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A Radioreceptor Assay for Mass Measurement of Inositol (1,4,5)-Trisphosphate Using Saponin-Permeabilized Outdated Human Platelets

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The binding of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] to saponin-permeabilized blood-bank-outdated human platelets, 6 days old, has been characterized (K_d = 3.8 nM; B_{max} = 1.7 pmol/mg protein) and used to develop a novel radioreceptor assay which allows the measurement of the Ins(1,4,5)P_3 content in resting or agonist-stimulated cells. This assay is as sensitive (0.25 pmol in a 0.25 ml volume), specific, and reproducible as previously proposed methods. In addition, obtaining large batches of the Ins(1,4,5)P_3 binding protein by treating outdated platelets with saponin is simple and quick and uses otherwise discarded material. Moreover, the assay is considerably cheaper than commercially available kits. Using this method we confirmed that thrombin evokes a rapid, transient, and dose-dependent increase in the platelet concentration of Ins(1,4,5)P_3. © 1998 Academic Press

Key Words: **inositol (1,4,5)-trisphosphate; radioreceptor assay; platelets.**

Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3][2] has been clearly established as a second messenger linking the activation of many cell-surface receptors to the increase in the intracellular Ca^{2+} level (1–5). As for other second messengers, such as cAMP, there is considerable interest in the specific and reliable quantification of the cellular Ins(1,4,5)P_3 content.

Determinations of Ins(1,4,5)P_3 have been mainly indirect, relying on the loading of cells with [*myo*-2-[^{3}H]inositol ([^{3}H]inositol), which incorporates to phosphatidylinositol (4,5)-bisphospate (PtdIns(4,5)P_2), thus rendering radiolabeled inositol phosphates that can be extracted and analyzed by a variety of detection systems (2, 6–10). The disadvantages of such procedures are the inevitable assumption that all pools of inositol phosphates have been labeled to equilibrium, and the complex, time-consuming, and relatively insensitive analysis.

Alternative methods for mass measurement of endogenous Ins(1,4,5)P_3 have been developed using a radioreceptor approach, which takes advantage of saturable, high-affinity binding sites for Ins(1,4,5)P_3 in microsomal fractions of bovine adrenal cortex or rat cerebellum (11–14). Indeed, a modification of such competition radiobinding assays led to the commercial development of Ins(1,4,5)P_3-quantification kits (New England Nuclear and Amersham International), meeting adequate properties of simplicity, sensitivity, and specificity, but being rather expensive.

Specific binding sites for Ins(1,4,5)P_3 have been reported in many tissues (3). In human platelets, few studies have examined Ins(1,4,5)P_3 binding, using partially or highly purified membrane fractions (15–20). Moreover, it is known that addition of Ins(1,4,5)P_3 to permeabilized platelets leads to activation and aggregation (21, 22), but direct evidence for Ins(1,4,5)P_3 binding on permeabilized platelets is scarce (23).

In the present study we have characterized the binding of Ins(1,4,5)P_3 in saponin-permeabilized human platelets. Blood bank platelet concentrates stored for 6 days under standard conditions, hence outdated for clinical use, were used. Moreover, we describe an alternative competition radiobinding assay for the measurement of the Ins(1,4,5)P_3 content of cells and changes in it upon agonist stimulation.

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[2] Abbreviations used: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PCs, platelet concentrates.

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MATERIALS AND METHODS

Materials

D-[^{3}H]Ins(1,4,5)P_3 (specific activity 34–38 Ci/mmol) was obtained from Amersham Ibérica SA (Madrid, Spain). Unlabeled inositol phosphates and low-molecular-weight heparin were from Sigma–Aldrich Quimica SA (Madrid, Spain). A commercial protein determination kit was used (Bio-Rad Laboratories SA, Madrid, Spain). Human thrombin (5459 NIH units/mg of protein) was from Calbiochem–Novabiochem Int. (La Jolla, CA).

Preparation of Saponin-Treated Platelets

Platelet concentrates (PCs) were obtained from human whole blood units as previously described (24). Platelet count in the PCs averaged 1.5×10^{12} cells/L, and contamination by white cells and erythrocytes was below 2×10^9 and 2×10^{10} cells/L, respectively. Following acidification to pH 6.45 with acid/citrate/dextrose, platelets were collected at 1200*g* for 30 min at RT. Subsequently, platelets were washed twice in Ca^{2+}-free Tyrode's buffer (0.04 mM NaH_2PO_4, 136 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 5.4 mM dextrose, 1.0 mM $MgCl_2$; pH 6.5), and resuspended in a modified Tyrode's solution also containing 0.35% human serum albumin, 0.1 mM PMSF, and 1 units/ml aprotinin (pH 7.35). Permeabilization was achieved by addition of 10 μg of saponin per 10^8 cells (15 min, RT; final concentrations 0.1 mg of saponin/ml, 10^9 platelets/ml). This treatment induced the release of more than 90% of the cytosolic enzyme lactate dehydrogenase. Finally, saponin-treated platelets were pelleted (1200*g*, 20 min, RT), resuspended in a binding buffer containing 50 mM Tris–HCl, 1 mM EDTA, 1 mM β-mercaptoethanol (pH 8.3), at a protein concentration of 0.5–1 mg/ml. Aliquots of 5 ml were stored at −80°C until use; no significant decrease in the Ins(1,4,5)P_3 binding activity was observed within 3 months.

Ins(1,4,5)P_3 Binding Assays

Saponin-treated platelets (50–100 μg of protein) were mixed with 0.5 nM D-[^{3}H]-Ins(1,4,5)P_3, in the presence of increasing concentrations of nonlabeled D-Ins(1,4,5)P_3 (0.1 nM to 1 μM), other inositol phosphates, or heparin, in a final volume of 0.25 ml with binding buffer. Nonspecific binding was determined in the presence of 5 μM Ins(1,4,5)P_3. Equilibrium binding was reached by incubation at 4°C for 15 min, followed by centrifugation at 12,000*g* for 5 min. The supernatants were aspirated out, and the amount of radioactivity retained within the pellet was determined by solubilization in 50 μl of tissue solubilizer (OptiSolv, FSA Lab, Loughborough, Leics, England), followed by liquid scintillation counting. Careful washing of the packed pellet decreased the radioactivity associated to pellets by less than 5%. The density of binding sites (B_{max}) and the dissociation constant (K_d) values for Ins(1,4,5)P_3 were determined by Scatchard transformation of binding data using the "cold" option of the EBDA-LIGAND program (Elsevier–Biosoft, Cambridge, UK). The displacement data of other competing agents were analyzed using the nonlinear curve-fitting package Ultrafit (Biosoft, Cambridge, UK), to obtain the IC_{50} values, defined as the concentration of inhibitor required to displace Ins(1,4,5)P_3-specific binding by 50%. In experiments designed to determine mass content of Ins(1,4,5)P_3 in platelet extracts (see below), binding assays were carried out as above, including tubes with either defined amounts Ins(1,4,5)P_3 as standards or an adequate volume of the test samples.

Thrombin Stimulation of Ins(1,4,5)P_3 Formation in Human Platelets and Ins(1,4,5)P_3 Extraction

Platelets from PCs were washed as described above and resuspended at 10^9 cells/ml in Tyrode's buffer, pH 7.35, also containing 0.35% human serum albumin and 2 mM $CaCl_2$. Aliquots (0.25 ml) of platelet suspensions were stimulated with 5 μl of thrombin. The reactions were terminated at selected times by addition of 0.25 ml of ice-cold 10% perchloric acid, followed by a 30-min incubation in an ice bath. After centrifugation (12,000*g* 5 min), 0.4 ml of the acidified supernatant was thoroughly mixed with 0.125 ml of 10 mM EDTA and 0.3 ml of trichlorotrifluorethane:trioctylamine (1:1, v/v) and pulsed to aid phase partition. Samples of the upper aqueous phase were taken for measurement of Ins(1,4,5)P_3. This procedure achieved extraction of more than 95% of the Ins(1,4,5)P_3 content, as demonstrated by the addition of exogenous Ins(1,4,5)P_3 to platelet suspensions. Platelet-free Tyrode's buffer samples were identically extracted to provide suitable medium for the preparation of the Ins(1,4,5)P_3 standard curve of the radioreceptor assay.

RESULTS

Characterization of Ins(1,4,5)P_3 Binding in Saponin-Treated Platelets

In experiments designed to establish the optimal binding assay, a known concentration of Ins(1,4,5)P_3 was incubated with a fixed amount of saponin-treated platelets, under varying conditions. Thus, we found that Ins(1,4,5)P_3 binding to saponin-treated preparations was temperature dependent, representing at RT about half of that obtained at 4°C. Moreover, the Ins(1,4,5)P_3 binding reached the maximum at pH 7.5–8.5, falling sharply outside this range. According to these results, subsequent experiments were conducted at 4°C and pH 8.0.

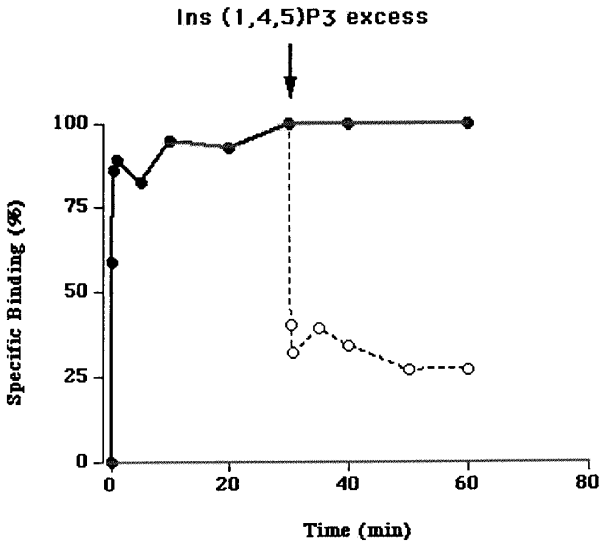

FIG. 1. Association and dissociation of [^{3}H]-Ins(1,4,5)P_3 binding to saponin-permeabilized outdated human platelets. For association experiments (●), saponin-permeabilized platelets were incubated with 0.5 nM [^{3}H]-Ins(1,4,5)P_3 and reactions were stopped at various time points by centrifugation, as described in the text. For the dissociation (○), 5 μM D-Ins(1,4,5)P_3 was added at the time indicated by the arrow. Each point shows the mean of values converted into percentage of maximal binding, from a representative experiment performed in triplicate. Two additional experiments provided similar results.

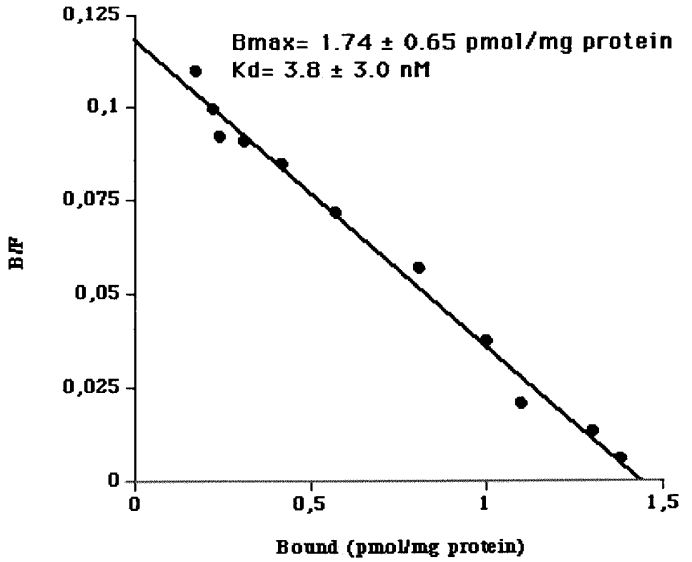

FIG. 2. Scatchard plot of [^{3}H]-Ins(1,4,5)P_3 binding to saponin-permeabilized outdated human platelets. Saponin-permeabilized platelets were incubated with 0.5 nM [^{3}H]-Ins(1,4,5)P_3 and increasing concentrations of unlabeled Ins(1,4,5)P_3. Using the COLD option of the computer program LIGAND, the displacement curves obtained were transformed into Scatchard plots, which are plotted as *B/F* [ratio of specifically bound to free ligand] against bound [the amount of specifically bound ligand, in pmol of Ins(1,4,5)P_3/mg of protein]. The points are means of duplicates from a typical experiment, which was repeated 10 times.

Consistent with our previous results in partially purified membranes from platelets and other cell types (16), the binding of D-[^{3}H]-Ins(1,4,5)P_3 to saponin-treated platelets was extremely fast, with maximum binding being reached in less than 1 min. Similarly, the dissociation of bound D-[^{3}H]-Ins(1,4,5)P_3 upon addition of cold Ins(1,4,5)P_3 was also very rapid (Fig. 1).

As described under Materials and Methods, saturation studies were performed by examining the reduction of D-[^{3}H]-Ins(1,4,5)P_3 binding with increasing concentrations of unlabeled Ins(1,4,5)P_3. Scatchard transformation of the competition curve demonstrated that binding best fitted to a single class of noninteracting sites, with an apparent K_d of 3.8 ± 3.0 and a density (B_{max}) of 1.7 ± 0.6 pmol/mg protein (mean ± SD, n = 10) (Fig. 2).

Table 1 summarizes the results of experiments designed to assess the ability of several inositol phosphates to compete for D-[^{3}H]-Ins(1,4,5)P_3 binding sites in saponin-treated platelets. As shown, we observed that this binding was highly selective for the D-[^{3}H]-Ins(1,4,5)P_3 isomer, displaying little cross-reactivity with other inositol phosphates studied. Thus, the strongest competitor Ins(1,3,4,5)P_4, which has also been involved in Ca^{2+} mobilization and possesses a specific receptor in platelets (25, 26), showed a binding affinity relative to D-Ins(1,4,5)P_3 of less than 5%. The extremely low cross-reactivity of L-Ins(1,4,5)P_3 shows a marked stereospecificity of Ins(1,4,5)P_3 binding sites in these saponin-treated platelet preparations. Finally, we also found that, as in several cell types (16), heparin acts as a competitive inhibitor at the same site as D-Ins(1,4,5)P_3 in saponin-permeabilized platelets.

TABLE 1

Effect of Inositol Phosphates and Low-Molecular-Weight Heparin on D-[^{3}H]-Ins(1,4,5)P_3 Binding in Saponin-Permeabilized Outdated Human Platelets

Compound	IC_{50} (nM)	RBA (%)
D-Ins(1,4,5)P_3	4.6 ± 0.3	100
L-Ins(1,4,5)P_3	4830 ± 184	0.095
Ins(2,4,5)P_3	139 ± 44	3.3
Ins(1,3,4,5)P_4	138 ± 2	3.3
InsP_6	2611 ± 256	0.17
Ins(1,4)P_2	$>10^4$	<0.05
Ins(1)P_1	$>10^4$	<0.05
Heparin	2100 ± 1000 (6 ± 3 μg/ml)	0.22

Note. IC_{50} values for the tested compounds were obtained from the curve-fitting computer program ULTRAFIT, and are expressed as the geometric mean ± SD of two different experiments, each performed in triplicate. RBA (%) refers to the binding affinity of each compound, relative to D-Ins(1,4,5)P_3.

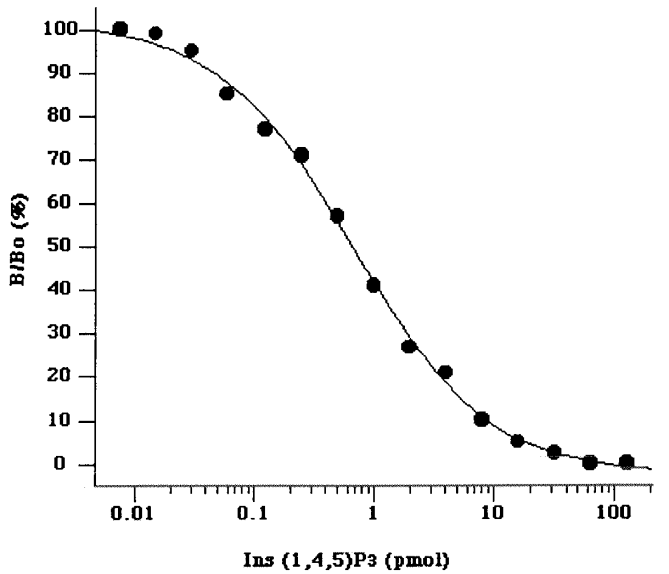

FIG. 3. Typical standard curve for Ins(1,4,5)P_3 radioreceptor assay. Binding of [^{3}H]-Ins(1,4,5)P_3 to saponin-permeabilized outdated platelets was assayed as described under Materials and Methods in the absence (B_0) or presence of defined amounts of Ins(1,4,5)P_3 or test samples (B). Fitting of the standard curve and determinations of the content in test samples was achieved using the computer program ULTRAFIT. The points are means of triplicates from a typical experiment.

A main purpose of this study was to develop a simple assay that would be optimal for measurement of Ins(1,4,5)P_3 mass in cells and tissues. Figure 3 represents an example of the standard curve for the Ins(1,4,5)P_3 radioreceptor assay using outdated platelets permeabilized with saponin. As shown, stepwise reduction of the D-[^{3}H]-Ins(1,4,5)P_3-specific binding is attained over a broad range of Ins(1,4,5)P_3 concentrations. Under our experimental conditions, the limit of detection of the radioreceptor assay was 0.25 pmol of Ins(1,4,5)P_3, an amount that consistently achieved a reliable 20% inhibition of D-[^{3}H]-Ins(1,4,5)P_3 binding. Measurement of Ins(1,4,5)P_3 content in test samples with this assay was highly reproducible, with intra- and interassay coefficients of variations of 6 ± 5 and 16 ± 4%, respectively.

Using this radioreceptor assay we determined that resting platelets have a basal Ins(1,4,5)P_3 mass level of 18.7 ± 12.8 pmol/10^9 platelets (mean ± SD; n = 5). As expected, we observed that thrombin induced a sharp and transient rise in Ins(1,4,5)P_3 mass content in platelets (Fig. 4A), and that this thrombin effect was dose-dependent (Fig. 4B).

DISCUSSION

In the present study, Ins(1,4,5)P_3 binding to saponin-permeabilized human platelets was examined and used in an alternative radioreceptor assay for Ins(1,4,5)P_3 mass measurement. Binding of Ins(1,4,5)P_3 to these platelet preparations displayed characteristics similar to those previously reported in partially or highly purified platelet membranes (15–20); i.e., binding was fast, reversible, and of high affinity and specificity for D-Ins(1,4,5)P_3. The concentration of Ins(1,4,5)P_3 binding sites in saponin-treated human platelets was also consistent with our previous data on platelets and in some other tissues (16).

Our present competition binding assay for Ins(1,4,5)P_3 mass measurement has certain advantages. First, it is as specific and sensitive (0.25 pmol in a 0.05 ml of test sample) as those previously reported assays (11–14), and the determination of Ins(1,4,5)P_3 mass in test samples was also highly reproducible. A major advantage of this

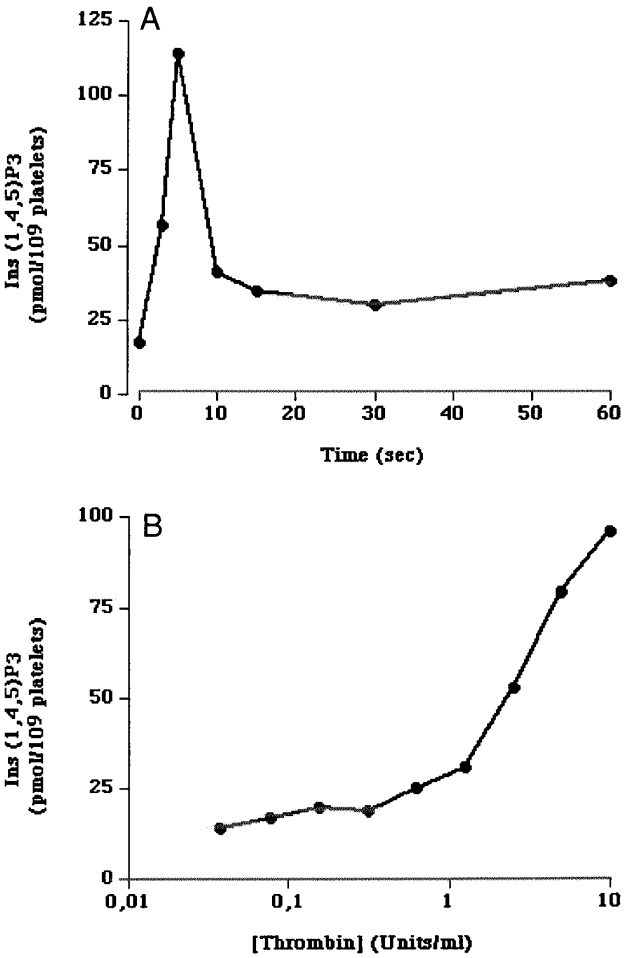

FIG. 4. Thrombin induced formation of Ins(1,4,5)P_3 in human platelets. Washed platelets in Tyrode's buffer were stimulated for various times with thrombin (5 units/ml) (A), or for 5 s with different concentrations of thrombin (B). The reactions were stopped at selected times by the addition of ice-cold 10% perchloric acid (v/v). Ins(1,4,5)P_3 was extracted from the acidic supernatant as described under Materials and Methods and quantified using the radioreceptor assay. Points are the mean of determinations of three different experiments conducted in triplicate.

method is that the preparation of an $Ins(1,4,5)P_3$ binding protein by simply treating platelets with saponin is considerably less complex and time-consuming than its achievement from adrenalcortical or cerebellar material (16). Moreover, the assay could be set up using otherwise discarded blood-bank-outdated PCs, which we found to contain concentrations and affinity of $Ins(1,4,5)P_3$ binding sites similar to fresh PCs (data not shown). By permeabilizing the cell content of 10 outdated PCs with saponin, in 2 h a binding protein batch sufficient for 2000 $Ins(1,4,5)P_3$ determinations could be obtained. The use of outdated platelets in the $Ins(1,4,5)P_3$ radioreceptor assay also obviates ethical concerns on the use of clinically useful blood components for research purposes. Finally, our procedure is considerably cheaper than the commercially available kits which measure $Ins(1,4,5)P_3$ by radioreceptor techniques (New England Nuclear or Amersham International). Thus, under our assay conditions we estimated a cost of less than $1 U.S. per $Ins(1,4,5)P_3$ determination.

In this study we used the radioreceptor assay to evaluate the mass content of $Ins(1,4,5)P_3$ in human platelets and to assess the changes in it upon thrombin stimulation. Consistently with previously reported data from other groups using different measurements (27–29), we found that thrombin induces a rapid and transient rise of the $Ins(1,4,5)P_3$ platelet content, in a dose-dependent manner (Fig. 4). The deficiency of $Ins(1,4,5)P_3$ formation has been recently described in some patients with inherited platelet function defects associated with mild to moderate bleeding symptoms (30, 31). The findings strengthen that $Ins(1,4,5)P_3$ mass determination might be helpful not only for a better understanding of the role of $Ins(1,4,5)P_3$ as a second messenger, but also for the identification of congenital or acquired defects in cellular signaling are strengthened. Because of its simplicity, specificity, sensitivity, and low cost, the present competition binding assay for $Ins(1,4,5)P_3$ could be a convenient procedure for that purpose.

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