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Characterization of phosphoinositide-specific phospholipase C from human platelets

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Phosphoinositide-specific phospholipase C (PI-PLC) from human platelet cytosol was purified 190-fold to a specific activity of 0.68 μmol of phosphatidylinositol (PI) cleaved/min per mg of protein. It hydrolyses PI and phosphatidylinositol 4,5-bisphosphate (PIP_2), but not phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine. The enzyme exhibits an acid pH optimum of 5.5 and has a molecular mass of 98 kDa as determined by Sephacryl S-200 gel filtration. It required millimolar concentrations of Ca^{2+} for PI hydrolysis, whereas micromolar concentrations are optimal for PIP_2 hydrolysis. Mg^{2+} could substitute for Ca^{2+} when PIP_2, but not PI, was used as the substrate. EDTA was more effective than EGTA in inhibiting the basal PI-PLC activity towards PIP_2. Sodium deoxycholate strongly inhibits the purified PI-PLC activity with either PI or PIP_2 as substrate. *Ras* proteins, either alone or in the form of liposomes, have no effect on PI-PLC activity.

INTRODUCTION

A wide variety of tissues and cells when exposed to hormones, neurotransmitters or growth factors respond by increased (poly) phosphatidylinositol (PI) turnover (for reviews, see Berridge, 1984; Majerus *et al.*, 1984; Berridge & Irvine, 1984) and a rapid (seconds in some cases) transient increase in diacylglycerol (DG) and water-soluble inositol products (Abdel-Latif *et al.*, 1977; Billah & Lapetina, 1982; Agranoff *et al.*, 1983; Imai *et al.*, 1983; Perret *et al.*, 1983; Creba *et al.*, 1983; Litosch *et al.*, 1983). Both DG and inositol triphosphate (IP_3) fulfil several criteria of second messengers much the same way as cyclic AMP: they form rapidly, are degraded rapidly and act at very low concentrations. Hence these immediate products of phosphatidylinositol 4,5-bisphosphate (PIP_2) degradation have been proposed as intermediates in the activation of cell function and growth (Berridge, 1984). IP_3 can release Ca^{2+} from intracellular stores (Streb *et al.*, 1983; Burgess, 1984; Joseph *et al.*, 1984), and DG is an activator of protein kinase C (reviewed by Nishizuka, 1984). The enzyme responsible for the hydrolysis of phosphoinositides to DG and inositol phosphates is a phosphodiesterase that acts much like phospholipase C, but shows specificity towards inositol lipids. This phosphatidylinositol-specific phospholipase C (PI-PLC) is widely distributed in mammalian tissues and cells (Dawson, 1959; Thompson & Dawson, 1964; Dawson *et al.*, 1971; Jungalwala *et al.*, 1971; Irvine *et al.*, 1977, 1978, 1979; Richards *et al.*, 1979; Kennerly *et al.*, 1979; Igarashi & Kondo, 1980; Bell *et al.*, 1980; Okazaki *et al.*, 1981; Habenicht *et al.*, 1981; Shukla, 1982; Hofmann & Majerus, 1982*a*). Typically, the enzyme is cytoplasmic in location and Ca^{2+}-dependent, even though there are reports of a membrane-bound PI-PLC activity (Friedel *et al.*, 1969; Lapetina & Michell, 1973; Majunder & Eisenberg, 1974; Lapetina *et al.*, 1976; Allan & Michell, 1974; Akhtar & Abdel Latif, 1978; Downes & Michell, 1981; Van Rooijen *et al.*, 1983; Cockcroft *et al.*, 1984). In platelets stimulated by thrombin, the concept of a direct receptor-mediated breakdown of membrane phosphoinositides by PI-PLC is increasingly accepted (Lapetina *et al.*, 1981*a*,*b*). However, in platelets PI-PLC is localized mainly in the soluble fraction (Mauco *et al.*, 1979; Rittenhouse-Simmons, 1979; Billah *et al.*, 1979, 1980; Hofmann & Majerus, 1982*a*). How and whether cytosolic PI-PLC recognizes the agonist occupation of receptors on the platelet cell surface and breaks down phosphoinositides that are in the membrane to generate the putative second messengers IP_3 and DG is totally unknown.

In spite of the central role that PI-PLC plays in transmembrane signalling, cell differentiation and tumour promotion, there has been only a limited characterization of this enzyme. Two PI-PLC enzymes from sheep seminal vesicles and one PI-PLC from rat liver were highly purified (Hofmann & Majerus, 1982*a*; Takenawa & Nagai, 1981), and factors modulating the activity of these enzymes were studied in great detail (Hofmann & Majerus, 1982*b*; Wilson *et al.*, 1984, 1985). The PI-PLC from platelets has been studied in several laboratories (Chau & Tai, 1982; Hakata *et al.*, 1982; Siess & Lapetina, 1983; Lenstra *et al.*, 1984; Nakamura *et al.*, 1985; Low *et al.*, 1984, 1986). There is uncertainty about the different forms (Chau & Tai, 1982; Low *et al.*, 1984, 1986) and optimum pH for the reaction (Chau & Tai, 1982; Siess & Lapetina, 1983; Low *et al.*, 1986). There is some evidence that a GTP-binding protein is involved in platelet activation via PI-PLC (Haslam & Davidson, 1984). Proteins encoded by *ras* genes are GTP-binding proteins (Scolnick *et al.*, 1979, 1980; Manne *et al.*, 1984) and have been speculated as possible modifiers of PI-PLC (Berridge & Irvine, 1984). It will be interesting to test this possibility directly with purified

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate; DG, diacylglycerol; PI-PLC, phosphoinositide-specific phospholipase C; PAGE, polyacrylamide-gel electrophoresis.

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PI-PLC and *ras* proteins. Ha-*ras* proteins expressed in *Escherichia coli* were highly purified in our laboratory (Manne *et al.*, 1984, 1985). We have now obtained highly purified PI-PLC from human platelets and investigated some of its characteristics, in view of several reported discrepancies about this enzyme, and have tested the effect of recombinant *ras* proteins on PI-PLC activity.

EXPERIMENTAL

Materials

Fresh human platelet-rich-plasma concentrate was obtained from the American Red Cross, Lansing, MI, U.S.A., and processed immediately on arrival. [γ-^{32}P]ATP (5000 Ci/mmol), L-3-phosphatidyl[2-^{3}H]-inositol (17.1 Ci/mmol), L-3-phosphatidyl[U-^{14}C]inositol (270 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]linoleoyl L-3-phosphatidylinositol (58 mCi/mmol), dioleoyl L-α-phosphatidyl-L-[U-^{14}C]serine (30 mCi/mmol) and 1,2-dioleoyl L-3-phosphatidyl[2-^{14}C]ethanolamine (49 mCi/mmol) were obtained from Amersham Corp. L-α-Dipalmitoyl phosphatidyl[*Me*-^{3}H]choline (51 Ci/mmol) and phosphatidyl[2-^{3}H(n)]inositol 4,5-bisphosphate (3.6 Ci/mmol) were purchased from New England Nuclear. Phosphoinositides (bovine brain), phosphatidylinositol 4-monophosphate (PIP) (bovine brain), phosphatidylinositol 4,5-bisphosphate (PIP_2) (bovine brain), phosphatidylcholine (egg), phosphatidylethanolamine (egg), phosphatidylserine (bovine brain), sodium deoxycholate, IP_3, inositol 2-phosphate, phenylmethanesulphonyl fluoride and EGTA were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. PI (plant) was a product of Avanti Polar Lipids, Birmingham, AL, U.S.A. Silica-gel H plates with 1% potassium oxalate were purchased from Analtech, Newark, DE, U.S.A. Purity of commercially obtained and prepared phospholipids was checked on oxalate-coated silica-gel H plates in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) or chloroform/methanol/water/conc. NH_3 (17:13.2:2.8:1, by vol.). Phospholipids were detected with iodine vapour, and radioactive lipids were detected by autoradiography and the spots quantified by liquid-scintillation counting.

Preparation of ^{32}P-labelled PIP_2

^{32}P-labelled PIP_2 (^{32}P-labelled in the 5-phosphate group) was prepared by using PIP and ATP-dependent PIP kinase. PIP kinase was partially purified from rat brain as described by Van Dongen *et al.* (1984) as far as the DEAE-cellulose chromatography step. Some 50 nmol of PIP was phosphorylated for 60 min in 1 ml of reaction mixture with 560 μg of partially purified PIP kinase and 100 μM-[γ-^{32}P]ATP under conditions described previously (Van Dongen *et al.*, 1984; Jolles *et al.*, 1980). The reaction was terminated with 20 ml of chloroform/methanol/12 M-HCl (800:800:3, by vol.), and 3.75 ml of 0.6 M-HCl was added to separate the phases. After a brief centrifugation (3000 *g*/5 min), the upper phase was discarded. The lower chloroform phase was washed repeatedly with 10 ml of chloroform/methanol/0.6 M-HCl (3:48:47, by vol.) until the upper phase was substantially free of radioactivity. The lower phase was subsequently dried under argon and redissolved in 250 μl of chloroform/methanol/water (75:25:2, by vol.) and analysed on oxalate-coated silica-gel H plates with PIP and PIP_2 standards. More than 98% of the radioactivity was found to be associated with PIP_2 spot. About 830 nmol of unlabelled PIP_2 was added to the labelled PIP_2 sample and dried under argon into a thin film. Then 0.8 ml of water was added and sonicated briefly in a bath sonicator, to obtain a white opaque suspension. The specific radioactivity of [^{32}P]PIP_2 was determined at this stage by measuring the total lipid phosphorus with amidol reagent (Kates, 1972), and ranged from 88740 to 95864 c.p.m./nmol in several preparations.

Assay for PI-PLC activity

The assay system that we adopted was based on a reported procedure (Rittenhouse, 1979). The standard assay mixture (final volume 20 μl) consisted of 50 mM-Mes, pH 5.5, 50 μM[^{32}P]PIP_2 (10000–80000 c.p.m.) or 200 μM-L-3-phosphatidyl[2-^{3}H]inositol (6660 c.p.m.) and enzyme. The mixture contained 1 mM-EDTA/0.9 mM-$CaCl_2$ when [^{32}P]PIP_2 was used as the substrate and 10 mM-$CaCl_2$ when [^{3}H]PI was used as the substrate. The reaction was initiated by the addition of enzyme, incubated at 37 °C for 10 min and terminated by the addition of 0.5 ml of ice-cold chloroform/butan-1-ol/12 M-HCl (500:500:3, by vol.) and 0.3 ml of 1 M-HCl. Mixtures were vortex-mixed and then centrifuged in a table-top Eppendorf centrifuge for 3 min to obtain a clear phase separation. Duplicate 100 μl samples of the upper aqueous phase (total 320 μl) were transferred to scintillation vials, 10 ml of Hydrofluor was added and the mixtures were counted for radioactivity in a Beckman LS 7800 liquid-scintillation counter.

Analysis of reaction products

After termination of the reaction and separation of the aqueous phase, a portion of the aqueous phase was neutralized to pH 7.5 with saturated sodium tetraborate and diluted to 5 ml with distilled water, and analysed by anion-exchange chromatography on small (approx. 1 ml) AG I-X8 columns as described by Downes & Michell (1981).

Other methods

Free Ca^{2+} concentrations in the range 1–40 μM were obtained by using $CaCl_2$/EDTA buffers as described by Wolf (1973), at pH 5.5. Free Ca^{2+} concentrations in the range 0.1–4.0 μM were achieved by using $CaCl_2$/EGTA buffers as described by Schatzmann (1973), when the activity was measured at pH 7.0. The total concentrations of EDTA and EGTA in the assays were 1 mM and 2.5 mM respectively. Reported free Ca^{2+} concentrations were uncorrected for temperature, ionic strength and other factors such as phosphoinositides, which are known to bind Ca^{2+}. SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) was carried out as described by Laemmli (1970). Protein was measured as described by Bradford (1976), with bovine serum albumin as the standard.

Purification of PI-PLC from human platelets

Five 50 ml packets (each packet corresponds to 450 ml of whole blood) of platelet-enriched serum were supplemented with EGTA to 1 mM and spun at 1000 rev./min in a table-top IEC centrifuge for 10 min. The supernatant was carefully separated from a small contaminating red-blood-cell pellet and spun in a Beckman centrifuge at 5000 rev./min in a JA17 rotor for 20 min. The pellet was washed twice with 120 mM-

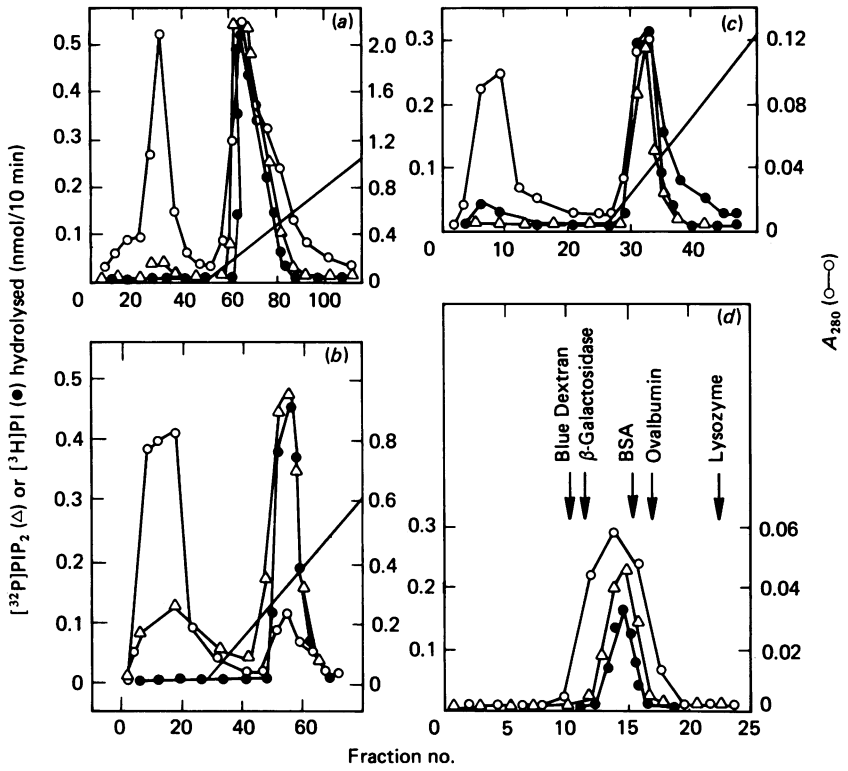

Fig. 1. Chromatography of PI-PLC

(*a*) DEAE-cellulose; (*b*) phosphocellulose; (*c*) heparin–Sepharose; (*d*) Sephacryl S-200. Details of the chromatographic conditions are given in the text. Protein was measured by the A_{280} (○), and 2 μl samples of the fractions were assayed for hydrolysis of PI (●) or PIP_2 (△) under optimal assay conditions. The continuous straight lines in (*a*), (*b*) and (*c*) represent the salt gradients used as described in the text. Arrow positions in (*d*) indicate the positions of various markers (BSA, bovine serum albumin).

NaCl/1 mM-EGTA/30 mM-Tris/HCl, pH 7.4, suspended in 65 ml of Buffer A [20 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol/5% (v/v)glycerol/0.5 mM-phenylmethanesulphonyl fluoride] and sonicated for 3 × 20 s with maximum output in a sonicator ultrasonic processor (Heat Systems Ultrasonics). The extract was spun for 20 min at 5000 rev./min in a JA17 rotor in a Beckman centrifuge, and the supernatant was removed and stored on ice. The pellets were suspended again in 32 ml of Buffer A and sonicated as above, and centrifuged at 5000 rev./min for 20 min in a JA17 rotor. The supernatants were pooled and spun at 33000 rev./min in a Beckman ultracentrifuge (Ti45 rotor) for 65 min at 4 °C. The clear supernatant was separated from the membrane fraction and designated 'S_{100} fraction': it was dialysed against Buffer A.

DEAE-cellulose chromatography

After dialysis, the sample was clarified by centrifugation at 15000 *g* for 10 min, and loaded on a DEAE-cellulose column (1.6 cm × 25 cm) pre-equilibrated with Buffer A. The column was first washed extensively until the A_{280} of the eluate was about 0.10 with Buffer A containing 50 mM-NaCl, and then developed with a linear gradient of 50–600 mM-NaCl in Buffer A in a total volume of 300 ml. Fractions (5 ml) were collected and small portions were assayed for PLC activity. About one-third of the loaded protein was washed unbound by Buffer A containing 50 mM-NaCl and contained very little, if any, PLC activity. Most of the bound protein was eluted between 0.12 mM- and 0.30 M-NaCl as a broad peak and contained PLC activity (Fig. 1*a*). Active fractions were pooled and dialysed against Buffer A.

Phosphocellulose chromatography

The dialysed sample from the DEAE-cellulose step was loaded on a column (1.6 cm × 25 cm) of phosphocellulose (P11; Whatman) equilibrated with Buffer A, and the column was washed with the same buffer until the A_{280} of the eluate fell to the baseline. The column was eluted with a linear gradient of 0–0.6 M-NaCl in Buffer A (total 200 ml); 5 ml fractions were collected. Most of the applied protein was not bound to the column. With PIP_2 as the substrate, two peaks of apparent PLC activity were detected, one in the unbound fraction and the other in fractions eluted by 0.25–0.36 M-NaCl (Fig. 1*b*). However, only one peak of PLC activity was detected when PI was used as the substrate (Fig. 1*b*). When the products of PIP_2 hydrolysis by the unbound fraction from phosphocellulose chromatography were analysed by anion-exchange chromatography on AG 1-X8, it was found that the radioactivity was not associated with IP_3. Most of the liberated radioactivity was identified as $[^{32}P]P_i$ (results not shown). On the other hand, when the products of PIP_2 hydrolysis by the bound fraction were analysed as above, all the water-soluble radioactivity was identified as IP_3; moreover, the activity in the unbound fraction was 3–4-fold higher when the reaction was carried out at pH 7.5

Table 1. Purification of PI-PLC from 27.5×10^{10} platelets

Activity was determined by using ^{3}H-labelled PI.

Step	Total protein (mg)	Total activity (nmol/min)	Sp. activity (nmol/min per mg)	Purification (fold)	Yield (%)
Total sonicated preparation	357.6	1287	3.6	1	100
S_{100}	190.4	1134	5.96	2	88
DEAE-cellulose	98.76	1037	10.5	3	81
Phosphocellulose	11.36	959	84.4	23	74
Heparin–Sepharose	1.472	522	354.8	98	40
Sephacryl-S-200	0.431	293	680	189	23

(results not shown). Presumably, the unbound fraction has a phosphatase activity which is acting on PIP_2, liberating a labelled phosphate moiety. As a result, we concluded that only the bound fraction contained PLC activity, and these fractions were pooled and dialysed against Buffer B [20 mM-Mops (pH 6.8)/100 mM-NaCl/1 mM-dithiothreitol/0.5 mM-phenylmethanesulphonyl fluoride/5% glycerol].

Heparin–Sepharose chromatography

The dialysed enzyme fraction from the phosphocellulose step was subjected to heparin–Sepharose chromatography, with a 0.9 cm × 10 cm column of heparin–Sepharose eluted with Buffer B. The enzyme was loaded on the column and washed until the eluate was substantially free of 280 nm-absorbing material. PLC activity was bound to the heparin–Sepharose column and eluted at about 0.17 M-NaCl when the column was eluted by a linear gradient of 0.1–0.6 M-NaCl in Buffer B (total volume 100 ml) (Fig. 1*c*). Active fractions containing more than 10% of the maximum activity were pooled and concentrated by Amicon PM 30 membrane filtration to about 2 ml.

Sephacryl S-200 chromatography

The concentrated sample from the previous step was then subjected to gel filtration on a column (1.6 cm × 100 cm) of Sephacryl S-200 pre-equilibrated with Buffer A containing 100 mM-KCl. PLC activity was eluted as a single symmetrical peak, whose position was between β-galactosidase (M_r 116250) and bovine serum albumin (M_r 66200) markers (Fig. 1*d*). Active fractions were pooled and concentrated by Amicon PM 10 membrane filtration to 2 ml and stored in liquid N_2 as small batches.

RESULTS

Purification of PI-PLC

Most of the PI-PLC activity was found in the soluble fraction after high-speed (100000 *g*) centrifugation of the platelet homogenate. When the S_{100} fraction was dialysed against Buffer A and loaded on a DEAE-cellulose column, all the PI-PLC activity was bound to the column and was eluted as a single peak of activity, in contrast with the reported observation (Chau & Tai, 1982) of resolution of platelet PI-PLC into two different forms, one not retained and the other retained on DEAE-cellulose under similar conditions. In four different purifications, no PI-PLC was found in the pass-through fractions from the DEAE-cellulose chromatography. We routinely dialysed platelet cytosol before application to DEAE-cellulose, whereas Chau & Tai (1982) loaded their cytosol preparation directly without dialysis. Before phosphocellulose chromatography, the enzyme preparation yielded P_i and IP_3 in approximately equal amounts when PIP_2 was used as the substrate (results not shown). Apparently a phosphatase is acting directly, on either PIP_2 or IP_3 to form P_i, and for this reason the specific activity of PI-PLC in the S_{100} fraction or the DEAE-cellulose fraction could not be obtained with PIP_2 as substrate. Phosphocellulose chromatography separates this phosphatase activity from the PI-PLC activity (Fig. 1*b*). PI-PLC activity

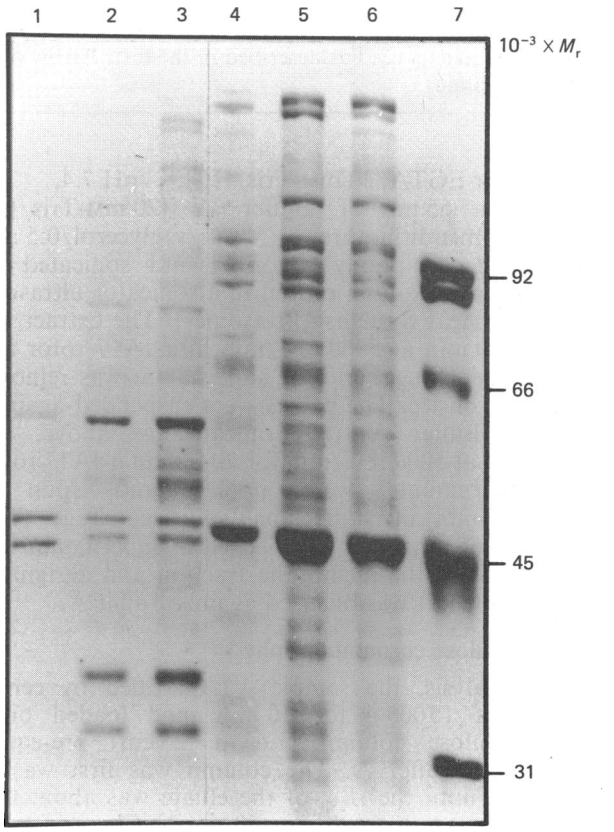

Fig. 2. SDS/PAGE of PI-PLC at various stages of purification

Lane: 1, Sephacryl S-200-purified PI-PLC; 2, heparin–Sepharose-chromatography preparation; 3, phosphocellulose-chromatography preparation; 4, DEAE-cellulose-chromatography preparation; 5, S-100; 6, total sonicated platelet preparation; 7, M_r standards.

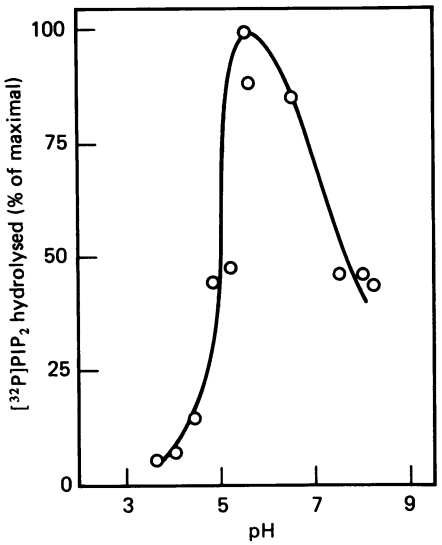

Fig. 3. Effect of pH on PI-PLC

PI-PLC activity of Sephacryl-S-200-purified fraction was determined with 100 ng of protein and PIP_2 as the substrate in the presence of 50 mM buffer of the indicated pH. Free Ca^{2+} concentrations of 40 μM were maintained constant by using a two-metal-ion buffer system, with Mg^{2+} as the secondary ion (4 mM), Ca^{2+} as the primary ion (0.5 mM) and EDTA as the chelator (1 mM). The EGTA buffer system is valid only between pH 7.5 and 10, whereas the EDTA buffer system used here holds good for pH 5.5–10. Acetic acid/sodium acetate for pH 3.6–5.6, Mes/KOH for pH 5.5–6.7, Mops for pH 6.9–7.9 and Hepes for pH 7.2–8.2 were used. The pH-versus-activity curve for PI hydrolysis with 10 mM-$CaCl_2$ in the assay is very similar to that shown.

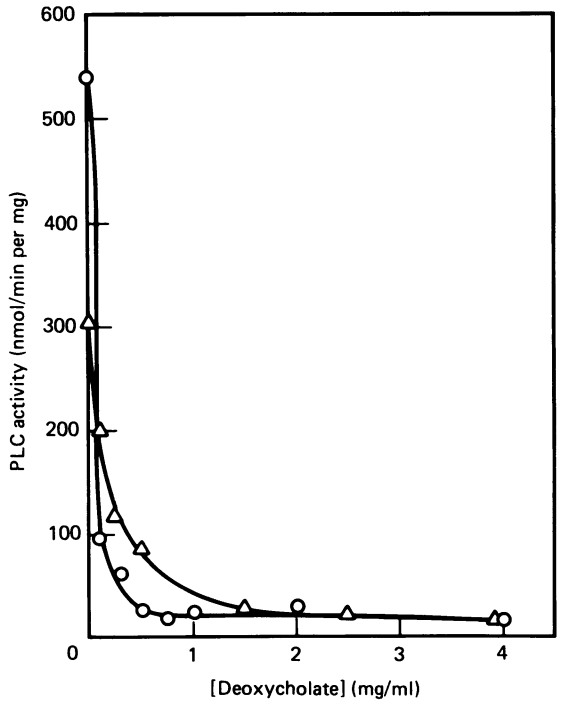

Fig. 4. Effect of sodium deoxycholate on PI-PLC activity

PI-PLC activity was measured as described in the Experimental section with either PI (○) or PIP_2 (△) as substrate, at the indicated concentrations of sodium deoxycholate.

was bound to the phosphocellulose column, whereas phosphatase was not retained under the conditions described. The purification is summarized in Table 1 and shows an overall purification of 190-fold with about 20% recovery. The SDS/PAGE pattern of PI-PLC at various stages of purification is shown in Fig. 2. The final enzyme preparation showed three major polypeptides, at 58, 45 and 38 kDa, when the gel was stained with Coomassie Blue. However, many minor contaminating bands were clearly visible when the gel was silver-stained.

Properties of PI-specific PLC

The purified enzyme is stable when stored in liquid N_2 or at −80 °C, and rapidly loses its activity at room temperature. There is considerable discrepancy in the literature as to the optimal pH for PI-PLC activity of platelets (Chau & Tai, 1982; Siess & Lapetina, 1983). We re-investigated this problem with purified PI-PLC, with both PI and PIP_2 as the substrates. Purified PI-PLC showed an acid pH optimum of 5.5, and the activity decreased sharply below pH 5.0 and above pH 7.0 (Fig. 3). Addition of detergents such as deoxycholate (0.1–4 mg/ml) had no effect on the pH optimum, in contrast with the sheep seminal-vesicle PI-PLC enzymes, which are reported to exhibit maximal activity around neutral pH in the presence of 1 mg of deoxycholate/ml

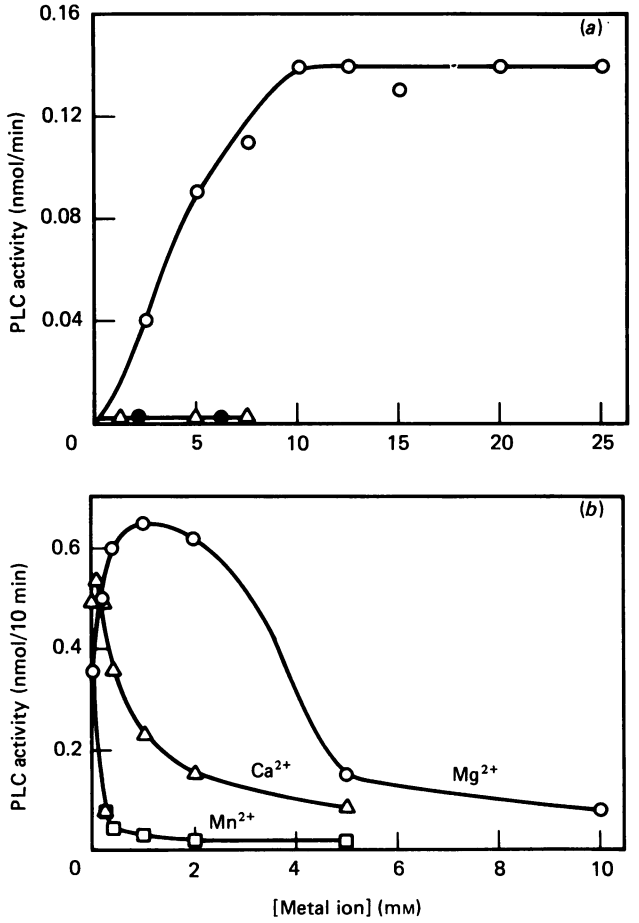

Fig. 5. Effect of Ca^{2+}, Mg^{2+} and Mn^{2+} on PI-PLC activity

PI-PLC activity was determined with PI (*a*) or PIP_2 (*b*) as the substrate at the indicated concentrations of different metal ions: ○, $CaCl_2$; △, $MgCl_2$; ●, $MnCl_2$.

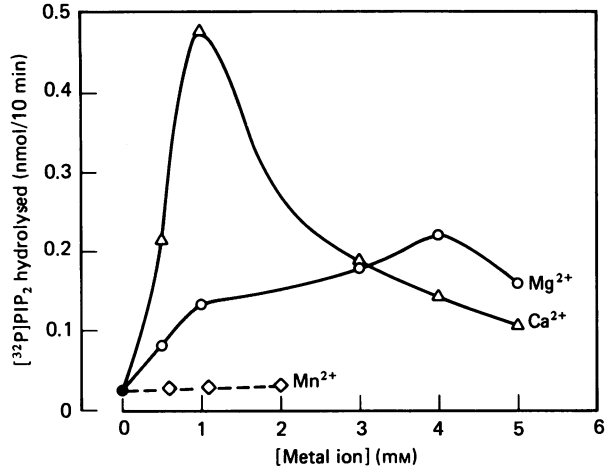

Fig. 6. Effect of Ca^{2+}, Mg^{2+} and Mn^{2+} on EDTA-inhibited PI-PLC activity

PI-PLC activity that was 95% inhibited by 1 mM-EDTA was measured as function of different concentrations of various metal ions, with PIP_2 as the substrate.

(Hofmann & Majerus, 1982*a*). In fact, the PI-PLC activity of human platelets is strongly inhibited by deoxycholate (Fig. 4). Addition of 0.5 mg of deoxycholate/ml to the assay mixture inhibited the PI-PLC activity towards PI and PIP_2 by 95% and 72% respectively. Both PI and PIP_2 are hydrolysed by the purified PI-PLC in a time- and enzyme-concentration-dependent manner. The reaction proceeded in a linear fashion with time until about 35–40% of the added PI or PIP_2 was converted into the products. About 60% of PIP_2 and 40% of PI in the assay mixture were converted into products with linear kinetics with increasing amounts of purified enzyme. The molecular mass of the purified enzyme was around 98000 kDa, as determined on a calibrated Sephacryl S-200 column. Since the enzyme after the final step of purification is not homogeneous, it is questionable whether the three major Coomassie-Blue-stainable bands (Fig. 2) constitute subunits of the native enzyme. However, no proteins of 70–100 kDa are detected in SDS/PAGE of the purified enzyme after silver staining. Hence, in all probability the enzyme is composed of subunits whose identity is not established.

Ca^{2+} requirement

Purified PI-PLC from human platelets exhibits some rather interesting and distinct metal-ion requirements that depend on the substrate in the assay mixture. Without any added Ca^{2+} (or other metal ion), PI-PLC activity towards PIP_2 was significant (Fig. 5*b*), whereas PI was not hydrolysed at all (Fig. 5*a*). The basal PI-PLC activity towards PIP_2 was substantially stimulated by increasing concentrations of Mg^{2+} and Ca^{2+}, but not Mn^{2+} (Fig. 5*b*). In fact, Mn^{2+} strongly inhibits the activity, as do higher concentrations of Mg^{2+} (3 mM) and Ca^{2+} (0.4 mM) (Fig. 5*b*). On the other hand, PI-PLC activity towards PI substrate showed a stringent Ca^{2+} requirement in the millimolar range (optimum 10 mM), and was not inhibited by further increases in Ca^{2+} concentration up to 25 mM (Fig. 5*a*). In addition, both Mg^{2+} and Mn^{2+} were ineffective in substituting for Ca^{2+} in the reaction. The basal PI-PLC activity towards PIP_2 could be abolished by either EDTA or EGTA. Addition of 0.5 mM-EDTA to the reaction mixture was enough to inhibit the basal activity completely, whereas EGTA required 10 mM final concentration (results not shown). It was decided to study how the EDTA-inhibited PI-PLC activity would respond to addition of exogeneous Ca^{2+}, Mg^{2+} and Mn^{2+}. The results of such an experiment are shown in Fig. 6. Both Ca^{2+} and Mg^{2+} are effective in restoring the initial PI-PLC activity, even though the former was able to give much greater stimulation. Again, higher Ca^{2+} concentrations (2 mM) were inhibitory. Free Ca^{2+} concentrations in the micromolar range were obtained by using various ratios of Ca^{2+} to EDTA in the assay mixture (Wolf, 1973), and the PI-PLC activity towards PI and PIP_2 is shown in Fig. 7. Only PIP_2 was hydrolysed under these conditions, with maximum activity in the range 10–20 μM free Ca^{2+}. These free Ca^{2+} concentrations should be regarded as high, since PIP_2 in the assay mixture binds Ca^{2+} and may decrease the effective free Ca^{2+} concentrations (Allan & Michell, 1974; Irvine & Dawson, 1978). PI was not hydrolysed when the free Ca^{2+} concentrations were in the micromolar range (Fig. 7), and only when the Ca^{2+} concentrations were raised to 10 mM in the assay did we observe PI hydrolysis (results not shown). When free Ca^{2+} concentrations in the micromolar range were achieved by using two metal-ion buffer systems (Ca^{2+} as the primary, Mg^{2+} as the secondary, and EDTA as the chelator), the PI-PLC activity did not respond in a saturable way as in Fig. 7, but showed the same activity (comparable with Ca^{2+} or Mg^{2+}-stimulated activity) at all free Ca^{2+} concentrations tested (Fig. 8). This may not be surprising, since the Mg^{2+}/EDTA ratio in the two-metal-ion buffer system used was 4, and at these concentrations Mg^{2+} is already shown to be an effective metal ion for

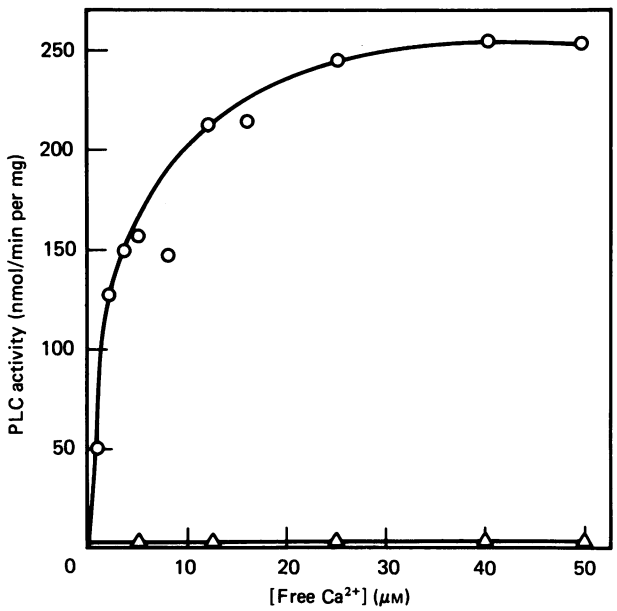

Fig. 7. PI-PLC activity as a function of free Ca^{2+} concentration

PI-PLC activity was measured with either PI (△) or PIP_2 (○) as the substrate at the indicated free Ca^{2+} concentrations obtained as described (Wolf, 1973) by using an EDTA/$CaCl_2$ buffer system. This system is useful to vary Ca^{2+} concentrations at pH 5.5 between 1 and 40 μM.

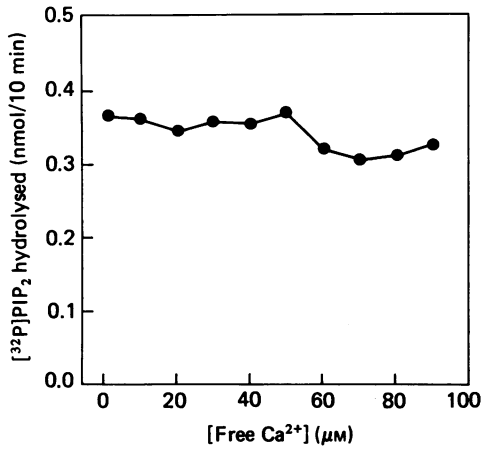

Fig. 8. PI-PLC activity as a function of free Ca^{2+} concentration in a two-metal-ion buffer system

PI-PLC activity was measured with PIP_2 as the substrate at the indicated free Ca^{2+} concentrations obtained as described (Wolf, 1973) by using a two-metal-ion buffer system, with Ca^{2+} as primary, Mg^{2+} as secondary and EDTA as the chelator. The concentration of free Ca^{2+} was varied by changing the total concentration of Ca^{2+}, with total concentrations of EDTA and Mg^{2+} fixed at 1 mM and 4 mM respectively.

PIP_2 hydrolysis by PI-PLC. The fact that the PI-PLC activity towards PIP_2, is not affected by increasing free Ca^{2+} concentrations in the presence of EDTA and Mg^{2+} supports the previous observations (Fig. 5*a*) that Mg^{2+} could effectively substitute for Ca^{2+} for PIP_2 hydrolysis by PI-PLC. Moreover, addition of Ca^{2+} to Mg^{2+}-stimulated PI-PLC (no EDTA) did not significantly increase its activity, and in fact the activity was strongly inhibited by Ca^{2+} concentrations in excess of 0.5 mM (results not shown). Finally, the product released from PIP_2 hydrolysis in the presence of Mg^{2+} or Ca^{2+} is in fact IP_3. The results shown in Fig. 9 clearly show that IP_3 is the only radioactive product formed from PIP_2 whether Ca^{2+} or Mg^{2+} was used in the assay.

Substrate requirement and specificity

As described above, PI-PLC activity from platelets exhibits similar properties (e.g. identical pH optimum, inhibition by deoxycholate), whether PI or PIP_2 is used as the substrate. However, the enzyme required millimolar Ca^{2+} concentrations to hydrolyse PI, whereas micromolar concentrations were optimal for PIP_2 hydrolysis. Under the optimal conditions for PIP_2 hydrolysis, the effect of PIP_2 on the rate of PIP_2 cleavage was determined (Fig. 10). The apparent K_m as obtained from a double-reciprocal plot (Fig. 10 inset) was 10–50 μM. The $V_{max.}$ values were 1.25–2.5 μmol of PIP_2 cleaved/min per mg of protein. The activity of the enzyme was tested against L-α-phosphatidyl-L-[U-^{14}C]serine, L-3-phosphatidyl[2-^{14}C]ethanolamine and [^{3}H]phosphatidylcholine under conditions that are optimal for hydrolysis of PI and PIP_2 in separate experiments. In no case was any activity found by assay of radioactive products, and an activity equivalent to 0.5% of that with PI or PIP_2 would have easily been detected by this assay.

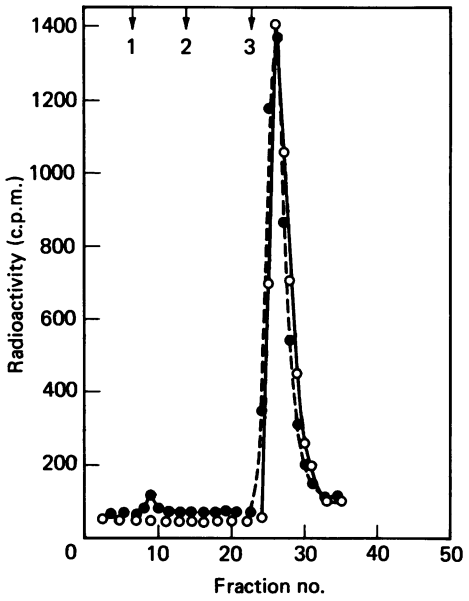

Fig. 9. Anion-exchange chromatography of water-soluble products of PIP_2 hydrolysis in the presence of Ca^{2+} (○) or Mg^{2+} (●)

PIP_2 was hydrolysed in the presence of Ca^{2+} or Mg^{2+} and PI-PLC. After termination of the reaction, the aqueous layer was adjusted to neutral pH with sodium tetraborate, diluted to 5 ml and chromatographed on an AG 1-X8 column as described by Downes & Michell (1981). Arrows indicate the positions where the indicated eluents were applied: inositol monophosphate is eluted by 1 (0.2 M-ammonium formate/0.1 M-formic acid), inositol by 2 (0.4 M-ammonium formate/0.1 M-formic acid) and IP_3 by 3 (1.0 M-ammonium formate/0.1 M-formic acid).

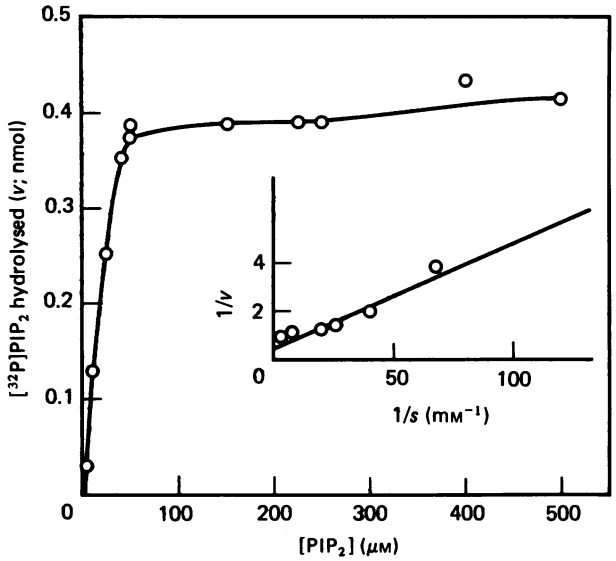

Fig. 10. Effect of PIP_2 on the rate of the reaction

Assays were performed as described in the Experimental section at the indicated concentrations of PIP_2. The inset shows the Lineweaver–Burk plot of the data.

Effect of *ras* proteins in PI-PLC activity

Ha-*ras* proteins expressed and purified from *E. coli* were tested for their effect on PI-PLC activity. Ha-*ras* proteins either in solution or in the form of liposomes

(lysophosphatidylcholine) had no significant effect on PI-PLC activity towards either PI or PIP_2. Also, *ras* proteins did not show any specific association with PI-PLC as tested by sucrose-density-gradient analysis. The studies on effect of *ras* proteins on PI-PLC activity were carried out in the presence of GTP as well as GDP. Purified *ras* proteins were initially incubated with 2 μM-GTP or -GDP under conditions that promote maximal guanine nucleotide binding (Manne *et al.*, 1984, 1985; Manne & Kung, 1986). The *ras* proteins in the form of ras·GTP or ras·GDP complex were then added to the PI-PLC assay mixture.

DISCUSSION

We have partially purified and studied some of the properties of PI-PLC from human platelets. Even though PI-PLC enzymes from several sources are reported to have acid pH (5.5) optima (Hofmann & Majerus, 1982*a*; Siess & Lapetina, 1983), the rat liver enzyme was shown to be optimally active at pH 7.0 (Takenawa & Nagai, 1981). In fact, the partially purified human platelet enzyme was first reported to be optimally active at pH 7.0 (Chau & Tai, 1982), but this was later challenged by Siess & Lapetina (1983). Our results with purified enzyme agree with those of Siess & Lepatina (1983) in that the enzyme is optimally active at pH 5.5 whether PI or PIP_2 is used as the substrate. Since Ca^{2+} is a necessary cofactor for PI-PLC activity, and Ca^{2+} concentration in single-metal-ion buffer systems using EGTA is strongly influenced by pH (Wolf, 1973), one has to be careful in interpreting the optimum-pH data. We have maintained constant Ca^{2+} concentrations in a two-metal-ion buffer system at different pH values (PIP_2 hydrolysis) and have clearly shown that the PI-PLC from platelets had an acid pH optimum.

The PI-PLC from human platelets exhibits some interesting properties with respect to its Ca^{2+} requirement for PI and PIP_2 hydrolysis. It requires millimolar concentrations of Ca^{2+} for PI hydrolysis which are non-physiological. The partially purified PI-PLC from platelets was reported to be active at 1–100 μM free Ca^{2+} concentrations for PI hydrolysis, and higher concentrations were inhibitory (Chau & Tai, 1982). Our results differ substantially in that we find that PI-PLC activity towards PI is not inhibited by Ca^{2+} concentrations up to 25 mM, and micromolar concentrations of free Ca^{2+} obtained by using either EGTA or EDTA buffer systems were not sufficient for PI hydrolysis. Our results on the Ca^{2+} requirement for PI hydrolysis by the platelet enzyme agree with those of Siess & Lapetina (1983), who studied crude enzyme preparations. PI-PLC from platelets hydrolysed PIP_2 not only in the presence of micromolar concentrations of free Ca^{2+} but also in the presence of Mg^{2+}. Even though Ca^{2+} is much more effective in restoring EDTA-inhibited PI-PLC, Mg^{2+} is also effective to a significant degree for PIP_2 hydrolysis. This may be an interesting and relevant observation, since the breakdown product of PIP_2, namely IP_3, is supposed to release Ca^{2+} from internal stores. If PI-PLC were active only in the presence of Ca^{2+}, how the enzyme could degrade PIP_2, before IP_3 raises the resting Ca^{2+} concentrations of $\simeq$ 150 nM to $\simeq$ 2 μM, has been a puzzling question. Even though several explanations have been given, our observation that PI-PLC breaks down PIP_2 in the presence of Mg^{2+} provides a mechanism for PIP_2 breakdown before Ca^{2+} is released from storage granules. The enzyme could further be activated by now-available Ca^{2+}. Since the enzyme is active in the presence of Mg^{2+}, it is possible that the PI-PLC is switched on permanently, owing to the presence of millimolar Mg^{2+} concentrations inside the cells. However, it may not be the case, if one assumes that the phosphoinositide substrates present in the membrane are not readily available for the enzyme before agonist stimulation. Agonist stimulation could lead to changes in the membrane perturbations which might expose the previously unavailable phosphoinositides to the enzyme for breakdown. Hence the critical step in the activation process through the agonist occupation of receptors is probably the accessibility of the substrate to the cytosolic enzyme. In fact, Irvine *et al.* (1984) proposed that perturbation of the lipid bilayer was more likely to be the controlling mechanism than changes in intracellular Ca^{2+} concentration for the breakdown of PIP_2 into IP_3 and DG.

Our original intention in purifying PI-PLC from platelets was to test the hypothesis that *ras* proteins may be the regulatory components of PI-PLC (Berridge & Irvine, 1984), but we could obtain no evidence for this notion at this stage. We have used *ras* proteins bound to maximal amounts of GTP or GDP in these assays. However, these results still do not exclude such a possibility, since our purified *ras* proteins are produced in *E. coli* and apparently lack lipidation at the *C*-terminal end, which may be important for this activity of *ras* proteins. In fact, it has been shown that *ras*-transformed cells (fibroblasts) contain elevated concentrations of IP_3 and DG compared with non-transformed cells (Fleischman *et al.*, 1986).

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