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Specific binding of tritium-labeled inositol 1,4,5-trisphosphate to human platelet membranes: ionic and GTP regulation

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Specific, saturable and reversible binding of tritium-labeled inositol 1,4,5-trisphosphate ([^{3}H]Ins(1,4,5)P_3) to human platelet membranes is demonstrated. The Ins(1,4,5)P_3-binding sites are abundant and display high selectivity for Ins(1,4,5)P_3. Other inositol phosphates exhibit much lower affinity for this site. The specific [^{3}H]Ins(1,4,5)P_3 binding was found to be modulated by pH, monovalent and divalent cations, and GTP. A sharp increase in binding occurs at slightly alkaline pH. The monovalent cations, Na^+, K^+ and Li^+ almost double the binding at 30 mM. Mg^{2+} inhibits the specific [^{3}H]Ins(1,4,5)P_3 binding. At low concentrations of Ca^{2+}, the binding is inhibited, but at concentrations higher than 5 mM the binding is potentiated and increases by almost 5-fold at 100 mM. Similar pattern of the effects is also observed for Mn^{2+} and Sr^{2+}. The specific [^{3}H]Ins(1,4,5)P_3 binding is specifically inhibited by GTP. Other nucleotides also inhibit the binding but at higher concentrations. From saturation binding studies, Ca^{2+} potentiation seems to be due to the conversion of the receptor from the low-affinity state to the high-affinity one. In the absence of Ca^{2+}, the Scatchard plot is nonlinear and concave, and statistically can be fitted best with two equilibrium dissociation constants (K_d values), 0.19 ± 0.11 and 13.2 ± 18.1 nM, respectively, for high- and low-affinity binding sites. However, in the presence of 100 mM $CaCl_2$, the Scatchard plot reveals only the high-affinity binding sites with a K_d value of 0.32 ± 0.15 nM. The specific Ins(1,4,5)P_3 receptor in human platelets could therefore exist in multiple conformational states to regulate the intracellular Ca^{2+} concentration.

The initial response of many cells to hormones, neurotransmitters, growth factors and other Ca^{2+}-mobilizing receptor agonists in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) catalyzed by phospholipase C to generate the comessengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol [1]. Diacylglycerol activates protein kinase C and Ins(1,4,5)-P_3 causes the release of Ca^{2+} from intracellular pools. The action of Ins(1,4,5)P_3 in mediating Ca^{2+}-signaling events is now well established in a wide variety of cell types [1–3]. In a recent series of studies by Snyder and co-workers [4–6], specific Ins(1,4,5)P_3-binding sites were identified and characterized. Binding of [^{3}H]Ins(1,4,5)P_3 to other cell types of membrane fractions has also been reported [7–11]. Thus, the release of intracellular stored Ca^{2+} induced by Ins(1,4,5)P_3 is a receptor-mediated process.

Release of Ca^{2+} into the cytosol from the dense tubular system [12] is though to be one of the important steps in platelet activation. Since agonists such as ADP and thrombin appear to interact with platelets solely at the level of the plasma membrane, a signal transduction mechanism is required to link events on the cell surface with events in the dense tubular system. The potential for Ins(1,4,5)P_3 to perform this role is suggested by the observation that thrombin [13–20], ADP [21,22], collagen [23], and platelet-activating factor [24,25] are able to decrease platelet PIP_2 levels and increase formation of Ins(1,4,5)P_3. Ins(1,4,5)P_3 has also been demonstrated

Abbreviations: PIP_2, phosphatidylinositol 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; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1)P_1, inositol 1-monophosphate; Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(4,5)P_2, inositol 4,5-bisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; Ins(1,4,5,6)P_4, inositol 1,4,5,6-tetrakisphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); p[NH]ppG, guanylyl imidodiphosphate; BSA, bovine serum albumin.

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to trigger Ca^{2+} release from internal stores and cause secretion of dense granular content [26] in saponin-permeabilized platelets. To data, no studies examining specific Ins(1,4,5)P_3 receptors in platelets have been reported. Here, we demonstrate the existence of a specific Ins(1,4,5)P_3 receptor in isolated human platelet membranes. The affinity of the receptor in human platelets is quite different from the one previously identified in rat cerebellar homogenates [5]. The specific Ins(1,4,5)P_3 binding to receptors is modulated by either monovalent or divalent cations. GTP also specifically regulates binding to the specific Ins(1,4,5)P_3 receptor, which appears to exist in multiple conformational states.

Materials and Methods

Materials

D-[1(n)-^{3}H]Inositol 1,4,5-trisphosphate ([^{3}H]Ins(1,4,5)P_3) was obtained from NEN-DuPont (Boston, MA) with a specific activity of 20 Ci/mmol. Unlabeled *myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), *myo*-inositol 2,4,5-triphosphate (Ins(2,4,5)P_3), *myo*-inositol 1,3,4-triphosphate (Ins(1,3,4)P_3), *myo*-inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), *myo*-inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4) and *myo*-inositol 4,5-bisphosphate (Ins(4,5)P_2) were purchased from Calbiochem. *myo*-Inositol 1,4-bisphosphate (Ins(1,4)P_2) and *myo*-inositol 1-monophosphate (Ins(1)P_1) were obtained from Sigma. Suramin, Trypan blue and 8-(4-anilino-5-sulfo-1-naphthylazo)-1-naphthol-3,6-disulfonic acid (L-451,167) were obtained from Chemical Data Department, Merck Sharp & Dohme Research Laboratories. Guanosine 5′-triphosphate (GTP) and adenosine 5′-triphosphate (ATP) were purchased from Pharmacia Inc. (Piscataway, NJ), Sigma, Calbiochem (San Diego, CA), and Boehringer Mannheim Biochem. (Indianapolis, IN). Chemically synthetic ATP by phosphorylation of adenosine was obtained from either Sigma or Pharmacia. Guanosine 5′-diphosphate (GDP), guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), guanylyl imidodiphosphate (p[NH]ppG), cytidine 5′-triphosphate (CTP), inosine 5′-triphosphate (ITP), and uridine 5′-triphosphate (UTP) were obtained from Boehringer Mannheim Biochem.

Methods

Preparation of human platelet membranes. Human platelets were prepared from freshly drawn venous blood into 0.1 volume of 3.8% sodium citrate following exactly the same procedure described previously for rabbit platelets [29]. The cells were then lysed in a Na^+-free medium containing 5 mM $MgCl_2$, 10 mM Tris and 2 mM EDTA at pH 7.0 [28]. The lysed membranes were then further fractionated with a 0.25, 1.03 and 1.5 M discontinuous sucrose density gradient at a speed of 63 500 × *g* for 2 h. The membranes fractionated at the interface of 0.25 and 1.03 M (membrane fraction A) and of 1.03 and 1.5 M (membrane fraction B) were collected separately [29]. Membrane fraction A contained higher activity of alkaline phosphatase and lower activity of antimycin-insensitive NADH–cytochrome-*c* reductase than membrane fraction B [29,30]. Also, membrane fraction A is enriched with either glycoprotein and/or glycolipid (Hwang, S.-B., unpublished data), therefore, membrane fraction A is likely a plasma membrane-enriched fraction, whereas membrane fraction B is enriched with the dense tubular system. Membrane fraction B was about 2-fold enriched in receptor sites for Ins(1,4,5)P_3 and was therefore used throughout the experiments. The protein content in the prepared membranes was determined by the method of Lowry et al. [31] with bovine serum albumin (BSA) as the standard. The prepared membranes were stored at −80 °C and thawed before use.

Binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes. Binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes was performed as previously described [27]. To assess the affinity of [^{3}H]Ins(1,4,5)P_3 and the maximal detectable receptor sites on the isolated human platelet membranes, 100 μg of membrane protein was incubated with 0.1 to 12 nM [^{3}H]Ins(1,4,5)P_3 in an incubation medium containing 20 mM Hepes, 1 mM EDTA at pH 8.0 (in the absence of Ca^{2+}) or in a medium containing 20 mM Hepes, 100 mM $CaCl_2$ at pH 8.0 (in the presence of Ca^{2+}). After 0.5 min (in the presence of Ca^{2+}) or 1 h (in the absence of Ca^{2+}) incubation at 0 °C, the bound and unbound [^{3}H]Ins(1,4,5)P_3 were separated through a Whatman GF/C filter under the house vacuum. The filter was then washed with 20 ml wash buffer containing 20 mM Hepes, 0.1% BSA at pH 8.0. The nonspecific binding was determined from total binding of [^{3}H]Ins(1,4,5)P_3 in the presence of excess (1000-fold) unlabeled Ins(1,4,5)P_3. The bound and unbound [^{3}H]Ins(1,4,5)P_3 were separated through a Whatman GF/C filter under the house vacuum. The filter was then washed with 20 ml wash buffer containing 20 mM Hepes, 0.1% BSA at pH 8.0. The nonspecific binding was determined from total binding of [^{3}H]Ins(1,4,5)P_3 in the presence of excess (1000-fold) unlabeled Ins(1,4,5)P_3. The specific binding was defined as the difference between total binding and nonspecific binding. Saturation binding data were analyzed by the method of Scatchard [32]. The K_d values and the number of the receptor sites were determined by using programs of EBDA and LIGAND from Elsevier-Biosoft, Cambridge, U.K. in an IBM AT computer.

To assess the comparative potencies of several selected competitors to displace [^{3}H]Ins(1,4,5)P_3 from binding sites, 0.2 nM [^{3}H]Ins(1,4,5)P_3 was incubated with 100 μg of membrane protein in the absence of Ca^{2+} at 0 °C for 1 h. The bound and unbound [^{3}H]Ins(1,4,5)P_3 were separated as described above. The

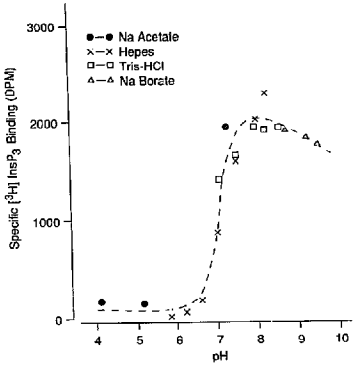


Fig. 1. pH dependence of [^{3}H]Ins(1,4,5)P_3 binding. 100 μg membrane protein was mixed with 1 pmol [^{3}H]Ins(1,4,5)P_3 (final concentration of 1 nM) with 50 mM sodium acetate buffer (●), Hepes buffer (×), Tris-HCl buffer (■), or sodium borate buffer (△) at the indicated pH in the presence of 1 mM EDTA. The reaction mixture was incubated for 2 h at 0°C. Data are from a single experiment performed in triplicate.

inhibition by the compound was normalized as percent inhibition by the equation described elsewhere [33]. The ED_{50} was defined as the concentration of inhibitor required to achieve 50% inhibition of the specific binding. The assays were routinely carried out in triplicate.

Results

pH dependence of [^{3}H]Ins(1,4,5)P_3 binding

At pH values below 6.0, no significant specific binding of [^{3}H]Ins(1,4,5)P_3 was observed (Fig. 1). However, specific binding increased dramatically at pH values above 6.0 with a half-maximum at around pH 7.0 and maximum at pH 8.0 and then slightly decreased at pH values above 8.0.

Specificity of [^{3}H]Ins(1,4,5)P_3 binding

The [^{3}H]Ins(1,4,5)P_3-binding site markedly differentiates among various inositol phosphates and is highly specific for Ins(1,4,5)P_3. As shown in Fig. 2, unlabeled Ins(1,4,5)P_3 inhibited [^{3}H]Ins(1,4,5)P_3 binding 50% at 0.3 nM. Of a variety of inositol phosphates examined, Ins(1,4,5)P_3 was the most potent inhibitor of binding with Ins(2,4,5)P_3 being the next potent, which inhibited [^{3}H]Ins(1,4,5)P_3 binding 50% at 6 nM. Ins(1,3,4)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(4,5)P_2, Ins(1,4)P_2 and Ins(1)P_1, were much less potent inhibitor of binding. The order of potency was Ins(4,5)P_2 > Ins(1)P_3 > Ins(1,3,4)P_3 > Ins(1,4,5,6)P_4 > Ins(1,3,4,5)P_4 > Ins(4,5)P_2 (Table I). Ins(1)P_1 was about 10-times more potent than Ins(1,4)P_2 in human platelet membranes, which is different from that in rat brain, where Ins(1,4)P_2 is a much more potent inhibitor than Ins(1)P_1 [5].

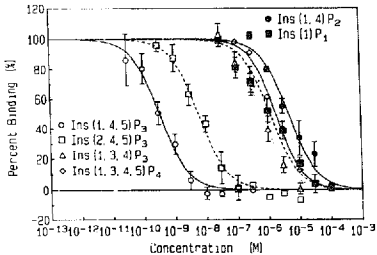


Fig. 2. Inhibition of [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. Membrane protein (100 μg), 0.2 nM [^{3}H]Ins(1,4,5)P_3 and the indicated concentration of inositol analogs were added to the medium containing 20 mM Hepes, 1 mM EDTA at pH 8.0. The data points are the averages of two or three experiments, each runs in triplicate. Error bars are S.D. values.

Several pharmacological agents known to influence intracellular calcium disposition were tested. Dantrolene (1 mM), verapamil (10 and 100 μM), and 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (10 and 100 μM) did not affect [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. However, heparin inhibited [^{3}H]-Ins(1,4,5)P_3 binding with an ED_{50} of approx. 1 μg/ml. Several other polyanions also inhibited the binding of [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. Suramin was the most potent inhibitor with an ED_{50} of 0.67 μM, followed with Trypan blue (ED_{50} = 5.4 μm) and then L-451 167 (ED_{50} = 15 μM) (Fig. 3).

Effects of nucleotides on the [^{3}H]Ins(1,4,5)P_3 binding

As shown in Fig. 4, GTP potently inhibited the binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes with an ED_{50} around 75 μm. Two nonhydrolyzable analogs, GTPγS and p[NH]ppG showed roughly about the same potency (data not shown). Other

TABLE I

Inhibition of binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes

Compound	ED_{50} (M)
Ins(1,4,5)P_3	$3.34 \cdot 10^{-10}$
Ins(2,4,5)P_3	$5.97 \cdot 10^{-9}$
Ins(1,3,4)P_3	$6.51 \cdot 10^{-7}$
Ins(1,3,4,5)P_4	$1.84 \cdot 10^{-6}$
Ins(1,4,5,6)P_4	$1.06 \cdot 10^{-6}$
Ins(1,4)P_2	$4.70 \cdot 10^{-6}$
Ins(4,5)P_2	$3.04 \cdot 10^{-7}$
Ins(1)P_1	$3.54 \cdot 10^{-7}$

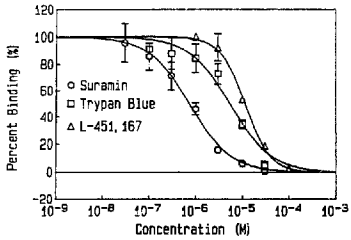


Fig. 3. Inhibition of [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes by sumarin, Trypan blue and L-451 167. Experimental conditions are identical to those described in Fig. 2. Data points are the means of triplicate determinations.

nucleotides also showed inhibitory effects but at a higher concentration. ATP, CTP, UTP and ITP showed roughly the same potency with an ED_{50} of 630 μM, which is about 10-times higher than that of GTP. GDP is even less potent with an ED_{50} value higher than 1 mM (Fig. 4). Nucleotides from different sources showed roughly identical potency to compete against the [^{3}H]Ins(1,4,5)-P_3 binding to its binding site.

Ionic modulation on the [^{3}H]Ins(1,4,5)P_3 binding

Ions modulated the specific binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes. As shown in Fig. 5, monovalent cations, including Na^+, K^+ and Li^+, potentiated the [^{3}H]Ins(1,4,5)P_3 binding in a concentration range between 1 and 100 mM with a maximum around 30 mM. However, at concentrations higher than 100 mM, monovalent cations inhibited the binding.

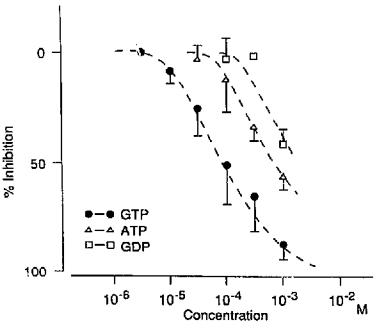


Fig. 4. Inhibition of [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes by nucleotides. Experimental conditions are identical to those described in Fig. 2. Data points and error bars are means and S.D. values of two or three experiments, each runs in triplicate.

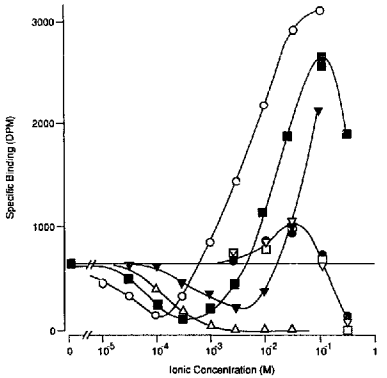
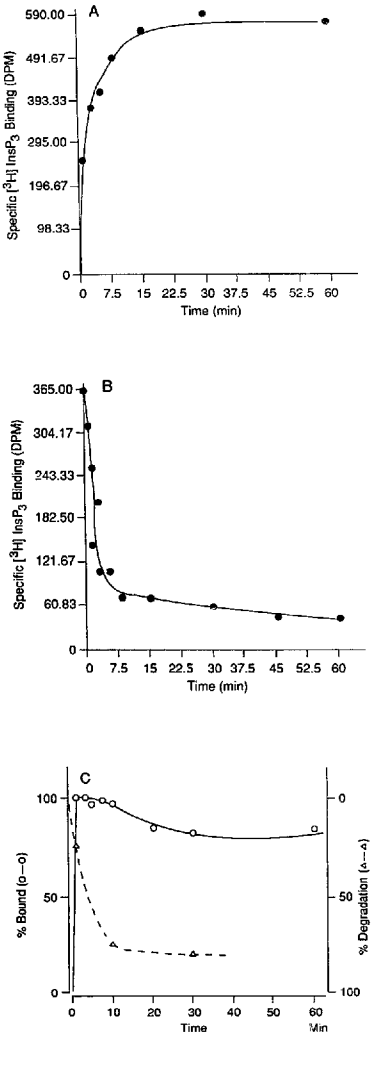


Fig. 5. Ionic modulation of specific [^{3}H]Ins(1,4,5)P_3 (0.2 nM) binding to human platelet membranes (100 μg membrane protein/ml). Ca^{2+} (■——■), Mg^{2+} (△——△), Mn^{2+} (○——○), Sr^{2+} (▼——▼), Na^+ (□——□), Li^+ (▽——▽), and K^+ (●——●) ions were added to a reaction mixture which contains 20 mM Hepes at pH 8.0. The point with zero concentration of ions was performed with the addition of 5 mM EDTA to the reaction mixture.

Divalent cations modulated the [^{3}H]Ins(1,4,5)P_3 binding in a different manner. At concentrations as low as 100 μM, Ca^{2+} and Mg^+ significantly inhibited the binding. The inhibitory effect by Mg^{2+} increased with increasing Mg^{2+} concentration. At 3 mM, Mg^{2+} totally abolished the specific [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. Ca^{2+}, on the other hand, inhibited the binding with concentrations between 30 μM and 3 mM and potentiated the binding at concentrations above 10 mM with a maximum around 100 mM. Similar patterns of the effects were also observed for Mn^{2+} and Sr^{2+} (Fig. 5). The inhibition and/or potentation of the [^{3}H]Ins(1,4,5)P_3 by Mn^{2+} occurred at lower concentrations than Ca^{2+}. A significant inhibition was observed at a concentration of Mn^{2+} as low as 10 μM, which was the Ca^{2+} concentration demonstrated by Pietri et al. [37] to be able to control the properties of the Ins(1,4,5)P_3 binding in the permeabilized hepatocytes and/or the liver plasma membranes. Sr^{2+} was less potent. It significantly inhibited the binding at concentration greater than 0.3 mM and potentiated the binding at or above 30 mM.



Reversibility of [^{3}H]Ins(1,4,5)P_3 binding and Ca^{2+}-induced degradation of [^{3}H]Ins(1,4,5)P_3

The binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes was fast. In the absence of 1 mM EDTA, the association of [^{3}H]Ins(1,4,5)P_3 reached a maximum around 30 min (Fig. 6A). The bound [^{3}H]Ins(1,4,5)P_3 could be displaced with unlabeled Ins(1,4,5)P_3 after the binding has reached an equilibrium (Fig. 6B). No degradation of [^{3}H]Ins(1,4,5)P_3 was observed even up to 2 h incubations at 0 °C in the absence of Ca^{2+} and with 1 mM EDTA.

In the presence of 100 mM $CaCl_2$, the association of [^{3}H]Ins(1,4,5)P_3 with human platelet membranes was too quick to be resolved with the filtration technique used here. Even at 30 s, the [^{3}H]Ins(1,4,5)P_3 binding already reached the maximum. However, the binding dropped after 5–10 min incubation. This could be due to the degradation of [^{3}H]Ins(1,4,5)P_3 in the presence of $CaCl_2$ (Fig. 6C). [^{3}H]Ins(1,4,5)P_3 was quickly metabolized. Even at 1 min incubations, about 25% of [^{3}H]Ins(1,4,5)P_3 was metabolized, mainly into Ins(1)P_1 in the presence of 100 mM $CaCl_2$. Degradation was not inhibited by 20 mM $LiCl_2$.

Saturation binding studies

To assess the affinity of Ins(1,4,5)P_3 binding and the number of recognition sites on the isolated human

Fig. 6. (A) Association of specific [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. [^{3}H]Ins(1,4,5)P_3 (0.17 nM) was incubated in a suspension of human platelet membranes (100 μg) at 0 °C, in a medium of 20 mM Hepes, 1 mM EDTA (pH 8.0), which was sampled at various time intervals for filtration and then radioactivity counting. The data points are the means of triplicate determinations. The solid line is the best fit of the data points with two observed association rate constants (k_{obs}) of 0.152 min^{-1} and 1.56 min^{-1} using the program KINETIC from Elsevier-Biosoft, Cambridge, U.K. in an IBM AT computer. (B) Dissociation of specific [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. Dissociation was started at equilibrium conditions (2 h incubation at 0 °C) by the addition of 0.2 μM unlabeled Ins(1,4,5)P_3 to an incubation medium to have 0.17 nM [^{3}H]Ins(1,4,5)P_3 and 100 μg membrane protein in a reaction mixture of 20 mM Hepes, 1 mM EDTA at pH 8.0. The data points are the means of triplicate determinations. The solid curve is the best fit of the data points with two dissociation rate constants (k_{-1}) of 0.566 min^{-1} and $1.144 \cdot 10^{-2}$ min^{-1} using the program KINETIC as described in Fig. 6A. (C) Association and degradation of [^{3}H]Ins(1,4,5)P_3. The experiments for the association of [^{3}H]Ins(1,4,5)P_3 (0.17 nM) to human platelet membranes were performed in an identical procedure as those described in Fig. 6A, except that the assays were performed in an incubation medium of 100 mM $CaCl_2$, 20 mM Hepes (pH 8.0). For the degradation experiments, the reaction mixture was incubated in the exact same way as described above, but 500 μl reaction mixture was added to 75 μl 10% trichloroacetic acid and then incubated for 10 min at 0 °C. The precipitate was removed by centrifugation and supernatant was repeatedly washed three times with 5 ml ethyl ether. The aqueous phase was then separated in a HPLC system with a column of Partisil 10-SAX (Whatman) eluted with a step gradient ammonium formate.

platelet membranes, 100 μg of membrane protein was incubated with 0.01 to 12 nM [^{3}H]Ins(1,4,5)P_3. Fig. 7 shows the binding isotherm of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes in the presence of 1 mM EDTA. The specific binding was saturable at around 10–15 nM (Fig. 7A). The nonspecific binding was nonsaturable and linear with the concentration of [^{3}H]Ins(1,4,5)P_3 and was about 5–10% of total binding. The Scatchard plot obtained by plotting the bound/free ratio of the labeled Ins(1,4,5)P_3 as a function of the Ins(1,4,3)P_3 concentration that is bound to the receptor

TABLE II

Equilibrium dissociation constants (K_d) and maximal detectable receptor sites (B_{max}) for [^{3}H]Ins(1,4,5)P_3 on human platelet membranes

Ionic conditions	K_d (nM)	B_{max} (fmol/mg protein)
1 mM EDTA		
High affinity	0.193 ± 0.113	295.8 ± 64 (n = 5)
Low affinity	13.15 ± 18.07	846.5 ± 413 (n = 5)
100 mM $CaCl_2$	0.32 ± 0.15	3365 ± 980 (n = 5)

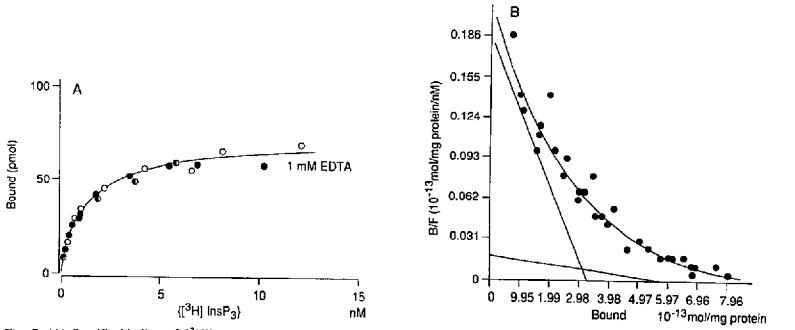


Fig. 7. (A) Specific binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes. 100 μg membrane protein was added to tubes containing [^{3}H]Ins(1,4,5)P_3 ranging from 0.01 to 12 nM in medium containing 20 mM Hepes, 1 mM EDTA at pH 8.0. Data points were the averages of triplicate and three independent experiments were given in the same curve. (B) Scatchard plot of the specific [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. Data points were obtained from Fig. 7A. K_d and B_{max} were calculated with the LIGAND program from Elsevier-Biosoft in an IBM AT computer. The solid curve is the best fit of the data points with two K_d values.

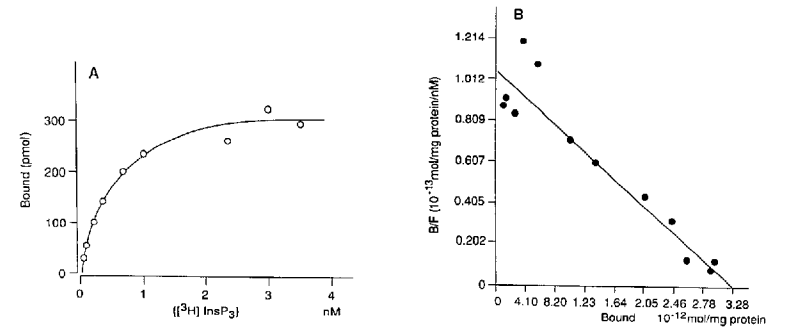


Fig. 8. (A) Specific binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes. 100 μg of membrane protein was added to tubes containing 0.01 to 4 nM [^{3}H]Ins(1,4,5)P_3 in a medium containing 20 mM Hepes, 100 mM $CaCl_2$ at pH 8.0. Data points were the averages of triplicate experiments. (B) Scatchard plot of the specific [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes. Data points were obtained from Fig. 8A.

revealed a nonlinear but concave curve (Fig. 7B), which can be statistically best fitted with two equilibrium dissociation constants (K_d) of 0.19 and 3.3 nM. The maximal detectable binding sites B_{max} for high and low affinity obtained from Fig. 7B are 332 and 567 fmol/mg protein, respectively. The mean and standard deviation (S.D.) from several repeated experiments were listed in Table II. The variations for the high-affinity binding site in the determination of K_d and B_{max} are less than that for the low-affinity binding site. This could be due to the filtration technique we used to separate the bound and unbound tritium labeled ligand. Since it takes about 30 s to wash the filter with 20 ml wash buffer, some of the ligand bound to the low-affinity site could be washed away.

In the presence of 100 mM $CaCl_2$, [^{3}H]Ins(1,4,5)P_3 was quickly degradated. However, the [^{3}H]Ins(1,4,5)P_3 binding reached a maximum within 30 s, where more than 80% of [^{3}H]Ins(1,4,5)P_3 remained in the original form. To access the affinity of Ins(1,4,5)P_3 binding to human platelet membranes, the bound and unbound [^{3}H]Ins(1,4,5)P_3 were separated after 30 s incubation of [^{3}H]Ins(1,4,5)P_3 with human platelet membranes. Under these conditions, the specific binding was highly potentiated. It was again readily saturable and reached near maximum around 3 nM [^{3}H]Ins(1,4,5)P_3 (Fig. 8A). The Scatchard plot of the specific binding in 100 mM $CaCl_2$ is linear (Fig. 8A) indicating a single class of high-affinity binding sites. Results from several repeated experiments are summarized in Table II. It showed a K_d value of 0.32 ± 0.15 nM ($n = 5$) and B_{max} of 3.365 ± 0.98 pmol/mg protein. The K_d value in 100 mM $CaCl_2$ is slightly higher than the one with high affinity in the absence of $CaCl_2$. This could be due to the nonequilibrium binding situation here by separating the bound and unbound [^{3}H]Ins(1,4,5)P_3 in 30 s. The affinity could therefore be underestimated. The B_{max} value in the presence of 100 mM $CaCl_2$ (Table II) is about 3-fold higher than the sum of B_{max} with both high and low affinity in the absence of Ca^{2+}. Again, this could be due to an underestimation of B_{max} of low affinity due to the disadvantage of filtration techniques.

Discussion

Here, we have demonstrated the specific Ins(1,4,5)P_3 binding to human platelet membranes and its modulation by pH, monovalent and divalent cations and GTP. The specific Ins(1,4,5)P_3 binding has an optimal pH around 8.0. Monovalent cations including Na^+, K^+ and Li^+ examined here potentiate the binding, whereas Mg^{2+} inhibits the binding. Ca^{2+}, Mn^{2+} and Sr^{2+} inhibit the bidning at low concentrations, but dramatically potentiate the binding at higher concentrations. As demonstrated in Scatchard plots, this potentiation effect by Ca^{2+} seems to be due to the increase in the affinity of binding sites but not the maximal number of receptor sites. Therefore, similar to the receptor of platelet-activating factor [36], multiple conformational states of a single type of Ins(1,4,5)P_3 receptor could also exist in human platelet membranes. Similar effects of Ca^{2+} on the control of the interconversion of the Ins(1,4,5)P_3 receptor from a low-affinity to a high-affinity state have also been reported in permeabilized rat hepatocytes and liver plasma membranes [37]. However, the Ca^{2+} concentration required to potentiate the [^{3}H]Ins(1,4,5)P_3 binding in human platelet membranes is about 10–100-times higher than that reported in liver plasma membranes. The physiological significance of this Ca^{2+}-induced potentiation apparently need to be further elucidated.

Monovalent cations have been demonstrated to alter Ins(1,4,5)P_3-stimulated Ca^{2+} release from liver microsomes [38]. Calcium release process stimulated by Ins(1,4,5)P_3 appears to be electrogenic [38,39]. Monovalent cationic channels of high conductance have also been detected in sarcoplasmic reticulum vesicles [40]. Ins(1,4,5)P_3-stimulated Ca^{2+} release is therefore thought to be charge compensated by the inward entry of monovalent cations [38,41]. Activation of platelets by ADP [42,43] or platelet-activating factor (Hwang, S.-B., unpublished data) is associated with Na^+ influx. Potentiation of the specific [^{3}H]Ins(1,4,5)P_3 binding to human platelet membranes by Na^+ suggests the possible role of the influxed Na^+ on the mobilization of the intracellular Ca^{2+} upon activation of platelets.

Similar to Ins(1,4,5)P_3 receptors in rat brains [5], binding of [^{3}H]Ins(1,4,5)P_3 to human platelet membranes is inhibited by low concentrations of Ca^{2+} and is sensitive to the pH value of the assay medium. Ca^{2+} sensitivity for the release of Ca^{2+} in response to Ins(1,4,5)P_3 from permeabilized cells [37,44,45] and isolated membrane vesicles [46] has been previously reported, although such effects are not universally observable [26,47]. Also, the elevation of intracellular pH appears to be intimately linked with stimulus-response coupling [48,49] and forms an essential step in the cascade of events required to increase cytoplasmic free Ca^{2+} in platelets [26,50]. A calcium-mediator protein, calmedin, has recently been isolated [48], which enables Ca^{2+} to inhibit Ins(1,4,5)P_3 binding to purified receptors from rat cerebellar membranes. Whether an identical or similar protein exists in human platelets is not yet known. But it seems to be clear that in human platelets the mechanism of Ins(1,4,5)P_3-mediated Ca^{2+} release is also self-regulated by levels of cytosolic-free Ca^{2+} and that the influence of pH on Ca^{2+} release [26] may directly affect on Ins(1,4,5)P_3 interactions with its receptors.

In spite of the similar inhibitory activity of the calcium and the pH sensitivity on the Ins(1,4,5)P_3 binding between rat brains and human platelets, binding of

$Ins(1,4,5)P_3$ to human platelet membranes is also inhibited by Mg^{2+}, but potentiated by Ca^{2+}, Mn^{2+} and Sr^{2+} at high concentrations. Mg^{2+} at a similar concentration shows an inhibitory effect. Also the differentiation between $Ins(1,3,4,5)P_4$ and $Ins(1)P_1$ or between $Ins(1.4)P_2$ and $Ins(1)P_1$ in human platelets (Fig. 2) is not as pronounced as that in rat brains (Fig. 3 in Ref. 5). In fact, $Ins(1)P_1$ is more potent than $Ins(1,4)P_2$ and $Ins(1,3,4,5)P_4$ in inhibiting the [^{3}H]$Ins(1,4,5)P_3$ binding to human platelet membranes. These results suggest that $Ins(1,4,5)P_3$ receptors are different between human platelets and rat brains. The liver $Ins(1,4,5)P_3$ receptors also displays some properties different from those of the brain receptor [37]. However, from the results we have, we cannot distinguish between whether the differences are due to the tissue specificity or due to the species variation.

Here, we observed that GTP and nonhydrolyzable analogs, GTPγS and p[NH]ppG specifically inhibit the specific [^{3}H]$Ins(1,4,5)P_3$ binding. Specificity of GTP on the inhibition of a radioligand receptor binding is generally correlated with those receptors coupled to guanyl nucleotide regulatory protein (G-protein) in receptors from plasma membranes [49,50]. Also, a series of polyanionic compounds that inhibit the coupling of the α_2-adrenergic receptors and the β_2-adrenergic receptors to G-proteins [51–53] also potently inhibit the binding of [^{3}H]$Ins(1,4,5)P_3$ to human platelet membranes with potencies comparable to those in inhibiting the binding of [^{3}H]UK 14304 to the α-adrenergic receptors [53]. The molecular cloned $Ins(1,4,5)P_3$ receptor [54] is also found to span the plasma membrane seven times and have an extracellular amino terminus and an intracellular carboxyl terminus, a general feature for those plasma membrane receptors coupled to G-proteins. However, in the reconstituted vesicles of the purified $Ins(1,4,5)P_3$ binding protein, a single protein is able to mediate both the recognition of $Ins(1,4,5)P_3$ and calcium transport [55]. Apparently, the biological significance of these effects of GTP on the binding of [^{3}H]$Ins(1,4,5)P_3$ needs to be further elucidated.

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