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Phospholipase C isoforms in vascular smooth muscle and their regulation by G-proteins

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1 We sought to reconstitute and characterize G-protein linked phosphatidyl-D-inositol 4,5-bisphosphate (PIP_2)-directed phospholipase C (PLC) isoform activity in pig aortic vascular smooth muscle.

2 Six soluble PLC isoforms, namely γ_1, δ_1 and β_1 to β_4 were partially separated by heparin affinity chromatography and were identified by Western blotting using specific antibodies.

3 In separate experiments, PLC activity was measured in the eluted fractions. Four of the partially resolved PLC isoforms γ_1, β_4, β_2 and β_1, showed corresponding activity using exogenous [^{3}H]-PIP_2 as substrate.

4 The isolated soluble PLC isoforms were reconstituted with receptors and guanyl nucleotide regulatory proteins (G-proteins) by addition of plasma membranes, the phospholipids which had been prelabelled with [^{3}H]-*myo*-inositol. When so reconstituted PLC β_2, β_3 and β_4 were inhibited (40 ± 9, 47 ± 12 and $40\pm5\%$ respectively $n=12$, $\pm$s.e.mean and each $P<0.05$) by the addition of 1 mM guanosine 5'[$\beta\gamma$-imido]triphosphate (p[NH]ppG).

5 By contrast, when plasma membranes were preincubated with pertussis toxin to inhibit the activity of G-protein subunits $G\alpha_i/\alpha_o$ the activities of PLC β_2, β_3 and β_4 were stimulated (46 ± 11, 31 ± 9 and $37\pm8\%$ respectively, $n=12$, $\pm$s.e.mean and each $P<0.05$) by the addition of p[NH]ppG.

6 Using well resolved fractions containing only PLC β_3, time-dependent activity in the presence of p[NH]ppG was measurable only with membranes pretreated with pertussis toxin.

7 PLC β_3 activity, measured with pertussis pretreated membranes, showed a dose-dependent increase in the presence of p[NH]ppG or guanosine 5'-[γ-thio]triphosphate (GTP[S]). This increase with 10 μM p[NH]ppG or GTP[S] $10\%\pm4$ and $12\%\pm5$ respectively (both $P<0.05$ vs control without GTP analogue $\pm$s.e.mean, $n=10$) was abolished by 50 μM guanosine 5'-[β-thio]diphosphate (GDP[S]) which also reduced constitutive PLC β_3 activity by $9\%\pm4$.

8 G-protein antibodies were used to neutralize PLC activity. Antibody to $G\alpha_q/\alpha_{11}$, added to membrane fractions pretreated with pertussis toxin and assayed with GTP[S], reduced PLC β_3 activity by $21\%\pm6$ $P<0.02$, $n=6$, but was without effect on non-pertussis pretreated membranes. Antibodies to $G\alpha_{i1}/\alpha_{i2}$ had no effect. Antibodies to G-protein β subunits had no effect on PLC β_3 activity with pertussis pretreated preparations but activity without pertussis pretreatment was increased by $30\%\pm10$, $P<0.03$, $n=6$. All results were expressed as % change from controls containing rabbit IgG.

9 In conclusion, pig aortic vascular smooth muscle contains six PLC isoforms. Activation of pertussis sensitive G-protein by GTP analogues results in inhibition of PLC β_3 activity from liberated G-protein $\beta\gamma$ subunits. Stimulation of PLC β_3 activity is associated with a G-protein of the $G\alpha_q$ family acting through the α subunit. The results suggest that the G-protein linked PLC β isoforms in vascular smooth muscle demonstrate dual regulation by an inhibitory pertussis-sensitive pathway and a stimulatory G-protein of the $G\alpha_q$ family, which is the case for PLC β_3. This dual regulation is analogous to that of adenyl cyclase.

Keywords: Phospholipase C isoforms γ_1, β_1, β_2, β_3, β_4 and δ_1; inhibition and stimulation by G-proteins; pertussis toxin; vascular smooth muscle

Introduction

Phosphatidyl-D-inositol, 4,5-bisphosphate (PIP_2)-directed phospholipase C (PLC) isoforms in vascular smooth muscle are activated by vasoconstrictor agonists acting through receptors coupled to guanyl nucleotide regulatory proteins (G-proteins) (Abdel-Latif, 1986; Fain, 1990). PLC isoforms have been classified into three distinct groups, β, γ and δ according to their cDNA sequences and these groups have been subdivided as additional isoforms have been described (Lee & Rhee, 1995). The major group associated with G-protein activation is PLC β, with molecular masses between 130 and 150 kDa; this group is subdivided into β_1 to β_4 (Katan & Parker, 1987; Park *et al.*, 1992; Jhon *et al.*, 1993; Lee *et al.*, 1993). PLC γ is associated with activation by tyrosine kinase (Liao *et al.*, 1992). The mechanism of regulation of PLC δ is unknown (Parker *et al.*, 1994). Our experiments aimed to separate and identify the soluble PLC isoform(s) present in pig aortic vascular smooth muscle, and to determine which are associated with responses to those vasoconstrictor agonists that act through receptors with seven membrane spanning regions. As such receptors invariably activate effectors via G-proteins, we sought to reconstitute the partially resolved soluble PLC isoforms with receptors and G-proteins present in plasma membrane fractions.

The biochemical study of G-protein linked PLC activity has proved difficult in *in vitro* systems because many isoforms of PLC are loosely associated with the membrane. Following fractionation of broken cells, these occur in the soluble fraction, whereas the natural inositolphospholipid substrates and

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the receptor coupled G-protein complex are fractionated with the plasma membranes (Fain, 1990; Thomas *et al.*, 1991). As a short cut, PLC activity has, therefore, often been measured in the absence of its physiological activation mechanism by using radiolabelled phosphatidylinositol (PI), phosphatidyl-D-inositol, 4-monophosphate (PIP) or PIP_2, and measuring the resultant production of radiolabelled inositol phosphates. To measure the involvement, in regulating PLC activity, of agonist and/or G-protein, these pathways have been reconstituted by adding purified PLC to membranes with prelabelled inositolphospholipids prepared by incubating cultured cells with [^{32}P]-phosphate (Thomas *et al.*, 1991; Baldassare *et al.*, 1993) or [^{3}H]-*myo*-inositol (Morris *et al.*, 1990; Vaziri *et al.*, 1993). These methods, however, required cell culture whereas our study aims to understand the activation of PLC by vasoconstrictor agents in contractile smooth muscle rather than the noncontractile proliferative phenotype generated in cell culture. We therefore used membrane fractions isolated from the same contractile pig aortic vascular smooth muscle as the soluble PLC isoforms. In order to label the inositol phospholipids *in vitro* (McPhee *et al.*, 1991b; Stephens *et al.*, 1993), we incubated isolated membrane fractions of pig aorta with [^{3}H]-*myo*-inositol. The separated soluble PLC isoforms were then added to the labelled microsomes in the presence or absence of the GTP analogues, guanosine 5'[$\beta\gamma$-imido]triphosphate (p[NH]ppG) or guanosine 5'-[γ-thio]triphosphate (GTP[S]), to activate G-proteins thereby reconstituting the G-protein/PLC pathways.

Methods

Separation of PLC isoforms

Porcine aortae were obtained fresh from an abattoir. Soluble fractions containing the PLC isoforms were isolated from a porcine aorta, as described previously (Blayney & Newby, 1990) and the final supernatant constituted the soluble fraction. The PLC isolation buffer used throughout was 20 mM HEPES pH 6.8 containing 5mM EDTA, 5 mM EGTA, 10 mM benzamidine, 1 mM dithiotreitol, 1 μg ml^{-1} soybean trypsin inhibitor, 5 μg ml^{-1} aprotinin, 2 μM pepstatin A, 100 μM 1-choro-3-tosylamido-7-amino-1-2-heptanone, 100 μM leupeptin, 1 mM phenyl methyl sulphonyl fluoride or [4-(2-aminoethyl)-benzene] sulphonyl fluoride, 15 μg ml^{-1} calpain I inhibitor and 7 μg ml^{-1} calpain II inhibitor. To separate the PLC isoforms the soluble fraction was loaded onto a 24 ml Heparin Affigel column and the column was subsequently washed with five column-volumes of PLC isolation buffer. The PLC isoforms were eluted with a linear NaCl gradient (0–700 mM) in PLC isolation buffer and 60 fractions each of 2 ml were collected overnight. All the procedures were carried out at 4°C.

Identification of PLC isoforms

SDS PAGE was used to identify the PLC isoforms separated by Heparin Affigel chromatography. The fractions were concentrated 40 fold by centrifugal ultrafiltration, using Amicon centricon 30 tubes. The concentrated fractions were dissolved in SDS buffer and run on a 10% polyacrylamide gel (Laemmli, 1970). They were transferred to nitrocellulose by a wet blotting technique (Towbin *et al.*, 1979). The Western blot blocking buffer was 5% w/v dried milk powder (Marvel) in Tris buffered saline, pH 7.4 with 0.2% v/v Triton X-100. The PLC antibodies were used at a dilution of 1 μg ml^{-1}. The secondary antibody was a peroxidase conjugated sheep anti-(rabbit Ig) used with an enhanced chemiluminescent (ECL) detection kit.

*Preparation, [^{3}H]-*myo*-inositol incorporation and pertussis treatment of microsomal membranes*

Microsomal fractions containing plasma membranes were isolated from five fresh pig aortae, as described previously (Blayney & Newby, 1990), and resuspended in 4 ml of Buffer A (20 mM Tris/maleate, 100 mM KCl pH 6.8) and stored overnight at 4°C. The labelling of the inositolphospholipids in the plasma membranes with [^{3}H]-*myo*-inositol was based on a method used in turkey erythrocyte membranes (McPhee *et al.*, 1991a,b) and pertussis treatment on a method of ribosylating platelet membranes (Brass *et al.*, 1988). The membranes were incubated in Buffer A (pH 8.0) containing 250 μM histamine, 1 mM cytidine 5'-monophosphate (CMP), 5 mM ATP, 10 mM $MgCl_2$, 3 mM phosphoenolpyruvate, 1 mM dithiotreitol, 10 mM thymidine, 2.5 mM nicotinamide adenine dinucleotide (NAD), 20 μg ml^{-1} pertussis toxin, 30 μg ml^{-1} pyruvate kinase and 100 μCi [^{3}H]-*myo*-inositol (12.3 Ci mmol) at 37°C for 1 h. The reaction was stopped at this time by the addition of 25 ml of ice cold Buffer A containing 20 mM LiCl. The membranes were centrifuged at 100,000 *g* for 45 min and resuspended three times at 4°C. They were resuspended in 4 ml of Buffer A containing 20 mM LiCl.

To determine whether pertussis treatment labelled a G-protein component [^{32}P]-NAD (125 μCi of specific activity 1000 Ci mmol^{-1}) was added to the reaction medium and pertussis toxin was omitted from the control. The washed pellets were resuspended at room temperature in 1 ml of SDS sample buffer (Towbin *et al.*, 1979) and left for 10 min. They were centrifuged at 13000 r.p.m. in a microfuge for 5 min and the supernatants were concentrated 20 fold by centrifugal ultrafiltration at 4°C as above. The concentrate was separated by SDS PAGE using a 10% gel. The gel was dried and exposed to X-ray film. To detect G-protein isoforms, microsomal fractions were solubilized and concentrated and separated by SDS PAGE as for pertussis-treated pellets. The isoforms were detected by Western blotting (see above) with 5% w/v bovine serum albumin substituted for Marvel. Antibodies recognizing $G\alpha_q/_{11}$, $G\alpha_{i3}/\alpha_o$, $G\alpha_{i3}$, $G\alpha_{i1}/\alpha_{i2}$, $G\alpha_{i1}$ and G-protein β subunit were used at 1000 fold dilution.

Otherwise membranes were used immediately to reconstitute PLC activity. The distribution of the tritium label in the membrane inositolphospholipids was determined by extracting with ice cold 6% w/v perchoric acid (PCA) and separating the deacylated derivatives using Dowex resin columns (Harden *et al.*, 1987).

Assay of PLC activity

PLC activity was measured with exogenous [^{3}H]-PIP_2 as described by Katan & Parker (1987): 50 μl of each eluted fraction was added to the [^{3}H]-PIP_2 containing buffer, pH 6.8, final assay volume 100 μl and incubated for 10 min at 37°C. G-protein linked PLC activity in the fractions was measured by adding 25 μl of the [^{3}H]-inositol labelled membranes (approximately 0.1–0.2 mg protein ml^{-1}, measured by the method of Bradford, 1976) to 50 μl of each soluble fraction and 25 μl of reaction medium. The reaction medium contained the GTP analogue p[NH]ppG or GTP[S] (final concentration 100 μM) in Buffer A containing 1 mM EGTA and 0.85 mM $CaCl_2$ [(calculated to give a free Ca^{2+} concentration of 5 μM, as described previously (Blayney & Newby, 1990)]. The final assay volume was 100 μl. After 30 min incubation at 37°C the reaction was terminated by the addition of 500 μl of PCA. The samples were kept at 4°C for 1 h and then centrifuged; 500 μl of supernatant was adjusted to pH 7.4 with 200 mM HEPES/2 M NaOH and 500 μl was added to a scintillation vial and the radioactivity measured. For assays using PLC β_3, fraction 49-55 were pooled. All assays were done in triplicate. Inositol phosphates were determined after neutralization on Dowex resin columns (Harden *et al.*, 1987). After the elution of glycerophosphoinositide with 60 mM ammonium formate the inositol phosphates, inositol monophosphate ($InsP_1$), inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$), were eluted with 180 mM ammonium formate, 400 mM ammonium formate/0.1 M formic acid and 800 mM ammonium formate/0.1 M formic acid respectively.

Effect on PLC β_3 activity of antibodies to $G\alpha_i/\alpha_o$, $G\alpha_q$ or G-protein β subunits

Broad spectrum antibodies (Santa Cruz) to G-protein subunits $G\alpha_i/\alpha_o$, $G\alpha_q/_{11}$ or β (75 μl containing 7.5 μg of antibody protein) were preincubated with prepared membrane fractions (200 μl), for 1 h on ice prior to assay, to give a concentration of 2.7 μg of antibody per 100 μl of membrane fraction and the controls for all antibody experiments contained the same volume and concentration of rabbit IgG. The membranes and antibodies were then diluted by a factor of 4 during assay of PLC activity in the presence of 100 μM GTP[S] as described above.

Calculation of data

PLC activity was measured as release of water soluble tritium (inositol phosphates) into the supernatant fraction. To normalize for variations in the incorporation of [^{3}H]-*myo*-inositol into the inositol phospholipids of microsomal membranes, activities were always expressed as a percentage change from the appropriate controls. All results were analysed by Student's t test for paired data and a one sample t test was performed on the percentage difference; the degrees of freedom were taken as $n-1$ pairs of data, where n = number of experiments, each performed with a different preparation of membranes and soluble fraction.

Materials

Mouse monoclonal antibodies to the PLC isoforms γ_1, δ_1 and β_1 were obtained from TCS Biologicals, Botolph Claydon, Buckingham. Each contained a mixture of monoclonal antibodies to specific peptides derived from sequences of bovine brain PLCs. Rabbit polyclonal antibodies to human PLCs β_2, (C terminal epitope 1170-1181), rat brain PLC β_3 (C terminal 20 amino acids) and rat brain PLC β_4 (C terminal epitope 1159-1176) were purchased from Santa Cruz Biotechnology, California, 95060, U.S.A. G-protein antibodies (all to the C terminal region), for Western blots, were purchased from Calbiochem-Novobiochem (UK) Ltd, Nottingham. To determine the G-protein interaction with PLC β_3 G-protein antibodies were obtained from Santa Cruz; anti $G\alpha_q/_{11}$ recognized both $G\alpha_q$ and $G\alpha_{11}$ of mammalian species (epitope 341-359 common to $G\alpha_q$ and $G\alpha_{11}$ of mouse origin), anti $G\alpha_i/_o/_t/_z$ with broad specificity to all members of the $G\alpha_i/_o$ family (epitope 325-344 of $G\alpha_z$ of rat origin) and anti G-protein subunit β (C terminal 20 amino acids of β_1 of mouse origin) with broad specificity to mouse rat and human $G\beta_1$ to β_4. [^{3}H]-PIP_2, [^{3}H]-*myo*-inositol, [^{32}P]-NAD, anti-(rabbit Ig) horseradish peroxidase linked whole antibody from sheep and ECL Western blotting detection kit were obtained from Amersham. Pertussis toxin was obtained from Fluka and [4-(2-aminoethyl)-benzene] sulphonyl fluoride and calpain I and II inhibitors were from Calbiochem-Novobiochem (UK) Ltd. ATP (disodium salt) was obtained from Boehringer. Heparin Affigel and Dowex AG-1X (200-400 mesh) formate form were obtained from Biorad. All other biochemicals were obtained from Sigma.

Results

Identification of PLC isoforms

PLC isoforms γ_1, δ_1 and β_1 to β_4 were partially separated by heparin affinity chromatography and detected after SDS PAGE and Western blotting using specific antibodies, (Figure 1). The specificity of the various antibodies was confirmed by

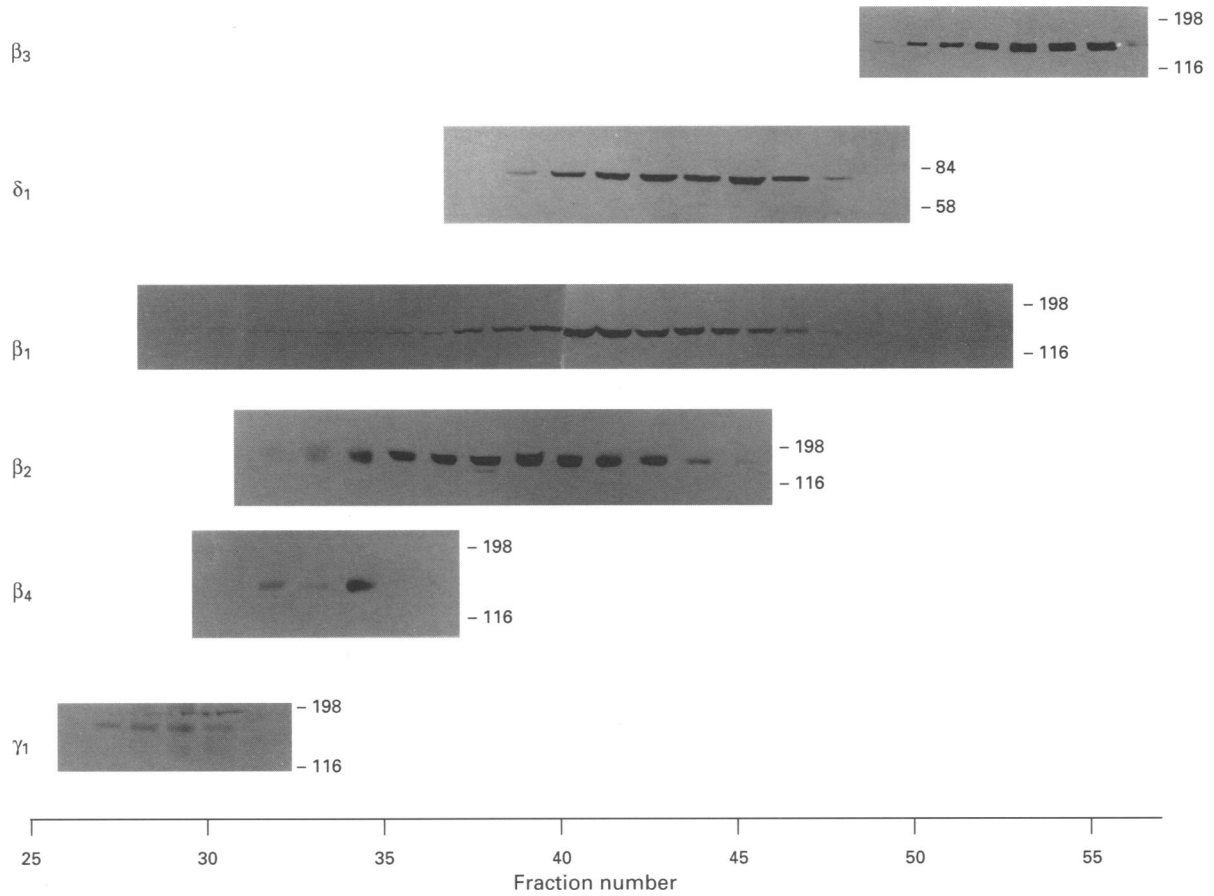

Figure 1 Distribution of PLC isoforms in eluted fractions from heparin affinity columns. The fractions eluted from the heparin affinity column were concentrated and separated by SDS PAGE and detected by Western blotting as described in the Methods section. The isoform distribution to antibodies recognizing specific rat brain PLCs γ_1, β_1, β_2, β_3, β_4 and δ_1 is shown against the fraction number and the position of the molecular mass markers is indicated alongside the individual blots.

the unique distribution obtained with each one. The antibodies to PLC γ_1 identified a clear band at the expected molecular mass of 150kDa but also detected additional bands. This mixture of monoclonal PLC γ_1 antibodies includes some antibodies that recognize the SH_2 and SH_3 (src homology) regions, within the enzyme (suppliers information). Such regions, found originally in the oncogene c-src which is a soluble tyrosine kinase, are targeting sequences for phosphotyrosine and proline rich regions respectively. Since SH_2 and SH_3 regions occur in many proteins involved in cell signalling the additional bands were attributed to cross reaction with other proteins containing these.

For each of the other antisera to PLCs δ_1, β_1 to β_4 a single protein band was detected (Figure 1). All the PLC β isoforms had the expected molecular masses of between 130 and 150 kDa and PLC δ_1 had the expected molecular mass of 85 kDa. Each isoform had a unique pattern of distribution although there was overlap in the distribution of PLCs β_1, β_2 and δ_1, however, PLC β_3 was well resolved.

PLC activity measured with [^{3}H]-PIP$_2$

The distribution of PLC isoforms was compared to the assay of PLC activity in eluted fractions, (Figure 2). The first peak of PLC activity corresponded to fraction 27 and the distribution of PLC γ_1, the second to fractions 31 to 34 which contained PLC β_4. PLC β_2 did not have corresponding activity assayed with exogenous [^{3}H]-PIP$_2$, perhaps because it was overwhelmed by the overlapping activity of PLC β_1. The third peak of activity followed closely the distribution of PLC β_1 (Figure 2a) and the fourth peak of activity corresponded to the distribution of PLC β_3.

*Incorporation of [^{3}H]-*myo*-inositol into plasma-membrane inositol phospholipids*

The time course of [^{3}H]-*myo*-inositol incorporation is shown in Figure 3. The assay contained about 10^6 d.p.m of [^{3}H]-*myo*-inositol of which 10% were incorporated into membrane fractions, in the presence of CMP, (Figure 3). The percentage distribution of the tritium-labelled membrane inositolphospholipids was 12.5 ± 1.7 in the intial wash, 81.8 ± 1.9, 4.8 ± 1.0 and 0.6 ± 0.06 in the PI, PIP and PIP$_2$ fractions respectively ($n = 4 \pm$ s.e.mean). The addition of pertussis toxin during labelling did not affect the distribution (data not shown).

Assay of G-protein associated PLC activity

Fractions containing well separated PLC isoforms were selected. PLC γ_1 activity was assayed by pooling data from fractions 27, 28 and 29 and PLC β_4 activity from fractions 32, 33 and 34. PLC β_2 distribution partly overlapped with PLC β_1 but was adequately resolved when data were pooled from fractions 35, 36 and 37. Because of its overlap with other isoforms it was not possible to select fractions representing PLC β_1 activity alone. PLC β_3 was, however, well

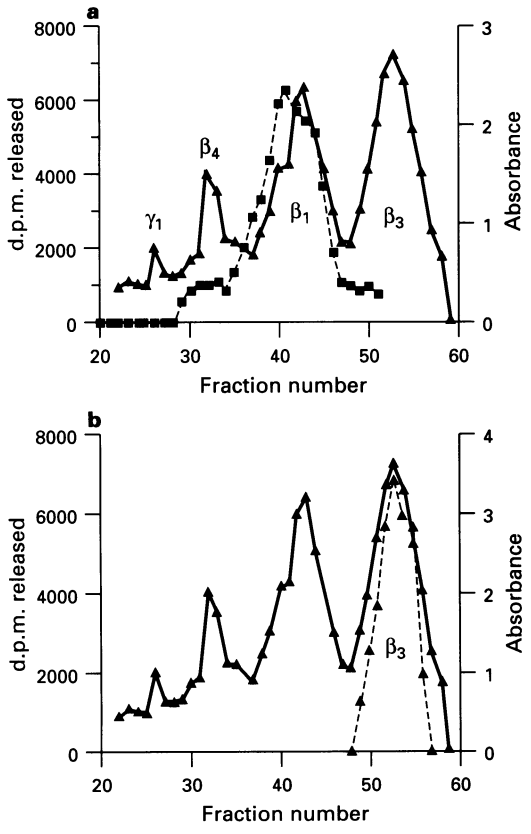

Figure 2 PLC activity measured with exogenous [^{3}H]-PIP$_2$ as substrate and comparison with PLC β_1 and PLC β_3 isoform distribution. Eluted fractions were concentrated forty fold and subjected to SDS PAGE and Western blotting to determine β_1 and β_3 isoform distribution. The intensity of the bands on the film was measured as absorbance using densitometry. PLC activity was assayed with [^{3}H]-*myo*-inositol labelled microsomes (▲), as described in the Methods section. (a) β_1 distribution (dotted line with ■) and (b) β_3 isoform distribution (dotted line and ▲).

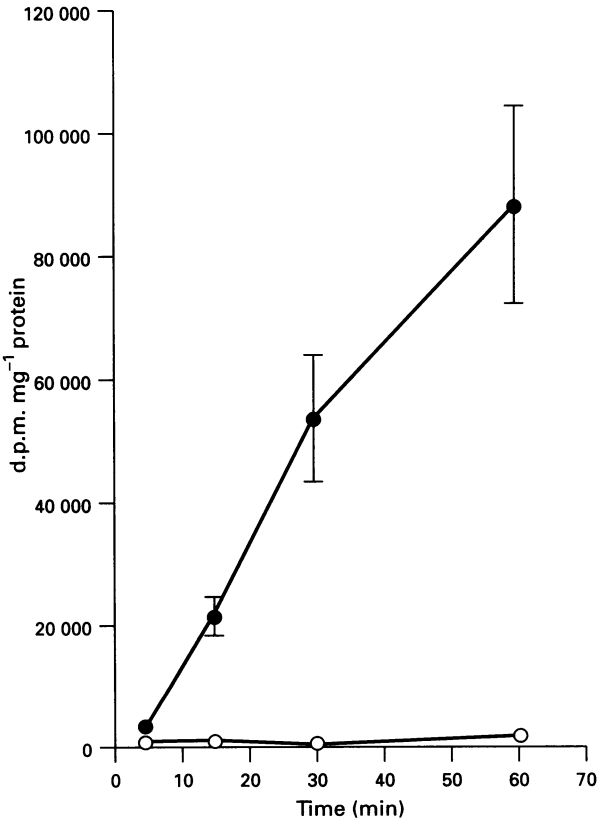

Figure 3 Time course of incorporation of [^{3}H]-*myo*-inositol into microsomal vesicles. The uptake of [^{3}H]-*myo*-inositol into plasma membranes was determined by sampling 100 μl of the reaction mixture, described in Methods and passing the samples through 0.45 μM Gelman filters at the time points indicated. The filters were placed in a scintillation vial and dissolved with 1 ml of 2-methoxyethanol before the addition of scintillant. The experiment was repeated with three separate membrane preparations. Uptake with CMP (●); control, i.e. uptake in the absence of CMP (○).

Table 1 PLC activity measured with [^{3}H]-*myo*-inositol labelled membranes

PLC isoform	PLC activity (% above control) −Pertussis toxin No addition	−Pertussis toxin p[NH]ppG	+Pertussis toxin No addition	+Pertussis toxin p[NH]ppG
γ_1	34±6	27±4	32±6	36±5
β_4	25±3	15±1*	30±5	41±6†
β_2	30±6	18±2*	26±5	38±5†
β_3	30±6	16±3*	29±8	38±5†

PLC activity was measured by adding the fractions corresponding to PLC isoform distribution as described in Figure 2 and the Methods section to membranes prelabelled with [^{3}H]-*myo*-inositol with or without preincubation with pertussis toxin (20 μg ml^{-1} of preincubation buffer). The release of water soluble ^{3}H was measured and expressed as % of control activity measured with membranes but without soluble fraction. $n=12\pm$s.e.mean.*$P<0.05$ vs no addition, †$P<0.05$ vs no addition+pertussis.

resolved when measured in fractions 53, 54 and 55. Addition of fractions containing PLC β_4, β_2 or β_3 to labelled membranes in each case increased the release of radioactive products by 20–40% above the control values measured with isolation buffer alone, (Table 1). The addition of p[NH]ppG caused a 40–50% decrease in each PLC activity, (Table 1). Conversely, after pretreatment of plasma membranes with pertussis toxin, p[NH]ppG caused a 30–50% activation of each isoform (Table 1). PLC γ_1 showed the same activity regardless of whether p[NH]ppG was present or of pertussis pretreatment, as expected for an isoform not associated with G-proteins.

A time course of activity of the well resolved PLC β_3 comparing membranes with and without prior pertussis treatment (Figure 4) confirmed the data in Table 1. Only after pertussis pretreatment was there any measurable stimulation of PLC activity by p[NH]ppG. Reconstitution of PLC β_3 activity with pertussis pretreated membranes showed a dose-dependent stimulation with GTP analogues p[NH]ppG and GTP[S]. The stimulation was inhibited by GDP[S], confirming its mediation by G-proteins, (Figure 5). Constitutive PLC β_3 activity measured in the absence of GTP analogue was also reduced by GDP[S], indicating that some basal activity was G-protein dependent, (Figure 5).

PLC activity was measured routinely by the increase in total water soluble tritium. To characterize the reaction products further, the [^{3}H]-inositol phosphates, $InsP_1$, $InsP_2$ and $InsP_3$ were resolved by ion exchange chromatography. These showed percentage increases of 87±19, 95±35 and 72±33 respectively ($n=6\pm$s.e.mean), when comparing 0 min with 30 min of incubation using pertussis pretreated membranes reconstituted with soluble fractions containing PLC β_3.

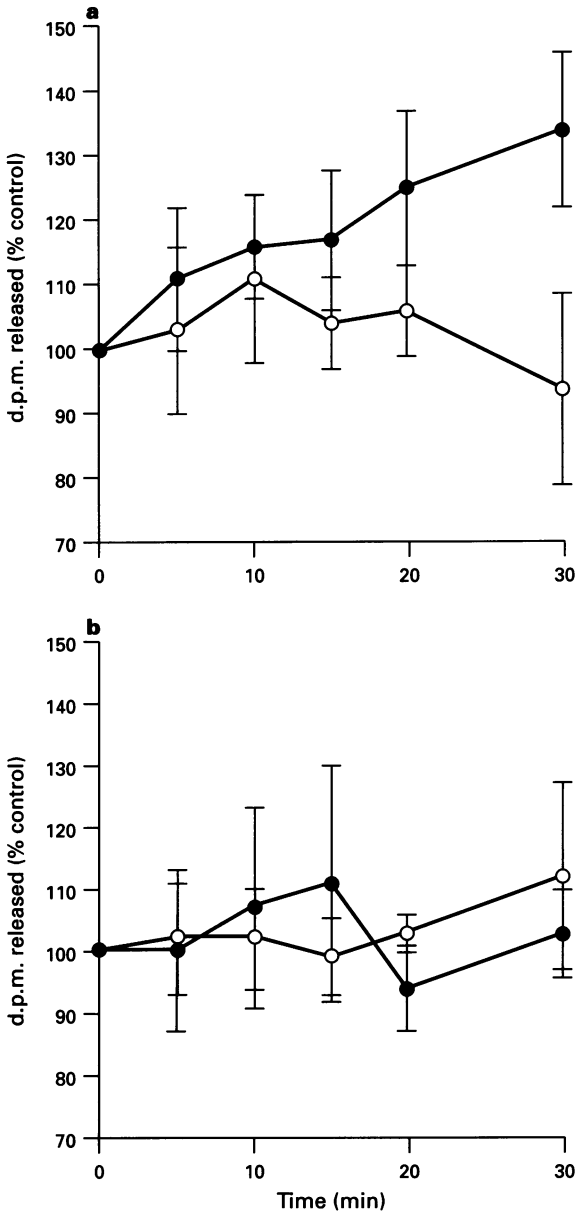

Figure 4 The time course of PLC β_3 activity assayed with microsomal fractions labelled with [^{3}H]-*myo*-inositol and with (a) or without (b) pertussis toxin pretreatment. Soluble fractions from the heparin affigel separation containing PLC β_3 were added to microsomal fractions and the time course of release of water soluble tritium was measured, as described in Methods and expressed as a percentage of the d.p.m. released by the $t=0$ control (100%). The ^{3}H released without added PLC β_3 (○) is compared to that released with added PLC β_3 (●) in the presence and absence of pertussis toxin, $n=3\pm$s.e.mean.

Presence of pertussis-sensitive G-proteins

SDS PAGE and Western blot analysis indicated that the plasma membrane fraction contained several G-protein α subunits of the $G\alpha_i/\alpha_o$ family (Figure 6). With antibodies to $G\alpha_{i1}$ a major band of 38 kDa was found. An antibody with dual specificity for $G\alpha_{i1}$ and $G\alpha_{i2}$ detected a doublet of molecular masses 38 and 36 kDa showing that both these α subunits were present. Antiserum to $G\alpha_{i3}$ detected a single band at 38 kDa, whereas dual specificity $G\alpha_{i3}/\alpha_o$ antibody detected a doublet. Antibodies to $G\alpha_q/_{11}$ also detected a doublet at molecular masses 36 and 38 kDa suggesting both α subunits of this family were present (Figure 6a). G-protein β subunit was also detected as a single band at its reported molecular mass of 36 kDa although one other major band was present at molecular mass of 27 kDa, which seemed to be a common band recognized to a varying degree by most of the G-protein antibodies. Pertussis treatment in the presence of [^{32}P]-NAD led to a single band of phosphorylated protein (Figure 6b) which is likely to reflect the phosphorylation of several unresolved subunits.

G-protein subtype interacting with PLC β_3

Antibodies with broad specificity to the $G\alpha_i$, $G\alpha_q/\alpha_{11}$ or G-protein β subunits families were added to assays of PLC activity as neutralizing antibodies (Figure 7). Anti-$G\alpha_q$ inhibited GTP[S] stimulated, PLC activity in pertussis pretreated membranes. Anti-$G\alpha_i/\alpha_o$ had no effect on PLC activity (a second

antibody was purchased from Calbiochem and this too was without effect (data not shown). In contrast anti-G-protein β subunit stimulated PLC activity in non-pertussis pretreated membranes but not in pertussis pretreated membranes.

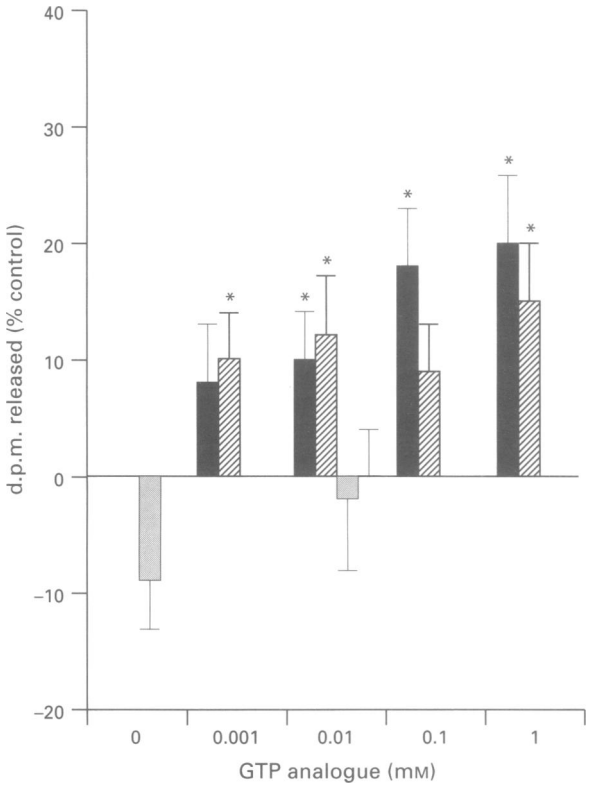

Figure 5 Dependence of PLC β_3 activity on the concentration of GTP analogue and GDP[S]. G-protein-dependent PLC β_3 activity was reconstituted by the addition of PLC β_3-containing soluble fractions to microsomal fractions prelabelled with [^{3}H]-*myo*-inositol and pretreated with pertussis toxin. The reaction medium contained different concentrations of the GTP analogues as described in the Methods section. The dose response to p[NH]ppG is shown as solid columns and to GTP[S] as hatched columns and the effect of adding GDP[S] is shown as stippled columns. The data are expressed as % change from the control value measure with soluble fraction and membranes but without other additions. All results are ±s.e.mean of $n=10$ experiments, $^*P<0.05$ vs control.

Discussion

In vascular smooth muscle from pig aorta we have partially resolved by heparin affinity chromatography and clearly identified six PLC isoforms γ_1, β_1 to β_4 and δ_1, present in the soluble fraction. Four of the PLC isoforms γ_1, β_1, β_3 and β_4 exhibited PLC activity towards exogenous [^{3}H]-PIP$_2$. Reconstitution of PLC activity in selected fractions from heparin affinity chromatography, with membranes containing [^{3}H]-*myo*-inositol incorporated into the inositol phospholipids, revealed a similar pattern of activity and regulation by G-protein for each of the PLC β isoforms. PLC γ_1 was not regulated by G-protein, in agreement with previous work (Lee & Rhee, 1995). Antibody neutralization experiments with well resolved fractions of PLC β_3 showed that the inhibition of activity was related to G-protein β subunits liberated by stimulation of pertussis sensitive G-proteins. Stimulation of activity in pertussis pretreated membranes was via a member of the Gα_q family of G-proteins.

The antibodies used to identify the PLC isoforms were obtained commercially. Their unique specifity for the corresponding PLC isoforms has been established in the laboratories where they were originally developed and is well documented (Lee *et al.*, 1987; Park *et al.*, 1992; Jhon *et al.*, 1993; Lee *et al.*, 1993; Lee *et al.*, 1994). Each antibody was raised to specific peptides derived from non homologous regions of individual bovine, rat or human PLC isoforms. These sequences of the PLC isoforms are well conserved across species documented in the suppliers literature. Anti-bovine PLC γ_1, δ_1 and β_1 cross react with the respective bovine, human, mouse, rat and rabbit isoforms. Anti-human PLC β_2 and β_3 cross react with mouse and rat isoforms and anti-rat PLC β_4 cross reacts with mouse and human isoforms. Although not previously characterized in pig, each of the PLC antibodies recognized antigens with a distinct pattern of distribution, in the eluted fractions from the heparin affinity column, at molecular masses consistent with those expected of the PLC isoforms. The precise correspondence between the immunological detection of the PLC β_1 and β_3 isoforms and their activity with [^{3}H]-PIP$_2$ further demonstrates the specificity of these antibodies. The immunological signal from the PLC β_4 antibody was the weakest being detectable in only a few peak fractions. The peak of PLC activity with exogenous [^{3}H]-PIP$_2$ coincided with the peak of immunological reaction but was measurable also in fractions bracketing these. PLC γ_1 showed good immunological activity which corresponded to a very weak peak of PLC activity measured with [^{3}H]-PIP$_2$. Only for PLC β_2 was there no separable peak of [^{3}H]-PIP$_2$ activity and the reason for

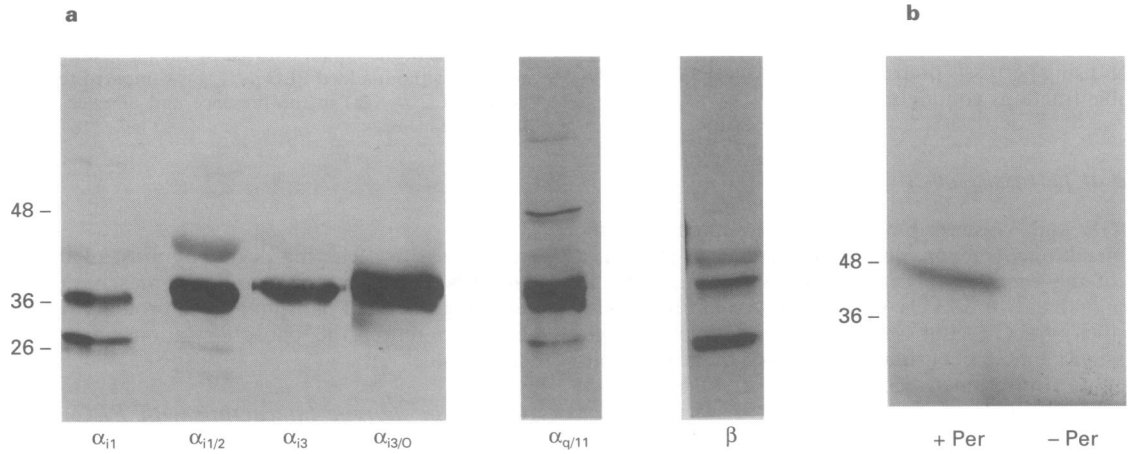

Figure 6 The presence of G-proteins in microsomal fractions. (a) G-proteins were solubilized, concentrated and subjected to SDS PAGE and Western blotting using antibodies recognizing different Gα_i/α_o, Gα_q/α_{11} and G-protein β subunit isoforms, as described in Methods. (b) G-proteins from membranes pretreated ± pertussis toxin in the presence of [^{32}P]-NAD were solubilized, concentrated and subjected to SDS PAGE. The gels were dried and exposed to X-ray film to detect ^{32}P incorporation.

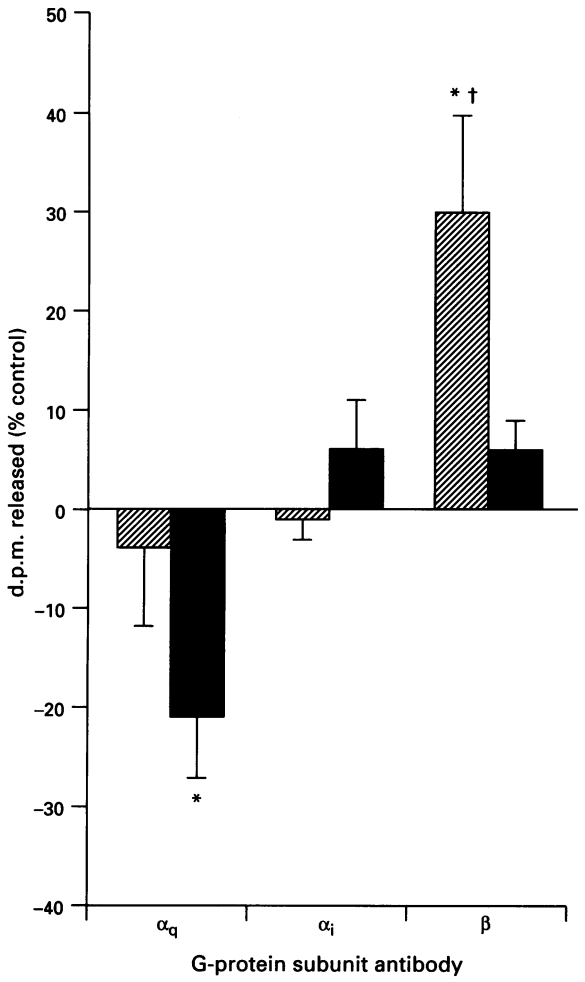

Figure 7 Effect of G-protein subunit antibodies on PLC β_3 activity. G-protein antibodies to the C-terminal regions of $G\alpha_q/\alpha_{11}$, $G\alpha_i$, or G-protein β subunits, were preincubated with non-pertussis (hatched columns) pertussis toxin-treated (solid columns) plasma membranes for 1 h on ice. These membranes (and antibodies) were diluted by a factor of 4 into the final assay mix containing soluble fractions rich in PLC β_3 and in the presence of 100 μM GTP[S]. The data are expressed as % change from the control value measured with the same concentration of rabbit IgG as antibody, as described in Methods. All results are $\pm$s.e.mean of $n=10$ experiments for anti-$G\alpha_i$ and $n=6$ experiments for anti-$G\alpha_q$ and anti-G-protein β subunit. *$P<0.02$ vs control and †$P<0.02$ vs anti-$G\alpha_q$.

this is not clear. It is not possible to equate the relative intensity of the immunological signals to two different antibodies to the quantity of the proteins present, thus the activity of PLC β_2 could be overwhelmed by the activity of PLC β_1 in adjacent fractions. PLC δ_1 was detectable not only at a characteristic position but had a clearly different molecular mass, 85 kDa as expected, compared with the value of 130–150 kDa for each of the other isoforms. Taken together these data strongly support the previously established specificity of the antibodies and demonstrate that all six known PLC isoforms are present in pig aortic vascular smooth muscle.

Our finding of so many PLC isoforms in a single homogeneous tissue appears to be novel. A similar number has been found in bovine retina (Lee *et al.*, 1993) and bovine brain (Suh *et al.*, 1988) but this might be because the isoforms are differentially distributed amongst the different cell types that make up these tissues. We removed both adventia and endothelium from our preparations and thus all these PLC isoforms are derived from the homogeneous smooth muscle cells comprising the pig aortic media. A specialist physiological role for PLC β_4 and, thereby, a limited tissue distribution, has been postulated from work on the retina. Since PLC β_4 is inhibited by ribonucleotides such as GMP but not by cyclic GMP, it could be regulated by the relative concentrations of these nucleotides and hence be linked to the activation of rhodopsin (Lee *et al.*, 1994). However, its detection here in vascular smooth muscle suggests a wider role. One reason for the diversity of PLC isoforms may be different intracellular distribution and function within vascular smooth muscle. In Swiss 3T3 cells, PLC β_1 is associated primarily with the nuclear matrix and PLC γ with the cytoskeleton filaments (Martelli, 1992; Zini *et al.*, 1993). However, the distribution of PLC β isoforms other than PLC β_1 is unknown. Different PLC isoforms might be linked selectively to different vasoconstrictor agonist receptors via different G-proteins or might subserve different regulatory roles, for example in contraction, hypertrophy, migration or proliferation. These exciting possibilities need now to be tested.

One of our principal objectives was to reconstitute the G-protein/PLC pathway using components derived entirely from smooth muscle, even though greater activity might have been achieved by reconstituting purified soluble PLCs from other tissues. We believe that it is necessary to obtain base-line data in a homogeneous vascular smooth muscle system before proceeding to heterologous systems. Moreover, we chose to study the regulation of PLC activities in a minimally resolved state to avoid the possibility that essential cofactors might be purified away. Using a single step chromatography, it was possible to separate adequately three of the four PLC β isoforms and a consistent pattern of activation was observed for each of these three isoforms.

Although we anticipated activation by GTP analogues, the effect observed with each of the PLC β_2, β_3 and β_4 isoforms was inhibitory. Inhibition of PLC β_1 activity has been observed in cells transfected with $G\alpha_{i2}$ (Watkins *et al.*, 1994) and the authors suggested a dual system of regulation analagous to $G\alpha_i$ inhibition of adenylate cyclase (Gilman, 1987) involving stimulation by a different G-protein. Our results are consistent with this hypothesis and extend it to an untransfected cell, and to include inhibition of the three other PLC β isoforms found in our preparation. Consistent with this dual regulation hypothesis, we observed significant p[NH]ppG stimulation of the PLC β isoforms when using pertussis pretreated membranes. Increase in PLC activity was measurable in membrane preparations that were pretreated with pertussis toxin but not without pertussis pretreatment suggesting that a pertussis-sensitive G-protein was exerting an inhibitory influence on PLC activity. The action of pertussis toxin is to ribosylate a cysteine residue four amino acids from the C terminal end of the G-protein α subunits of the $G\alpha_i/\alpha_o$ family. The effect of this ribosylation is to couple tightly the holotrimeric configuration of G-protein $\alpha\beta\gamma$ subunits (Birnbaumer *et al.*, 1990). Thus pertussis toxin pretreatment could remove the influence of either the G-protein α_i/α_o or G-protein $\beta\gamma$ subunits on effector coupling. This issue was resolved by the use of neutralizing antibodies to G-protein α_i/α_o and G-protein β subunits. A broad spectrum polyclonal antibody to the C-terminal region recognizing the $G\alpha_{i1,2}$ and $_3$ isoforms and $G\alpha_o$ had no effect on GTP[S] stimulated PLC activity in either pertussis or non-pertussis treated membranes, whereas a broad spectrum polyclonal antibody to G-protein β subunit enhanced activity in non pertussis-treated membranes but was without effect on pertussis pretreated membranes. These data thus demonstrate that the inhibition of PLC β_3 activity in these experiments resulted from interaction of PLC β_3 with G-protein $\beta(\gamma)$ subunits. We have confirmed directly that our membrane preparation contains several members of the $G\alpha_i/\alpha_o$ family and that pertussis toxin ribosylates one or more of these. Several other workers have shown inhibition of PLC activity consistent with dual regulation and these studies taken together suggest stimulation of PLC activity by one receptor pathway, acting through a non-pertussis sensitive G-protein, which is attenuated by a second receptor pathway acting through a

pertussis-sensitive G-protein (Litosch, 1989; Bizzarri *et al.*, 1990; Van Geet *et al.*, 1990; Cherifi *et al.*, 1992; Litosch *et al.*, 1993).

The increase in PLC β_3 activity seen in our study, in the presence of GTP analogues using pertussis toxin pretreated preparations, was attributed to stimulation via a pertussis toxin-insensitive G-protein. Furthermore, we demonstrated that antibodies which recognize the C terminal of $G\alpha_q/\alpha_{11}$ could inhibit this activity. Thus our results confirm much previous work with purified PLC β isoforms showing that $G\alpha_q$ directly activates these enzymes (Jhon *et al.*, 1993 Lee *et al.*, 1993). Purified PLC β_3 (and also PLC β_1 and PLC β_2) activity can be stimulated also by G-protein $\beta\gamma$ subunit families acting through a different domain from purified $G\alpha_q$ (Sternweis, 1994). Pertussis-sensitive activation of PLC, which has been reported in some cell types (Gerwins & Fredholm, 1992a,b; Dickenson & Hill, 1993) may be explained through independent direct activation by G-protein $\beta\gamma$ subunits liberated from pertussis-sensitive $G\alpha_i$ subunits (Camps *et al.*, 1992; Boyer *et al.*, 1992; Sternweis, 1994; Dickenson *et al.*, 1995). The reasons why, and the mechanism by which, a pertussis-sensitive G-protein inhibits PLC activity in some systems but stimulates it in others remain to be elucidated.

In conclusion, we have demonstrated the presence of six soluble PLC isoforms in vascular smooth muscle. Reconstitution with plasma membranes, particularly of PLC β_3, demonstrates both G-protein-mediated pertussis toxin-insensitive activation and pertussis toxin-sensitive inhibition of these PLC β isoforms.

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