Specific binding sites for inositol 1,3,4,5-tetrakisphosphate are located predominantly in the plasma membranes of human platelets

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In the present study we describe the characterization and localization of Ins(1,3,4,5)P_4-binding sites in human platelet membranes. Specific binding sites for Ins(1,3,4,5)P_4 have been identified on mixed, plasma and intracellular membranes from neuraminidase-treated platelets using highly purified carrier-free [^{32}P]Ins(1,3,4,5)P_4. The displacement of Ins(1,3,4,5)P_4 from these sites by Ins(1,4,5)P_3 and InsP_6 occurs at greater than two orders of magnitude higher concentrations and with Ins(1,3,4,5,6)P_5 at about 40-fold higher concentrations than with Ins(1,3,4,5)P_4. The membranes were further separated by free-flow electrophoresis into plasma and intracellular membranes. The Ins(1,3,4,5)P_4-binding sites separated with plasma membranes, and showed similar affinities and specificities as mixed membranes, whereas Ins(1,4,5)P_3-binding sites were predominantly in the intracellular membranes. These results suggest a predominantly plasma membrane location for putative Ins(1,3,4,5)P_4 receptors in human platelets.

INTRODUCTION

The suggested second-messenger role for Ins(1,3,4,5)P_4 remains a matter of some controversy, both as to whether it has a role at all, and if so, what that role might be [1–3]. One factor supporting the possibility that it is a second messenger is that, in a wide range of neural and peripheral tissues, binding sites for Ins(1,3,4,5)P_4 have been identified which show a high specificity for this ligand [4–10]. The specificity of these sites is that they can, and have, been used to set up mass assays for Ins(1,3,4,5)P_4 in crude cell extracts [11,12], and the recent observation that this quantitative recognition still holds if the binding assays are done under physiological conditions [10] implies that Ins(1,3,4,5)P_4 is the likely principal ligand on these sites in an intact cell. These Ins(1,3,4,5)P_4-specific sites are distinct from InsP_n-binding sites that can bind Ins(1,3,4,5)P_4 but that favour InsP_6 (the two sets of proteins are actually separable [8]) and are also distinguishable from any known InsP_n metabolizing enzymes [4]. Although some Ins(1,4,5)P_3 receptors will bind Ins(1,3,4,5)P_4 [13], these are distinct from the putative Ins(1,3,4,5)P_4 receptors we are considering here in that the latter greatly favour Ins(1,3,4,5)P_4 over Ins(1,4,5)P_3 [4–10].

Overall, there is a similarity between these binding sites in various tissues [1,4–10], which, with the properties summarized above, implies that there may exist in cells a protein or proteins which could act as receptors for a second-messenger function for Ins(1,3,4,5)P_4. What that function is, is still controversial, but the most consistent collection of evidence (see [1] and [2] for discussion) points to a role in the control of Ca^{2+} homoeostasis at the plasma membrane [14–17], and current hypothesis on this action [1,2,18], along with a demonstration of a direct effect of Ins(1,3,4,5)P_4 on channels in the plasma membrane of endothelial cells [17], all invoke a location of an Ins(1,3,4,5)P_4 receptor in, or close to, the plasma membrane.

One way to address the subcellular location of putative Ins(1,3,4,5)P_4 receptors directly is to monitor the distribution of Ins(1,3,4,5)P_4-binding sites in subcellular fractions. This has not been addressed yet, partly because there are few really efficient purifications of plasma membranes from tissues extracts, and principally because with the low frequency of Ins(1,3,4,5)P_4-binding sites in peripheral tissues (in which such subcellular fractionations are best characterized), it is difficult to quantify such sites in cell fractions using [^{3}H]Ins(1,3,4,5)P_4 probes of low specific activity; it is also true that because other proteins in addition to these putative receptors are known to bind Ins(1,3,4,5)P_4 (discussed above), it is a potential complication of measuring Ins(1,3,4,5)P_4 binding to an isolated membrane fraction that the probe may no longer be binding predominantly to the correct (putative receptor) protein.

In this set of experiments we have sought to overcome these problems by using a [^{32}P]Ins(1,3,4,5)P_4 probe of high specific radioactivity and purity and applying it to platelet membranes for which there exists a well-characterized and efficient separation of plasma and intracellular membranes [19–22]. This separation method has already been used to show that Ins(1,4,5)P_3 receptors are located, as expected, predominantly intracellularly as opposed to in plasma membranes [22]. Here we show that platelets have binding sites for Ins(1,3,4,5)P_4 with the characteristics consistent with putative receptors for that ligand, and that membrane purification reveals that these specific binding sites fractionate differently from Ins(1,4,5)P_3 receptors, being primarily located in the plasma membrane.

MATERIALS AND METHODS

Production of [^{32}P]Ins(1,3,4,5)P_4

The strategy employed in the production of [5-^{32}P]Ins(1,3,4,5)P_4 is based on the method described by Letcher et al. [23]. Briefly, bovine brain phosphatidylinositol 4-monophosphate is phosphorylated by a purified bovine brain phosphatidylinositol 4-phosphate 5-kinase [24] using γ^{32}P-labelled ATP (4500 Ci/mmol; ICN Flow), to form 5-^{32}P-labelled PtdIns(4,5)P_2. This lipid is then deacylated and deglycerated by standard chemical procedures (see [23]) in order to produce [5-^{32}P]Ins(1,4,5)P_3, which is subsequently purified by h.p.l.c. This purification stage is essential to remove contaminating radiolabelled species which co-chromatograph with Ins(1,3,4,5)P_4 and which compromise the detection of specific binding (P. Cullen, unpublished work).

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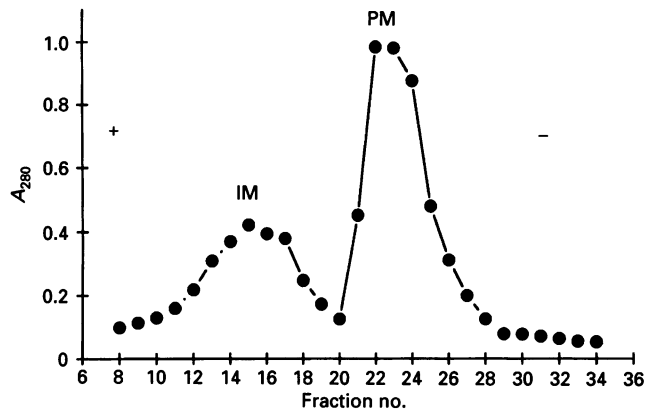

Figure 1 Separation of platelet intracellular and plasma membranes by free-flow electrophoresis

A typical protein (measuring absorbance at 280 nm) of free-flow electrophoresis fraction is shown. Abbreviations: IM, intracellular membranes; PM, plasma membranes. + and − indicate the polarity of electrophoresis chamber.

Phosphorylation of the radiolabelled InsP_3 using a purified bovine brain InsP_3 3-kinase (C. Brooksbank, N. Divecha and R. Irvine, unpublished work) results in [5-^{32}P]Ins(1,3,4,5)P_4, which is finally purified by h.p.l.c.

Preparation of platelet membranes

The procedure for the preparation of platelet plasma and intracellular membranes has been described in detail previously [19–21]. Briefly platelets are separated from blood, washed, treated with neuraminidase (0.05 unit/ml, 20 min, 37 °C) and sonicated in the presence of the proteinase inhibitors aprotinin (0.3 unit/ml), pepstatin A (5 μg/ml), phenylmethanesulphonyl fluoride (0.2 mM) and dithiothreitol (1 mM). A mixed membrane fraction is obtained (free from cross-contamination by granular constituents) from sonicated platelets by centrifugation (40000 *g*; 90 min) on a linear (1–3.5 M) sorbitol density gradient. After concentration of the mixed membrane fraction by centrifugation (100000 *g*; 60 min), they are further separated into plasma and intracellular membranes by application to a Bender Hobein VAP 5 electrophoresis unit running at 1000 V and 100 mA. Protein profiles of the fractions obtained reveals two discrete protein peaks (Figure 1), of which the smaller, more electronegative, peak represents intracellular membranes ('IM') and the larger, less electronegative, peak (the effect of neuraminidase treatment cleaving sialic acid residues from surface glycoproteins at the intact-cell stage) are plasma membranes ('PM'). Fractions from the free-flow-electrophoresis profile are taken from the tops of the peaks and pooled rather that across the whole peaks, in order to minimize cross-contamination, concentrated by centrifugation (100000 *g*, 60 min) and resuspended in 0.34 M sorbitol/10 mM Hepes, pH 7.2, for subsequent evaluation. Both peaks have been extensively characterized with respect to a range of marker enzymes, protein and lipid profiles (for a summary, see [21]). Recently we have demonstrated, using an antiserum to the platelet plasma-membrane glycoprotein GP1b, that this is localized in our plasma-membrane fractions with no recognition of any protein in the intracellular membranes using Western-blotting techniques (S. S. El-Daher and K. S. Authi, unpublished work). With relevance to the relationship of inositol phosphate function in Ca^{2+} homoeostasis, we have previously demonstrated that the intracellular membrane fraction exhibits ATP-dependent Ca^{2+} sequestration with the associated Ca^{2+}-ATPase activity, which is absent in the plasma-membrane fraction [20,21] and is the predominant location for the binding site for Ins(1,4,5)P_3 [22]. The 20 min neuraminidase treatment of intact platelets required to reduce the surface negative charge does not lead to platelet activation or effect the responses of platelets to the agonists collagen, thrombin and the ionophore A23187 (results not shown).

Binding assay

An individual Ins(1,3,4,5)P_4 binding assay mixture contained 100 mM KCl, 20 mM NaCl, 10 mM Hepes/NaOH, pH 7.0, 1 mM EDTA, 0.1% (w/v) BSA, 22000 d.p.m. of [^{32}P]Ins(1,3,4,5)P_4, 70–320 μg of protein, and various concentrations of competing inositol phosphates in a final volume of 0.5 ml. Equilibrium binding was reached by incubation for 20 min, followed by centrifugation for a further 10 min. All manipulations were performed in a coldroom at 4 °C. The amount of radioactivity retained within the pellet was determined by liquid-scintillation counting.

For InsP_3 binding assays the equilibrium binding was reached by incubation for 10 min at 4 °C, followed by centrifugation for 5 min. Each assay mixture contained 50 mM Tris/HCl, pH 8.3, 1 mM EDTA, 20000 d.p.m. of [^{32}P]InsP_3, 35 μg of protein and various concentrations of competing inositol phosphates in a final volume of 0.5 ml.

Materials

Highly purified PtdIns4*P* was prepared from a brain inositide fraction [25] by the method of Palmer [26]. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were prepared as in [27]. InsP_6 was from Sigma, was purified by h.p.l.c. [28] and was converted into its sodium salt. Ins(1,3,4,5,6)P_5 was from Boehringer, and was further purified by ionophoresis [29]. Fresh buffy-coat fractions of human blood were obtained from the Blood Transfusion Laboratories, Tooting, London, U.K. Neuraminidase (type X) was purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The results presented focus on the fractionation of a mixed membrane preparation essentially devoid of secretory granules. We did investigate Ins(1,3,4,5)P_4 binding on fractions from the sorbitol density gradient containing secretory granules and found a decrease in Ins(1,3,4,5)P_4 binding with respect to mixed membranes; all the binding in these fractions could be accounted for by contamination with the mixed membranes (results not shown).

Characterization of the [^{32}P]Ins(1,3,4,5)P_4-binding site(s) present within human platelets was performed on mixed membranes containing components of both plasma and intracellular membranes. As can clearly be seen from Figures 2(a) and 2(b), Ins(1,3,4,5)P_4 bound to the membranes with a high affinity and specificity. The K_d value for Ins(1,3,4,5)P_4 {i.e. the concentration required to compete for 50% of bound [^{32}P]Ins(1,3,4,5)P_4} was 24.6±4.5 nM (n = 3), with the other inositol phosphate isomers tested, namely Ins(1,3,4,5,6)P_5, InsP_6 and Ins(1,4,5)P_3, having K_d values of 1 μM, 3 μM and 10 μM respectively. Detailed Scatchard

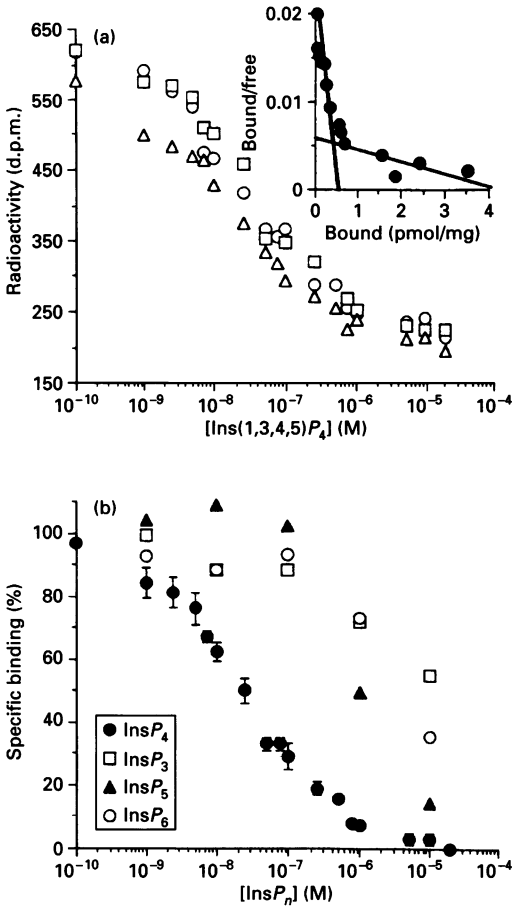

Figure 2 Characterization of the Ins(1,3,4,5)P_4-binding site(s) present within human platelet mixed membranes

(**a**) Data from three individual determinations of the competition curve for Ins(1,3,4,5)P_4. The inset shows Scatchard transformation of all three sets of data analysed simultaneously in order to give the best accurate transformation possible using the EBDA-LIGAND computer program. Binding was performed as described in the Materials and Methods section, using 320 μg of mixed membranes. (**b**) Specificity of the described Ins(1,3,4,5)P_4-binding site, where InsP_3, InsP_5 and InsP_4 are Ins(1,4,5)P_3, Ins(1,3,4,5,6)P_5 and Ins(1,3,4,5)P_4 respectively. Again, binding was performed using 320 μg of mixed membranes. Non-specific binding was defined as the binding remaining in the presence of 20 μM Ins(1,3,4,5)P_4. Data for the InsP_4 competition curve were obtained from three separate determinations and are means ± S.E.M. For other competing Ins$P$ species data shown are means of duplicate determinations, with similar binding affinities observed in two other preparations.

Table 1 Distribution of Ins(1,3,4,5)P_4- and Ins(1,4,5)P_3-binding sites in human platelet plasma and intracellular membranes

Data are expressed in relation to the parent mixed membranes used for free-flow electrophoresis. For InsP_4 binding the data reflect analysis of three different membrane preparations and for InsP_3 binding one membrane preparation (for the latter, see also [22]).

Membrane fraction	Enrichment of binding	
	InsP_4 (n = 9)	InsP_3 (n = 3)
Mixed	1.00 ± 0.15	1.00 ± 0.07
Intracellular	0.76 ± 0.09	3.57 ± 0.32
Plasma	3.33 ± 0.32	0.797 ± 0.14

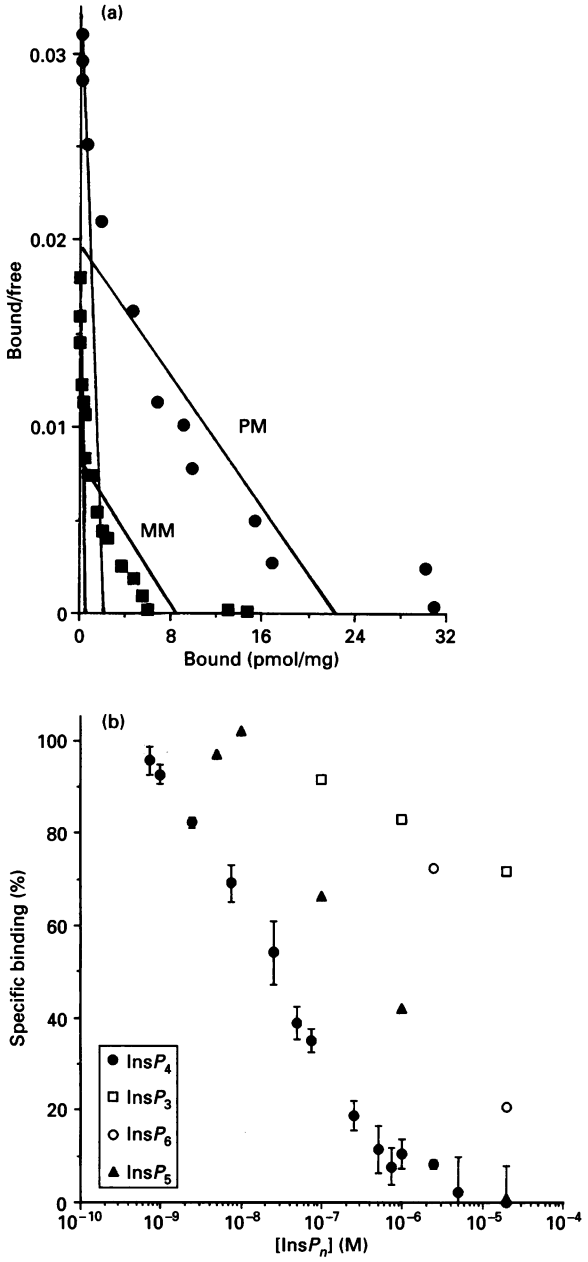

Figure 3 Characterization of the Ins(1,3,4,5)P_4-binding site(s) present on highly purified platelet plasma membranes

(**a**) Scatchard transformation of the Ins(1,3,4,5)P_4 binding to mixed membranes (MM) (■) and plasma membranes (PM) (●). For each membrane fraction, sets of data from three competition curves were simultaneously analysed again using the EBDA-LIGAND computer program in order to give the best transformation possible. Similar data was obtained with two other membrane preparations. (**b**) Specificity of the Ins(1,3,4,5)P_4-binding site present in platelet plasma membranes. Binding was performed with 40 μg of membrane protein, and non-specific binding and incubation conditions were as defined in Figure 2(b). Data shown for InsP_4 competition are means ± S.E.M. ($n = 3$); results for other Ins$P$ species are mean of duplicate determinations, with similar results obtained in two other membrane preparations.

transformation of the Ins(1,3,4,5)P_4 competition curve using the EBDA-LIGAND data-analysis computer program, resolves two apparent binding sites ($P = 0.001$ compared with a one-site fit) with affinities of 9.3 ± 3.6 nM and 225 ± 151 nM (see the inset to Figure 2a). In these characteristics the binding closely resembles

Table 2 Equilibrium constants from Scatchard transformations of the Ins(1,3,4,5)P_4-binding site(s) present within platelet mixed and plasma membranes

The Table documents the affinities and binding densities of the Ins(1,3,4,5)P_4-binding sites present within mixed and plasma membranes that was obtained from the Scatchard transformations depicted in Figure 3(a). K_d^H and K_d^L refer to the high- and low-affinity binding sites respectively, and $B_{max.}^H$ and $B_{max.}^L$ describe the maximum binding densities of each of the binding sites. The data are mean ± S.E.M. from three independent experiments on one set of membranes (see the legend to Figure 3a).

Constant	Mixed membranes	Plasma membranes
K_d^H (nM)	6.2 ± 1.9	8.2 ± 5.6
$B_{max.}^H$ (pmol/mg)	0.47 ± 0.17	1.9 ± 1.6
K_d^L (nM)	200 ± 109	100 ± 55.2
$B_{max.}^L$ (pmol/mg)	8.18 ± 3.29	21.3 ± 5.1

putative Ins(1,3,4,5)P_4 receptors documented in several other tissues ([1,4–10]; for reviews on current concepts, see [30]).

Subcellular distribution of Ins(1,3,4,5)P_4-binding site(s) was performed on membranes fractionated using free-flow electrophoresis as described in the Materials and methods section. However, owing to the small amounts of purified membranes harvested from the free-flow-electrophoresis apparatus, a lesser amount of characterization was possible. The data in Table 1 show conclusively that most of the Ins(1,3,4,5)P_4 binding resides with the plasma membranes, with a depletion of the binding activity in the intracellular membranes when compared with the parental mixed membranes. Moreover, this distribution is completely opposite to that observed for the InsP_3-binding site, for which the intracellular membranes is the enriched fraction (see Table 1 and a previous study [22]). Analysis of the Ins(1,3,4,5)P_4-binding sites within the purified plasma-membrane fraction revealed binding sites displaying affinities similar to those described in the parental mixed membranes. Figure 3(a) shows the Scatchard analysis of Ins(1,3,4,5)P_4 binding to both mixed- and the corresponding plasma-membrane fractions, and the equilibrium constants are expressed in Table 2. Again, the binding resolves into two sites ($P < 0.01$) with K_d values similar in plasma and mixed membranes, but with an enhancement of the maximal binding ($B_{max.}$) values in the plasma membranes, reflecting the increase in binding seen in Table 1. Presently it is not known whether the two affinity states reflect two proteins or are different conformations of the same protein, but our results indicate a similar subcellular distribution. Figure 3(b) demonstrates that the binding to purified plasma membranes again exhibits specificity for Ins(1,3,4,5)P_4 when tested against the other InsP_n (InsP_3, InsP_5 and InsP_6). The data again indicate a similar order of potency for [^{32}P]Ins(1,3,4,5)P_4 displacement, as with mixed membranes, i.e. InsP_4 > InsP_5 > InsP_6 > InsP_3.

In conclusion, we have shown that human platelets, when assayed under physiological ionic strength and pH, possess InsP_4-binding sites with the following characteristics: (i) an apparent dissociation constant (K_d) of approx. 25 nM; (ii) resolvable into two apparent binding sites, as determined by Scatchard transformation; and (iii) displacement by InsP_4 and InsP_n reflecting putative InsP_4 receptors rather than InsP_n-binding proteins that bind InsP_4. We have demonstrated that the subcellular distribution of these sites is clearly different from Ins(1,4,5)P_3 binding, and overall our data suggest that if these sites do indeed reflect receptors for a second-messenger function of Ins(1,3,4,5)P_4, then such receptors are predominantly associated with the plasma membrane.

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