Isolation of InsP_4 and InsP_6 binding proteins from human platelets: InsP_4 promotes Ca^{2+} efflux from inside-out plasma membrane vesicles containing 104 kDa GAP1^{IP4BP} protein

Flavia O'ROURKE*, Eileen MATTHEWS and Maurice B. FEINSTEIN

Department of Pharmacology, University of Connecticut School of Medicine, Farmington, CT 06030, U.S.A.

A low-density membrane fraction from human platelets contained the plasma membrane marker glycoprotein Ib (GpIb) and selective binding sites for InsP_4 and InsP_6. It was separated from the bulk of InsP_3-receptor-containing membranes, but was heterogeneous, probably also containing surface-connected canalicular system and some lighter elements of the internal dense tubule system. After loading with calcium oxalate and re-centrifugation on Percoll gradients, this mixed fraction was subfractionated into light membranes containing all of the GpIb, high-affinity InsP_4 binding sites (K_D = 18 nM) and phosphate-stimulated Ca^{2+} transport activity. InsP_4 (EC_{50} 0.6 μM), but not InsP_3 or InsP_6, released up to 35 % of the accumulated Ca^{2+} from these vesicles, which were shown to be inside-out plasma membrane vesicles by a biotinylation labelling technique and selective removal of right-side-out plasma membrane vesicles with streptavidin–agarose. Most of the InsP_4, and all of the InsP_6, binding was present in the much denser calcium oxalate-loaded subfractions, which were free of GpIb. InsP_6 binding activity was chromatographically purified as a 116 kDa protein (K_D for InsP_6 = 5.9 nM), with an amino acid content and two internal peptide sequences identical to those of 116 kDa vinculin. A 104 kDa InsP_4 binding protein (K_D for InsP_4 = 12 nM), probably identical to GAP1^{IP4BP} described by Cullen, Hsuan, Truong, Letcher, Jackson, Dawson and Irvine [(1995) Nature (London) **376**, 527–530], was also isolated. This InsP_4 receptor may mediate Ca^{2+} influx in platelets that occurs subsequent to receptor-stimulated production of InsP_3 and unloading of internal Ca^{2+} stores.

INTRODUCTION

The importance of Ins(1,4,5)P_3 as a second messenger for intracellular Ca^{2+} signalling is well documented in many cell types, including platelets. Other inositol phosphates such as Ins(1,3,4,5)P_4 and InsP_6 are found in many cells, and it has been suggested that they are also involved in signal transduction processes, including the control of cytosolic Ca^{2+} levels and clathrin assembly, but their specific functions are largely either unclear or unknown. InsP_4 is formed intracellularly by the phosphorylation of InsP_3, and InsP_4 binding sites have been identified in cerebellum [1,2], HL-60 cells [3], adrenal cortex [4], liver [5] and platelets [6]. A specific InsP_4 receptor has been purified from pig platelet plasma membranes [7], and recently its cDNA was sequenced and shown to code for a member of the GTPase-activating protein (GAP) protein family, and designated as GAP1^{IP4BP} [8]. InsP_4 has various effects on Ca^{2+} in cells; e.g. it may (1) control Ca^{2+} entry into sea urchin eggs as a mechanism to replenish internal Ca^{2+} stores [9], (2) contribute to the release of Ca^{2+} from internal stores in brain [10], (3) enhance Ca^{2+} uptake into internal storage sites [11], and (4) activate a Ca^{2+}-permeable channel in endothelial cell plasma membranes [12]. Several inositol polyphosphate binding proteins that bind InsP_4, but with lesser affinity than for InsP_6, have also been described [13–15].

InsP_3 releases Ca^{2+} from vesicles derived from the internal membrane system (internal dense tubule system; DTS) of human platelets [16–20], but nothing is known about the functions of either InsP_4 or InsP_6 in platelets. Here we report on the binding of InsP_4 and InsP_6 to platelet membranes, and the purification of a 104 kDa InsP_4 receptor protein and its separation from a 116 kDa InsP_6 binding protein. We also show that the InsP_4 binding protein is found in two fractions, with the majority in a membrane fraction containing the InsP_6 binding protein and exhibiting oxalate-stimulated Ca^{2+} transport. Lastly, we demonstrate the ability of InsP_4 to increase Ca^{2+} efflux across inside-out membrane vesicles derived from the plasma membrane fraction of human platelets. Therefore the plasma membrane InsP_4 receptor may mediate Ca^{2+} influx in agonist-stimulated platelets, which occurs subsequent to the production of InsP_3 and unloading of internally sequestered Ca^{2+} stores by InsP_3 [21].

MATERIALS AND METHODS

Materials

Radiolabelled [^{3}H]InsP_4 (17 Ci/mmol), InsP_6 (21 Ci/mmol) and $^{45}CaCl_2$ were from New England Nuclear, [^{3}H]InsP_3 (35 Ci/mmol) was purchased from Amersham, and non-radioactive inositol phosphates were from the Rhode Island Chemical Group (University of Rhode Island, Kingston, RI, U.S.A.). The sources of other reagents were as follows: monoclonal antibody against glycoprotein Ib (GpIb) (Immunotech), peroxidase-labelled second antibodies (Boehringer Mannheim), sulphosuccinimidyl-6-(biotin amido)hexanoate (sulpho-NHS-LC-biotin), peroxidase-labelled streptavidin and Immunopure immobilized streptavidin linked to agarose (Pierce Chemical Co.), chemiluminescence reagents (Kirkegaard and Perry), concanavalin A (ConA)–agarose (Sigma Chemical Co.).

Assays of Ca^{2+} uptake and release in platelet membrane vesicles

Platelets obtained from the Connecticut Red Cross were washed in buffer containing 0.135 M NaCl, 5 mM KCl, 10 mM Hepes,

Abbreviations used: GAP, GTPase-activating protein; DTS, dense tubule system; GpIb, glycoprotein Ib; sulpho-NHS-LC-biotin, sulphosuccinimidyl-6-(biotin amido)hexanoate; DTT, dithiothreitol; ConA, concanavalin A; SCCS, surface-connected canalicular system.

* To whom correspondence should be addressed.

pH 7.6, and then washed in 0.125 M NaCl, 5 mM KCl, 10 mM Pipes, 0.2 mM EGTA, pH 6.8. Washed platelets were sonicated, and membrane fractions were separated on a 12 ml continuous 40 % Percoll gradient by the method of O'Rourke and Feinstein [17] to yield 1.0 ml fractions numbered 1–11 from the top of the gradient. Membranes from the Percoll gradients were washed twice in a buffer of 100 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT) and 10 μM each of pepstatin and leupeptin. Membranes (0.2 mg of protein/ml) were incubated for 1 h at 23 °C in Ca^{2+} uptake buffer, i.e. the above wash buffer plus 0.5 μCi/ml $^{45}CaCl_2$, 0.56 mM EGTA, 0.4 mM $CaCl_2$, 10 mM potassium phosphate and 2 mM disodium ATP (free $Ca^{2+} = 1$ μM). Duplicate aliquots were added at various time points to 25 μl of a formalin/EDTA solution [17] to immediately stop all transport, and centrifuged for 2 min at 463000 ***g*** in a TL-100 centrifuge. Supernatants were aspirated and the pellets were extracted with 50 μl of 50 % (w/v) trichloroacetic acid and assayed for $^{45}Ca^{2+}$ by liquid scintillation counting. Ca^{2+} release induced by InsP_4 was studied using vesicles preloaded with $^{45}Ca^{2+}$. These reactions were also stopped by formalin/EDTA and the pellets analysed for $^{45}Ca^{2+}$ as above.

Separation of plasma membrane vesicles from internal membrane vesicles by loading with calcium oxalate

Fractions 1–3 from Percoll gradients were pooled and incubated for 1 h at 25 °C in Ca^{2+} uptake buffer (without $^{45}Ca^{2+}$) plus 5 mM potassium oxalate and subfractionated into 'heavy' and 'light' membrane fractions by centrifugation on a 40 % Percoll gradient for 50 min at 40000 ***g*** in a Sorvall centrifuge using an SS34 rotor. One-quarter of the total membranes were incubated in medium with $^{45}Ca^{2+}$, which thereby served as a marker to locate on the gradient those membrane subfractions that were capable of oxalate-stimulated Ca^{2+} uptake. Fractions of 1.0 ml each, from the top of the gradient down, were removed and designated as subfractions 1a–11a. Non-radioactive subfractions isolated in the same way were washed twice and resuspended in Ca^{2+} uptake buffer supplemented with 10 mM phosphate, 1 mM ATP and $^{45}Ca^{2+}$, and incubated for 60 min at 23 °C. The effect of InsP_4 on $^{45}Ca^{2+}$ accumulation by these vesicles was measured as described above.

Ligand binding assays

Platelet sonicates were centrifuged on 12 ml Percoll gradients, the upper 3.0 ml (fractions 1–3) of the gradients was pooled, and 2.0 ml fractions were collected from the remaining gradient. All membranes were washed and resuspended at 1.0 mg of protein/ml. The binding of radioactive InsP_4, InsP_6 and InsP_3 to membranes, at equilibrium, was evaluated over the pH range 5.8–8.3 at 4 °C. A pH 5.8 buffer (25 mM Mes, 1 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF) was optimal for [^{3}H]InsP_4 binding, whereas [^{3}H]InsP_3 binding was greatest in bicarbonate buffer at pH 8.3 [17] and [^{3}H]InsP_6 binding, which is unaffected by pH, was measured at pH 7.4. Non-specific binding was measured in the presence of 1μM of the appropriate non-radioactive inositol phosphate. Duplicate aliquots of 100 μl were centrifuged for 4 min in a Beckman TL-100 centrifuge at 463000 ***g***. Supernatants were aspirated and the pellets were extracted with 50 μl of 50 % trichloroacetic acid. [^{3}H]Inositol phosphate in the extracts was assayed by liquid scintillation counting.

[^{3}H]InsP_4 binding to solubilized receptor protein was assayed using aliquots (75 μl) of chromatographic fractions that were diluted with an equal volume of pH 5.8 Mes buffer without CHAPS and incubated with [^{3}H]InsP_4 for 15 min at 4 °C, followed by addition of 8 μl of rabbit IgG (50 mg/ml) as carrier. Non-specific binding was determined in the presence of 1 μM non-radioactive InsP_4. Duplicate aliquots of 75 μl were added to 100 μl of 30 % poly(ethylene glycol) 6000 in pH 5.8 Mes buffer to precipitate proteins.The samples were held at 4 °C for 10 min, followed by centrifugation at 463000 ***g*** in a TL-100 Beckman centrifuge for 10 min. The protein pellets were extracted with 50 % trichloroacetic acid and centrifuged to obtain supernatants that were assayed by liquid scintillation counting. Similarly, binding of [^{3}H]InsP_6 was measured at pH 7.4. All ligand binding data were analysed using the non-linear regression program BIOSOFT (Elsevier) and displayed as Scatchard plots.

Labelling of the platelet cell surface by sulpho-NHS-LC-biotin

All procedures were carried out at 25 °C. Three units of platelets (1 unit is 60 ml of platelet-rich plasma) were washed with 0.135 M NaCl, 5 mM KCl, 10 mM Hepes, pH 7.6, and then with 0.125 M NaCl, 5 mM KCl, 10 mM Pipes, 0.2 mM EGTA, pH 6.8. The platelet pellets were resuspended in PBS (138 mM NaCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) with 5 mM sulpho-NHS-LC-biotin and incubated for 45 min. After two washes with PBS to remove unreacted biotin, the platelets were resuspended in 2 ml of ice-cold buffer containing 100 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μM leupeptin and 10 μM pepstatin, pH 7.1. The platelets were sonicated and the membranes were washed with the above buffer and then incubated with Immunopure immobilized streptavidin for 1 h. The suspensions were centrifuged for 10 min at 12000 ***g*** to remove the biotin-labelled membranes adhered to streptavidin–agarose beads. Unlabelled cells, treated in the same manner, served as a control. Thus the net result was to specifically remove biotin-labelled (right-side-out) vesicles, leaving inside-out vesicles plus internally derived membranes in the suspension. The latter were loaded with calcium oxalate, as described above, followed by centrifugation on a 40 % Percoll gradient. Membrane subfractions (1b–11b) were washed and assayed for GpIb, $^{45}Ca^{2+}$ uptake in the presence of phosphate, and release of Ca^{2+} by InsP_4. The membrane vesicles that were not removed by streptavidin–agarose were subjected to SDS/PAGE (7.5 % gels), blotting with peroxidase-labelled streptavidin and detection by enhanced chemiluminescence.

Western blotting for GpIb

Membrane proteins separated by SDS/PAGE (7.5 % gels) were semi-dry blotted on to Immobilon nylon membranes and incubated for 45 min with a blocking solution of 5 % powdered milk and 1 % goat serum in PBS/Tween (i.e. PBS, pH 7.4, with 0.1 % Tween 20). The blots were incubated with a 1:10000 dilution of anti-GP1b monoclonal antibody in blocking buffer for 1 h at 23 °C, and then washed with PBS/Tween for 2×15 min and 3×5 min. The blots were incubated for 45 min with peroxidase-labelled goat anti-mouse IgG_1 diluted 1:15000 in PBS/Tween, followed by washing as described for the first antibody. The washed immunoblots were incubated with enhanced chemiluminescence reagent and exposed to BioMax-MR X-ray film (Kodak) for 1 min. The images were quantified by a Molecular Dynamics laser densitometer using Image Quant software.

Purification of InsP_4 and InsP_6 binding proteins

All procedures were carried out at 4 °C. Platelet membrane fractions 1–3 (80–100 mg of protein) were pooled from Percoll gradients and washed twice with buffer containing 1 M NaCl,

20 mM bicarbonate, 10 μM each of leupeptin and pepstatin, 0.5 mM PMSF, 0.5 mM DTT, pH 7.4, to remove non-integral membrane proteins. The membranes were washed twice more in the same buffer without added NaCl and then solubilized in 50 ml of the same buffer plus 5% CHAPS. The protease inhibitors and DTT stabilized the binding activity of detergent-solubilized protein. The solubilized membranes were stirred for 1 h and centrifuged for 1 h at 40000 ***g*** to remove particulate matter. The supernatant was passed through a 5 ml ConA–agarose column to remove glycoproteins. The ConA–agarose flow-through was adjusted to 0.025 M NaCl and pH 5.8 with Mes and applied to an HPLC anion-exchange column (Supelco) at a flow rate of 1 ml/min. Proteins were eluted with a gradient of 0.025–1.0 M NaCl in 1% CHAPS, 1 mM EDTA, 25 mM Mes at pH 5.8. Fractions (1.0 ml) were collected and assayed for binding of [^{3}H]InsP_4 and [^{3}H]InsP_6. The peak [^{3}H]InsP_4 and [^{3}H]InsP_6 binding fractions were pooled, concentrated and dialysed by a Centricon device (molecular mass cut-off of 30 kDa) and then applied to a heparin-TSK HPLC column (ToyoHaas) in the elution buffer described above (with 25 mM NaCl) at a flow rate of 0.2 ml/min. [^{3}H]InsP_6 binding activity was all found in the heparin column flow-through, which was concentrated using a 30 kDa cut-off Centricon device and chromatographed on an HPLC gel filtration column (Zorbax G450; DuPont). Protein adsorbed on to the heparin-TSK column was eluted with a 0.025–1.0 M NaCl gradient at a flow rate of 0.5 ml/min and collected in 0.5 ml fractions. The peak [^{3}H]InsP_4 binding fractions were pooled, concentrated and dialysed overnight in elution buffer without NaCl, and assayed for ligand binding and analysis by SDS/PAGE.

RESULTS

The various inositol phosphates bound to sonicated platelet membranes with different dependencies on pH. InsP_3 binding was greatest at alkaline pH [17], the binding of InsP_4 was greatest at moderately acid pH, whereas InsP_6 binding was virtually unaffected from pH 5.8 to 8.3 (results not shown). Membranes isolated by centrifugation on Percoll gradients were divided into

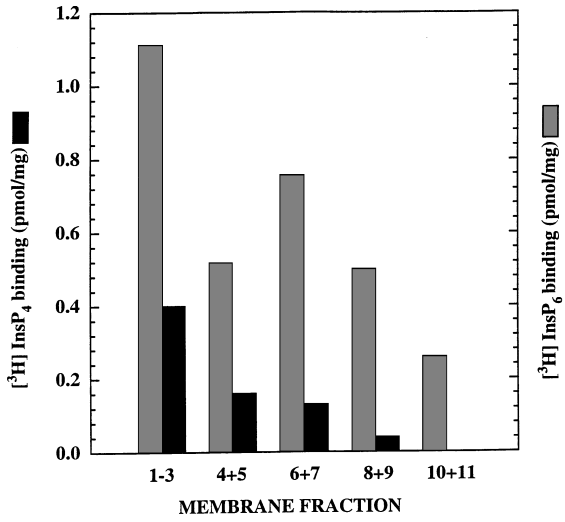

Figure 1 Binding of inositol phosphates to platelet membrane fractions

Membrane fractions (100 μg of protein) from a Percoll gradient were incubated with 10 nM [^{3}H]InsP_4 or [^{3}H]InsP_6 and specific binding was measured as described in the Materials and methods section. Data are from a single experiment repeated three times with similar results.

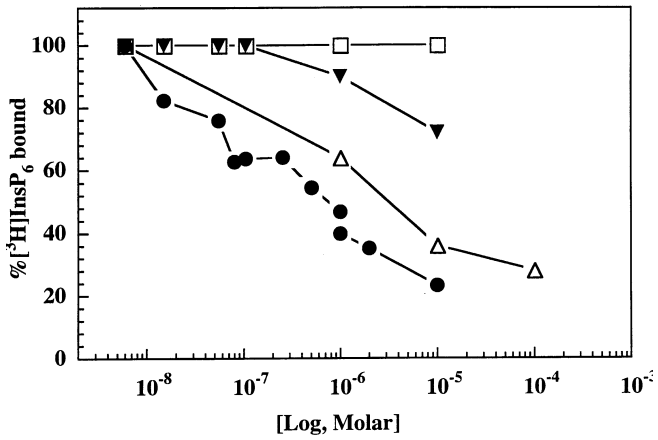

Figure 2 Displacement of bound [^{3}H]InsP_6 from platelet membranes

Membrane fractions 1–3 (50 μg of protein) were incubated with 5 nM [^{3}H]InsP_6 and various concentrations of non-radioactive displacer ligands, and analysed for bound radioactive ligand (% of control) as described in the Materials and methods section: ●, non-radioactive InsP_6; △, heparin; ▼, InsP_4; □, InsP_3. IC_{50} values for displacement: InsP_6, 100 nM; heparin, 5 μM; InsP_4 and InsP_3, > 10 μM.

12 × 1 ml fractions and assayed for [^{3}H]InsP_4 and [^{3}H]InsP_6 binding. A total of 52% of the total [^{3}H]InsP_4 binding was found in combined fractions 1–3, with a lesser amount in fractions 4 + 5 (21%) and 5 + 6 (17%) (Figure 1). Peak [^{3}H]InsP_6 binding (37%) was in fractions 1–3 (Figure 1). In contrast, most (50%) of InsP_3 binding was concentrated in the heavier fractions 4 + 5 [17,22], suggesting that the InsP_4 and InsP_6 receptors might reside in a different membrane compartment than the InsP_3 receptor. The binding of [^{3}H]InsP_4 was highly specific for the Ins(1,3,4,5)P_4 isomer; e.g. IC_{50} values for displacement of 10 nM [^{3}H]InsP_4 from fractions 1–3 were 40 nM for non-radioactive InsP_4, 8 μM for Ins(1,4,5,6)P_4, 50 μM for Ins(1,3,4,6)P_4, 30 μM for InsP_3 and 6 μM for InsP_6. The IC_{50} for heparin was 1 μM. Direct binding of [^{3}H]InsP_4 to membrane fractions 1–3, measured over a concentration range of 5–50 nM radioactive ligand, yielded an average K_D of 18 nM (results not shown). Similarly, the displacement of 5 nM [^{3}H]InsP_6 from membranes was highly specific; i.e. IC_{50}s were 100 nM for InsP_6, > 10 μM for InsP_4 and InsP_3, and 5 μM for heparin (Figure 2).

To test for functions of InsP_4 and InsP_6, we isolated membrane vesicle fractions 1–3 and 4 + 5 from 17 individual batches of human platelets and loaded them with $^{45}Ca^{2+}$ in phosphate-containing buffer plus ATP. InsP_4 released 15–25% of the $^{45}Ca^{2+}$ from fractions 1–3, but had little or no effect on fractions 4 + 5 (maximum 3–5% release by 10 μM InsP_4). In contrast, 5 μM InsP_3 released 25–40% of the accumulated $^{45}Ca^{2+}$ from fractions 4 + 5 (results not shown). $^{45}Ca^{2+}$ was released from fractions 1–3 by both InsP_3 and InsP_4, but their effects were additive, suggesting that each acted on different compartments. For example, the maximum release induced by a combination of InsP_3 + InsP_4 was 40%, the maximum by InsP_3 alone was 15% and the maximum by InsP_4 alone was 25%. InsP_6 (10–20 μM) did not release Ca^{2+} from any vesicles.

Fractions 1–3 from the Percoll gradients also showed some [^{3}H]InsP_3 binding and oxalate-stimulated Ca^{2+} uptake (results not shown). The latter is a specific characteristic of the Ca^{2+} transport system of the internal platelet membranes, but not that of the plasma membrane [23]. These findings, and the release of Ca^{2+} by both InsP_4 and InsP_3, indicated that fractions 1–3

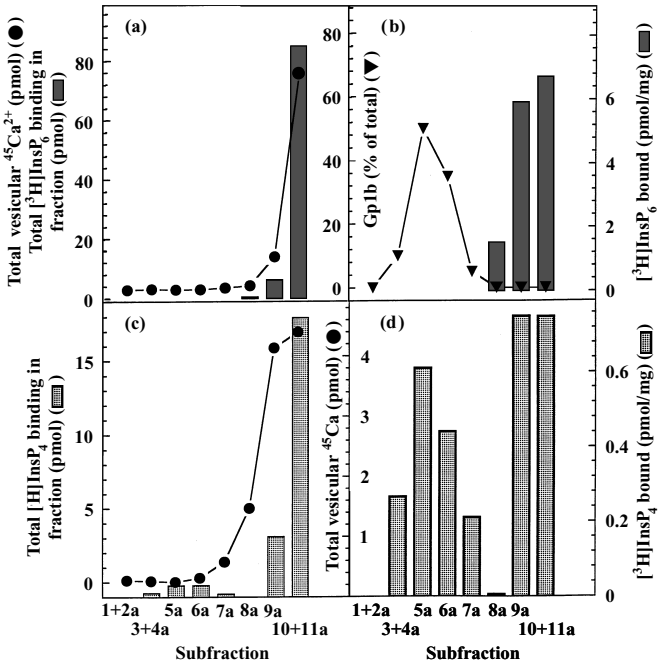

Figure 3 Binding of [^{3}H]InsP_4 and [^{3}H]InsP_6 to platelet membrane subfractions obtained after loading with calcium oxalate

Membrane fractions 1–3 were loaded with $^{45}Ca^{2+}$ in the presence of 5 mM oxalate and 1 mM ATP and recentrifuged on a Percoll gradient (see the Materials and methods section). Subfractions 1a–11a (30 μg of protein) were assayed for binding of [^{3}H]InsP_4 and [^{3}H]InsP_6, and for content of $^{45}Ca^{2+}$ and GpIb. (**a**) Bar graph: binding of [^{3}H]InsP_6; ●, $^{45}Ca^{2+}$ uptake. (**b**) Bar graph: specific activity of [^{3}H]InsP_6 binding; ▼, GP1b content of 10 μg membrane protein samples (average of three experiments). (**c**) Bar graph: binding of [^{3}H]InsP_4; ●, $^{45}Ca^{2+}$ uptake. (**d**) Specific activity of InsP_4 binding.

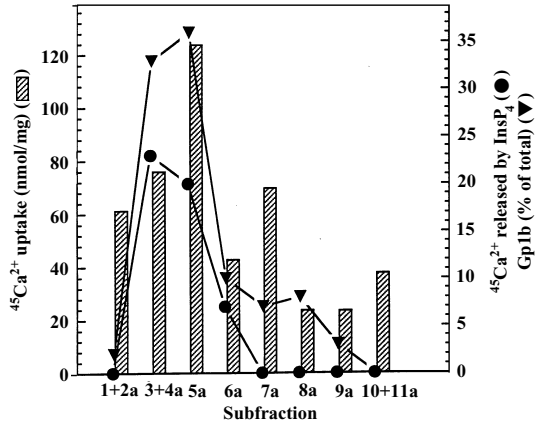

Figure 4 Phosphate-stimulated Ca^{2+} uptake by GpIb-containing membrane vesicle fractions 1a–6a and release of accumulated Ca^{2+} by InsP_4

Membrane subfractions (1a–11a) loaded with calcium oxalate (but no $^{45}Ca^{2+}$) were washed and resuspended in medium with 10 mM phosphate, 1 mM ATP and $^{45}Ca^{2+}$. ▼, GP1b content of 10 μg membrane protein samples (average of three experiments). The bar graph represents $^{45}Ca^{2+}$ content of membrane vesicles, and ● is the $^{45}Ca^{2+}$ released from membrane vesicles by 10 μM InsP_4 (average of three experiments). No Ca^{2+} was released from fractions 1a–6a by 10 μM InsP_3.

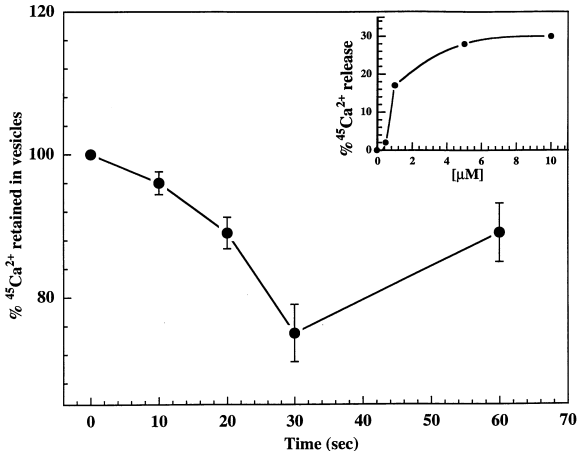

Figure 5 Ca^{2+} release from pooled subfractions 3a–5a by InsP_4

Membrane subfractions 3a–5a (15 μg of protein) were incubated in assay medium with 10 mM phosphate, 1 mM ATP and $^{45}Ca^{2+}$ for 1 h as described in the Materials and methods section. The vesicles accumulated 77 nmol of Ca^{2+}/mg of protein, and 60% of the $^{45}Ca^{2+}$ was released by ionophore A23187. InsP_4 was added and release of $^{45}Ca^{2+}$ was determined at the time points indicated. Data represent the means of four experiments. Inset: dose–response curve for Ca^{2+} release by InsP_4. Data are from a single experiment repeated four times with similar results.

contained elements of both the surface and internal membranes. Plasma membranes were further separated from vesicles derived from the internal membrane system by taking advantage of the tendency of calcium oxalate to accumulate and precipitate within internal membrane vesicles, thereby increasing their specific gravity. This was confirmed by loading fraction 1–3 vesicles with $^{45}Ca^{2+}$ in the presence of 5 mM oxalate and 1 mM ATP and recentrifuging them on a Percoll gradient. $^{45}Ca^{2+}$ and InsP_6 binding activity were found virtually exclusively in fractions 8a–11a, corresponding to a much denser part of the gradient (Figures 3a and 3b), whereas Gp1b was entirely confined to the lighter membranes in subfractions 3a–7a (Figure 3b). The dense membrane fractions also contained 90% of the total InsP_4 binding activity (Figure 3c), but the gradient had two areas with high specific activity for InsP_4 binding, i.e. the GpIb-containing fractions 3a–7a and the $^{45}Ca^{2+}$-labelled heavy fractions that contained all of the InsP_6 binding activity (Figure 3d). In the latter fractions we determined that InsP_4 and InsP_6 were binding to different entities, because 200 nM InsP_6 did not displace bound [^{3}H]InsP_4 (InsP_4 present at 10 nM; $K_D = 12$ nM) and 10 μM InsP_4 did not displace bound [^{3}H]InsP_6 (the latter present at 10 nM; $K_D = 18$ nM).

The inositol phosphates were then tested for their effects on Ca^{2+} release from vesicles of the 'light' membrane fractions obtained after calcium oxalate loading as described above. Membrane fractions 1–3 were loaded with calcium oxalate (but no $^{45}Ca^{2+}$) and centrifuged on Percoll to obtain fractions 1a–11a. The membrane fractions were individually washed and resuspended in medium with 10 mM phosphate, 1 mM ATP and $^{45}Ca^{2+}$. Phosphate-stimulated Ca^{2+} uptake was greatest in the light subfractions 1a–7a that contained virtually all of the GpIb. InsP_4 (10 μM) released Ca^{2+} from fractions 3a–6a (Figure 4). The latter were slightly denser on the gradient than the original fractions 1–3 from which they were derived, possibly due to some small accumulation of calcium oxalate. InsP_3 (10 μM) did not release $^{45}Ca^{2+}$ from fractions 3a–6a, whereas InsP_4 consistently released 15–30% of the vesicle $^{45}Ca^{2+}$ within 30 s, and ionophore A23187 released 55% of the calcium (Figures 4 and 5). $^{45}Ca^{2+}$ release was maximal at 6 μM InsP_4 ($EC_{50} = 0.6$ μM) (Figure 5, inset). Thapsigargin, an inhibitor of the internal membrane Ca^{2+}-

Table 1 Purification of an InsP_4 binding protein in human platelets

Platelet membranes from fractions 1–3 were isolated and solubilized as described in the Materials and methods section. Samples from each purification step were incubated with 10 nM [^{3}H]InsP_4 (specific radioactivity 17 Ci/mmol) and assayed for the amount of specific ligand binding to protein (pmol/mg). Data shown are from a single purification repeated three times with similar results.

Purification step	Protein (mg)	Specific binding (pmol/mg)	Total binding (pmol)	Yield (%)	Purification (fold)
Solubilized membranes	69	0.46	32.4	100	–
ConA–agarose flow-through	28	0.48	13.7	42	1
HPLC ion-exchange eluate	0.340	12.8	4.3	13	24
HPLC heparin-TSK eluate	0.0075	532	3.9	12	1136

ATPase [24], blocked ATP-dependent Ca^{2+} uptake by the DTS membranes (fractions 4+5) and released 62% of the Ca^{2+} accumulated by this InsP_3-sensitive fraction. Thapsigargin also released 55% of accumulated $^{45}Ca^{2+}$ from the original fractions 1–3, but failed to release any $^{45}Ca^{2+}$ from the InsP_4-sensitive fractions 1a–6a ($n = 3$ experiments).

The origin and orientation of vesicles exhibiting phosphate-stimulated Ca^{2+} uptake and Ca^{2+} release by InsP_4 was determined by labelling the surface proteins of intact platelets with sulpho-NHS-LC-biotin. This reagent has the advantages of reactivity with free protein amino groups at neutral pH and an inability to penetrate the cell membrane, resulting in specific labelling of plasma membrane proteins with low background and high sensitivity compared with other protein labelling reagents [25]. The procedure, described in detail in the Materials and methods section, involved biotinylation of intact platelets, washing to remove unreacted reagent, membrane sonication, and the selective precipitation and removal by streptavidin–agarose beads of membrane vesicles labelled with biotin on their outer surfaces. The remaining membranes were loaded with calcium oxalate and centrifuged on a Percoll gradient to obtain 1.0 ml subfractions designated 1b–11b and assayed for GpIb. Streptavidin–agarose beads (streptavidin concentration < biotin concentration) removed a maximum of 40% of the GpIb-containing membranes from biotinylated platelets, but did not remove any GpIb-containing membranes originating from platelets that had not been biotinylated. The remaining GpIb (60%) that was not removed by streptavidin–agarose was contained in the 'light' fractions 1b–5b of the gradients. GpIb was not present in the denser calcium oxalate-loaded internal membrane subfractions 6b–11b. Although not removed by streptavidin–agarose, many proteins in membrane fractions 1b–5b were found to be labelled with biotin by Western blot analysis after SDS/PAGE (see the Materials and methods section). From these data we conclude that: (1) fractions 1b–5b were largely of plasma membrane origin, and (2) since they were not precipitated by streptavidin–agarose beads, the biotin in them was oriented facing into the lumens of the vesicles, i.e. the membrane vesicles were inside-out. Subfractions 1b–5b took up $^{45}Ca^{2+}$ from the medium when incubated with 10 mM phosphate plus ATP, and they released 15–35% of accumulated $^{45}Ca^{2+}$ when exposed to InsP_4 (results not shown, similar to those in Figure 5).

Based on the evidence of specific and unique ligand binding sites for each of the inositol phosphates InsP_4, InsP_3 and InsP_6, we undertook the purification of the individual binding proteins. Using a radioactive ligand binding assay for detergent-solubilized receptors, three proteins were purified from platelet membrane fractions 1–3 by a sequence of column chromatography steps. Isolation of the human platelet InsP_3 receptor is described elsewhere [22]. The scheme for purification of the InsP_4 (Table 1)

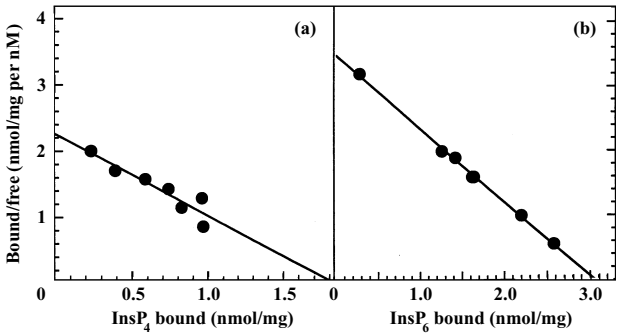

Figure 6 [^{3}H]InsP_4 and [^{3}H]InsP_6 binding to purified proteins

(**a**) Scatchard plot of [^{3}H]InsP_4 (5–50 nM) binding to 70 ng of solubilized 104 kDa protein. $B_{max} = 1.8$ nmol/mg of protein; $K_D = 12$ nM. (**b**) Scatchard plot of [^{3}H]InsP_6 (5–75 nM) binding to 250 ng of solubilized 116 kDa protein fraction. $B_{max} = 3$ nmol/mg of protein; $K_D = 5.9$ nM.

and InsP_6 binding proteins (see the Materials and methods section) employed a ConA–agarose column as the first step to remove membrane glycoproteins, followed by application of the flow-through fraction, containing all of the ligand binding activity, to an HPLC anion-exchange column. Both InsP_4 and InsP_6 binding activities were adsorbed on to this column and were eluted by a salt gradient with a peak at 0.12 M NaCl. The inositol phosphate binding fractions were concentrated and chromatographed on a heparin-TSK HPLC column. At pH 5.8 the[^{3}H]InsP_4 binding activity was adsorbed on to the column and was eluted by a salt gradient with a peak at 0.1 M NaCl. The peak binding fractions were combined and concentrated. This fraction bound InsP_4 with a K_D of 12 nM and a B_{max} of 1.8 nmol/mg of protein (Figure 6), and contained a single polypeptide of molecular mass 104 kDa by SDS/PAGE analysis (Figure 7).

All [^{3}H]InsP_6 binding activity was found in the flow-through fraction of the heparin-TSK column at pH 5.8, and was subsequently concentrated and further purified by gel filtration. [^{3}H]InsP_6 binding peaked in fractions which chromatographed with retention times intermediate between those of standards phosphorylase b (97.5 kDa) and myosin (206 kDa) that were chromatographed independently. The concentrated gel filtration fraction bound [^{3}H]InsP_6 with a K_D of 5.9 nM and a B_{max} of 3 nmol/mg of protein at pH 7.4 (Figure 6). There was no displacement of bound [^{3}H]InsP_6 (present at 10 nM concentration) by 10 μM InsP_3, and only 20% displacement by 10 μM InsP_4. Heparin inhibited the binding of 10 nM [^{3}H]InsP_6 with an

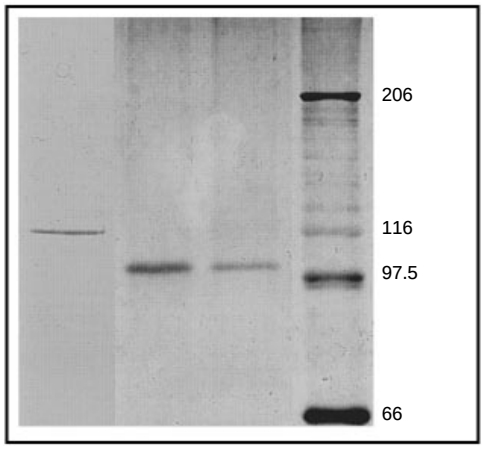

Figure 7 SDS/PAGE of purified InsP_4 and InsP_6 binding proteins from human platelets

Left lane, SDS/PAGE (7.5% gels) of InsP_6 binding fraction (1 μg of protein) isolated by HPLC gel filtration (colloidal Coomassie Blue stain). Middle lanes, SDS/PAGE (7.5% gels) of InsP_4 binding fractions eluted from the heparin-TSK HPLC column (silver stain). Right lane, molecular mass markers (kDa): myosin, 206; β-galactosidase, 116; phosphorylase b, 97.5; BSA, 66 (positions indicated).

IC_{50} of 2 μM. The concentrated InsP_6 binding fraction contained a single polypeptide band at 116 kDa by SDS/PAGE analysis (Figure 7). Bands were cut from gels and analysed for total amino acid content and for sequences of protease-generated peptides. The amino acid composition matched that of 116 kDa human vinculin [26], and two internal peptides (Val-Glu-Gly-Ile-Gln-Ala-Ser-Val-Lys and Ala-Arg-Met-Gln-Glu-Ala-Met-Thr-Gln-Glu-Val) were identical to vinculin amino acids 658–666 and 585–595 respectively.

DISCUSSION

As part of an endeavour for the concurrent purification of the various inositol phosphate receptors of human platelets we isolated the solubilized InsP_3 receptor [22], and in the present paper we have described: (1) unique and specific ligand binding sites for InsP_4 and InsP_6, (2) the purification of a specific InsP_4 receptor protein, and (3) the preliminary identification and partial purification of a specific InsP_6 binding protein. Furthermore, we demonstrate that InsP_4 promotes the efflux of Ca^{2+} across inside-out plasma membrane vesicles, further substantiating its putative role as a second messenger that promotes Ca^{2+} influx into cells.

Our findings are in agreement with Cullen et al. [6–8], who established the localization of InsP_4 binding in the plasma membrane fraction of human platelets. Those membranes were isolated from neuraminidase-treated platelets by a sequence of sonication, density gradient centrifugation and high-voltage free flow electrophoresis. The surface membrane, depleted of negatively charged surface sialic acid, undergoes a cathodal shift during electrophoresis due the change in the membrane zeta potential [27]. During the sonication process membrane vesicles of either right-side-out or inside-out orientations can be formed, and the electrophoretic method isolates vesicles of both orientations.

In our studies, the establishment of InsP_4 binding and effects on Ca^{2+} permeability also required methods to separate elements of the internal membranes from the plasma-membrane-containing fractions. In these procedures we employed GpIb as a specific marker for the plasma membrane and took advantage of the different properties of the Ca^{2+} pumps of the plasma membrane and the internal membranes [23]. The plasma membrane fraction, unlike the internal membrane fraction, was reported not to transport calcium in the presence of oxalate [23], a property that is shared by plasma membrane Ca^{2+} pumps from other cells [28,29]. Therefore we obtained membrane fractions 1–3 that were enriched in the plasma membrane marker GpIb, but released Ca^{2+} in response to both InsP_4 and InsP_3. Our strategy was to load the membrane vesicles with Ca^{2+} plus oxalate, and then recentrifuge them on a Percoll gradient to separate the denser membranes containing calcium oxalate deposits (fractions 6a–11a) from the 'light' plasma membrane vesicles (1a–5a). The latter subfractions (1a–5a) contained all of the membrane GpIb, had a high specific activity for [^{3}H]InsP_4 binding and were capable of phosphate (but not oxalate)-stimulated Ca^{2+} transport. These more purified plasma membrane vesicles released a substantial amount of their Ca^{2+} when exposed to InsP_4 (EC_{50} = 0.6 μM), but showed little or no response to InsP_3 or InsP_6. Furthermore, thapsigargin was unable to affect Ca^{2+} uptake or to cause release of Ca^{2+} from the InsP_4-sensitive membrane vesicles. In contrast, thapsigargin blocked ATP-dependent Ca^{2+} uptake by the DTS membranes (fractions 4 + 5) and released 62% of the Ca^{2+} accumulated by this InsP_3-sensitive fraction. Thus the characteristics of the InsP_4-sensitive membrane fractions were distinctly different from those of the internal membranes.

The denser, calcium oxalate-loaded, membrane fractions (7a–11a), derived from fractions 1–3, contained the bulk of the total InsP_4 binding activity (90%), and all of the InsP_6 binding, but were devoid of GpIb. This finding suggests that InsP_4 receptors are present in at least two different membrane compartments, but an alternative explanation (discussed below) is that these compartments may be two subdomains of the plasma membrane. The InsP_6 receptor, on the other hand, appears to be entirely associated with one compartment that may be part of, or connected to, a major InsP_4-receptor-containing compartment.

We consider three hypotheses that could account for these results. The first hypothesis holds that one compartment for InsP_4 receptors is in the plasma membrane, and the other compartment, containing most of the InsP_4 receptors and all of the InsP_6 receptors, is comprised of vesicles derived from internal DTS membranes that are capable of oxalate- and phosphate-stimulated Ca^{2+} transport. The second hypothesis places all of the InsP_4 and InsP_6 receptors in the plasma membrane, with the bulk of them in the surface connected canalicular system (SCCS), and requires that the SCCS is devoid of GpIb and is capable of oxalate-stimulated Ca^{2+} transport. The SCCS is the site of the greatest cytochemically detectable Ca^{2+}-ATPase activity in the plasma membrane [30], and it is possible that its properties differ from those of the plasma membrane Ca^{2+}-ATPase activity described by Enouf et al. [23]; however, GpIb has been found in the SCCS [31], indicating that the second hypothesis is untenable. Lastly, the InsP_6 receptor and most of the InsP_4 receptors may be located in subdomains of the plasma membrane that are devoid of GpIb, adherent to regions of the DTS and tightly associated with each other even after sonication of whole platelets. Such plasma membrane–DTS complexes would become much denser when the DTS component is loaded with calcium oxalate, leading to their separation from vesicles that are derived purely from the plasma membrane. The SCCS, representing invaginations of the plasma membrane, may comprise this plasma membrane

domain, since such an intimate association of DTS and SCCS membranes has been demonstrated by electron microscopy in intact platelets [32]. Further work is necessary to resolve these different possibilities.

The plasma membrane location of $Ins P_4$ receptors and the Ca^{2+}-releasing effect of $Ins P_4$ were further established by biotinylation of platelet surface proteins with a reagent that does not penetrate the cells. After sonication of the platelets the membrane vesicles with biotin exposed on their surfaces (right-side-out vesicles) were removed with streptavidin–agarose beads. The remaining membranes were loaded with calcium oxalate and centrifuged on a density gradient to separate 'heavy' membrane vesicles from the 'light' membrane fractions 1b–5b. The latter were largely plasma membrane vesicles, based on the fact that they contained all the GpIb, and they were in an inside-out orientation since, although not removed by streptavidin–agarose, they contained biotinylated proteins as shown by SDS/PAGE analysis. These fractions 1b–5b exhibited phosphate (but not oxalate)-stimulated active uptake of Ca^{2+}, a plasma membrane property. These plasma membrane vesicles could release as much as one-third of their accumulated Ca^{2+} in response to $Ins P_4$. Since the lumen of the inside-out plasma membrane vesicles represents the 'extracellular space', the action of $Ins P_4$ is consistent with other findings that $Ins P_4$ promotes Ca^{2+} influx into cells [9,12].

Proteins capable of selective high-affinity $Ins P_4$ binding have been isolated from platelets and brain [7,14,33]. The purified 104 kDa $Ins P_4$ binding protein of pig platelets [7] as well as that from human platelets is highly specific for Ins(1,3,4,5)P_4 compared with other isomers, $Ins P_6$ or $Ins P_3$. [^{3}H]$Ins P_4$ bound to the purified human platelet protein with a K_D of 12 nM and B_{max} of 1.8 nmol/mg of protein, compared with values of K_D = 9.6 nM and B_{max} = 2.5 and 6.0 obtained by Cullen et al. [8]. The predicted B_{max} is 9.6 nmol/mg of protein assuming one ligand binding site per molecule. In all respects, including the pH dependence for ligand binding [34], the human platelet protein appears to be identical to the 104 kDa protein from pig platelet plasma membranes isolated by Cullen et al. [7]. These platelet proteins differ from all other putative $Ins P_4$ binding proteins, such as the two fractions from brain with a high specific affinity for $Ins P_4$, which contained polypeptides of 182 plus 123 kDa and 174 plus 84 kDa respectively [14].

The platelet 104 kDa $Ins P_4$ binding protein was recently identified as a member of the GAP family and designated GAP1^{IP4BP} [8]. Important regions of this protein include a Lys/Arg-rich sequence in the Ca^{2+}-independent phospholipid binding C2B domain, also found in GAP1 and GAP1^m, which comprises the selective ligand binding site, a C2A Ca^{2+}-dependent phospholipid binding domain, and a pleckstrin homology domain. GAP1^{IP4BP} stimulated the GTPase activity of the low-molecular-mass GTP-binding proteins H-Ras, R-Ras and Rap1A, but not K-Ras, Q61L, Rap3A or Rac. GTPase activation exerted on Ras (but not Rap1A) by GAP1^{IP4BP} was inhibited by phospholipids commonly found in the inner leaflet of the plasma membrane, and this effect was reversed by $Ins P_4$. $Ins P_4$ did not affect Rap or Rap–GAP interactions. Platelets contain members of the Ras-related family such as Rap1A, Rap1B, Rap2B, Rac1 and Ral [35]. Rap1B is present in the plasma membrane and SCCS [36] and is a prominent substrate for protein kinase A. Rap1B has been implicated in cAMP-stimulated Ca^{2+} transport by plasma membrane vesicles [37]. Since $Ins P_4$ increases the Ca^{2+} permeability of plasma membrane vesicles, it is possible that $Ins P_4$–GAP1^{IP4BP}, in conjunction with a small G-protein, controls the opening of a plasma membrane channel that is permeable to Ca^{2+}. Indeed, this is also suggested by the finding that in v-Ki-*ras*-transformed NIH/3T3 cells, but not in control cells, the intracellular injection of 10 μM $Ins P_4$ produced sustained Ca^{2+} oscillations requiring the presence of extracellular Ca^{2+} [38].

A number of proteins that bind inositol polyphosphates such as $Ins P_4$ have an even higher affinity for $Ins P_6$. Many of these proteins are involved in membrane cycling of secretory vesicles or clathrin-coated vesicles. Platelets contain clathrin which is associated with the plasma membrane, the SCCS and the surface of α-granules [39], so it was important to also characterize the $Ins P_6$ binding proteins in platelets, and to discriminate between them and the specific 104 kDa $Ins P_4$ binding protein. $Ins P_6$ binding proteins include 65 kDa synaptotagmin II [15,40], a synaptic vesicle membrane protein involved in exocytosis [41], and adaptins, which are components of the adaptor complexes that link clathrin to receptors in coated vesicles, i.e. the α-subunit (AP-2, α-c large chain, alpha adaptin) of the clathrin assembly heterotetramer protein AP-2 [42] and clathrin assembly protein 3 (AP-3, AP180). AP-2 cDNAs code for proteins of 108 and 104 kDa [43], and were isolated from rat brain membranes as an $Ins P_6$ receptor complex of 115, 105 and 50 kDa polypeptides with K_D values for $Ins P_6$ of 12 nM and 120 nM, and the same sequence of affinities as AP-3, i.e. $Ins P_6 > Ins P_5 > Ins P_4 > Ins P_3$ [44]. AP-3 is a 91.4 kDa protein from brain that runs anomalously at 155–185 kDa by SDS/PAGE and has a K_D for $Ins P_6$ of 1.2 μM [45,46]. Some effects of inositol phosphates on the functions of these proteins have been described [45,47–49].

We identified a class of $Ins P_6$ binding sites in human platelet membranes with the relative affinities: $Ins P_6 > Ins P_5 > Ins P_4 > Ins P_3$. By appropriate chromatographic methods the $Ins P_6$ binding activity was totally separated from the 104 kDa $Ins P_4$ binding protein. The final purified $Ins P_6$ binding activity had a nominal molecular mass by gel filtration chromatography that was greater than 90 kDa, but less than 200 kDa, and contained one band of 116 kDa by SDS/PAGE analysis.The protein bound [^{3}H]$Ins P_6$ with a K_D of 5.9 nM and a B_{max} of 3 nmol/mg of protein. The expected B_{max} for a 116 kDa protein with one ligand binding site is 8.6 nmol/mg of protein, indicating that the $Ins P_6$ binding activity was highly purified. The total amino acid composition of the protein band corresponded to the 1066 amino acids of 116732 Da human vinculin, and two peptides generated from the protein by proteolysis were identical to sequences in human vinculin [26].

Vinculin is a component of the platelet plasma membrane cytoskeleton [50] that may associate with the membrane through myristoylation or palmitoylation [51,52]. Although the C-terminus of vinculin is extremely basic [53], which might confer ligand binding properties comparable with those of the Arg/Lys-rich inositol polyphosphophosphate binding regions in GAP1^{IP4BP} and synaptotagmin II, its ability to bind $Ins P_6$ has not been reported previously. However, we cannot rule out the possibility that the actual $Ins P_6$ binding protein is another component of similar molecular mass that co-purified with vinculin, such as the heavy chain of AP-2. Further work is required to fully establish the identity of the high-affinity $Ins P_6$ binding protein in platelets.

In conclusion, human platelet plasma membranes contain a specific $Ins P_4$ binding protein of apparent molecular mass 104 kDa and a specific $Ins P_6$ binding protein with an apparent molecular mass of 116 kDa. Inside-out plasma membrane vesicles containing the $Ins P_4$ receptor accumulated Ca^{2+} in the presence of phosphate anion and ATP, and they released a significant portion of that Ca^{2+} when exposed to submicromolar concentrations of $Ins P_4$, but not $Ins P_3$ or $Ins P_6$. This effect is consistent with other findings indicating that $Ins P_4$ can act as a second messenger to increase the Ca^{2+} permeability of the plasma membrane and permit entry of Ca^{2+} from the extracellular space. This action of $Ins P_4$ may be a significant mechanism in platelets

to account for the influx of Ca^{2+} that occurs subsequent to agonist-induced production of InsP_3 and its release of internal Ca^{2+} stores [54].

This work was supported by NIH grants HL 18937 and HL 42454-05 and by grants from the American Heart Association and the University of Connecticut Research Foundation.

REFERENCES

1 Theibert, A., Supattapone, S., Worley, P., Baraban, J., Meek, J. and Snyder, S. (1987) Biochem. Biophys. Res. Commun. **3**, 1283–1289
2 Reiser, G., Schafer, R., Donie, F., Hulser, E., Nehls-Sahabandu, M. and Mayr, G. (1991) Biochem. J. **280**, 533–539
3 Bradford, P. and Irvine, R. (1987) Biochem. Biophys. Res. Commun. **149**, 680–685
4 Enyedi, P. and Williams, G. (1988) J. Biol. Chem. **263**, 7940–7942
5 Koppler, P., Mersel, M. and Malviya, A. (1994) Biochemistry **33**, 14707–14713
6 Cullen, P., Patel, Y., Kakkar, V., Irvine, R. and Authi, K. (1994) Biochem. J. **298**, 739–742
7 Cullen, P., Dawson, A. and Irvine, R. (1995) Biochem. J. **305**, 139–143
8 Cullen, P., Hsuan, J., Truong, O., Letcher, A., Jackson, T., Dawson, A. and Irvine, R. (1995) Nature (London) **376**, 527–530
9 Irvine, R. and Moor, R. (1986) Biochem. J. **240,** 917–920
10 Joseph, S., Hansen, C. and Williamson, J. (1989) Mol. Pharmacol. **36**, 391–397
11 Hill, T., Dean, M. and Boynton, L. (1988) Science **242**, 1176–1178
12 Luckhoff, A. and Clapham, D. (1992) Nature (London) **355**, 356–358
13 Chadwick, C., Timerman, A., Saito, A., Mayrleitner, M., Schindler, H. and Fleischer, S. (1992) J. Biol. Chem. **267**, 3473–3481
14 Theibert, A., Estevez, V., Mourey, R., Marecek, J., Barrow, R., Prestwich, G. and Snyder, S. (1992) J. Biol. Chem. **267**, 9071–9079
15 Niinobe, M., Yamaguchi, Y., Fukuda, M. and Mikoshiba, K. (1994) Biochem. Biophys. Res. Commun. **205**, 1036–1042
16 O'Rourke, F., Halenda, S., Zavoica, G. and Feinstein, M. (1985) J. Biol. Chem. **260**, 956–962
17 O'Rourke, F. and Feinstein, M. (1990) Biochem. J. **267**, 297–302
18 Authi, K. and Crawford, N. (1985) Biochem. J. **230**, 247–253
19 White, G., II, Barton, D., White, T. and Fischer, T. (1989) Thromb. Res. **56**, 575–581
20 Authi, K. (1992) FEBS Lett. **298,** 173–176
21 Hughes, A. and Putney, J. (1990) J. Environ. Health Perspect. **84**, 141–147
22 O'Rourke, F., Matthews, E. and Feinstein, M. (1995) Biochem. J. **312**, 499–503
23 Enouf, J., Bredoux, R., Bourdeau, N. and Levy-Toledano, S. (1987) J. Biol. Chem. **262**, 9293–9297
24 Papp, B., Enyedi, A., Kovacs, T., Sarkadi, B., Wuytack, F., Thastrup, O., Gardos, G., Bredoux, R., Levy-Toledano, S. and Enouf, J. (1991) J. Biol. Chem. **266**, 14593–14596
25 Lantz, L. and Holms, K. (1995) Biotechniques. **18**, 56–58

Received 25 October 1995/20 December 1995; accepted 3 January 1996

26 Weller, P., Ogryzko, E., Corben, E., Zhidkova, N., Patel, B., Price G., Spurr, N., Koteliansky, V. and Critchley, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5667–5671
27 Crawford, N. (1985) in Cell Electrophoresis (Schutt, W. and Klinkmann, H., eds.), pp. 225–246, Walter de Gruyter & Co., Berlin
28 Grover, A. (1985) Cell Calcium. **6**, 227–236
29 Mickelson, J., Beaudry, T. and Louis, C. (1985) Arch. Biochem. Biophys. **242**, 127–136
30 Cutler, L., Rodan, G. and Feinstein, M. (1978) Biochim. Biophys. Acta **542**, 357–371
31 Escolar, G., Clemetson, K. and White, J. (1994) J. Lab. Clin. Med. **123**, 536–546
32 White, J. (1971) Am. J. Pathol. **66**, 295–311
33 Donie, F. and Reiser, G. (1991) Biochem. J. **275**, 453–457
34 Cullen, P. (1995) FEBS Lett. **358**, 240–242
35 Manning, S. and Brass, L. (1991) Thromb. Haemostasis **66**, 393–399
36 Berger, G., Quarck, R., Levy-Toledano, S., deGunzburg, J. and Cramer, E. (1994) Br. J. Haematol. **88**, 372–382
37 Corvazier, M., Enouf, J., Papp, B., de Gunzburg, J., Tavitian, A. and Levy-Toledano, S. (1992) Biochem. J. **281**, 325–331
38 Hashii, M., Nozawa, Y. and Higashida, H. (1993) J. Biol. Chem. **268**, 19403–19410
39 Klinger, M. and Kluger, H. (1995) Cell Tissue Res. **279**, 453–457
40 Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. and Mikoshiba, K. (1994) J. Biol. Chem. **269**, 29206–29211
41 Perin, M., Fried, V., Mignery, G., Jahn, R. and Sudhof, T. (1990) Nature (London) **345**, 260–263
42 Voglmaier, S., Keen, J., Murphy, J., Ferris, C., Prestwich, G., Snyder, S. and Theibert, A. (1992) Biochem. Biophys. Res. Commun. **187**, 158–163
43 Robinson, M. (1989) J. Cell Biol. **108**, 833–842
44 Theibert, A., Estevez, V., Ferris, C., Danoff, S., Barrow, R., Prestwich, G. and Snyder, S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3165–3169
45 Norris, F., Ungewickell, E. and Majerus, P. (1995) J. Biol. Chem. **270**, 214–217
46 Morris, S., Schroder, S., Plessman, U., Weber, K. and Lafer, E. (1995) J. Biol. Chem. **270,** 1564–1568
47 Ye, W., Bembenek, M., Shears, S. and Lafer, E. (1995) J. Biol. Chem. **270**, 1564–1568
48 Kijima, Y., Mayrleitner, M., Timerman, A., Saito, A., Schindler, H. and Fleischer, S. (1993) J. Biol. Chem. **268**, 16253–16258
49 Timerman, A., Mayrleitner, M., Lukas, T., Chadwick, C., Saito, A.,Watterson, D., Schindler, H. and Fleischer, S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 8976–8980
50 Fox, J., Lipfert, L., Clark, E., Reynolds, C., Austin, C. and Brugge, J. (1993) J. Biol. Chem. **268**, 25973–25984
51 Coutu, M. and Craig, S. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8535–8539
52 Fausser, J., Ungewickell, E., Ruch, J. and Lesot, H. (1993) J. Biochem. (Tokyo) **114**, 498–503
53 Strasser, P., Gimona, M., Herzog, M., Geiger, B. and Small, J. (1993) FEBS Lett. **317**, 189–194
54 Doni, M., Cavallini, L. and Alexandre, A. (1994) Biochem. J. **303**, 599–605