Succinate–cytochrome *c* reductase activity in the microsomal and cytosolic fractions amounted to 12 ± 2 and 1.5 ± 0.8% respectively of that in the mitochondrial fraction ($n = 3$).

Binding studies

Unless otherwise stated, the microsomal fraction or other subcellular fractions, containing 200–300 μg of protein (determined according to Miller, 1959) were incubated in a cytoplasmic-type medium (pH 7.2) (Burgess *et al.*, 1983) of the following composition (mM): NaCl, 20; KCl, 100; $MgSO_4$, 5; NaH_2PO_4, 0.96; $NaHCO_3$, 25; EGTA, 1.0; $CaCl_2$, 0.51, 2,3-bisphosphoglycerate, 1; ATP (sodium salt) 2; phosphocreatine, 5. The medium

Abbreviations used: Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(4,5)P_2, inositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(2,4,5)P_3, inositol 2,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

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also contained 10 μg of creatine kinase, 0.5 mg of bovine serum albumin, 40000 c.p.m. of [^{32}P]Ins(1,4,5)P_3 and 200 nCi of [^{3}H]mannose. The total volume of the incubation medium was 500 μl. Non-specific binding was determined in the presence of 10 μM-Ins(1,4,5)P_3. Incubations were carried out in an ice-bath, under an atmosphere of O_2/CO_2 (19:1), for 5 min (except for the kinetic studies). The calculated free Ca^{2+} concentration at this temperature (4 °C) was 180 nM. When the effect of Ca^{2+} concentration was studied, the required total Ca^{2+} concentration was calculated according to the procedure of Burgess *et al.* (1983). Incubations were terminated by filtration through phosphate-presoaked GF/C filters (Whatman), followed by a rapid washing with 5 ml of 0.25 M-sucrose in 10 mM-phosphate buffer (pH 7.0).The filtering efficiency with regard to retention of protein was $42 \pm 1\%$ (mean $\pm$ S.E.M., $n = 8$). The filters were placed in plastic scintillation vials and counted in an LKB (1211) Minibeta liquid-scintillation counter. All samples were counted for 10 min by liquid-scintillation spectrometry, and maximum counting error never exceeded 5%. ^{32}P values were corrected for trapped volume by using a [^{3}H]mannose marker (Burgess *et al.*, 1983).

RESULTS AND DISCUSSION

The liver microsomal fraction possesses Ins(1,4,5)P_3 receptors that obey the criterion for specific binding in that competition for the binding site(s) by unlabelled Ins(1,4,5)P_3 can be demonstrated (Fig. 1). A 50% displacement of the labelled ligand was attained with 8 nM of unlabelled ligand. The displacing activity of the artificial analogue Ins(2,4,5)P_3 was identical with that of Ins(1,4,5)P_3, while Ins(1,4)P_2 and Ins(4,5)P_2, at concentrations as high as 1 μM, had no significant effect on binding ($n = 2$–3) (results not shown).The differential effects of the inositol polyphosphates on radiolabelled ligand binding correspond to their respective Ca^{2+}-releasing activities. Thus, Ins(1,4,5)P_3 and Ins(2,4,5)P_3 are comparably efficacious in evoking $^{45}Ca^{2+}$ release from permeabilized hepatocytes (Burgess *et al.*, 1984), whereas, at concentrations of 1 μM or less, Ins(1,4)P_2 or Ins(4,5)P_2 are ineffective in releasing Ca^{2+} from either permeabilized hepatocytes or a liver microsomal preparation (Burgess *et al.*, 1984; Dawson & Irvine, 1984; Joseph *et al.*, 1984*a*). No changes were observed in the binding of the tracer by altering the Ca^{2+} concentration in the physiological range (0.5–570 nM). But, in the presence of a supraphysiological concentration (1.8 μM-Ca^{2+}), binding was decreased by an average of one-third.

Scatchard-analysis of the data (Fig. 1, inset) demonstrated a curvilinear plot, which could be resolved into two specific binding components. The mean values for the apparent K_D, calculated from slope of the high-affinity component, was 7.9×10^{-9} M and for the intercept of the x-axis, representive of receptor concentration, was 60 fmol/mg of protein ($n = 3$). The apparent K_D of the low-affinity component was 1.6×10^{-7} M, and the receptor concentration was 447 fmol/mg of protein. The apparent concentration of binding sites is a minimal value, since it does not take into account loss of microsomal protein through the filters.

Kinetic analysis of two preparations demonstrated that [^{32}P]Ins(1,4,5)P_3 rapidly binds to the microsomal fraction; half-maximal saturation was attained in 20 and 26 s, respectively (Fig. 2). In two separate experiments, when

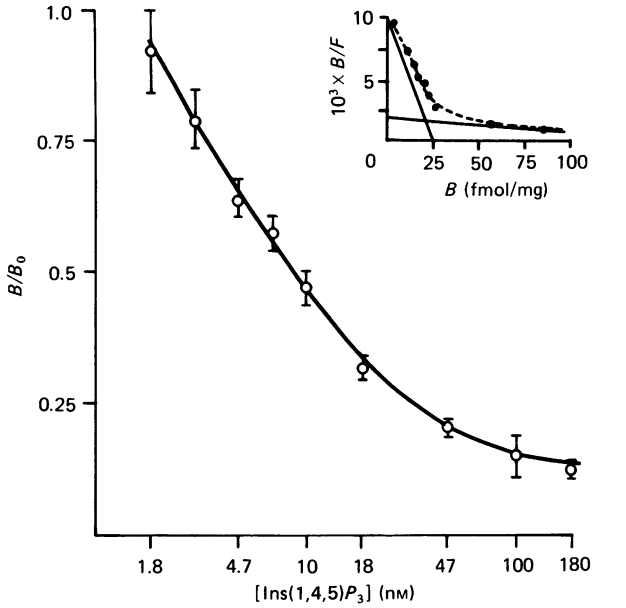

Fig. 1. Displacement of [^{32}P]Ins(1,4,5)P_3 by unlabelled Ins(1,4,5)P_3

Rat liver microsomes were incubated at 4 °C in a cytosol-like medium containing 0.9 nM labelled ligand, in the absence (B_0) or presence (B) of various concentrations of unlabelled ligand. After 5 min, the samples were filtered and washed, and the filters counted for radioactivity. The ^{32}P activity retained on the filter was corrected for trapped volume. The data are expressed as values relative to the total binding observed without unlabelled ligand (730 ± 135 c.p.m./mg of protein) and corrected for non-specific binding (220 ± 56 c.p.m./mg of protein). Values are means $\pm$ S.E.M. of three separate preparations, each done in duplicate. Inset: Scatchard plot of the specific binding data. Values are means of duplicate determinations and represent a typical result obtained from the three preparations.

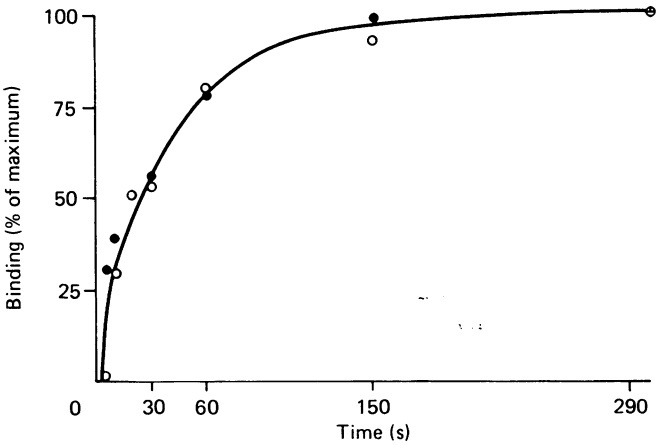

Fig. 2. Association of [^{32}P]Ins(1,4,5)P_3 with microsomal binding sites as a function of time

Binding is initiated by the addition of the microsomal suspension to buffer containing isotope. The data, derived from two separate experiments, are expressed as percentages of maximal specific binding (349 and 357 c.p.m./mg of protein) (corrected for non-specific binding).

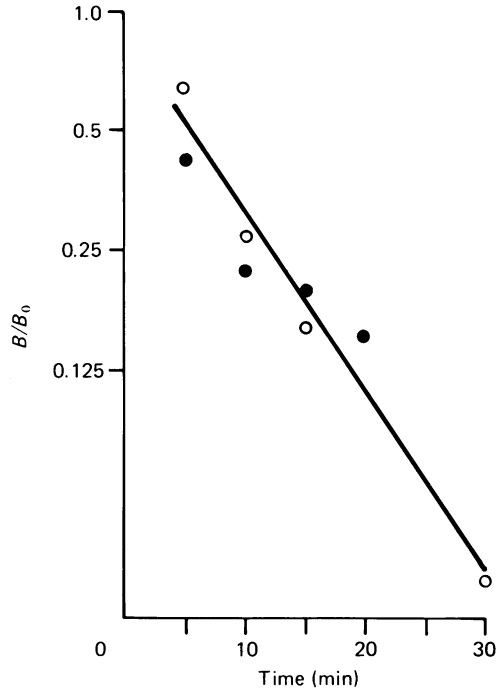

Fig. 3. Dissociation of [^{32}P]Ins(1,4,5)P_3 from microsomal binding sites

After incubation in the presence of labelled ligand for 5 min at 4 °C (as detailed in the Materials and methods section), unlabelled Ins(1,4,5)P_3 was added to give a final concentration of 10 μM. Samples were filtered at various time intervals. The two sets of symbols represent data derived from two separate preparations. Zero-time binding prior to dissociation was 478 and 306 c.p.m./mg of protein.

dissociation of the labelled ligand was induced by excess unlabelled Ins(1,4,5)P_3, a monoexponential fall in the binding was observed, with half-times of 5.9 and 4.7 min, respectively (Fig. 3). In view of this observation, it is unlikely that the upward concavity of the Scatchard curve is indicative of two separate receptor populations, but is more likely a reflection of a complex ligand–receptor interaction taking place at a single site.

Binding of labelled ligand to other subcellular fractions was also examined in three different preparations. While negligible binding to the cytosolic fraction was observed relative to the microsomal fraction (less than 7%), significant binding to the mitochondrial fraction was detected. The binding activity in the mitochondrial fraction correlated well with relative activity of NADH–cytochrome *c* reductase, a marker for endoplasmic reticulum (see the Materials and methods section). Thus, the mitochondrial fraction possessed binding and NADH–cytochrome *c* reductase activities that averaged 37 ± 6 and 38 ± 6%, respectively, of those found in the microsomal fraction. This suggests that Ins(1,4,5)P_3 binding to the mitochondrial fraction may be due to contamination with fragments of endoplasmic reticulum. Both mitochondrial and microsomal fractions probably contain inositol trisphosphate 5-phosphatase, which is associated with the plasma membrane (Seyfred *et al.*, 1984; Storey *et al.*, 1984), and potentially capable of binding Ins(1,4,5)P_3. Yet, the presence of the competitive enzyme inhibitor 2,3-bisphosphoglycerate (Downes *et al.*, 1982; Seyfred *et al.*, 1984) militates against the possibility that the observed high-affinity binding is associated with the hydrolysing enzyme.

In conclusion, our results demonstrate the presence of a specific, high-affinity binding site for Ins(1,4,5)P_3 in a microsomal fraction of rat liver. The rapid course of receptor binding corresponds to the rapid effects of exogenous Ins(1,4,5)P_3 on Ca^{2+} release (Joseph *et al.*, 1984*a*; O'Rourke *et al.*, 1985). The concentration of unlabelled Ins(1,4,5)P_3 required to displace half of the labelled ligand (8 nM) is about an order of magnitude lower than the concentrations required to induce half-maximal release of Ca^{2+}, as reported for several types of permeabilized cells, including rat hepatocytes (Berridge, 1984; Joseph *et al.*, 1984*a*). This apparent disparity may, in part, be attributed to the fact that the ligand binding assays and Ca^{2+} release experiments were carried out at 4 and 37 °C, respectively. However, 25 nM-Ins(1,4,5)P_3 is able to evoke half-maximal Ca^{2+} release from permeabilized insulinoma cells (Joseph *et al.*, 1984*b*), and recent studies in our laboratory using permeabilized peritoneal neutrophils have demonstrated Ca^{2+} release in response to 7 nM-Ins(1,4,5)P_3 (P. G. Bradford, A. Spät & R. P. Rubin, unpublished work). Nevertheless, further studies are required to determine the biological significance of the binding described here.

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