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A novel, specific binding protein assay for quantitation of intracellular inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) using a high-affinity $InsP_4$ receptor from cerebellum

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A membrane preparation from porcine cerebellum displays high-affinity binding sites for [^{3}H]inositol 1,3,4,5-tetrakisphosphate ([^{3}H]$InsP_4$) with a dissociation constant (K_d) of 1.0 nM and a density of 220 fmol/mg protein. Specific binding was maximal in the presence of 25 mM phosphate and at pH 5.0. The receptor site was specific for $InsP_4$, since Ins(1,3,4,5,6)P_5 and Ins(1,4,5,6)P_4 displaced binding of $InsP_4$ with EC_{50} values of 0.2 and 0.3 μM, respectively. Ins(1,4,5)P_3 and other inositol phosphates were less effective. Using this $InsP_4$ receptor, an assay for measuring tissue content of $InsP_4$ was developed. The detection limit of the assay was 0.1 pmol. In the same tissue samples the amount of Ins(1,4,5)P_3 was determined in parallel with a similar assay using a binding protein preparation from beef liver.

Inositol 1,3,4,5-tetrakisphosphate; Inositol 1,4,5-trisphosphate; Receptor, inositol phosphate; Bradykinin

1. INTRODUCTION

In a large variety of cells, cellular signal transduction involves activation of phosphoinositide-specific phospholipase C [1] yielding the second messengers Ins(1,4,5)P_3 and diacylglycerol. $InsP_3$, which releases Ca^{2+} from the endoplasmatic reticulum [1], is rapidly phosphorylated to yield Ins(1,3,4,5)P_4 [2]. $InsP_4$ has been proposed to mediate receptor-activated Ca^{2+} influx from the extracellular environment [3] and to regulate cytosolic Ca^{2+} activity by promoting replenishment of intracellular Ca^{2+} stores either from the extracellular space [4] or from the cytosol [5].

The concentrations of two prominent inositol phosphates, $InsP_3$ and $InsP_4$, in tissue extracts must be determined in order to establish the activation of inositol phosphate metabolism by hormones. In most cases, the various inositol phosphates are quantitated by HPLC analysis of the inositol phosphate metabolites from cells prelabelled with [^{3}H]inositol. A receptor assay for Ins(1,4,5)P_3 has recently been reported [6-8], which greatly facilitates the measurement of $InsP_3$. Here, we report the development of a novel, specific receptor protein assay for $InsP_4$. Furthermore, we use a binding protein for $InsP_3$ that is more readily available than that employed in current procedures for $InsP_3$ assay [6,8]. Thus, in the same tissue sample both $InsP_3$ and $InsP_4$ can be measured without further purification.

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Abbreviations: $InsP_4$, D-*myo*-inositol 1,3,4,5-P_4; $InsP_3$, D-*myo*-inositol 1,4,5-P_3

2. MATERIALS AND METHODS

[^{3}H]Ins(1,3,4,5)P_4 (spec. act. 50-52 Ci/mmol) and [^{3}H]Ins(1,4,5)P_3 (spec. act. 42-50 Ci/mmol) were from Amersham (Braunschweig); Ins(1,3,4,5)P_4, Ins(1,3,4)P_3 and Ins(1,4,5)P_3 (as the potassium salts), were from Calbiochem (Frankfurt); Ins(1)P, Ins(4,5)P_2, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(2,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4,5,6)P_5 (as the ammonium salts) and adenosine and guanosine 5′-triphos-

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phates were from Boehringer (Mannheim); phytic acid [Ins(1,2,3,4,5,6)P_6] and heparin being obtained from Sigma (Taufkirchen).

Binding protein for Ins(1,3,4,5)P_4 was prepared from freshly collected pig cerebellum. The tissue was homogenized in 10 vols buffer containing (mM) Tris-HCl (50), EDTA (1), mercaptoethanol (1), pH 7.7, 4°C. The homogenate was centrifuged for 30 min at 35 000 × g, the pellet resuspended and homogenized again, centrifuged (30 min, 35 000 × g), washed with buffer and centrifuged. The final pellet, resuspended in buffer at a concentration of 10–20 mg/ml, was homogenized using a Potter-Elvejhem and kept at −20°C. A similar procedure was applied to obtain the binding protein for Ins(1,4,5)P_3 from fresh beef liver.

For radioligand-binding studies, binding protein from cerebellum (0.3–1.0 mg per test tube) was added to a solution containing (final concentrations in mM), EDTA (1), bovine serum albumin (2.5 mg/ml), sodium acetate (25) and potassium phosphate (25), pH 5.0, [^{3}H]Ins(1,3,4,5)P_4 (approx. 10 000 dpm = 84 fmol) as tracer to give a final concentration of 0.22 nM and, where indicated, unlabelled Ins(1,3,4,5)P_4, tissue sample or other reagents. Incubations were carried out on ice. A solution of sucrose (5%), sodium acetate (25 mM), pH 5.0, was carefully injected into the reaction vial to obtain a sucrose density gradient with the reaction mixture (400 μl) forming the upper phase. After 20 min the reaction vials were centrifuged (10 000 × g) in a cooled Heraeus biofuge A for 3 min, thus separating the membranes from unbound ligand. This separation of bound from unbound ligand through a gradient improved the reproducibility. Thus, deviations of duplicate incubations were maximally 4% from the mean. The mean deviation for all samples used for 6 calibration curves (cf. fig.1A) was 2.5%. Finally, the pellet containing the bound label was quantitatively transferred to 5-ml scintillation vials by centrifugation for 5 min at 1500 × g and 3.5 ml scintillation liquid (Rotiszint 22, Roth, Karlsruhe) were added. Quenching was taken into account by automatic correction with a quench standard curve (LKB, 1214 Rack-beta) giving the final results in dpm. Ins(1,4,5)P_3 was determined similarly except that the reaction mixture contained (mM) EDTA (1), Tris-HCl (25), pH 9.0, bovine serum albumin (1 mg/ml). The binding reaction was initiated by adding binding protein isolated from bovine liver.

For the experiments neuroblastoma × glioma hybrid cells were cultured as described [9]. Cells were preincubated for 10 or 20 min in incubation medium [10]. After the challenge incubation with bradykinin the medium was aspirated, 1 ml ice-cold trichloroacetic acid (10%) was added, the mixture kept on ice for more than 20 min and then centrifuged (2 min, 5000 × g). The supernatant was extracted 4 times with 2 vols diethyl ether. In the samples, which had been neutralized with the appropriate volume of 1 M $NaHCO_3$, the content of $InsP_4$ and $InsP_3$ was determined.

3. RESULTS

$InsP_4$ binds with high affinity to a receptor in membranes from pig cerebellum. Specific binding of tracer is reduced by 50% in the presence of 1.8 nM $InsP_4$ and by 90% at concentrations above 25 nM (fig.1A). Scatchard analysis of [^{3}H]Ins(1,3,4,5)P_4-binding data (fig.1A, inset) revealed a high-affinity binding site with a dissociation constant (K_d) of 1.0 ± 0.3 nM (n = 7) and a binding capacity (B_{max}) of 220 ± 50 fmol/mg pro-

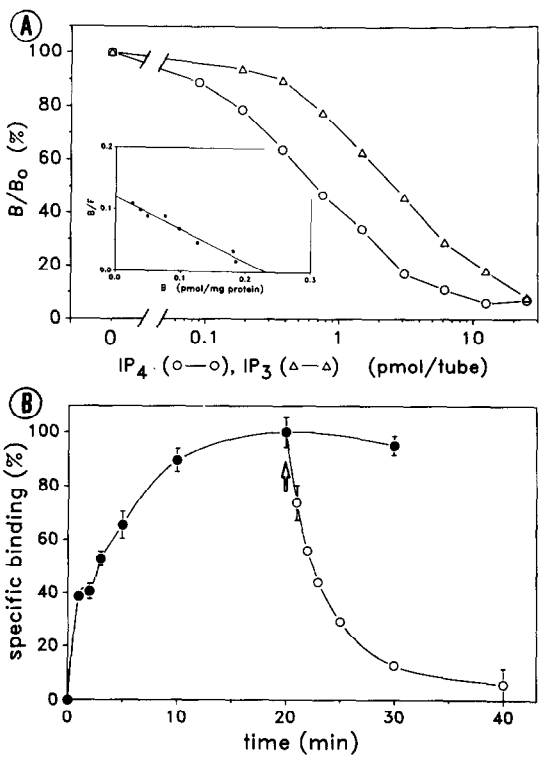

Fig.1. (A) Specific binding of [^{3}H]$InsP_4$ [0.2 nM (○)] and [^{3}H]$InsP_3$ [0.26 nM (Δ)], respectively, as a function of increasing concentration of unlabelled ligand using binding protein from cerebellum (○) or from liver (Δ). Maximal specific binding (B_0) was 2060 dpm for (○) and 1170 dpm for (Δ); reaction volume 400 μl. Unspecific binding, in the presence of 1 μM unlabelled ligand, was 34% (for $InsP_4$) and 14% (for $InsP_3$) of total maximal binding. (Inset) Scatchard analysis of binding data for high-affinity binding of $InsP_4$ from another experiment. Concentration of [^{3}H]$InsP_4$: 0.18 nM, 0.3 mg protein per assay. Specifically bound ligand was obtained by correcting for unspecific binding in the presence of 1 μM unlabelled $InsP_4$. (B) Kinetics of association and dissociation of [^{3}H]$InsP_4$ with binding sites from cerebellum (100%: binding at 20 min). Binding was initiated by adding the binding protein to an assay mixture containing 0.18 nM [^{3}H]$InsP_4$ kept at 0°C. Dissociation was started by adding 1 μM $InsP_4$ at the time point indicated by an arrow. k_{off}/k_{on} gave a K_d value of 1.1 ± 0.4 nM (n = 3). Incubations were terminated by immediate centrifugation through a sucrose density gradient (see section 2). Here and in fig.2, data points give the means of duplicate incubations. Similar results were obtained in 3 and 2 further experiments for A and B, respectively.

tein. A second low-affinity site with a K_d of about 100 nM was not characterized further.

Some of the properties of the high-affinity $InsP_4$-binding site were determined to optimize the conditions for binding. On varying the pH between 3 and 8, a 4-fold increase in specific binding was obtained at pH 5.0 (not shown). Specific binding of $InsP_3$, however, is maximal at pH 8.0–9.0 for the receptor from parathyroid [11] and from liver (not shown). Variation in the Ca^{2+} concentration from 10 nM to 10 mM did not alter binding of $InsP_4$ by more than 20%. However, when the concentration of phosphate was varied from 1 to 100 mM a clear maximum of specific binding of [^{3}H]$InsP_4$ was obtained at around 25 mM (not shown).

Analysis of the kinetics showed that specific binding of $InsP_4$ was half-maximal within 140 s and remained constant between 10 and 30 min after addition of ligand (fig.1B). Addition of 1 μM unlabelled $InsP_4$ caused displacement of bound ligand with a half-time for dissociation of 165 s.

The specificity of the $InsP_4$-binding site was determined in competition binding experiments performed with several inositol phosphates (fig.2). Among the compounds tested, the pentakisphosphate Ins(1,3,4,5,6)P_5 had the highest interfering affinity, but the EC_{50} of about 0.2 μM was 2 orders of magnitude greater than that of Ins(1,3,4,5)P_4. Ins(1,4,5,6)P_4 had an EC_{50} of 0.3 μM. However, the supplier of Ins(1,4,5,6)P_4 indicates that the substance contains 12% Ins(2,4,5,6)P_4 and a further contamination with less than 0.5% Ins(1,3,4,5)P_4 would suffice to account for the inhibition observed here. The inositol trisphosphates tested had a very low activity in comparison with Ins(1,3,4,5)P_4 with EC_{50} values of 0.4 μM for Ins(1,3,4)P_3, 6 μM for Ins(1,4,5)P_3 and above 10 μM for Ins(2,4,5)P_3. Ins(1)P, Ins(4,5)P_2 and Ins(1,4)P_2 did not affect specific binding of Ins(1,3,4,5)P_4 even at 10 μM (not shown). An important requirement concerning a suitable test system for $InsP_4$ is the ability to discriminate clearly between Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3, whose synthesis is stimulated considerably by many hormones [1]. Both of the nucleotides ATP and GTP reduced specific binding at 60 to 80 μM by 50% (fig.2). Heparin caused 50% displacement at about 25 μg/ml (not shown).

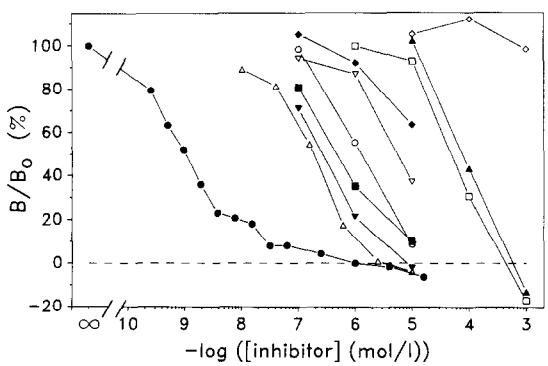

Fig.2. Specificity of $InsP_4$ binding to porcine cerebellar membranes. Displacement of [^{3}H]$InsP_4$ binding by various inositol phosphates, ATP, GTP and inositol. Specific binding, relative to B_0 (1690 dpm) of 0.18 nM [^{3}H]$InsP_4$ in the presence of various concentrations of Ins(1,3,4,5)P_4 (●), Ins(1,3,4,5,6)P_5 (△), Ins(1,4,5,6)P_4 (▼), Ins(1,3,4)P_3 (■), phytic acid (Ins(1,2,3,4,5,6)P_6) (○), Ins(1,4,5)P_3 (▽), Ins(2,4,5)P_3 (◆), GTP (□), ATP (▲) and inositol (◇), corrected for unspecific binding in the presence of 1 μM unlabelled Ins(1,3,4,5)P_4. Deviations (mean error 1.9%, max. 5%) were omitted for clarity. Comparable results were obtained for the various inhibitors in 2 or 3 further experiments.

A neuronal cell line in which bradykinin has been shown to induce rapid phosphatidylinositol response [12] was taken as proof of the applicability of our test assay system. The basal level of 2.7 pmol/mg protein $InsP_4$ was close to the detection limit. In the presence of 2 μM bradykinin, $InsP_4$ increased to a nearly constant level of 25.9 pmol/mg protein between 20 and 60 s after adding the peptide. In the same cells, $InsP_3$ rose transiently from 5.9 to maximally 145 pmol/mg protein at 15–20 s after addition of bradykinin, declining sharply thereafter. The $InsP_4$ assay was shown to be linear by testing different volumes from the same samples (not shown).

4. DISCUSSION

In porcine cerebellum membranes, there is a population of receptors which rapidly and reversibly bind $InsP_4$ with an apparent dissociation constant of 1.0 nM. Binding sites for $InsP_4$ have been described previously for HL-60 cells [13] and various rat tissues [14]. However, the receptors detected had a much lower affinity (K_d 90 and 300 nM, respectively). Moreover, the receptor from rat tissue did not really discriminate between $InsP_4$ and $InsP_3$ [14]. The receptor for $InsP_4$ characterized here, which shows similarities with

the $InsP_4$-binding site in bovine parathyroid glands [11], opens up the possibility of investigating the still elusive biological function of $InsP_4$ in the signal transduction mechanism.

With optimal conditions for $InsP_4$ binding, the receptor preparation from porcine cerebellum is suitable for measuring $InsP_4$ by radioligand displacement, since there is strong selectivity for Ins(1,3,4,5)P_4. The conditions used for the binding studies (0°C, addition of 1 mM EDTA in the absence of Mg^{2+}) were designed to reduce any residual phosphatase activity which could degrade the label. The lower limit of sensitivity of the assay is about 0.1 pmol $InsP_4$ per sample of 400 μl. Thus, a significant component of the inositol phosphate response can be easily measured, which was hitherto detectable only after separation of the inositol phosphates by anion-exchange chromatography using mostly a HPLC system [2]. However, this technique requires prelabelling of the inositol lipid pool in the cells with a radioactive tracer, which is only possible for tissues which can be maintained in culture. Mass measurements of $InsP_x$ [15,16] involving prior separation by HPLC followed by analysis on various detection systems are laborious and not applicable for routine tests of multiple samples. The radioreceptor assay described here provides the opportunity to quantitate $InsP_4$ in tissue extracts easily and directly.

Furthermore, both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 can be assayed in the same tissue sample by analogous procedures. The previously described assays for $InsP_3$ [6,8] have been modified here by improving the separation of bound from free ligand and by employing a more readily obtainable binding protein than that from rat cerebellum [6] or bovine adrenal cortex [8]. Bovine liver contains $InsP_3$ receptors with similarly high affinity (K_d 4.8 ± 0.9 nM, B_{max} 440 ± 70 fmol/mg protein, $n = 4$) and comparable selectivity among inositol phosphates and was stable during radioreceptor assays for at least 3 months (not shown).

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