Purification and characterization of the human type 1 $\text{Ins}(1,4,5)\text{P}_3$ receptor from platelets and comparison with receptor subtypes in other normal and transformed blood cells

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We report the first purification of a native human form of the $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) receptor. This receptor, isolated from platelets, has an apparent molecular mass on SDS/PAGE of 252 kDa and is chromatographed by gel filtration as an oligomer of about 1×10^6 kDa. [^3H] InsP_3 bound to a single class of sites on the purified receptor protein with a K_d of 27 nM and a B_{max} of 2.2 nmol/mg of protein. The platelet InsP_3 receptor, like the rodent cerebellar receptors, was identified immunochemically as a type 1 receptor, but unlike its brain counterparts bound poorly to concanavalin A and other lectins and was not significantly phosphorylated by protein kinase A. All cultured megakaryocytic leukaemia cell lines (e.g. Dami, CHRF-288 and Meg-01) and HEL cells were also immunopositive for type 1 receptor, which was substantially increased in some cases by DMSO or phorbol 12-myristate 13-acetate (PMA) which induce further megakaryo-

cyclic differentiation. Normal mixed lymphocyte and granulocyte fractions and an enriched T-cell fraction from human blood had measurable InsP_3 -binding activity, but no detectable type 1 protein. In contrast, Jurkat E6-1 (T-cell lymphoma) cells and the transformed B-cell line RPMI 8392 were immunopositive for type 1 receptor. HL-60 (human promyelocytic leukaemia) cells had no detectable type 1 receptor unless they were stimulated to differentiate along monocyte/macrophage lines by PMA. We conclude that: (1) of the major normal blood cells only platelets contain type 1 InsP_3 receptors; (2) some neoplastic transformed blood cell lines also express type 1 receptors, in contrast to their normal counterparts; and (3) increased levels of type 1 InsP_3 receptor are induced in some transformed cells under conditions that favour their further terminal differentiation.

INTRODUCTION

$\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) is an important second messenger for calcium mobilization in many cells including platelets. Specific receptors for InsP_3 have been purified from rat and mouse cerebellum [1,2] bovine aorta smooth muscle [3] and rat vas deferens [4]. The cDNA of the mouse cerebellar InsP_3 receptor codes for an integral membrane protein of molecular mass 313 kDa with eight transmembrane domains and consensus sites for phosphorylation by cyclic AMP-dependent protein kinase A (PKA) on serine residues 1588 and 1755 [5]. A similar type 1 InsP_3 receptor cDNA has been described in rat brain [6], *Drosophila* [7], *Xenopus* [8] and human Jurkat (T-cell lymphoma) cells [9]. The finding of type 2 cDNA in rat brain [10] and human umbilical cord endothelial cells [11] and a type 3 receptor in rat pancreatic islets [12], human intestinal epithelium cells [13] and human umbilical endothelial cells [11], established that these proteins comprise a family of InsP_3 receptor proteins. Moreover, the human type 2 and type 3 InsP_3 receptors are products of different genes [11].

The InsP_3 receptor types have areas of structural similarity which are conserved particularly in the N-terminus and transmembrane domains, specific variable regions in the cytoplasmic domain and the C-terminus, and sites S1 and S2 in type 1 that are alternatively spliced or deleted. Although the rodent receptors, the alternately spliced non-neuronal form in vas deferens, and the *Xenopus* receptor [8] all bear consensus sites for PKA, the type 1 InsP_3 receptor in *Drosophila* lacks these sites [7] and is not a substrate for PKA. Various species of the InsP_3 receptor family may possess unique properties with respect to intracellular localization, ligand binding, ion gating or regulation by other signal-transduction processes, such as covalent modification by

protein kinases. In this paper we describe the first purification of a native human InsP_3 receptor protein, as prepared from platelets. We also compare the InsP_3 receptor subtypes in normal human blood cells and several blood cell tumour lines, and the effects of phorbol 12-myristate 13-acetate (PMA) and DMSO on receptor concentration in several cell lines.

EXPERIMENTAL

Materials

Human platelets were obtained from the Connecticut Red Cross Blood Center, and lymphocytes and granulocytes, with informed consent, from human donors. The cell lines were cultured, and their sources, were: HEL human erythroleukaemia cells, the T-cell lymphomas Jurkat E6-1 and Jurkat CaMI-6, and HL-60 human promyelocytic leukaemia cells (all from American Type Tissue Culture, Rockville, MD, U.S.A.), RPMI 8392 transformed B-cells (Dr. P. Epstein, University of Connecticut Medical School, CT, U.S.A.) and the megakaryoblastic cell lines Meg-01 (Dr. M. Takeuchi, Institute for Fermentation, Osaka, Japan), CHRF-288 cells (Dr. M. Lieberman, University of Cincinnati, OH, U.S.A.) and Dami (Dr. S. Greenberg, Harvard University, Cambridge, MA, U.S.A.). Heparin-agarose type II and concanavalin A-agarose type III, wheat germ lectin-agarose, lentil lectin-agarose, the protease inhibitors leupeptin, pepstatin and PMSF, the catalytic subunit of PKA, PMA, DMSO and Histopaque were all from Sigma Chemical Co. InsP_3 was obtained from the University of Rhode Island Chemical Group, RI, U.S.A. [^3H] InsP_3 (specific activity 34–55 Ci/mmol) and [$\gamma\text{-}^{32}\text{P}$]ATP (6000 Ci/mmol) were products of Amersham and New England Nuclear respectively. Dr. J. Watras (University of

Connecticut School of Medicine) provided affinity-purified anti-body to the type 1 InsP_3 receptor, which was raised in rabbits against the 29 amino acid C-terminal peptide of the cerebellar type 1 receptor. The enhanced chemiluminescence (ECL) reagent came from Kirkegaard and Perry and goat anti-rabbit, peroxidase-labelled, second antibody was from Calbiochem.

METHODS

Membrane preparations

Platelet membranes enriched in [^3H] InsP_3 -binding activity and InsP_3 -induced calcium release were prepared by sonication of washed platelets and purified by centrifugation on a Percoll gradient, as described by O'Rourke and Feinstein [14]. Dami cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine and 5% w/v fetal bovine serum. Meg-01, CHRF-288, HL-60 and HEL cells were grown in Rosewell Park Memorial Institute medium (RPMI) supplemented with L-glutamine and 10% w/v fetal bovine serum. All cultured cells were harvested (4.0×10^8 cells), centrifuged for 5 min at 100 *g* and washed twice at 4 °C with PBS: 2.7 mM KCl/1.2 mM KH_2PO_4 /138 mM NaCl/8.1 mM Na_2HPO_4 (pH 7.4). Cell pellets were suspended in 50 ml of hypotonic swelling buffer (1 mM Na-bicarbonate, 1 mM EDTA, 10 μM each of pepstatin and leupeptin and 0.5 mM PMSF) and kept on ice for 30 min. After centrifugation for 30 min at 10000 *g* the pellets were resuspended in 5 ml of hypotonic buffer and homogenized with 20 strokes of pestle A in a Dounce homogenizer. Homogenates were centrifuged for 10 min at 3500 *g* to remove nuclei and cellular debris and the supernatants were centrifuged at 463000 *g* for 10 min in a Beckman TL-100 centrifuge to obtain the crude post-nuclear membrane fraction. Freshly collected donor blood was fractionated into mononuclear and granulocyte cell fractions on a Histopaque step gradient. The cells were washed twice with PBS and cooled to 4 °C. Lymphocyte and granulocyte species were identified by staining with Wright's Geimsa stain. Lymphocyte fractions contained 89.8% w/v lymphocytes, 8.8% w/v monocytes and 1.4% w/v granulocytes; granulocyte fractions were 97.6% w/v neutrophils, 1.2% w/v eosinophils, 0.4% w/v monocytes and 0.8% w/v lymphocytes. Membranes from human blood cell fractions were prepared as described above for cultured cells.

Assay of [^3H] InsP_3 binding to membranes

Membranes were incubated with various concentrations of [^3H] InsP_3 for 10 min at 4 °C and centrifuged at 463000 *g* for 4 min in a Beckman TL-100 and the supernatants were aspirated. [^3H] InsP_3 was extracted from the pellets with 50 μl of 50% w/v trichloroacetic acid (TCA) after centrifugation to remove protein precipitates and assayed by liquid-scintillation counting (LSC). Non-specific binding was determined in the presence of 1 μM non-radioactive InsP_3 .

Solubilization and purification of the platelet InsP_3 receptor

All procedures were carried out at 4 °C. InsP_3 receptor-enriched platelet membrane fractions (200 mg of protein) were washed twice with a solution of 20 mM Na-bicarbonate, 1.0 M NaCl, 1 mM EDTA, 10 μM each of leupeptin and pepstatin, 0.5 mM PMSF and 0.5 mM dithiothreitol (DTT), at pH 7.4, and then twice more in the same buffer without NaCl. Membrane proteins were solubilized by stirring for 2 h in 50 ml of the low-salt wash buffer with 5% w/v Chaps. Particulates were removed by centrifugation for 45 min at 40000 *g* in a Sorvall RC-5B. The

supernatant was passed through a 1×10 cm concanavalin A-agarose column equilibrated with solubilization buffer. The flow-through, which contained all of the InsP_3 -binding activity, was collected and passed through a 1×10 cm heparin-agarose column followed by washing with 20 ml of 0.2 M NaCl in solubilization buffer with 1% w/v Chaps. InsP_3 -binding protein was batch-eluted from the heparin-agarose column with a solution of 40 mM Na-bicarbonate, 0.5 M NaCl, 1 mM EDTA, 0.5 mM DTT, 1% w/v Chaps and protease inhibitors, at pH 8.3. The eluant volume was reduced to 200 μl with a Centricon 100 concentrator (Amicon) and then diluted with 4.8 ml of elution buffer without NaCl. The sample was injected at a flow rate of 0.2 ml/min onto a 1×7.5 cm heparin-TSK column connected to an HPLC apparatus. Protein in the effluent was monitored by absorbance at 280 nm and conductivity was measured in-line by a conductivity meter. The column was washed at 0.5 ml/min with a degassed mobile phase containing solubilization buffer plus 1% w/v Chaps. Adsorbed protein was eluted at a flow rate of 0.5 ml/min by a linear gradient of 0.1–1.0 M NaCl added to the mobile phase. InsP_3 -binding fractions were pooled (1.0 ml) and dialysed for 48 h with a 500 kDa cut-off membrane (Spectrapore) against mobile-phase buffer containing 0.5 M NaCl and 0.5% w/v Chaps. The dialysed samples were concentrated to 100 μl with a 100 kDa cut-off Centricon device (Amicon) and applied to a calibrated 9.4 mm $\times$ 25 cm Zorbax G-450 HPLC gel-filtration column at a flow rate of 1 ml/min using a degassed mobile phase of 40 mM Na-bicarbonate, 0.5 mM NaCl, 1 mM EDTA, 1% w/v Chaps and the protease inhibitors, at pH 8.3, and 0.5 ml fractions were collected. Standards used singly for calibration of the gel-filtration column were: IgM (950 kDa), thyroglobulin (667 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), phosphorylase *b* (92 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa).

Partial purification of rat cerebellum InsP_3 receptor

Rat cerebellar membranes, prepared according to Supattapone et al. [1] were solubilized by the same procedure used for platelet membranes. Partially purified cerebellar receptor was obtained by batch elution from a heparin-agarose column followed by salt-gradient elution from an HPLC heparin-TSK column. Cerebellar InsP_3 receptor was assayed in the samples by the same radioligand-binding assay used for platelets, as described below.

[^3H] InsP_3 binding to solubilized receptor protein

Aliquots (75 μl) of chromatographic fractions were diluted with an equal volume of bicarbonate buffer without Chaps and incubated with [^3H] InsP_3 for 10 min at 4 °C, followed by addition of 8 μl of rabbit IgG (50 mg/ml) as carrier. Duplicate aliquots (75 μl) were added to 100 μl of 30% w/v polyethylene glycol 6000 in pH 8.3 bicarbonate buffer to precipitate proteins. Non-specific binding was determined in the presence of 1 μM non-radioactive InsP_3 . After 10 min at 4 °C the samples were centrifuged for 10 min at 463000 *g* in a TL-100 Beckman centrifuge. Protein pellets were extracted with 50% w/v TCA, centrifuged, and the supernatants assayed by LSC. The data were analysed using the non-linear regression program BIOSOFT (Elsevier) and displayed as Scatchard plots.

Phosphorylation of InsP_3 receptors *in vitro*

Purified platelet InsP_3 receptor protein (0.5 μg) was incubated in 50 μl of a buffer containing 25 mM Tris/HCl, pH 7.5, with 20 mM MgCl_2 , 25 μM Na ATP and 10 μCi of [γ - ^{32}P]ATP plus 0.5 μg of PKA catalytic subunit (25 units). Partially purified

cerebellar InsP₃ receptor was used as a positive control. After 3 h at 37 °C the reactions were stopped by 1/3 vol. of solution containing 0.18 M Tris/HCl at pH 6.9, 6% w/v SDS, 45% w/v glycerol, 15% w/v 2-mercaptoethanol and 0.3% w/v Bromophenol Blue. The samples were separated by SDS/PAGE on a 6% w/v gel and the dried gels, with appropriate ³²P standards, were exposed for 3 h to a Molecular Dynamics phosphorimager screen followed by analysis of phosphorylated protein bands using Image Quant software.

Western blots of InsP₃ receptor types

Proteins separated by SDS/PAGE on a 6% w/v gel were semi-dry blotted onto Immobilon membranes using purified canine cerebellar membranes as a positive control for type 1 receptor. Blots were incubated for 45 min with a blocking solution of 5% powdered milk in PBS, pH 7.4, with 0.1% Tween 20 (PBS-Tween) and then with a 1:30000 dilution of antibody to the C-terminal domain of the type 1 InsP₃ receptor for 2 h in blocking buffer at room temperature. The membranes were washed twice with PBS-Tween for 15 min and then three times for 5 min each. The blots were then incubated for an additional 45 min with goat anti-rabbit, peroxidase-labelled, second antibody, diluted 1:12000 in PBS-Tween, followed by washing as described for the first antibody. The washed immunoblots were incubated with ECL reagent and exposed to film for 1 min. The images were analysed by a Molecular Dynamics laser densitometer.

RESULTS

Purification of the human platelet InsP₃ receptor protein

The platelet InsP₃ receptor was purified from the InsP₃-sensitive Ca²⁺-releasing membrane fraction obtained as described by O'Rourke and Feinstein [14]. The membranes were stored frozen at -20 °C at a concentration of 5 mg of protein/ml in buffer composed of 20 mM Na-bicarbonate, pH 7.4, 20 μM each of leupeptin and pepstatin, 0.5 mM DTT and 0.5 mM PMSF, and retained full binding activity for more than 6 months. Upon thawing, membranes were washed twice to remove non-integral membrane proteins with a buffer containing 1.0 M NaCl. Two additional washings in the same solution minus NaCl were required to remove the high salt which interfered with protein solubilization. Chaps, Triton X-100 and *N*-octylglucoside detergents were assessed as candidates for protein solubilization. Chaps detergent was selected because, unlike the others, it did not interfere with spectrophotometric protein detection at 280 nm and the non-specific binding of InsP₃ was low. The combination of the protease inhibitors leupeptin, pepstatin and PMSF and the

SH reducing agent DTT was found to stabilize the InsP₃-binding activity in solubilized extracts for up to 10 days.

The solubilized InsP₃ receptor was purified by a sequence of chromatographic steps employing concanavalin A-agarose, heparin-agarose, heparin-TSK HPLC and a gel-filtration HPLC column. Fractions from the chromatographic steps were analysed for protein concentration, [³H]InsP₃ binding and for protein composition by SDS/PAGE. The purification scheme and the compilation of the results are shown in Table 1. Platelet InsP₃ receptor activity did not bind to concanavalin A-agarose, but this column was employed as a first step as it effectively removed many membrane glycoproteins and provided a 2-fold purification of the InsP₃ receptor. The final highly purified platelet InsP₃ receptor also bound not more than 5–10% to columns of concanavalin A, wheat germ agglutinin or lentil lectins, compared with 50–60% binding in the case of the cerebellar receptor. This behaviour of the platelet receptor was in marked contrast to the InsP₃ receptors from rat cerebellum, vas deferens and bovine aorta, all of which were reported to bind substantially to concanavalin A-agarose [1,4] lentil lectin-Sepharose [2] or wheat germ agglutinin-agarose [3].

The platelet receptor is similar to other InsP₃ receptors in its affinity for heparin [14] so a heparin-agarose column was employed in the next step. The receptor was batch-eluted from the heparin-agarose column with a bicarbonate buffer at pH 8.3 containing 0.5 M NaCl, which yielded a further 4-fold receptor purification. The receptor-containing fraction was then adsorbed on a heparin-TSK HPLC column and eluted with a salt gradient, which resulted in an additional 11-fold enrichment of the InsP₃-binding activity. Fractions 10–12, which contained the InsP₃-binding activity were dialysed overnight with a Spectraphore 500 kDa cut-off membrane to remove additional proteins. The retained receptor was finally isolated by gel-filtration HPLC. [³H]InsP₃-binding activity eluted earlier than the IgM standard and thus had a nominal molecular mass greater than 950 kDa. This last step yielded a nearly 2000-fold final purification of the InsP₃ receptor, with a yield of 10–15%. The InsP₃-binding fraction from the final gel-filtration step contained a single major protein band at approximately 250 kDa and a minor band at 200 kDa (Figure 1a). Both bands reacted positively with type 1 receptor antiserum (described below), indicating that the minor protein component was most likely a proteolytic fragment of the native receptor protein.

[³H]InsP₃ binding to purified receptor

Specific binding of [³H]InsP₃ to the protein obtained by gel filtration fitted a model with a single ligand-binding site: $K_d = 27$

Table 1 Purification of the InsP₃ receptor from human platelets

Platelet membrane vesicles were isolated and proteins solubilized as described in the Methods section. Samples from each purification step were incubated with 10 nM [³H]InsP₃ and assayed for the amount of specific ligand-binding to protein (pmol/mg). Data represents a single purification which was repeated three additional times with similar results.

Sample	Protein (mg)	Specific activity (pmol/mg)	Total (pmol)	Fold purification	Yield (%)
Solubilized vesicles	240	0.325	78.0	—	100
Concanavalin A flow through	154	0.520	80.0	1.6	100
Heparin-agarose eluate	9	1.36	12.2	4.2	15
Heparin-TSK eluate	3.4	3.7	12.6	11	15
Dialysate	0.3	40.0	12.1	120	15
Gel filtration	0.018	645.0	11.6	1926	14

Figure 1 (a) SDS/PAGE of purified InsP_3 receptor protein from human platelets

(a) Lane 1, 1 μg of protein stained with Colloidal Coomassie Blue from fraction 16 chromatographed on the HPLC gel-filtration column. Lane 2, X-ray film image of immunoblot of protein in lane 1 using type 1 InsP_3 receptor antibody. A very faint positive band seen in the original blot at 200 kDa may not be visible in the photograph. Lane 3, molecular mass markers stained with Colloidal Coomassie Blue: myosin, 206 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97.5 kDa; BSA, 66 kDa. SDS/PAGE was on a 6% gel. Lanes 1 and 3 were stained with Colloidal Coomassie Blue. (b) X-ray film image of immunoblot of blood cells using type 1 InsP_3 receptor-specific antibody. Lanes: 1, platelets; 2, Meg-01; 3, HL-60; 4, CHRF-288; 5, Dami; 6, HEL; 7, RPMI 8392; 8, lymphocytes; 9, granulocytes; 10, Jurkat E6-1; 11, Jurkat CaMI-1. (c) Increase of type 1 InsP_3 receptor subtype by DMSO (right-hand lanes) in leukaemic cell lines as described in the Methods section. Lanes: 1, Meg-01; 2, HEL; 3, Dami; 4, CHRF-288.

Figure 2 Scatchard plot of $[^3\text{H}]\text{InsP}_3$ binding to purified platelet InsP_3 receptor

InsP_3 receptor obtained from the gel-filtration step was incubated with 0–50 nM radiolabelled $[^3\text{H}]\text{InsP}_3$ for 10 min at 4 $^\circ\text{C}$ and assayed for binding as described in the Methods section. The data was best fit to a one-site model with a K_d of 27 nM and a B_{max} of 2.2 nmol/mg of receptor protein. The data represent a single experiment repeated two additional times with similar results.

(± 5.4) nM; $B_{\text{max}} = 2.2 (\pm 0.4)$ nmol InsP_3 /mg of protein (Figure 2). The K_d agrees with the value of 23.3 nM reported for the dominant (85% of total) InsP_3 -binding site in platelet membranes [14], which is a higher affinity for InsP_3 than the mouse or rat cerebellar receptors; i.e. $K_d = 83$ nM [15,16], but lower than the rat vas deferens and bovine aortic smooth muscle receptors which had K_d s of 9.6 and 2.4 nM respectively [3,4].

Comparative phosphorylation of platelet and cerebellar InsP_3 receptors

Purified platelet receptor and partially purified rat cerebellar InsP_3 receptor were incubated with $[^32\text{P}]\text{ATP}$ and the catalytic subunit of PKA. The cerebellar receptor, isolated by two steps of heparin-agarose chromatography, was about 50% pure, based on the B_{max} for $[^3\text{H}]\text{InsP}_3$ binding. PKA catalysed the incorporation of 1.02 pmol of ^{32}P /pmol of cerebellar receptor protein (corrected for an estimated 50% purity), which is similar to the values of 1.1 to 1.2 reported for the purified rat and mouse cerebellar receptor [15,16]. In contrast, only 0.088 pmol of ^{32}P was incorporated per pmol of platelet receptor protein, which is less than 10% of the extent of phosphorylation of the cerebellar receptor.

Immunoblotting of InsP_3 receptor subtype 1 in platelets, normal blood cells and neoplastic blood cell lines

The platelet InsP_3 receptor reacted positively with an affinity-purified rabbit polyclonal antibody raised to the 29 C-terminal amino acids of the type 1 rat cerebellar receptor, a sequence that is not present in rat type 2 and human type 3 receptors. The type 1 antibody consistently reacted with two polypeptides in chromatographic fractions, the major band at 252 kDa and the minor band at 200 kDa (Figure 1a), showing that both polypeptides share common epitopes. The lower molecular mass polypeptide, which accounted for 10–21% of the total protein in the final purified gel-filtration fraction, probably represents a proteolytic fragment of the native receptor, and was also present in rat and canine cerebellar membranes (not shown) and in the rat cerebellum receptor purified by Supattapone [15]. Normal lymphocytes, T-cell-enriched fractions of 60% B- and 40% T-cells respectively (from Dr. S. Padula, University of Connecticut Medical School, CT, U.S.A.), and the granulocyte fractions that were almost entirely comprised of neutrophils, did not react with type 1 antibody (Figure 1b). However, we cannot exclude the presence of the type 1 receptor in minor blood cell components, such as monocytes, eosinophils and basophils, or specific subsets of T-cells. Further analysis of enriched populations of these rarer cell types are necessary. On the other hand, membranes from the transformed B-cell line RPMI 8392 and the T-cell lymphoma lines Jurkat E6-1 and Jurkat CaMI-6 reacted well with type 1 antibody (Figure 1b), demonstrating a striking difference in receptor expression between normal lymphocytes and certain transformed lymphocytic cell lines. Membranes from the megakaryocytic leukaemia cell lines Dami, Meg-01 and CHRF-288 also reacted positively with the type 1 antibody. The concentration of the type 1 InsP_3 receptor was substantially increased when these cell lines were incubated for 48 h with 1.25% v/v DMSO, and in HEL and Dami cells treated with 5 nM PMA (Figure 1c). In Dami cells DMSO and PMA elevated type 1 InsP_3 receptor concentration by 9.74-fold and 4.25-fold respectively (Table 2). Type 1 receptor was detectable in HL-60 membranes only after exposure to PMA, which differentiates these cells along a monocyte/macrophage line, but not by DMSO which causes granulocytic differentiation.

DISCUSSION

The only native InsP_3 receptor proteins that have been purified and characterized to date are the type 1 forms from the rat [1] and mouse [2] cerebellum, bovine aortic smooth muscle [3] and rat vas deferens [4]. The platelet InsP_3 receptor protein, whose purification we report in this paper, is the first human form of the protein to be isolated. This receptor from platelets displays some

Table 2 Expression of the type 1 InsP₃ receptor protein in three human megakaryocytic leukaemia cell lines and the HEL cell line stimulated with DMSO and PMA.

Cultured cell lines were incubated with 1.25% v/v DMSO or 5 nM PMA for 48 h. Membranes (20 µg of protein) were prepared, subjected to SDS/PAGE and immunoblotted with type 1 antibody as described in the Methods section. The data are expressed as averages of the fold-stimulation over untreated control cells in two experiments.

Blood cell type	DMSO	PMA
Meg-01	3.24	1.35
HEL	2.55	2.20
CHRF-288	3.23	1.13
Dami	9.74	4.25

characteristics which are similar to the type 1 receptor of the rat and mouse cerebellum, specifically with respect to its approximate molecular mass, ligand-binding stoichiometry and immunoreactivity, and is chromatographed by gel filtration in detergent as an oligomer of about 1×10^6 kDa, indicating that the solubilized platelet protein, like the rodent brain receptors, probably forms tetramers [2–4]. These receptors also share the same dependence of ligand binding on pH. The ligand-binding site of the platelet receptor has higher affinity for InsP₃ than has the cerebellar receptor (i.e. $K_d = 27$ nM, versus 83 nM for the purified cerebellar proteins) [1,2], but has lower affinity than the vas deferens and bovine aortic smooth muscle receptors [3,4]. The calculated number of binding sites on the purified platelet receptor was 2.2 nmol/mg of protein, which is comparable with that found for the proteins purified from cerebellum, vas deferens and smooth muscle; i.e. $B_{max} = 1.9$ – 2.7 nmol/mg of protein. These values are all somewhat less than the theoretical 3.2 nmol/mg of protein, assuming one mole of InsP₃ is bound per monomer of 313 kDa receptor protein [5]. The explanation for this deviation from the theoretical stoichiometry is not clear [3].

Platelets and the megakaryocytic leukaemia cell lines Dami, CHRF-288 and Meg-01 all express a type 1 receptor protein characterized by immunoblotting with type 1-specific receptor antibody. The receptor concentration was increased in the cells of megakaryoblastic or erythroleukaemic lineage when they were treated with DMSO, and in Dami and HEL cells treated with PMA. Membranes of the normal, peripheral, circulating lymphocyte and neutrophil fractions of human blood failed to react with type 1 receptor antibody. In contrast, membranes from cultured T-cell lymphoma lines (i.e. Jurkat E6-1, Jurkat CaMI-6) and the transformed B-cell line RPMI 8392 were positive for type 1 receptor. The presence of type 1 receptor in platelets and its lack in neutrophils demonstrates that receptor expression can differ despite the common origin from a myeloid stem cell. In addition, membrane protein blots sent to Dr. T. Maranto (Tufts University, Boston, MA, U.S.A.) for analysis with his type 3 receptor antibody were negative with platelets, normal lymphocytes, granulocytes and HL-60 cells, but showed weak reactivity with the other cells (results not shown).

There are two notable differences between the type 1 platelet and cerebellar InsP₃ receptors. The first is the low affinity of the platelet protein for concanavalin A, wheat germ agglutinin and lentil lectin, all of which bind substantial fractions of the InsP₃ receptor in other tissues, which is evidence for likely heterogeneity of receptor glycosylation. The second difference is the insignificant phosphorylation of the platelet receptor by PKA. Inhibition of the Ca²⁺ mobilizing response to InsP₃ by cyclic

AMP/PKA is a possible mechanism to explain some of the inhibitory actions of endothelium-derived prostacyclin on platelets. Indeed, Supattapone et al. [15] observed that PKA substantially decreased the potency of InsP₃ to release Ca²⁺ from cerebellar membranes. Quinton and Dean [17] also observed that PKA caused a 30% decrease of Ca²⁺ release from platelet membrane fractions by 10 µM InsP₃. In contrast, White et al. [18] and we [19] found that PKA did not affect either InsP₃ binding or Ca²⁺ release from platelet membranes. Moreover, phosphorylation of InsP₃ receptors by PKA has only been observed in cerebellar microsomes and with purified InsP₃ receptor protein [15,20], but not in primary cerebellar cell cultures [20], so that the ability of PKA to phosphorylate the InsP₃ receptor in intact cells remains unsubstantiated.

In conclusion, of the major human blood cell types, only platelets appear to contain type 1 receptors, and some characteristics of this receptor differ from the brain type 1 receptor, as evidenced by its lack of binding to lectins and phosphorylation by PKA. Megakaryoblastic cell lines also contain type 1 receptor, whose expression can be markedly increased in some cases by DMSO or PMA. In contrast to the major normal human lymphocyte and neutrophil populations, a transformed B-cell line and two T-cell lymphoma cell lines express type 1 receptors. It is evident that haematopoietic lineage-specific cells provide very interesting models to study the relationships between the expression of specific InsP₃ receptor subtypes and stages of cell development and proliferation and the specific signalling pathways that control expression of the receptor proteins.

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