Phospholipase D in rat myometrium: occurrence of a membrane-bound ARF6 (ADP-ribosylation factor 6)-regulated activity controlled by $\beta\gamma$ subunits of heterotrimeric G-proteins

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Both protein kinase C and protein tyrosine kinases have been shown to be involved in phospholipase D (PLD) activation in intact rat myometrium [Le Stunff, Dokhac and Harbon (2000) *J. Pharmacol. Exp. Ther.* **292**, 629–637]. In this study we assessed the involvement of monomeric G-proteins in PLD activation in a cell-free system derived from myometrial tissue. Both the PLD1 and PLD2 isoforms were detected. Two forms of PLD activity, essentially membrane-bound, were found in myometrial preparations. One form was stimulated by oleate and insensitive to guanosine 5'-[γ -thio] triphosphate (GTP[S]). The second required ammonium sulphate to be detected and was stimulated by GTP[S]. ADP-ribosylation factors (ARF1 and ARF6) and RhoA were immunodetected in myometrial preparations. ARF1 and RhoA were present in the membrane and cytosolic fractions whereas ARF6 was detected exclusively in the membrane fraction. A synthetic myristoylated peptide corresponding to the N-

terminal domain of ARF6 [myrARF6_(2–13)] totally abolished PLD activation in the presence of ammonium sulphate and GTP[S], whereas myrARF1_(2–17) and the inhibitory GDP/GTP-exchange factor, Rho GDI, did not. These data are consistent with a membrane-bound ARF6-regulated PLD activity. Finally, the stimulation of PLD by ARF6 was inhibited by AlF₄⁻ and this inhibition was counteracted by the fusion protein glutathione S-transferase- β -adrenergic receptor kinase 1 (495–689) and by the QEHA peptide (from adenylate cyclase ACII), which act as G-protein $\beta\gamma$ -subunit scavengers. It is concluded that G-protein subunits $\beta\gamma$ are involved in a pathway modulating PLD activation by ARF6, illustrating cross-talk between heterotrimeric and monomeric G-proteins.

Key words: fluoroaluminates, oleate-activated phospholipase D, phospholipase D1, phospholipase D2, small G proteins.

INTRODUCTION

Phospholipase D (PLD) is becoming increasingly recognized as an important signal-transducing enzyme involved in the regulation of several cellular processes. PLD hydrolyses the terminal phosphodiester bond of phosphatidylcholine, the major membrane phospholipid to release choline and phosphatidic acid (PA). The increase in PA, a possible second messenger, is associated with several important cellular modifications such as the activation of protein kinases, phospholipase C- γ and cytosolic phospholipase A₂, Ca²⁺ influx, DNA synthesis and c-Fos and c-Myc transcription [1,2]. In cells, PA is dephosphorylated by phosphatidate phosphohydrolase to produce diacylglycerol, which activates members of the Ca²⁺- and phospholipid-dependent protein kinase C (PKC) family. Other PA-derived molecules with regulatory properties include lysoPA and arachidonic acid metabolites, both of which act on specific cell-surface receptors. Two mammalian PLDs, PLD1 and PLD2, have recently been cloned [3]. Both are widely expressed in many cell types and tissues [4], and both require phosphatidylinositol 4,5-bisphosphate (PIP₂) for enzymic activity [3]. In addition to PIP₂-

dependent PLD isozymes, oleate-dependent PLD activity has been reported by several research groups [5,6]. A large variety of receptor tyrosine kinases and receptors coupled to heterotrimeric G-proteins in a wide range of cell types has been demonstrated to mediate PLD stimulation in response to specific agonists. Agonist-stimulated PLD activity appears to be tightly regulated by a complex group of proteins, including PKC, protein tyrosine kinase (PTK) and monomeric G-proteins [1,2]. However, the exact order of events leading from receptor occupancy by ligand to PLD activation and their relative importance have not yet been completely defined.

In our earlier studies [7,8], we reported that PLD in intact rat myometrium is activated by endothelin-1 (ET-1) via ETA receptor subtypes coupled to pertussis toxin-insensitive G-protein(s) and that two distinct pathways make a major contribution to the peptide-mediated responses. One pathway involves PKC activation that occurs after the breakdown of PIP₂ by phospholipase C and the second, independent of PKC activation, involves PTK activity. We have also provided evidence [8] that the activation of PLD is under the negative control of ceramides, which affect the PKC-dependent process, and of the cAMP-

Abbreviations used: PLD, phospholipase D; PBut, phosphatidylbutanol; ARF, ADP-ribosylation factor; PKC, protein kinase C; PTK, protein tyrosine kinase; PKA, cAMP-dependent protein kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; GST- β ARK1, glutathione S-transferase- β -adrenergic receptor kinase 1 (495–689) fusion protein; QEHA, peptide encoding residues 956–982 in the C2a region of adenylate cyclase ACII; GTP[S], guanosine 5'-[γ -thio] triphosphate; GDP [β -S], guanosine 5'-[β -thio] diphosphate; ET-1, endothelin-1; Rho GDI, Rho-guanine nucleotide-exchange inhibitor.

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dependent protein kinase (protein kinase A; PKA) cascade, which interferes specifically with the PTK-dependent cascade. Although phosphorylation-dependent mechanisms clearly play a major role in the regulation of PLD activity, most of the phosphorylated targets have yet to be identified. Both PKC and PTK have been reported to be involved in the recruitment of ADP-ribosylation factor (ARF) and Rho GTPases from the cytosol to the membrane compartment for PLD activation [2,9]. A recent study [10] provided some evidence that PKA-mediated phosphorylation of RhoA in human neutrophils enhances the dissociation of RhoA from the membrane, thereby reducing its ability to activate PLD. Other possible targets for PTKs include the guanine nucleotide-exchange factors for the small G-proteins that activate PLD [9].

The aim of this study was to assess the involvement of small G-proteins in PLD activation, using a cell-free system derived from rat myometrium. We detected PLD1 and PLD2 isoforms and identified both ARF6-sensitive and oleate-dependent PLD activities in myometrium. Most importantly, we demonstrated that $\beta\gamma$ subunits of heterotrimeric G-proteins display negative control of PLD activation by ARF6.

EXPERIMENTAL

Materials

[^3H]Myristic acid (30–40 Ci/mmol) and Western-blot enhanced chemiluminescence (ECL) reagent, the Renaissance product line, were purchased from Dupont NEN (Les Ulis, France). β -Oestradiol 3 benzoate, leupeptin, aprotinin, PMSF, guanosine 5'-[γ -thio] triphosphate (GTP[S]), guanosine 5'-[β -thio]diphosphate (GDP[β -S]) and sodium salts of oleic, linoleic, arachidonic, palmitic and stearic acids were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Myristoylated N-terminal ARF6 peptide [myrARF6₍₂₋₁₃₎] and myristoylated N-terminal ARF1 peptide [myrARF1₍₂₋₁₇₎] were synthesized as described previously [11]. QEHA, a peptide encoding residues 956–982 in the C2a region of adenylate cyclase ACII (95% pure), was synthesized by Eurogentec (Seraing, Belgium) and was provided kindly by Dr S. Mhaouty-Kodja (CNRS ESA 7080, Paris, France). RhoA antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal anti-ARF1, anti-PLD1 and anti-PLD2 antibodies and monoclonal anti-ARF6 antibody were generated as described previously [9,12–14]. Secondary antibodies (Conjugated with goat anti-mouse and anti-rabbit IgG1) were from Bio-Rad SA (Ivry-sur-Seine, France). NitroBind nitrocellulose, 0.45 μm pore size, was from Micron Separations (Westborough, MA, U.S.A.). All other chemicals were of the highest grade available. GST- β ARK1 [glutathione S-transferase- β -adrenergic receptor kinase 1 (495–689) fusion protein] expressed in *Escherichia coli* was kindly provided by Dr H. Chap (INSERM U-326, Toulouse, France). The fusion protein was purified as reported in [15], and was checked for integrity by SDS/PAGE and Coomassie Brilliant Blue staining. Recombinant Rho GDI (Rho-guanine nucleotide-exchange inhibitor) was purified from bacteria as described previously [16].

Animals and tissue processing

Immature female Wistar rats (4–5 weeks of age) were treated with 30 μg of oestradiol for 2 days and were killed the following day by 1 min of CO_2 inhalation. Uteri were removed and the myometrium was prepared free of endometrium [7,8]. Histological sections of myometrial preparations showed the samples to consist almost exclusively of longitudinal muscle.

Tissue labelling of phospholipids

Incubations were carried out as described previously [7,8] with minor modifications. Briefly, myometrial strips (about 50 mg) were equilibrated by incubation for 20 min in 5 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 117 mM NaCl, 4.7 mM KCl, 1.1 mM MgSO_4 , 1.2 mM KH_2PO_4 , 2.4 mM NaHCO_3 , 0.8 mM CaCl_2 and 1 mM glucose (gas phase 95% O_2 /5% CO_2) with constant agitation. Tissues were then incubated with 10 $\mu\text{Ci/ml}$ [^3H]myristic acid in 800 μl of fresh buffer for 5 h. Tissue strips were washed once with non-radioactive buffer containing 1 mg/ml serum albumin, and then twice with non-radioactive buffer without serum albumin.

Preparation of myometrial homogenates, membranes and cytosolic components

Prelabelled myometrial strips (100 mg) were homogenized with an Ultra-Turrax homogenizer in 0.7 ml of buffer A consisting of 50 mM Hepes/NaOH (pH 7.4), 1 mM EGTA, 0.5 M sucrose, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin and 1 mM PMSF. The tissue extract was centrifuged for 5 min at 700 g (4 $^{\circ}\text{C}$). The 700 g supernatant corresponded to total homogenate. In some experiments, the 700 g supernatant was centrifuged for 20 min at 100 000 g , using a Beckman TL-100 ultracentrifuge. The resulting supernatant corresponded to the cytosolic fraction, and the pellet (membrane fraction) was suspended in 100 μl of buffer A. An aliquot of each fraction was used for protein determination using the Lowry reagent [17].

Assay of PLD activity

To assay PLD activation in myometrial homogenate, 170 μl of the 700 g supernatant (400 μg of protein; 200 000–250 000 c.p.m.) were transferred to an Eppendorf tube containing sufficient MgCl_2 to give a final concentration of 10 mM and sufficient CaCl_2 to give a final free Ca^{2+} concentration of 1 μM . Samples were incubated for various periods of time with the indicated concentrations of GTP[S], ammonium sulphate and oleic acid in a total reaction volume of 500 μl . Butanol (0.3%) was included in the assay to permit detection of the transphosphatidylated product, [^3H]phosphatidylbutanol ([^3H]PBut), as a metabolically stable index of PLD activity. To assess PLD activation in membrane fractions, 25 μl (100 μg of protein; 200 000–250 000 c.p.m.) of the 100 000 g pellet were transferred to buffer A containing 10 mM MgCl_2 and 1 μM free Ca^{2+} . Samples were incubated at 37 $^{\circ}\text{C}$ for 15 min in the absence or presence of 170 μl of cytosol (300 μg of proteins), 25 μM GTP[S], 1.5 M ammonium sulphate or various concentrations of fatty acids in a total reaction volume of 500 μl supplemented with 0.3% butanol. To terminate the reaction, samples were mixed with 1.8 ml of ice-cold chloroform/methanol/10 M HCl (50:100:1, by vol.) and left at 4 $^{\circ}\text{C}$ overnight [18]. H_2O (0.5 ml) was then added, the mixture homogenized briefly and the monophasic split by adding 0.6 ml of H_2O and 0.6 ml of chloroform. After vigorous mixing, the phases were separated by centrifugation for 10 min at 1000 g and the aqueous phase was removed. The chloroform extract was dried using a speed vacuum concentrator and the residue was suspended in 50 μl of methanol/chloroform (19:1, v/v) before TLC on heat-activated pre-coated 20×20 cm silica-gel plates. To analyse the production of [^3H]PBut, plates were developed in the organic phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, by vol.) [19] and analysed with a computerized Berthold radiochromatocan. The production of [^3H]PBut was expressed as a percentage of the total radioactivity recovered on the plate for the same sample.

Immunodetection of ARF6, ARF1, RhoA, PLD1 and PLD2

Frozen myometrial strips (100 mg) were homogenized with an Ultra-Turrax homogenizer in 0.6 ml of a buffer consisting of 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM PMSF and centrifuged for 5 min at 700 *g* (4 °C). The 700 *g* supernatant was centrifuged for 20 min at 100 000 *g*, using a Beckman TL-100 ultracentrifuge. The resulting supernatant corresponded to the cytosolic fraction and the pellet (membrane fraction) was suspended in 100 μ l of the extraction buffer. In some experiments, detergent-extracted proteins were prepared by including 1 % Triton X-100 in the extraction buffer. Protein samples were heated for 10 min at 95 °C with Laemmli's buffer and were resolved by SDS/PAGE, with 7.5 % (w/v) acrylamide, for the detection of PLD isoforms and 15 % (w/v) acrylamide for small G-proteins. Proteins were then transferred to nitrocellulose and the membranes were blocked as described previously [20]. The following primary antibodies were used: polyclonal anti-PLD1 and anti-PLD2 (dilution 1:2000), polyclonal anti-ARF1 (dilution 1:2000) and anti-RhoA (dilution 1:2000) and monoclonal anti-ARF6 (dilution 1:5000) in 5 % non-fat dried milk in TBS (Tris-buffered saline; 20 mM Tris/HCl, pH 7.5/500 mM NaCl). The immunoreactive bands were visualized by the ECL detection system after incubation with mouse anti-IgG coupled to horseradish peroxidase (dilution 1:10 000) for anti-ARF6 and with rabbit anti-IgG coupled to horseradish peroxidase (dilution 1:4000) for the polyclonal antibodies.

Data analysis

Results are expressed as means $\pm$ S.E.M. and were analysed statistically using Student's *t* test. *P* < 0.05 was considered significant.

RESULTS

Immunodetection of PLD1 and PLD2 in myometrium

Detergent-extracted proteins derived from myometrial samples were subjected to electrophoresis and immunoblotted with PLD1 and PLD2 isoform-specific antibodies (Figure 1). PLD1-specific

Figure 1 Immunodetection of PLD1 and PLD2 in myometrial preparations

Detergent-extracted proteins (50 μ g) were resolved by SDS/PAGE (7.5 %, w/v, acrylamide) and immunoblotted with anti-PLD1 and anti-PLD2 antibodies (dilution 1:2000). The immunoreactive bands were visualized by the ECL detection system. Data are from one of three similar experiments.

Figure 2 Stimulatory effects of oleate and other fatty acids on PLD activity in myometrial membranes

Membrane fractions (100 μ g of protein) were incubated for 15 min with the indicated concentrations of oleate (A) or with 250 mM sodium fatty acid salts: PAL, palmitic; STE, stearic; OLE, oleic; LIN, linoleic; ARA, arachidonic acid (B). The production of [3H]PBut was expressed as a percentage of total label recovered from the plate of the corresponding sample. Results are given as means $\pm$ S.E.M. for three to six separate experiments, each done in duplicate.

antibodies recognized a major protein band of $\approx$ 120 kDa, similar to the molecular mass established for PLD1 [21], and another band at $\approx$ 80 kDa, which may have been a contaminant protein. PLD2-specific antibodies recognized a protein of $\approx$ 105 kDa, similar to the molecular mass established for PLD2 [22]. Another contaminant band ($\approx$ 130 kDa) was also recognized by PLD2 antibodies. The 120 kDa PLD1 and the 105 kDa PLD2 were not recognized by peptide-neutralized PLD-specific antibodies (results not shown). In another series of experiments, the distribution of PLD isozymes in isolated myometrial cells has been evaluated by immunofluorescence using PLD1 and PLD2 antibodies, and was visualized by confocal microscopy. Both PLD1 and PLD2 were detected almost exclusively on intracellular membranes with a minimal staining in the cytosol (results not shown).

Oleate-stimulated PLD activity in myometrial preparations

The treatment of crude myometrial membranes with low concentrations of oleate increased PLD activity, as estimated by [3H]PBut production (Figure 2A). Stimulation of PLD activity was optimum at 0.25 mM and decreased thereafter, being undetectable at a concentration of 3 mM oleate. PLD activity was increased 3–7-fold over control values. Other unsaturated fatty acids (linoleic and arachidonic acids) at concentrations of 0.25 mM increased PLD activity but to a lesser extent than oleate (Figure 2B). In contrast, saturated fatty acids (palmitic and stearic acids) had no effect. The use of 0.1 % Triton X-100 had almost no effect (results not shown). This indicated that PLD activation by oleate could not be due to a detergent-like action on myometrial membranes. Oleate-dependent PLD activity in myometrial membranes was totally insensitive to the addition of GTP[S], in both the absence (Figure 2A) and presence (results not shown) of cytosol.

Figure 3 Dose- and time-dependent stimulation of PLD by ammonium sulphate in a myometrial cell-free system

Aliquots of a myometrial homogenate (400 μ g or protein) were incubated with 1.5 M $(\text{NH}_4)_2\text{SO}_4$ for the times indicated (A), or for 15 min with various concentrations of $(\text{NH}_4)_2\text{SO}_4$ (B), or with MgSO_4 or NH_4Cl , both at concentrations of 1.5 M (C). $[\text{H}]\text{PBut}$ was expressed as a percentage of total label recovered from the plate of the corresponding sample. Results are given as means $\pm$ S.E.M. for three to six separate experiments, each done in duplicate.

Ammonium sulphate revealed the presence of G-protein-regulated PLD activity in myometrial homogenates

In myometrial homogenates, PLD activity was revealed progressively by the addition of increasing concentrations of ammonium sulphate, with a maximal effect observed at 1.5 M, with a mean of 1.5% of total label being recovered as $[\text{H}]\text{PBut}$ (Figure 3B). This dramatic salt effect, which has been described previously for PLD activity in various cell extracts [23], was not due simply to high ionic strength. At 1.5 M, MgSO_4 and NH_4Cl had almost no effect. In the presence of 1.5 M ammonium sulphate, the production of $[\text{H}]\text{PBut}$ was linear up to 15 min (Figure 3A).

In the absence of ammonium sulphate, the non-hydrolysable analogue of GTP, GTP[S], was unable to stimulate PLD activity. However, PLD activity detected in the presence of 1.5 M ammonium sulphate was increased further by adding GTP[S]. The time and concentration dependence of the activating effect of GTP[S] are shown in Figure 4. Activation was linear up to 15 min (Figure 4A) and product formation plateaued in the presence of 25 μM GTP[S] (Figure 4B). Activation by GTP[S] was counteracted by GDP[β -S]. Furthermore, AlF_4^- which is known to activate heterotrimeric G-proteins but is reportedly unable to activate small GTP-binding proteins [24], could not substitute for GTP[S]; in contrast AlF_4^- had an inhibitory effect (see below). This is consistent with the notion that in myometrium, as commonly described for other cell systems, small G-proteins are required for PLD activation.

Co-localization of PLD and the activating GTP-binding protein in myometrial membranes

To determine which myometrial fractions were required for PLD activity, isolated crude membrane and cytosolic fractions were tested alone and in combination for $[\text{H}]\text{PBut}$ formation. Membrane fractions that were washed thoroughly so as to be free of cytosol displayed ammonium sulphate-sensitive PLD activity similar to that observed in the corresponding total homogenate

Figure 4 Dose- and time-dependent stimulation by GTP[S] of PLD in the presence of ammonium sulphate: inhibitory effect of GDP[β -S]

(A) Aliquots of a myometrial homogenate (400 μ g of protein) were incubated in the presence of 1.5 M ammonium sulphate without ($\circ$) or with 25 μM GTP[S] ($\bullet$) for the times indicated. (B) Incubations were carried out for 15 min in the presence of 1.5 M ammonium sulphate and various concentrations of GTP[S], or in the presence of both 25 μM GTP[S] and 250 μM GDP[β -S] (black bar). Results are given as means $\pm$ S.E.M. for three to six separate experiments, each done in duplicate.

Table 1 Activation by ammonium sulphate and GTP[S] of PLD in membrane preparations: a cytosol-independent effect

PLD activity was assayed in the presence of 1.5 M ammonium sulphate with and without GTP[S] (25 μM) in a myometrial homogenate (400 μ g of protein) or in membranes (100 μ g of protein) alone or combined with cytosol fractions (300 μ g of protein). Incubations were conducted for 15 min. The production of $[\text{H}]\text{PBut}$ was expressed as the percentage of total label recovered from the plate of the corresponding sample. Results are given as means $\pm$ S.E.M. for eight separate experiments, each done in duplicate.

Fractions added	Ammonium sulphate (1.5 M)	$[\text{H}]\text{PBut}$ (% of total counts)	
		— GTP[S]	+ GTP[S]
Homogenate	+	1.08 $\pm$ 0.1	2.65 $\pm$ 0.25
Crude membranes	+	1.11 $\pm$ 0.09	2.24 $\pm$ 0.2
Crude membranes + cytosol	+	1.0 $\pm$ 0.11	2.33 $\pm$ 0.21

(Table 1). No additional PLD activity could be measured when membranes were reconstituted with the corresponding cytosolic fraction. PLD activity stimulated by GTP[S] was also totally recovered in membrane fractions free of cytosol. Addition of the corresponding cytosol did not further increase the GTP[S]-dependent formation of $[\text{H}]\text{PBut}$. Thus in myometrium, PLD activity and the activating GTP-binding protein appear to co-localize with the membranes.

Identification and subcellular location of ARF1, ARF6 and RhoA in myometrium

Among the small G-proteins, members of the ARF family, including ARF1 and ARF6, and a member of the Rho family, RhoA, have been reported to be efficient PLD activators in a variety of cellular systems [1,2]. No information is yet available

Figure 5 Immunological identification and localization of ARF1, ARF6 and RhoA in myometrial extracts

Membranes (40 μ g of protein; Mb) and cytosol fractions (40 μ g of protein; Cyt) were resolved by SDS/PAGE (15%, w/v, acrylamide) and immunoblotted with the polyclonal anti-ARF1 antibody, the polyclonal anti-RhoA antibody or the monoclonal anti-ARF6 antibody. The immunoreactive bands were visualized by the ECL detection system. Data are from one of three similar experiments.

concerning the nature of the small G-proteins present in the myometrium. Western-blot analysis of myometrial fractions with antibodies specific for ARF1 and ARF6 detected two proteins with an apparent molecular mass of 20 kDa (Figure 5). Antibody specific for RhoA recognized a protein with an apparent molecular mass of 20 kDa. Both ARF1 and RhoA were present in membranes and cytosol. In contrast, we found that ARF6 was present almost exclusively in the membrane fraction and was undetectable in the cytosol (Figure 5).

Figure 6 Effects of N-terminal ARF1 [myrARF1₍₂₋₁₇₎] and ARF6 [myrARF6₍₂₋₁₃₎] synthetic peptides and Rho GDI on PLD activity stimulated by ammonium sulphate and GTP[S]

(A) Aliquots of a myometrial homogenate (400 μ g of protein) were incubated for 15 min in the presence of 1.5 M ammonium sulphate with various concentrations of myrARF6 peptide (●), 50 μ M myrARF1 peptide (hatched bar) or 6 μ M Rho GDI (black bar). In (B) incubations were carried out in the presence of 1.5 M ammonium sulphate with 25 μ M GTP[S] in the absence (white bar) or the presence of myrARF6 peptide (bar with dot), myrARF1 peptide (hatched bar) or Rho GDI (black bar). Results are given as means $\pm$ S.E.M. for three to six separate experiments, each done in duplicate.

Figure 7 Inhibitory effect of sodium oleate on the PLD activity stimulated by ammonium sulphate and GTP[S]

(A) Aliquots of a myometrial homogenate (●) and membrane fractions (○) were incubated for 15 min with 1.5 M ammonium sulphate in the absence (0 mM) or presence of the indicated concentrations of sodium oleate. (B) Membrane fractions were incubated with 1.5 M ammonium sulphate and 25 μ M GTP[S] in the absence (hatched bar) or presence (white bars) of the indicated concentrations of sodium oleate. Results are given as means $\pm$ S.E.M. for four to six separate experiments, each done in duplicate.

ARF6-regulated PLD activity in myometrial homogenates

To assess ARF-regulated PLD activity, we used synthetic peptides corresponding in sequences to the N-terminal regions of ARF1 and ARF6. These peptides have been reported to block ARF activities in various cellular processes [11,25–27]. Figure 6 shows the effects of the myristoylated myrARF6₍₂₋₁₃₎ peptide and myrARF1₍₂₋₁₇₎ peptide on [³H]PBut formation in myometrial homogenates. myrARF6₍₂₋₁₃₎ produced a dose-dependent inhibition of PLD activity in the presence of 1.5 M ammonium sulphate, with 50% inhibition at 30 μ M and almost total inhibition at 50 μ M peptide. myrARF6₍₂₋₁₃₎ also totally counteracted the stimulatory effect of GTP[S]. In contrast, myrARF1₍₂₋₁₇₎ used at a concentration of 50 μ M did not affect the formation of [³H]PBut in the presence of ammonium sulphate or prevent the stimulatory effect of GTP[S] (Figure 6). Thus, the strong inhibition observed in the presence of myrARF6₍₂₋₁₃₎ was not due to a non-specific effect of the myristoyl group and provided evidence that ARF6 constitutes a potential regulator of PLD activity in the *in vitro* myometrial assay. The inhibitory GDP/GTP-exchange factor, Rho GDI, has been reported to inhibit several functional Rho-related responses, including PLD activation [28,29]. Under our experimental conditions (Figure 6), Rho GDI, used at a concentration of 6 μ M, did not affect the formation of [³H]PBut in the presence of ammonium sulphate. Also, it did not prevent the stimulatory effect of GTP[S], eliminating the possibility of the contribution of Rho-related proteins in the regulation of PLD activity in myometrial homogenates.

A number of reported findings have shown clearly that ARF-dependent PLD activity is inhibited by oleate, particularly if used at high concentrations [5,6]. Data in Figure 7 show that high

Table 2 Inhibitory action of AlF_4^- on PLD activity in the presence of ammonium sulphate

Homogenates (400 μg of protein) were incubated for 15 min in the presence and absence of 1.5 M ammonium sulphate with AlCl_3 (10 μM) and NaF (10 mM), added alone or combined (AlF_4^-). Incubations were conducted for 15 min. The production of $[\text{H}]$ PBuT was expressed as a percentage of total label recovered from the plate of the corresponding sample. Results were given as means $\pm$ S.E.M. for three separate experiments, each done in duplicate. ND, not determined.

Treatment	$[\text{H}]$ PBuT (% of total counts)	
	– $(\text{NH}_4)_2\text{SO}_4$	+ $(\text{NH}_4)_2\text{SO}_4$
None	0.15 ± 0.06	1.18 ± 0.1
AlCl_3	ND	1.4 ± 0.12
NaF	ND	1.06 ± 0.09
AlF_4^- (AlCl_3 + NaF)	0.1 ± 0.05	0.41 ± 0.06

concentrations of sodium oleate strongly inhibited the production of $[\text{H}]$ PBuT supported by ammonium sulphate in both homogenate and crude myometrial membranes. The inhibitory effect of oleate was concentration-dependent, with about 70% inhibition at 6 mM. Inhibition by oleate was even more pronounced when the production of $[\text{H}]$ PBuT was stimulated by GTP[S]. At 1 mM, oleate only slightly inhibited PLD activity in the presence of ammonium sulphate alone, but markedly reduced (60%) the stimulatory effect of GTP[S] (Figure 7). Inhibition by oleate is thus consistent with the presence of an ARF-stimulated PLD in the myometrium.

Evidence that G-protein $\beta\gamma$ subunits mediate the AlF_4^- -induced inhibition of ARF6-regulated PLD in myometrium

In the presence of ammonium sulphate the formation of $[\text{H}]$ PBuT, which appeared to involve an ARF6-mediated process, was strongly decreased by AlF_4^- (Table 2). NaF and AlCl_3 used alone had no effect. Thus, the inhibitory effect of AlF_4^- was probably due to the activation of heterotrimeric G-proteins present in the myometrial homogenates. Recent *in vitro* [30,31] and *in vivo* [11] studies demonstrating a possible interaction between ARF proteins and $\text{G}\beta\gamma$ subunits led us to hypothesize that AlF_4^- may act by releasing $\text{G}\beta\gamma$ subunits from heterotrimeric G-proteins and that ARF6, complexed with $\text{G}\beta\gamma$, would be unable to support PLD activation. To test this hypothesis, we used GST- βARK1 , a recombinant protein encoding the $\text{G}\beta\gamma$ -binding domain of βARK1 in fusion with GST, which has been reported to interact with and tie up $\text{G}\beta\gamma$ subunits [15,32]. Figure 8 shows that GST- βARK1 at a concentration of 24 μM totally counteracted the inhibitory effect of AlF_4^- on the formation of $[\text{H}]$ PBuT. GST- βARK1 has been reported to bind not only $\text{G}\beta\gamma$ subunits but also PIP_2 , an important cofactor for PLD activity. More specific evidence for a direct involvement of $\text{G}\beta\gamma$ subunits in the inhibitory effect of AlF_4^- was obtained by using the synthetic peptide QEHA, encoding residues 956–982 in the C2a region of type-ACII adenylate cyclase. QEHA interacts with $\text{G}\beta\gamma$, and thereby prevents $\text{G}\beta\gamma$ -mediated events [33,34]. As demonstrated in Figure 8, QEHA peptide efficiently counteracted, in a dose-dependent manner, the inhibitory effect of AlF_4^- on the formation of $[\text{H}]$ PBuT. Overall, our results are consistent with $\text{G}\beta\gamma$ subunits being involved in AlF_4^- mediated inhibition of PLD activity in the presence of ammonium sulphate. This inhibition is most probably the consequence of ARF6 becoming bound to $\text{G}\beta\gamma$, rendering ARF6 unable to support PLD activity.

Figure 8 Reversal by GST- βARK1 and QEHA peptide of the inhibitory effect of AlF_4^- on PLD activity in the presence of ammonium sulphate

Aliquots of a myometrial homogenate (400 μg of protein) were incubated for 15 min in the absence (black bar) or the presence of 1.5 M ammonium sulphate with AlCl_3 (10 mM) + NaF (10 mM), and in the left-hand panel, in the absence or presence of GST- βARK1 at 24 μM , and in the right-hand panel, in the presence of various concentrations of QEHA peptide. Results are given as means $\pm$ S.E.M for three to five separate experiments, each done in duplicate.

DISCUSSION

We have reported previously that several signalling pathways including PKC and PTK are implicated in the regulation of PLD and its activation by ET-1 in the intact rat myometrium [7,8]. Using a cell-free system derived from myometrial tissue, we report here that there are at least two forms of PLD activity in the myometrium. One form is oleate-activated, insensitive to GTP[S] and does not require the presence of ammonium sulphate for activity. The second is GTP[S]-dependent, ARF6-mediated and requires the presence of high concentrations of ammonium sulphate. Furthermore, our data implicate $\text{G}\beta\gamma$ subunits in the negative regulation of PLD activation by ARF6, illustrating the cross-talk between heterotrimeric and monomeric G-proteins in this process.

In addition to PIP_2 and ARF-dependent PLD isozymes, oleate-stimulated PLD activities have been reported by several research groups [5,35]. Our findings demonstrate the existence of an oleate-activated PLD activity in myometrium, located mostly in membranes and sensitive to low concentrations of oleate (0.1–0.25 mM). Consistent with previous observations [6,36], the possibility that oleate may be acting as a non-specific detergent has been ruled out. The oleate-stimulated PLD activity in myometrial membranes was totally insensitive to the addition of GTP[S], suggesting that the oleate-stimulated and the ARF6-regulated activities represent two distinct PLDs in rat myometrium. Accordingly, it has been reported previously that solubilized brain PLD can be chromatographically separated into oleate-stimulated and ARF-stimulated activities [5]. Similarly, L1210 cells (a leukaemic cell line) express an oleate-activated PLD that can be distinguished from the ARF-dependent PLD [37]. Until recently, it was thought that PLD1 and PLD2 were inhibited and not stimulated by oleate. However, a recent report [38] provided evidence that purified human PLD2, but not

human PLD1, was specifically stimulated by unsaturated fatty acids, including oleate. This led the authors to propose PLD2 as a likely candidate for the oleate-dependent PLD activity. In line with these observations, stimulation of PLD activity by oleate can be attributable to PLD2 in Jurkat T cells [39], since these cells do not express PLD1 [40]. Moreover there was little oleate-dependent PLD activity in undifferentiated HL-60 cells expressing PLD1 but not PLD2 [40]. In a recent report [41], PLD activity stimulated by oleate has been characterized in rat heart sarcolemma and it has been found that PLD2 is the major myocardial PLD associated with this fraction [42]. The identification of PLD2 by Western-blot analysis and our biochemical data suggest that the oleate-stimulated PLD activity of rat myometrium is characteristic of PLD2.

Several studies have provided evidence implicating small G-proteins of the ARF and Rho families in the activation of PLD [1,2]. Here, we investigated the presence and distribution of ARF and Rho proteins in the myometrium. ARF1 and RhoA were found in both membrane and cytosolic fractions, whereas ARF6 was localized exclusively in the membrane compartment. Although ARF and Rho proteins are believed to cycle on and off the membrane in a manner that is tightly coupled to the binding and hydrolysis of GTP [43,44], various reports have demonstrated, in a number of cell types, that ARF6 is almost exclusively present on membranes [11,14,45]. Our findings in the myometrium are in line with these observations. Moreover, GTP[S]-activated PLD was also predominantly detected in the membrane fraction, indicating that PLD and the activating G-protein are probably associated with the same membrane compartment in myometrium.

Consistent with the involvement of small G-proteins [24], we observed that PLD activity detected in the presence of ammonium sulphate was increased by adding GTP[S] but not AlF_4^- . Rho-related proteins are probably not involved in the regulation of this PLD activity in myometrial homogenates, since Rho GDI, the inhibitory GDP/GTP-exchange factor that has been reported to inhibit several Rho-related responses, including PLD activation [28,46], did not affect the stimulation of PLD by GTP[S]. The occurrence of an ARF-regulated PLD activity was probed by the use of synthetic peptides, corresponding in sequence to the N-terminal regions of ARF6 and ARF1 [myrARF6₍₂₋₁₃₎ and myrARF1₍₂₋₁₇₎]. These peptides have been used successfully to block ARF activities in various cellular processes [11,25-27]. Under our experimental conditions, myrARF1₍₂₋₁₇₎ did not affect PLD activation whereas myrARF6₍₂₋₁₃₎ had a strong inhibitory effect. These findings support the notion that ARF6 is a key regulator of PLD activity in the myometrium. Surprisingly, myrARF6₍₂₋₁₃₎ not only counteracted the stimulatory effect of GTP[S], but it also inhibited, in a dose-dependent manner, the PLD activity measured in the sole presence of ammonium sulphate. It has been reported previously [23] that PLD activity in a number of mammalian cell extracts is mostly latent but can be revealed by the addition of high concentrations of ammonium sulphate. Although the mechanism underlying this salt effect remains unclear, the data reported here raise the intriguing possibility that ARF6 is involved in ammonium sulphate-mediated PLD activation. Indeed, it is possible that high concentrations of ammonium sulphate specifically induce some conformational changes in PLD and/or may counteract the association of PLD with putative inhibitory factors [3], rendering the enzyme accessible to activation by ARF6. Although activation by ammonium sulphate occurred in the absence of added GTP[S], it is worth recalling that some ARF6 may be present in myometrial extracts in a GTP-bound state [47]. It is also possible that the conformation of ARF6 itself may be affected by ammonium

sulphate, resulting in the release of bound GDP and an ARF6 in a different conformation with an empty guanine nucleotide site, capable of activating PLD and supporting GTP[S]-mediated activation.

We found that AlF_4^- , which activates heterotrimeric G-proteins [48], strongly reduced PLD activity in the presence of ammonium sulphate. Li and Fleming [49] recently reported an inhibitory effect of AlF_4^- on PLD in submandibular extracts via a mechanism that appeared to be independent of its ability to stimulate heterotrimeric G-proteins. In a subsequent study [50], these authors suggested that AlF_4^- is a competitive inhibitor of purified cabbage PLD via a mechanism based on its phosphate-mimicking property. Our findings do not support such a mechanism for the AlF_4^- effect in the myometrium. In contrast, it seems likely that the inhibitory input emanating from AlF_4^- on ARF6-regulated PLD activity involves $G\beta\gamma$ subunits of heterotrimeric G-proteins. The demonstration was achieved using GST- $\beta\text{ARK}1$ and the QEHA peptide: both factors, well known to complex $G\beta\gamma$ subunits and to interfere with $G\beta\gamma$ -mediated processes [32,33], counteracted the inhibitory effect of AlF_4^- . A number of recent *in vitro* observations have demonstrated an interaction between ARF proteins and $G\beta\gamma$ [30,31]. Galas et al. [11] have described a possible interaction of ARF6 with $G\beta\gamma$ subunits on secretory granules. These authors also reported that AlF_4^- inhibited Ca^{2+} -induced ARF6-dependent PLD activation in permeabilized chromaffin cells [25], most likely by preventing the uncoupling of ARF6- $G\beta\gamma$ on the secretory granule membranes [11]. In line with these observations, our results suggest that ARF6 is bound to $G\beta\gamma$ subunits in an inactive form in the myometrial membranes and that ammonium sulphate disrupts this association, enabling ARF6 to mediate PLD activation.

Both PLD1 and PLD2 are distributed widely in many cell types and tissues [4]. The catalytic activity of PLD1 is dependent on PIP_2 as an essential cofactor and is activated by ARF (ARF1, ARF6) and Rho family proteins. PLD2 also requires PIP_2 for activity and has frequently been shown to be activated poorly by ARF and Rho proteins *in vitro*. However, in a recent report, while confirming that an intact full-length PLD2 is stimulated minimally by ARF proteins, removal of most of the non-core N-terminal region resulted in a truncated PLD2 form (66 kDa) that was stimulated by ARF proteins, including ARF5 and ARF1. ARF6 was a relatively poor stimulator of both the full-length and the truncated PLD2 forms [51]. It is thus reasonable to propose that it is the PLD1 and not the PLD2 isoform immunodetected in myometrial preparations that is involved in the ARF6-mediated activation. However, the contribution of an as yet unidentified ARF6-sensitive PLD activity cannot be ruled out totally.

We have reported recently that in intact myometrial preparations, PLD activity is stimulated by PTK- and PKC-mediated events and that both contribute to the activation of PLD triggered by ET-1. A number of observations have demonstrated that the activation of small G-proteins, including ARF, may be controlled by PTK as well as by PKC [9]. We are currently investigating the role of small G-proteins in the agonist-mediated activation of PLD as well as the regulation of PLD by oleate in myometrial preparations *in vivo*. Further studies are also required to prove a direct collaboration between the $G\beta\gamma$ subunits of heterotrimeric G-proteins and ARF6 for the control of PLD activity in intact myometrial cells.

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