Purification and characterization of an $\text{Ins}(1,3,4,5)\text{P}_4$ binding protein from pig platelets: possible identification of a novel non-neuronal $\text{Ins}(1,3,4,5)\text{P}_4$ receptor

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A novel $\text{Ins}(1,3,4,5)\text{P}_4$ -binding protein has been purified to apparent homogeneity from solubilized membranes derived from pig platelets. It has a high affinity for $\text{Ins}(1,3,4,5)\text{P}_4$ (K_d 6.3 ± 0.4 nM), a B_{max} of 2.5–6.0 nmol/mg of protein, and a high specificity for $\text{Ins}(1,3,4,5)\text{P}_4$ [K_d values for $\text{Ins}(1,3,4,5,6)\text{P}_5$, InsP_6 , $\text{GroPtdIns}(3,4,5)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(3,4,5,6)\text{P}_4$ and L-

$\text{Ins}(1,3,4,5)\text{P}_4$ of 85.0 ± 4.1 nM, 800.0 ± 20.2 nM, 65.6 ± 2.6 nM, > 10 μM , 793.3 ± 55.6 nM and 81.0 ± 5.9 nM respectively]. The protein has an apparent molecular mass of 104 kDa, suggesting that this peripheral tissue protein may be different from $\text{Ins}(1,3,4,5)\text{P}_4$ binding proteins previously isolated from neuronal tissues.

INTRODUCTION

There has been considerable controversy over whether there is a physiological function for $\text{Ins}(1,3,4,5)\text{P}_4$, and, if there is a second-messenger function, what that function may be (Irvine, 1992; see also Putney and Bird, 1993). One piece of evidence that supports a second-messenger function for this inositol phosphate is that, in a wide range of neuronal and non-neuronal tissues, binding sites exist for $\text{Ins}(1,3,4,5)\text{P}_4$ that have high affinity and specificity for this ligand (Irvine and Cullen, 1993). These $\text{Ins}(1,3,4,5)\text{P}_4$ -specific sites are: (i) distinct from InsP_n -binding sites that favour InsP_6 over $\text{Ins}(1,3,4,5)\text{P}_4$; (ii) distinguishable from any known InsP_n -metabolizing enzymes; (iii) distinct from $\text{Ins}(1,4,5)\text{P}_3$ receptors that appear to bind $\text{Ins}(1,3,4,5)\text{P}_4$, since the putative $\text{Ins}(1,3,4,5)\text{P}_4$ receptors greatly favour $\text{Ins}(1,3,4,5)\text{P}_4$ over $\text{Ins}(1,4,5)\text{P}_3$ (see Irvine and Cullen, 1993). Two further points should be noted concerning these putative $\text{Ins}(1,3,4,5)\text{P}_4$ receptors. First, under physiological conditions the specificity of these sites has allowed the development of $\text{Ins}(1,3,4,5)\text{P}_4$ mass assays using crude cell extracts, which implies that $\text{Ins}(1,3,4,5)\text{P}_4$ is the likely principal ligand on these sites in an intact cell (Cullen and Irvine, 1992). Secondly, the putative $\text{Ins}(1,3,4,5)\text{P}_4$ receptor present within human platelets has recently been shown to have a subcellular distribution distinct from that of the $\text{Ins}(1,4,5)\text{P}_3$ receptor located on the intracellular membrane, i.e. it is predominantly located within the plasma membrane (Cullen et al., 1994).

$\text{Ins}(1,3,4,5)\text{P}_4$ -binding proteins have been purified from two neuronal sources: pig (Donié and Reiser, 1991) and rat cerebellum (Theibert et al., 1991, 1992). The binding site purified from pig cerebellum has an apparent molecular mass of 42 kDa, but is relatively unspecific in that $\text{Ins}(1,3,4,5,6)\text{P}_5$ displaces with an equal affinity to $\text{Ins}(1,3,4,5)\text{P}_4$. In rat cerebellum, however, the situation is more complex. Using an $\text{Ins}(1,3,4,5)\text{P}_4$ affinity column, Theibert et al. (1991, 1992) have identified two apparently distinct $\text{Ins}(1,3,4,5)\text{P}_4$ -binding fractions. The first is

apparently a single binding protein with a K_d for $\text{Ins}(1,3,4,5)\text{P}_4$ of 4 nM and a molecular mass of 182 kDa, and the other has an affinity for $\text{Ins}(1,3,4,5)\text{P}_4$ of 1.5 nM and contains proteins of 174 and 84 kDa. All of these binding proteins are specific for $\text{Ins}(1,3,4,5)\text{P}_4$ in that the next best displacing ligand is $\text{Ins}(1,3,4,5,6)\text{P}_5$ with an approximately 10-fold lower affinity.

Although $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase and $\text{Ins}(1,3,4,5)\text{P}_4$ -binding proteins are found at highest levels in neuronal tissues (Mailleux et al., 1991; Theibert et al., 1992), they are nevertheless expressed in nearly all animal tissues (Irvine, 1992). If $\text{Ins}(1,3,4,5)\text{P}_4$ were to have one or more physiological function, at least some of these functions should manifest themselves in peripheral tissues, and in this study we describe for the first time the purification and molecular identification of a putative peripheral $\text{Ins}(1,3,4,5)\text{P}_4$ receptor.

MATERIALS AND METHODS

Preparation of platelet membranes

Fresh pig blood (15 litres) was collected directly into 0.1 vol. of 3.8% (w/v) sodium citrate. Platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min followed by aspiration of the resulting plasma. The erythrocytes were re-extracted with an equal volume of buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 90 mM NaCl, 10 mM EDTA, 2 mM EGTA, pH 6.5) with the resulting suspension being re-centrifuged as above. Again the supernatant was aspirated, combined with the plasma fraction and acidified to pH 6.4 with 150 mM citric acid. This procedure yielded approx. 13 litres of platelet-rich plasma. The platelets and contaminating erythrocytes were pelleted at 1200 *g* for 20 min before resuspension in 1.5 litres of ice-cold resuspension buffer [150 mM NaCl, 4 mM KCl, 3 mM EDTA, 10 mM Hepes (pH 7.2), 2 mM EGTA, 1 mM 2-mercaptoethanol, 0.4 mM phenylmethanesulphonyl fluoride (PMSF), 5 mM leupeptin]. All of the above centrifugations were performed in an IEC DPR

6000 centrifuge (6×1 litre buckets) at room temperature. Contaminating erythrocytes were removed by centrifugation in a Sorvall RC-5B at 200 g for 10 min with the resulting supernatant being centrifuged at 1200 g for 10 min to pellet the platelets. Platelets were resuspended in 160 ml of resuspension buffer and sonicated at 4 °C (3×15 s with 30 s cooling between each sonication using an Ultrasonic W-225 Processor at full power fitted with a 1 cm diameter probe). The suspension was re-centrifuged at 1200 g with the supernatant being retained. The pellet was subjected to another cycle of sonication and centrifugation. The two supernatants were combined and the platelet membranes were pelleted at 45000 g for 45 min before resuspension in 25 mM Tris/HCl (pH 7.2) buffer containing 1 mM EGTA, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.4 mM PMSF and 5 mM leupeptin at a protein concentration of 7–8 mg/ml (total protein per preparation approx. 350 mg). Platelet membranes were stored at –20 °C.

Purification of Ins(1,3,4,5) P_4 -binding protein

Platelet membranes (total of approx. 1 g of protein at 4.5 mg/ml) were solubilized in 25 mM Tris/HCl (pH 7.2) containing 1 mM EGTA, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.4 mM PMSF and 1% (w/v) CHAPS for 1 h at 4 °C with constant stirring. Unsolubilized material was removed by centrifugation at 45000 g for 1 h at 4 °C. The solubilized binding activity was loaded at 1 ml/min on to a CM-cellulose column (2.5 cm diameter $\times$ 15 cm) pre-equilibrated with buffer A [25 mM Tris/HCl (pH 7.2), 1 mM EGTA, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, 0.25% (w/v) CHAPS]. The column was washed with 3 column vol. of buffer A before elution of binding activity with a 10 column vol. linear gradient of 0–1.0 M NaCl at 1 ml/min. Peak fractions were pooled, diluted 2-fold in buffer A before loading at 1 ml/min on to a 5 ml Hi-Trap Heparin column. The column was washed with 4 column vol. of buffer A containing 0.2 M NaCl and then attached to the f.p.l.c. A linear 6 column vol. gradient of 0.2–1.0 M NaCl at 1 ml/min was used to develop the column. The peak fractions were pooled and concentrated to 0.5 ml with an Amicon Centriprep 10 concentrator. Activity was diluted to 10 ml in buffer A before loading on to a 1 ml Mono Q column. The column was washed with 5 ml of buffer A and then developed with a 10 ml gradient to 1.0 M NaCl. Peak fractions were pooled and concentrated to 0.5 ml before loading via a 10 ml dilution in buffer A on to a 1 ml Mono S column. The column was washed and developed as described for the Mono Q column. All columns were pre-equilibrated with a minimum of 10 column vol. of buffer A and salt gradients were based on buffer A. Tris-based buffers were adjusted with respect to pH at 4 °C.

Binding assays

For platelet membranes an individual Ins(1,3,4,5) P_4 -binding assay mixture contained 100 mM KCl, 20 mM NaCl, 10 mM Hepes (pH 7.0), 1 mM EDTA, 0.1% (w/v) BSA 50000–60000 d.p.m. [32 P]Ins(1,3,4,5) P_4 (4500 Ci/mmol), 200–300 μ g of membrane protein and various concentrations of competing inositol phosphates in a final volume of 0.5 ml. Equilibrium binding was reached by incubation at 4 °C for 15 min, followed by centrifugation for 5 min. The amount of radioactivity retained within the pellet after removal of the supernatant was determined by solubilization in 300 μ l of Soluene 350 tissue solubilizer followed by liquid-scintillation counting using Emulsifier Scintillator 299.

Binding to solubilized proteins were determined by a poly(ethylene glycol) precipitation protocol. The individual assay

conditions were as described above with the addition, however, of 1% (w/v) CHAPS and 10–100 μ l of solubilized protein. Equilibrium binding was reached by incubation at 4 °C for 15 min. Receptor–ligand complex was precipitated by the addition of 500 μ g of γ -globulin (as carrier protein) and 1 ml of 25% (w/v) poly(ethylene glycol) (average molecular mass 3350 Da) for 5 min at 4 °C before centrifugation at 12000 g for 5 min. The supernatant was removed and the pellet was counted for radioactivity as previously described.

Materials

[32 P]Ins(1,3,4,5) P_4 was prepared as previously described (Cullen et al., 1994), except that phosphorylation of [32 P]Ins(1,4,5) P_3 was by a bacterially expressed Ins(1,4,5) P_3 3-kinase (Takazawa et al., 1990), a generous gift from Dr. C. Erneux (Universite Libre de Bruxelles, Bruxelles, Belgium). Fresh pig blood was obtained from a local abattoir. CM-cellulose (C-0806) was from Sigma Chemical Co. Hi-Trap Heparin was from Pharmacia Biotech. Inositol phosphates were obtained as previously described (Cullen et al., 1994) for the following gifts: Ins(3,4,5,6) P_4 from Dr. S. Shears (NIEHS, Research Triangle Park, NC, U.S.A.); L-Ins(1,3,4,5) P_4 from Professor S. Nahorski (University of Leicester, Leicester, U.K.); GroPIns(3,4,5) P_3 from Dr. P. Hawkins (Babraham Institute, Cambridge, U.K.) made using synthetic PtdIns(3,4,5) P_3 produced by Dr. R. Gigg (NIMR, Mill Hill, London, U.K.). All other reagents were of analytical grade.

RESULTS

Characterization of pig platelet Ins(1,3,4,5) P_4 -binding site(s)

Characterization of the [32 P]Ins(1,3,4,5) P_4 -binding site(s) present within pig platelet membranes was carried out as described in the Materials and methods section. As can clearly be seen in Figure 1, Ins(1,3,4,5) P_4 bound to the membranes with 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

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

Data from three individual determinations of the competition curve for Ins(1,3,4,5) P_4 (●). The inset shows Scatchard transformation of the depicted data using the EBDA-LIGAND computer program. Binding was performed as described in the Materials and methods section. Specificity of the binding site(s) is demonstrated: ■, Ins(1,4,5) P_3 ; ▲, Ins(1,3,4,5,6) P_5 ; ○, Ins(P_2). Non-specific binding was defined as the binding remaining in the presence of 10 μ M Ins(1,3,4,5) P_4 . Data are means $\pm$ S.E.M.

Figure 2 Purification of Ins(1,3,4,5) P_4 receptor

Solubilized pig platelet membranes were initially fractionated on a CM-cellulose column, which yielded a single peak of activity. On all subsequent columns, no significant Ins(1,3,4,5) P_4 -binding activity was detected other than in the peaks shown. The fraction from CM-cellulose containing the activity was loaded on to the Hi-Trap Heparin column and eluted with a linear salt gradient of 0.2–1.0 M NaCl (a). Ins(1,3,4,5) P_4 -binding activity (●) was concentrated and applied to a Mono Q column (b). This yielded a single peak of activity (●) at approximately 0.4 M NaCl which was concentrated and loaded on to a Mono S column (c). Development of the Mono S column resulted in a single peak of activity (●) which when subjected to SDS/PAGE (9% gel; 40 μ l of each column fraction) showed a single 104 kDa polypeptide that co-migrated with the Ins(1,3,4,5) P_4 -binding activity (d). Gels were run under reducing conditions essentially as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250 (0.25% in 40% methanol/10% acetic acid) overnight and destained with several washes of the above solution without the Coomassie Blue. In all data from (a) to (c) ---- represents protein concentration as determined by A_{280} and — is salt concentration (M).

24.8 ± 6.9 nM ($n = 3$) with Ins(1,3,4,5,6) P_5 , Ins P_6 and Ins(1,4,5) P_3 having K_d values of 0.53 ± 0.04 , 5.47 ± 0.47 and 8.10 ± 0.75 μ M respectively ($n = 3$ in each case). Detailed Scatchard transformation of the Ins(1,3,4,5) P_4 competition curve (using four to five points per log unit) resolves two apparent binding sites ($P = 0$ compared with a one-site fit) with affinities of 11.98 ± 1.97 and 900 ± 488 nM (inset in Figure 1) and B_{max} values of 0.90 ± 0.17 and 15.64 ± 7.35 pmol/mg respectively. With respect to these parameters the Ins(1,3,4,5) P_4 -binding site(s) within pig platelets closely resemble putative Ins(1,3,4,5) P_4 receptors described in a number of other tissues (Irvine and Cullen, 1993) including human platelets (Cullen et al., 1994).

Characterization of solubilized pig platelet Ins(1,3,4,5) P_4 -binding site(s)

It was important to document initially the effect that solubilization had on the characteristics of the Ins(1,3,4,5) P_4 -binding site(s). As before solubilization, [32 P]Ins(1,3,4,5) P_4 bound to a protein(s) with high affinity and specificity. The K_d value of Ins(1,3,4,5) P_4 was 72.4 ± 13.3 nM ($n = 3$; results not shown) with Ins(1,3,4,5,6) P_5 , Ins P_6 and Ins(1,4,5) P_3 having K_d values of 0.93 ± 0.006 μ M, 16.0 ± 0.6 μ M and only $9.9 \pm 6.9\%$ displacement with 20 μ M respectively ($n = 3$ for each; results not shown). Once more, Scatchard analysis revealed two apparent binding sites ($P = 0.065$ compared with a one-site fit) with affinities of 33.0 ± 28.6 and 213.6 ± 20.5 nM and B_{max} values of 1.66 ± 1.32 and 6.13 ± 1.66 pmol/mg respectively (results not shown).

Purification of Ins(1,3,4,5) P_4 -binding protein

Ins(1,3,4,5) P_4 -binding activity could be successfully solubilized from pig platelet membranes using 1% (w/v) CHAPS with high yield (400%) and stability. Application of the solubilized protein to CM-cellulose resulted in approx. 25% ($n = 4$) of activity adhering to the resin. In later purifications, we could increase the amount of activity binding to the column [approx. 75% ($n = 2$)] by simply increasing the column size to 250 ml (results not shown). A linear salt gradient applied to the CM-cellulose eluted a single peak of activity at approx. 0.2 M salt (results not shown). All the activity from the CM-cellulose was found to bind to the Hi-Trap Heparin column and was eluted as a single peak of activity at approx. 0.8 M NaCl (Figure 2a). The activity from the Hi-Trap Heparin was concentrated, rediluted and applied to a Mono Q column. Binding activity was again eluted as a single peak at approx. 0.4 M NaCl (Figure 2b). Concentration and redilution before loading on Mono S allowed activity to adhere which could be eluted as a single peak at approx. 0.65 M NaCl (Figure 2c). Analysis of the Mono S elution profile by SDS/PAGE demonstrated the presence of a major protein of molecular mass 104 kDa ($n = 5$; Figure 2d) which was co-eluted with Ins(1,3,4,5) P_4 -binding activity. Table 1 summarizes the scheme of an individual purification.

Ins(1,3,4,5) P_4 -binding to the purified protein

[32 P]Ins(1,3,4,5) P_4 bound to the purified protein with a K_d of 9.3 ± 0.6 nM, with the other inositol phosphates tested (see Table 2) having K_d values of greater than or equal to 10-fold lower affinity. Scatchard analysis from two separate purifications revealed an apparent single class of binding sites ($P = 0.001$ compared with a two-site fit) having an affinity of 6.3 ± 0.4 nM ($n = 6$ from two purifications) and B_{max} values of 6.0 and

Table 1 Summary of the purification of the Ins(1,3,4,5) P_4 receptor

The data shown are from a single experiment typical of at least three different purifications.

Purification step	Protein (mg)	Specific binding (d.p.m./mg)	Total binding (d.p.m.)	Yield (%)	Purification (fold)
Solubilized membranes	640.9	7.87×10^4	5.04×10^7	100	1
CM-cellulose peak	39.6	3.20×10^5	1.27×10^7	25.2	4.1
Hi-Trap Heparin peak	1.8	5.40×10^6	9.72×10^6	19.3	68.6
Mono Q peak	0.51	1.10×10^7	5.61×10^6	11.1	139.8
Mono S peak	0.026	1.05×10^8	2.73×10^6	5.4	1334.2

Table 2 Pharmacology of purified Ins(1,3,4,5) P_4 receptor

Results are shown as means $\pm$ S.E.M. ($n = 3$) and were determined as described in the Materials and methods section.

Inositol phosphate	K_d (nM)
D-Ins(1,3,4,5) P_4	6.3 ± 0.4
D-Ins(3,4,5,6) P_4	793.3 ± 55.6
L-Ins(1,3,4,5) P_4	81.0 ± 5.9
GroPIns(3,4,5) P_3	65.6 ± 2.6
D-Ins(1,3,4,5,6) P_5	85.0 ± 4.1
Ins P_6	800.0 ± 20.2
D-Ins(1,4,5) P_3	> 10000

Figure 3 Characterization of Ins(1,3,4,5) P_4 binding to the purified 104 kDa protein

Data are from three individual determinations of the competition curve for Ins(1,3,4,5) P_4 to purified protein obtained from one purification. Similar data were obtained in one other purification. The inset depicts the Scatchard transformation of the data performed as described in Figure 1.

2.5 nmol/mg purified protein for two separate purifications (see Figure 3).

DISCUSSION

Pig platelets, when assayed at physiological pH, possess an Ins(1,3,4,5) P_4 -binding protein (or, proteins) which display characteristics consistent with Ins(1,3,4,5) P_4 receptors, namely a

high affinity and specificity for Ins(1,3,4,5) P_4 over other inositol phosphates. Solubilization of the binding activity with 1 % (w/v) CHAPS does not appear to affect dramatically the fundamental characteristics of the binding protein(s) (i.e. affinity, specificity and two-site Scatchard transformation). After purification of the present Ins(1,3,4,5) P_4 -binding protein, we observed a similar affinity and specificity as for the platelet membranes (compare Figure 1 and Table 2). However, Scatchard transformation now yields a single class of high-affinity binding sites (compare insets in Figures 1 and 3). This can be explained by either the existence of two or more physically distinct proteins of high and low affinity or one individual protein that possesses both these affinity states and during purification shifts to the high-affinity state. At present we cannot distinguish between these two possibilities.

The purified putative Ins(1,3,4,5) P_4 receptor appears on SDS/PAGE to have a molecular mass of 104 kDa, as revealed by the coincidence between Ins(1,3,4,5) P_4 binding and the relative abundance of this protein band. Assuming therefore a stoichiometry of one Ins(1,3,4,5) P_4 -binding site per receptor molecule we would predict that a protein of 104 kDa would have a maximum specific binding of 9.6 nmol/mg of protein. In two preparations in which we specifically addressed this question, we obtained B_{max} values of 6 and 2.5 nmol/mg of protein. This stoichiometry adds support to our contention that the putative Ins(1,3,4,5) P_4 receptor is indeed the 104 kDa protein, as there are insufficient contaminating proteins to account for the binding activity other than in the 104 kDa band. We do not at present know the relationship between this protein and others with similar pharmacologies purified from cerebellum (Theibert et al., 1992; Donié and Reiser, 1991). These have masses of 42 (Donié and Reiser, 1991), 182, 174 and 84 kDa (Theibert et al., 1992). Given that the pig blood was collected fresh in the presence of protease inhibitors, and that we were unable to detect any higher-molecular-mass Ins(1,3,4,5) P_4 -binding proteins in our preparation, we do not consider it very likely that the 104 kDa protein is a breakdown product of a higher-molecular-mass precursor. Correlation of this peripheral Ins(1,3,4,5) P_4 -binding protein (the first of its kind to be purified) with the various neuronal proteins must await the cloning of these different species.

The observation of the *in vivo* production of PtdIns(3,4,5) P_3 (Traynor-Kaplan et al., 1988; Stephens et al., 1993) leads to the possibility that, since Ins(1,3,4,5) P_4 is the head group of PtdIns(3,4,5) P_3 , Ins(1,3,4,5) P_4 -binding sites may in fact represent PtdIns(3,4,5) P_3 receptors. We have tried to address this question by investigating the binding of GroPIns(3,4,5) P_3 to our purified protein. It can clearly be seen (Table 2) that GroPIns(3,4,5) P_3 is an order of magnitude less efficacious at displacing

[^{32}P]Ins(1,3,4,5) P_4 than Ins(1,3,4,5) P_4 itself, suggesting that the protein described in this report is more likely to have Ins(1,3,4,5) P_4 as its natural ligand. Understanding the structure and function of this protein should prove a valuable advance in our elucidation of inositide signalling.

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