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Characterization of a membrane-associated, receptor and G-protein responsive phosphoinositide-specific phospholipase C from avian erythrocytes

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We describe the reconstitution and purification of a membrane-associated phosphoinositide-specific phospholipase C (PIC) from turkey erythrocyte ghosts. This PIC is responsive to a G-protein coupled to P_{2y} purinergic receptors which are expressed in turkey erythrocytes. Reconstitution is achieved by adding partially purified PIC to [^{3}H]inositol-prelabelled turkey erythrocyte membranes depleted of their endogenous PIC (acceptor membranes). PIC activity is associated with a 52 kDa polypeptide on SDS-polyacrylamide gel electrophoresis. Addition of a 307-fold purified enzyme to the acceptor membranes has no effect on basal PIC activity, but markedly increases the response to GTPγS and P_{2y}-purinergic receptor activation.

Phosphoinositide, Phospholipase-C; G-protein; Turkey erythrocyte, Cytoskeleton; Purinergic receptor

1. INTRODUCTION

The hydrolysis of phosphatidylinositol(4,5)-biphosphate (PtdIns(4,5)P_2) into diacylglycerol (DG) and inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) is a common response to hormones, growth factors and other agents in a number of different cells [1]. The phosphoinositide-specific phospholipase C (PIC) responsible for this hydrolysis is a crucial point in this bifurcating signal pathway. Turkey erythrocytes have been shown to possess a P_{2y} purinergic receptor that regulates phosphoinositide metabolism [2], and membranes prepared from these cells have proven to be a valuable model system for studying this signal transduction mechanism as they demonstrate a PIC that remains efficiently coupled to a G-protein and the purinoreceptor [3,4]. After hypotonic lysis of turkey erythrocytes, PIC activity is found in both cytosolic and membrane fractions. The cytosolic PIC has been purified [5] and has been shown to reconstitute with erythrocyte ghosts [6]. In vitro reconstitution of PIC activation by a G-protein has been achieved in a few systems. Berstein et al. [7,8] reconstituted phosphoinositide-specific phospholipase C-β1 with purified m1 muscarinic receptor and $G_{q/11}$, and Wu et al. [9] used a similar system to reconstitute $G_{q/11}$ and phospholipase C-β1. Blank et al. [10] purified two G- proteins that activated in vitro phospholipase C-β1 and Smrcka et al. [11] purified a G_q α subunit that activated in vitro a partially purified PIC from bovine brain membranes. The other member of this family, phospholipase C-β2, seems to escape activation by the G_q α subunit and probably the $\beta\gamma$ complex of the G-protein would play an important role in its activation [12]. Nevertheless, there is still no evidence for a mechanism of translocation of cytosolic forms of PIC onto membranes and coupling to corresponding G-protein as a response to agonists. In the case of turkey erythrocyte membranes, it is not clear whether the membrane-associated PIC is the same species as the cytosolic form. In this work we purify the membrane associated PIC from turkey erythrocytes, extracting it from agonist-responsive clean ghosts, which loose their endogenous PIC activity, and we reconstitute them by adding back the extracted and 307-fold purified membrane-associated PIC.

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2. MATERIALS AND METHODS

2.1. *Preparation of washed, packed cells*

Turkey blood (800 ml) was mixed with heparin sodium (approx. 15,000 units) and the washed erythrocytes were prepared within two hours of collection. This whole blood was initially centrifuged at 1.000 × *g* for 5 min and the supernatent and buffy coat removed by aspiration. The packed cells were washed twice in cold (4°C) washing solution (150 mM NaCl, 1.5 mM HEPES pH 7.2)

2.2. *Lysis and preparation of white ghosts*

Washed cells were lysed with 20 vols. of lysis buffer (3 mM NaCl, 2 mM $MgCl_2$, 8.5 mM Tris, pH 7.8) They were stirred with a glass rod for 20 min at 4°C and then centrifuged at 12,000 × *g* for 10 min The washing and centrifugation process was repeated until the pellet

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was creamy white (5–7 times) and these clean ghosts were washed once in low Mg^{2+} buffer (3 mM NaCl, 0.2 mM $MgCl_2$, 8.5 mM Tris, pH 7.8) which made the cytoskeleton much more fragile.

2.3. *Extraction and chromatographic purification of PIC activity*

The pellet of clean ghosts, treated with low Mg^{2+} buffer, was resuspended in 1 vol. of the same buffer Mechanical disruption of the cytoskeleton and extrusion of the nuclei was achieved by homogenization in a Dounce homogeniser. After 20 vigorous passes of a tight-fitting motor-driven Teflon pestle operating at 400 rpm, the homogenate was centrifuged at 14,000 × *g* for 10 min and the pellet rehomogenised and recentrifuged. Supernatants were mixed together and centrifuged at 38,000 × *g* for 20 min. The creamy-brown pellet was highly enriched in clean membranes, which were seen to be free of nuclei when observed under electron microscopy. The supernatant was centrifuged at 100,000 × *g* for 1 h, filtered through a 0.4 μm pore size filter and loaded onto a 50 ml column packed with Fractogel TSK DEAE-650 (S) (Merck). The column was washed with 150 ml of buffer A containing: 20 mM Tris, pH 7.5, 1 mM EGTA and 0.1 mM DTT, and eluted with a 300 ml linear gradient of 0–700 mM KCl. The eluant was collected in 4 ml fractions which were assayed for PIC activity (see exogenous substrate assay, below). The active fractions were pooled, diluted 3-fold with buffer A and loaded onto a TSK gel-Heparin 5PW column. This column was eluted with a 45 ml linear gradient of 0–900 mM KCl in buffer A. As before, the active fractions were pooled, concentrated and loaded onto a Superose 12 column. The PIC activity was eluted from this column in the void volume, probably associated with other proteins in a hydrophobic aggregate.

2.4. *PIC assay: endogenous substrate*

[^{3}H]Inositol labelled turkey erythrocyte membranes with a very high specific radioactivity were used for PIC depletion, reconstitution of purified PIC and for assays of residual endogenous activity. 0.5 ml of washed erythrocytes were incubated with 1.5 ml of DMEM, penicillin/streptomycin and 1.0–2.0 mCi of [^{3}H]inositol. Cells were lysed and membranes prepared exactly as described previously [3]. Membranes were resuspended in 2.5 ml of 10 mM HEPES, pH 7.0 (concentrated ghosts) and diluted 50-fold in HEPES buffer for use in reconstitution assays. Each assay contained 50 μl of dilute membranes in 200 μl of the previously defined assay buffer [4] designed to mimic intracellular ionic conditions, with or without partially purified PIC (3 μg). Residual endogenous PIC activity was measured in 50 μl aliquots of membranes diluted to varying degrees in different buffers as described for reconstitution, but without addition of partially purified PIC. Inositol phosphates released by PIC activity were extracted and quantified as previously described [3].

2.5 *PIC assay exogenous substrate*

A substrate of [^{3}H]PtdIns(4,5)P_2 (30 μM) was prepared by sonication in 10 mM HEPES buffer (pH 7) containing 0.5% cholate (10 μm intensity peak-to-peak for 60 s). The incubation medium contained cholate up to 0.5%, 1 mM free Ca^{2+}, 2 mM EGTA, 1 mM free Mg^{2+} and 120 mM KCl Incubations were performed in a water bath at 37°C with continuous shaking The inositol phosphates produced by PIC activity were extracted and quantified as previously described [3]. Free Ca concentrations and kinetic parameters and were determined as described in [4] and [5], respectively.

2.6. *Renaturation treatment*

The extract obtained after low-Mg^{2+} treatment and mechanical disruption of clean ghosts, was partially purified by DEAE chromatography (see above). The preparation obtained was incubated with non-reducing Laemmli buffer at 50°C for 10 min and loaded onto a 1.5 mm thick 7.5% SDS-PAGE gel. After electrophoresis with refrigeration, the gel was excised into 2.5 mm thick slices and each slice crushed into 200 μl of 10 mM HEPES buffer, pH 7, containing 10^{-7} free Ca^{2+}, 1 mM free Mg^{2+}, 2 mM EGTA, 120 mM KCl, 5 mM phosphate, 0.5 mM PMSF and 5% sodium cholate. Samples were kept rotating at 4°C overnight and centrifuged to pellet the gel pieces. Supernatents were collected and assayed for PIC activity following the standard procedure described below. Active samples were analyzed again by SDS-PAGE and silver stained to determine protein molecular weights.

2.7. *Materials*

Myo-2-[^{3}H]inositol (17.1 Ci/mmol) and [^{3}H]PtdIns(4,5)P_2 (5.3 Ci/mmol) were obtained from DuPont-New England Nuclear, phosphocreatine kinase from Sigma, and ATP, GTPγS, GTP and phosphocreatine from Boehringer Mannheim. 2MeSATP was from Research Biochemicals Inc , Natic, MA. Chicken serum and DMEM were from Gibco.

3. RESULTS

Clean turkey erythrocyte ghosts, prepared as described above, contained a very active endogenous PIC which was responsive to purinergic receptors and G-

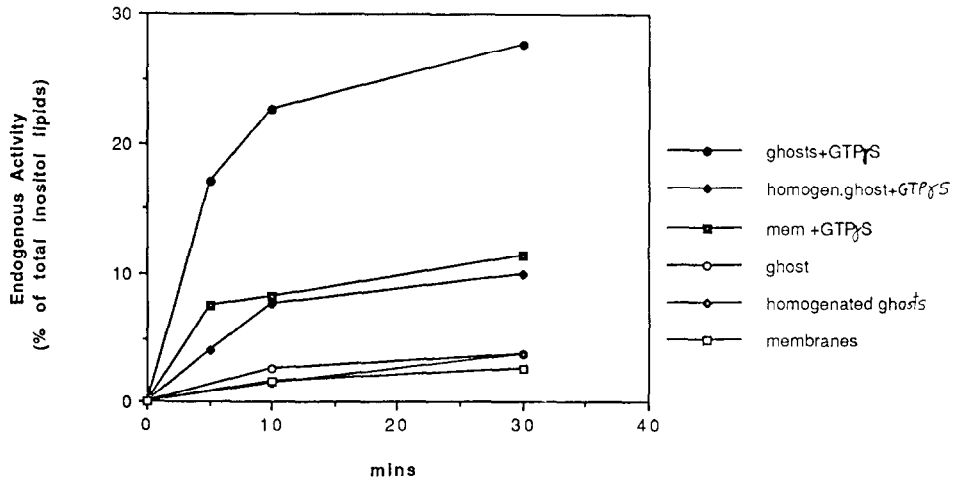

Fig. 1. Endogenous PIC activity of different erythrocyte membranes. The PIC activities of the three different preparations (clean ghosts, homogenized ghosts and membranes; see section 2) were assayed with endogenous [^{3}H]inositol-labelled lipids in the absence (open symbols) or presence (closed symbols) of 5 μM GTPγS, as described in section 2.

protein activation, as previously reported [2,3,4]. When the pelleted erythrocyte ghosts were resuspended in a low-Mg^{2+} hypotonic buffer, which is known to loosen the structure of the cytoskeleton [15], and subsequently mechanically disrupted by homogenisation, a large proportion of their PIC activity was lost (Fig. 1). After a further wash of these homogenised ghosts in the low-Mg^{2+} buffer, a membrane preparation was obtained that was free of nuclei (as shown in the electron micrograph), and which showed a further slight reduction in PIC activity (Fig. 1). During the final centrifugation step in the preparation of these clean erythrocyte membranes, it was seen that the highest specific PIC activity was recovered in the supernatent from these homogenised ghosts (Fig. 2). A similar loss of PIC response in clean erythrocyte ghosts was also seen if they were simply diluted into increasing volumes of a Mg^{2+}-free hypotonic buffer, as illustrated in Fig. 3. However, when these ghosts were diluted into increasing volumes of either a hypotonic buffer (10 mM HEPES, pH 7.0) containing 1 mM Mg^{2+}, or the PIC assay buffer (see [4]) that was designed to mimic intracellular ionic conditions, a large proportion of the membrane-associated PIC was retained. This was demonstrated by the constant value of the proportion of endogenous substrate hydrolysed through an increasing series of these dilutions of the high specific radioactivity membranes (Fig. 3). Under the conditions of depleted PIC activity described above, it was also possible to confirm that these membranes still retained their purinergic receptors coupled to the

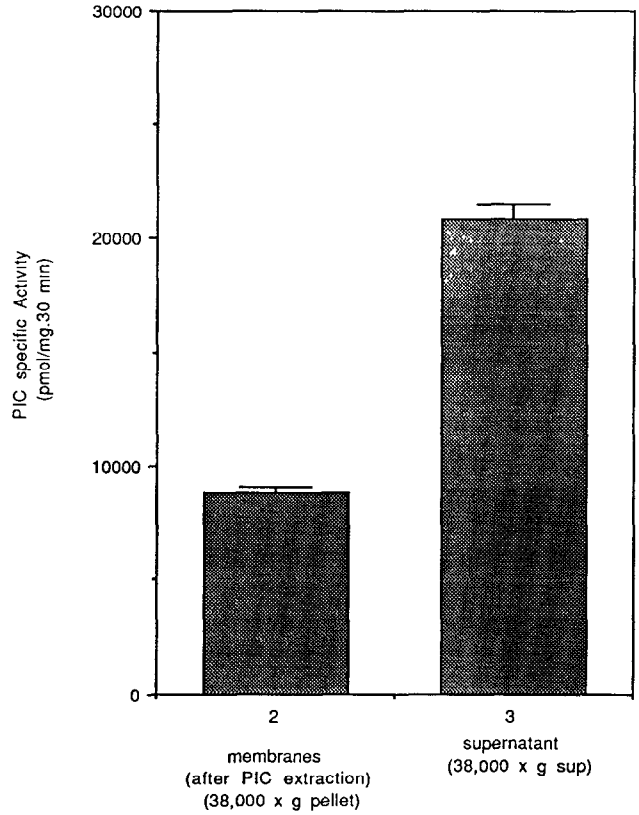

Fig. 2. Specific PIC activity in two subfractions of clean erythrocyte ghosts. Membrane and supernatent fractions were obtained from clean erythrocyte ghosts and assayed for PIC activity with exogenous substrate, as described in section 2.

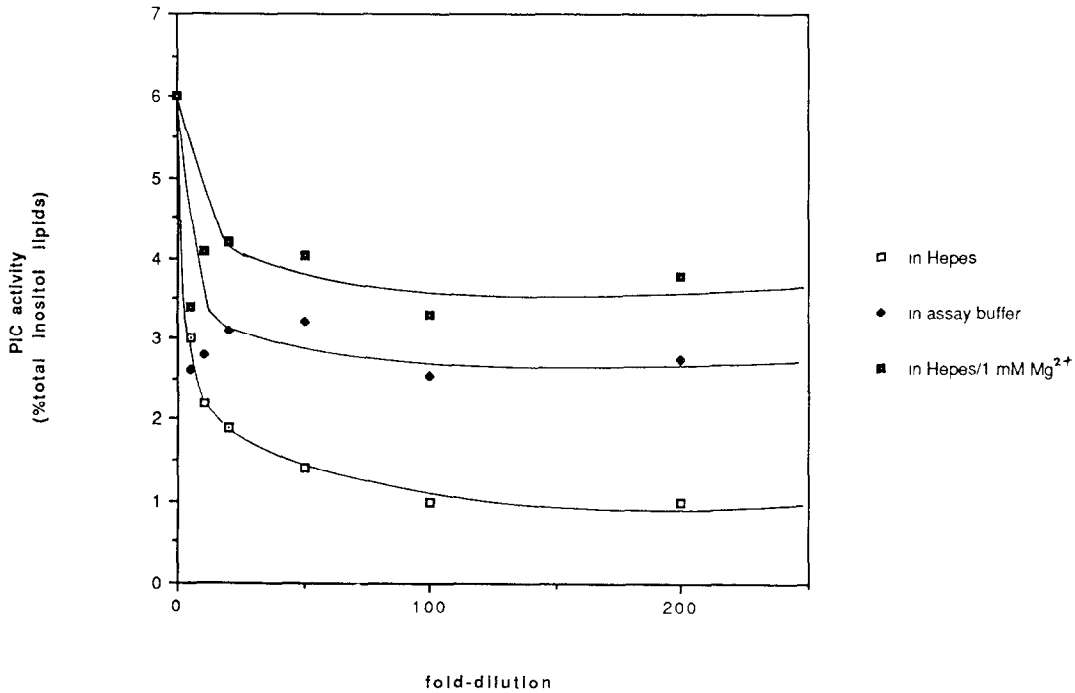

Fig. 3. Dilution effect on the endogenous PIC activity of clean ghosts. Clean erythrocyte ghosts were diluted to different degrees in 10 mM HEPES, pH 7, 1 mM $MgCl_2$ (■), PIC assay buffer (♦), or 10 mM HEPES, pH 7 (□). PIC activities were measured with endogenous [^{3}H]inositol-labelled lipids as described in section 2.

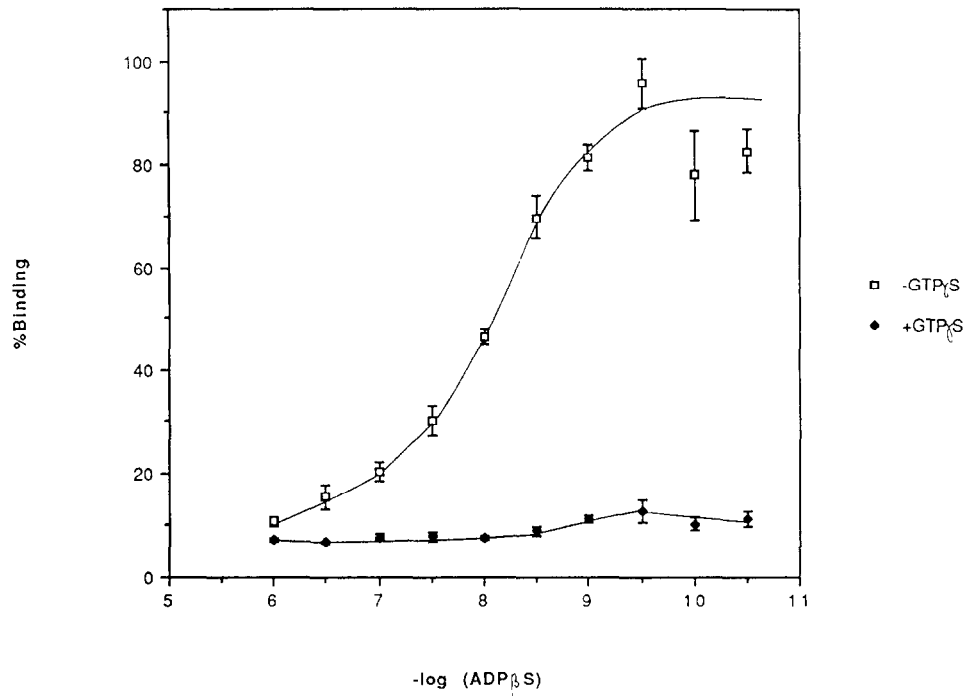

Fig 4 Agonist binding to PIC-depleted membranes and effect of GTPγS on the binding Membranes were depleted of their endogenous PIC activity following the procedure described in section 2. Binding of [^{35}S]ADPβS was determined in the absence (open symbols) or presence (closed symbols) of 5 μM GTPγS. The procedure was as reported in [3].

G-protein, as shown by the guanine nucleotide sensitivity of the binding of the purinergic agonist ADPβS to its receptor (Fig. 4). As these membranes (when prepared from [^{3}H]inositol-loaded turkey erythrocytes) demonstrated a 75% loss of endogenous PIC activity when diluted 50-fold in Mg^{2+}-free hypotonic buffer (see Fig. 3), they were used as acceptor membranes for the reconstitution of partially purified PIC activity.

The soluble extract obtained after low-Mg^{2+} treatment and mechanical disruption of the cytoskeleton of clean and fully active turkey erythrocyte ghosts was used as the source for purification of the membrane-associated PIC. The 307-fold purified PIC was used for the reconstitution studies. The first stage of purification involved the elution of the extract from a Fractogel TSK DEAE column using a 300 ml linear gradient of 0–700 mM KCl, a typical example of which is illustrated in Fig. 5. After pooling of the PIC activity peak, a 24-fold purification over the PIC activity of the hypotonic extract was obtained (see also Table I). This sam-

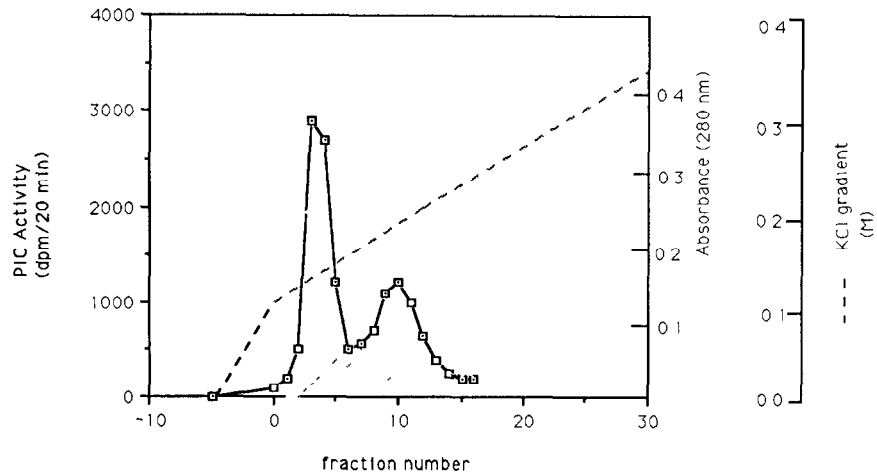

Fig. 5. Chromatography on DEAE Fractogel. The low-Mg^{2+} extract from clean erythrocyte ghosts was loaded onto a 50 ml DEAE Fractogel column. It was eluted with a 300 ml linear gradient of 0–700 mM KCl (---) and 4 ml fractions were collected and assayed for PIC activity (□) as explained in section 2. Protein elution was determined by absorbance at 280 nm (...).

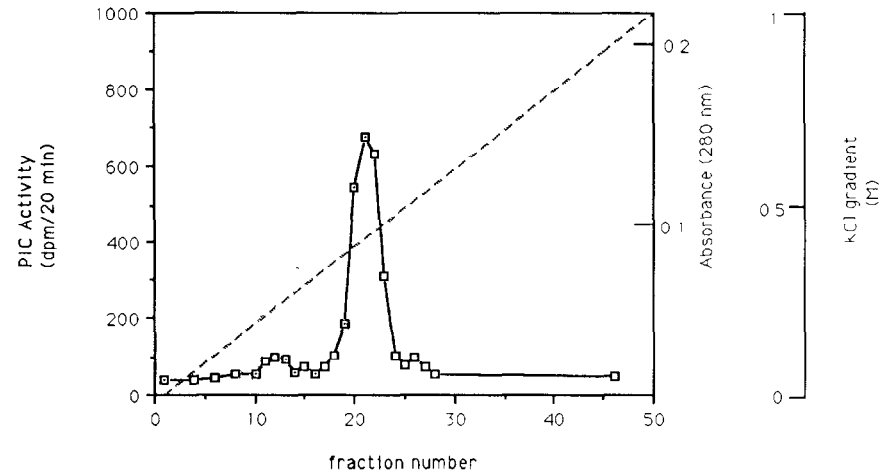

Fig. 6. Chromatography on heparin. Active fractions from the DEAE column were pooled together, diluted 3-fold and loaded onto a TSK gel-heparin 5PW column. It was eluted with a 45 ml linear gradient of 0–900 mM KCl (---) and 1 ml fractions were collected and assayed for PIC activity (□) as explained in section 2. Protein elution was determined by absorbance at 280 nm (...).

ple was diluted, applied to a TSK-heparin column and eluted with a 45 ml linear gradient of 0–900 mM KCl (Fig. 6). The pooled fractions from the PIC activity peak showed a further 1.8-fold purification over the DEAE pool, giving a 307-fold purification in the PIC activity over the original erythrocyte ghost preparation (Table I). This purification could be improved slightly if the sample was concentrated and loaded onto a Superose 12 column, allowing a final 346-fold purification to be obtained. However, it was the pooled PIC activity obtained after the heparin chromatography that was used to investigate some of the kinetic properties of this enzyme. As shown in Table II, the K_m values obtained for both substrates, PtdIns(4,5)P_2 and PtdIns(4)P, were 8 μM and 15 μM respectively. These values, and the higher activity of the PIC towards PtdIns(4,5)P_2, were in accordance with the results obtained in intact ghosts (see Table II), where the endogenous substrate PtdIns(4,5)P_2 was hydrolysed more rapidly than PtdIns(4)P. An investigation of the Ca^{2+}-dependence of this PIC activity (Fig. 7) demonstrated its activation from 10 nM to 1 μM free Ca^{2+}, with a plateau in the activity from 1 μM to 1 mM free Ca^{2+}. This profile was analogous to the one obtained with endogenous substrate in intact ghosts (see also Fig. 7), indicating that the membrane-bound PIC of clean ghosts was extracted and enriched by this procedure.

Reconstitution of the 307-fold purified PIC activity was performed using membranes with endogenous [^{3}H]inositol-labelled lipids that had been prepared with a 50-fold dilution in Mg^{2+}-free hypotonic buffer, as detailed above. Addition of 3 μg of the partially purified PIC restored up to 60% of the agonist and guanine nucleotide response of the PIC-depleted membranes (using 10 μg membrane protein), as compared to the concentrated membrane preparation, as illustrated in Fig. 8. This thus demonstrates that the partially purified enzyme retained the property of G-protein-dependent regulation. In order to determine the molecular weight of this reconstituted membrane-bound PIC, the preparation was analyzed by non-reducing SDS-PAGE and

Table I

Purification of PIC

	PIC specific activity (nmol/mg·30 min)	Recovery (%)	Purification (-fold)	Protein (mg)
Ghosts	0.45	100	1	2000
Hypotonic extract	3.27	31	7	125
DEAE-column	77.25	14	170	1.75
Heparin-column	139 51	5	307	0.24

The procedure followed for purification of membrane-bound turkey erythrocyte PIC is detailed in section 2 The table is derived from a single purification and is representative of more than six separate preparations using the same protocol. Enzyme activity was determined with pure phosphatidylinositol(4,5)-biphosphate as described in the text.

Table II

Kinetic constants of PIC

Substrate	K_m (μM)	V_{max} (nmol/min·mg)	Endogenous activity in ghosts (a.u.)
PtdIns(4,5)P_2	8	10	100
PtdIns 4 P	15	7	60

Purified PIC was assayed with pure phosphoinositides and the PIC activity of clean ghosts was assayed with endogenous [^{3}H]inositol-labelled lipids as described in section 2.

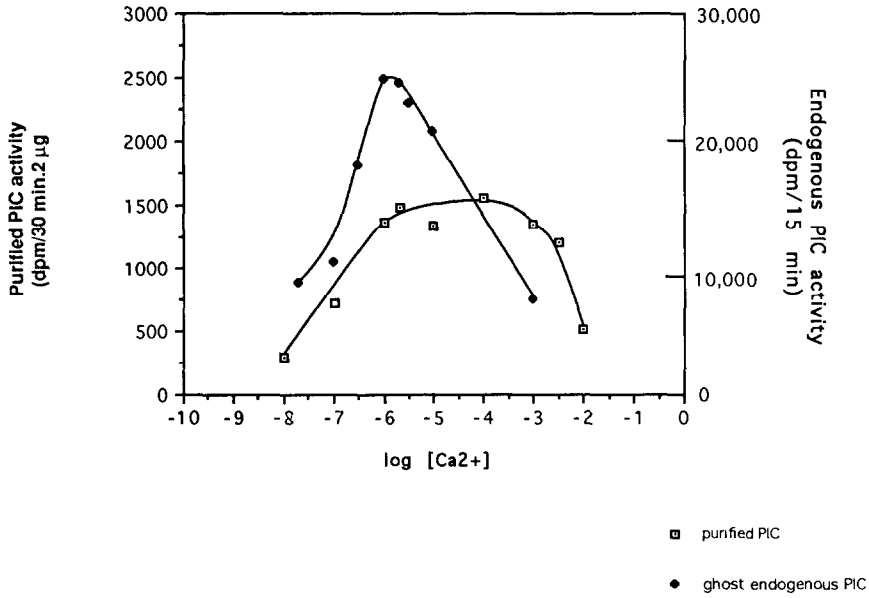

Fig. 7. Effect of calcium ions on PIC activity. PIC activity of the pooled fractions following heparin chromatography (□) was assayed with pure PtdIns(4,5)P_2 as described in section 2. PIC activity in clean erythrocyte ghosts (♦) was measured with endogenous substrate in the presence of 5 μM GTPγS as described in section 2.

renatured following the method described above. Three gel slices revealed PIC activity after this procedure and a 52 kDa protein correlated with renatured PIC activity (Fig. 9).

4. DISCUSSION

A 150 kDa PIC has been purified to homogeneity from the cytosolic fraction of turkey erythrocytes and

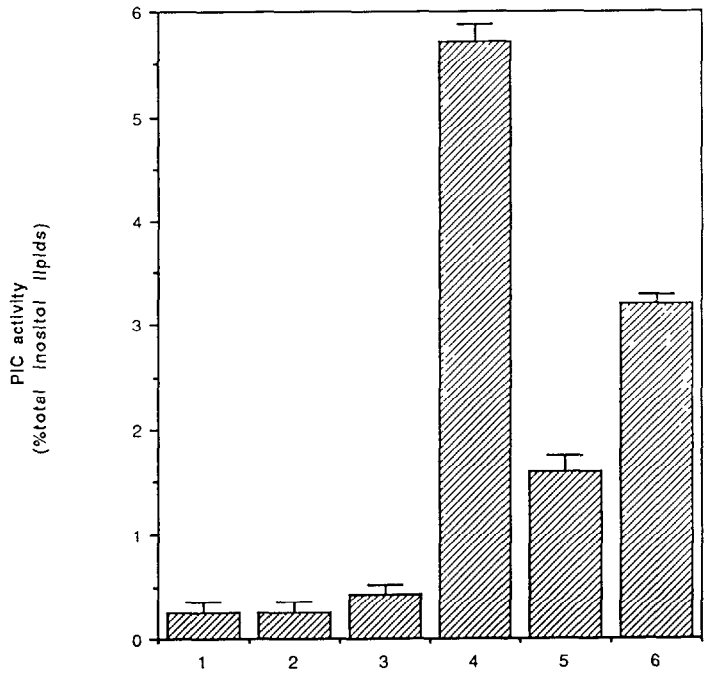

Fig. 8. Reconstitution of a G protein responsive PIC activity onto PIC-depleted membranes. Assays were carried out with endogenous [^{3}H]inositol-labelled lipids as described in section 2. 1 = basal PIC activity of concentrated membranes; 2 = basal PIC activity of dilute membranes; 3 = basal PIC activity of dilute membranes reconstituted with purified PIC; 4 = PIC activity of concentrated membranes under stimulation with 5 μM GTPγS; 5 = PIC activity of dilute membranes under stimulation with 5 μM GTPγS; 6 = PIC activity of dilute membranes reconstituted with purified PIC under stimulation with 5 μM GTPγS.

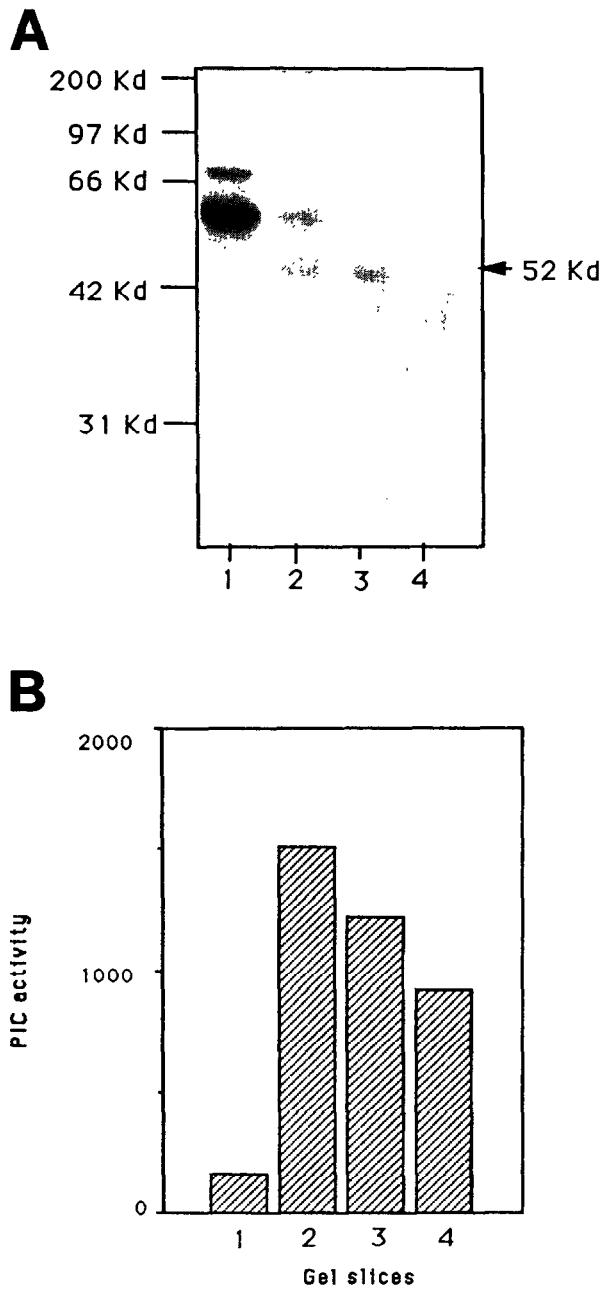

Fig. 9. Purified membrane-associated PIC. (A) Silver stained SDS-PAGE gel of the purified PIC after the renaturation treatment. (B) PIC activity of the corresponding fractions. PIC activities were measured using pure PtdIns(4,5)P_2, with all procedures as described in section 2.

has been proven to reconstitute onto membranes [6], but the possible translocation of cytosolic PIC onto the plasma membrane and the mechanism of its binding to the correct site on the membrane so that activation by the corresponding G-protein occurs as a response to agonists, is still to be studied. In this work the starting material for isolation of the G-protein responsive PIC from turkey erythrocytes was active clean erythrocyte ghost membranes depleted of soluble cytosolic proteins. These ghosts possess a fully active system responsive to purinergic agonists [2,3,4] and this response is mediated by a G-protein which activates a PIC strongly associated with the structure of these ghosts. We show that this PIC is not an intrinsic protein of the membrane. It can be extracted by low-Mg^{2+} treatment, by high concentrations of salt (data not shown), or by disruption of the erythrocyte cytoskeleton, which suggests an important role for this subcellular structure consisting of microfibers, microtubules and intermediate filaments in the signal transduction. It appears that the cytoskeleton is the structure that keeps PIC bound to the correct active site on the membrane in the proximity of the G-protein and the purinergic receptor. Disruption of the cytoskeleton released the active PIC from its plasma membrane binding site and rebinding was achieved by addition of a high concentration of the active PIC to diluted membranes previously depleted of their endogenous PIC.

Cytoskeleton-free membranes, which lacked their intrinsic PIC, were observed under electron microscopy (Plate 1). They still retained their adrenergic receptor coupled to a G-protein (Fig. 4). The purinergic agonist ADPβS bound PIC depleted membranes and its receptor was still coupled to the corresponding G-protein which was activated in the presence of GTPγS (Fig. 4). We released PIC from active ghosts by two methods (1) On a large scale, for PIC purification, we washed ghosts in a low-Mg^{2+} buffer, which rendered a very fragile cytoskeleton [15], and then homogenized them with a tight-fitting motor-driven Teflon pestle in fresh low-Mg^{2+} buffer. The soluble fraction after this treatment contained the highest specific PIC activity (Fig. 2) and was the starting material for PIC purification. (2) On a smaller scale, in order to obtain PIC-depleted membranes for the rebinding of the partially purified enzyme, [^{3}H]inositol-labelled ghosts were diluted 50-fold in a Mg^{2+}-free medium (10 mM HEPES) (Fig. 3). Addition of 3 μg of purified PIC to these labelled membranes restored 60% of their response to GTPγS (Fig. 8).

Kinetic parameters, such as affinity for different phosphoinositides and dependence on calcium ions, of the endogenous PIC activity in clean ghosts and the purified enzyme coincided (Table II). After five purification steps of the membrane-extracted PIC activity, including non-reducing SDS-PAGE and renaturation treatment, a 52 kDa protein correlated with PIC activity, indicating that this 52 kDa protein contained the catalytic site for binding and hydrolysis of the lipid. This result did not exclude the possibility that there was another higher molecular weight protein that also contained PIC activity but which was not detected because its refolding after SDS-PAGE and renaturation treatment was not efficient enough to regain activity. Renaturation from the random coil state varies from one protein to another and this fact should be taken into account [13]. A 57 kDa PIC was purified to homogene-

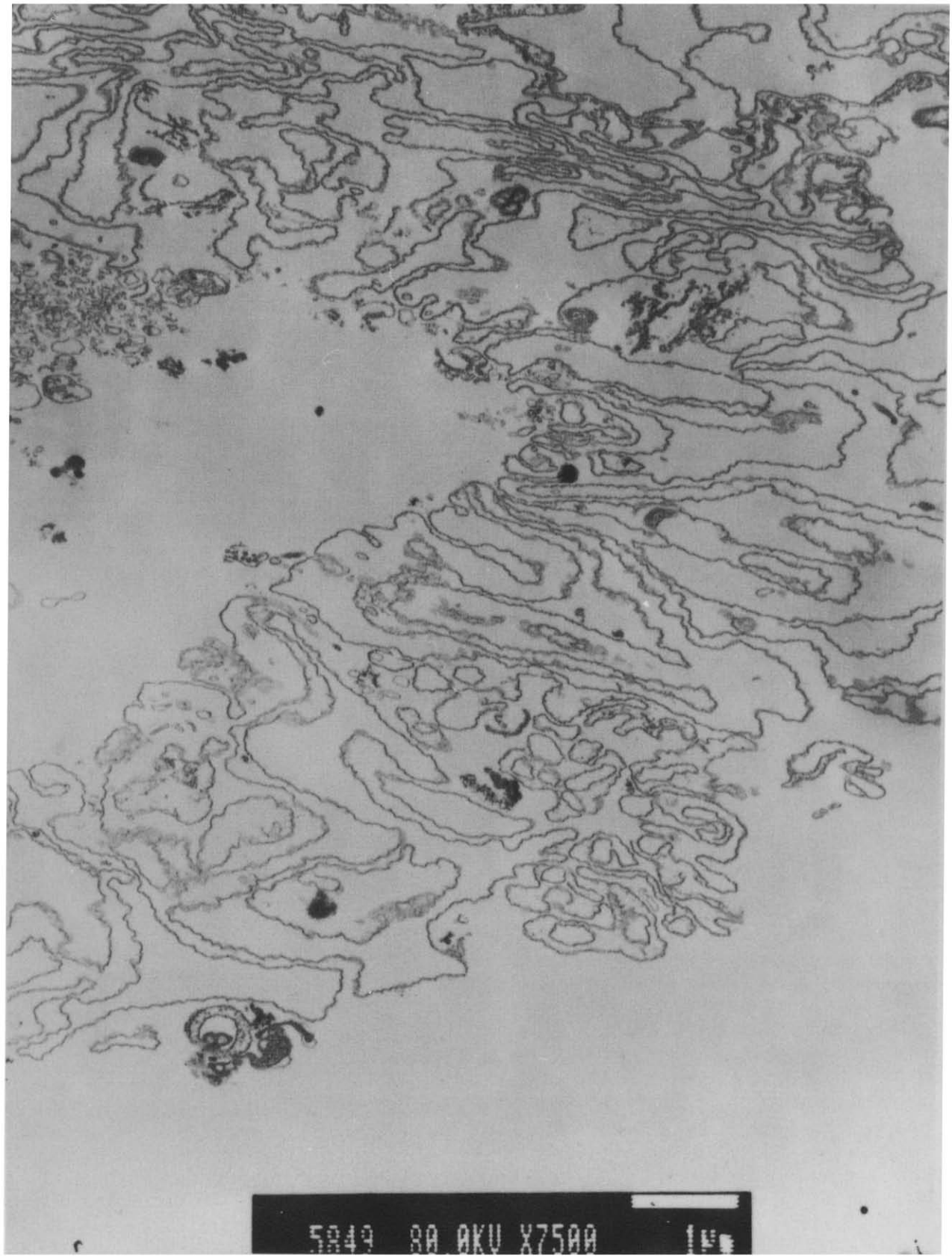

Plate 1. Plasma membranes of turkey erythrocytes. After PIC extraction the cytoskeleton is completely broken and cell shape is lost. Electron micrograph (1:7500 enlargement).

ity from bovine brain [14] that showed a requirement for calcium and a K_m of 90 μM towards PtdIns(4,5)P_2. This 57 kDa PIC was associated in a ratio 1:1 with a myelin basic protein, was stimulated by it and phosphorylation/dephosphorylation processes appeared to be involved in regulating its activity. We don't exclude the possibility that the 52 kDa active protein we find associated to membranes in turkey erythrocytes could be a proteolytic fragment containing the catalytic domain of a higher molecular weight and full-length PIC which did not recover activity after renaturation treatment.

A point that should be made is that this membrane-associated PIC is not an intrinsic protein of the plasma membrane but is attached to it through electrostatic forces, which can be neutralized by changing the ionic strength of the medium. Mechanical disruption of the cytoskeleton also releases the enzyme, indicating that this spectrin-actin network plays an important role not only in maintaining structures but in the transduction of extracellular signals. It seems that the cyskeleton maintains PIC bound to the correct active site on the membrane in the proximity of the G-protein. More studies are in progress in order to find possible mechanisms of translocation of PIC onto membranes, regulation of that process, possible modification of the enzyme prior to translocation and comparison of aminoacid sequences of two related isoforms of this enzyme, all of which are still important goals to be achieved.

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