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ARF1(2–17) does not specifically interact with ARF1-dependent pathways

Inhibition by peptide of phospholipases Cβ, D and exocytosis in HL60 cells

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Abstract

The small GTP-binding protein ARF has been shown recently to regulate phospholipase D (PLD). In order to investigate the role of ARF proteins in regulated exocytosis, we have used the N-terminal peptide ARF1(2–17) of the ARF1 protein. ARF1 reconstituted PLD activity in cytosol-depleted HL60 cells was inhibited by ARF1(2–17). In the presence of endogenous cytosol, ARF1(2–17) also inhibited GTP-γ-S-stimulated PLD activity and exocytosis. Mastoparan *Politses jadwagae* and mastoparan *Vespula lewisii* which exhibit similar structural properties to ARF1(2–17) also inhibited GTP-γ-S-stimulated PLD and exocytosis. GTP-γ-S-stimulated phospholipase C-β (PLC-β) was also inhibited by ARF(2-17) and mastoparan. In cytosol-depleted HL60 cells, the ARF(2–17) inhibited the reconstitution of GTP-γ-S-stimulated PLC-β activity with exogenously-added PLC-β1 and phosphatidylinositol transfer protein. We conclude that the widely-used ARF1(2–17) peptide inhibits both ARF-independent (i.e. PLC-β) and ARF-dependent pathways (i.e. PLD) and therefore cannot be regarded as a specific inhibitor of ARF function.

Key words: ADP-ribosylation factor; Exocytosis; Phospholipase D

1. Introduction

Activation of phospholipase D (PLD) by cell-surface receptors is a widespread response observed in many cell types [1]. Studies in cell-free systems and in permeabilized cells has indicated that PLD activity is regulated by ADP-ribosylation factors, ARF1 and ARF3 [2, 3]. ADP-ribosylation factor (ARF), a small GTP-binding protein, was originally identified as the protein co-factor required for efficient ADP-ribosylation of the α sub-unit of G_s by cholera toxin [4]. More recently, ARF has been shown to be involved in vesicular trafficking between the ER and cis-Golgi compartment [5], endosome-endosome fusion [6] and budding of coated vesicles from the Golgi [7]. In yeast two genes encode the ARF proteins – ARF1 and ARF2. The deletion of both genes is lethal, while the deletion of ARF1 alone results in the disruption of the secretory pathway [8]; this can be rescued by the addition of human ARF1 or ARF4 proteins [9] indicating the highly conserved nature of these proteins.

In many cell types including HL60 cells and neutrophils, GTP-γ-S activates exocytosis as well as PLD activity when added to permeabilized cells [10]. Exocytosis in many cell-types is inhibited by alcohols including ethanol [10]. Since alcohols are known to interfere with the production of PA by PLD by diverting it to form phosphatidylalcohols, this suggested that a strong link existed between exocytosis and PLD activity [10]. Since alcohols are not very specific in their actions, it was desirable to find an alternative means of inhibiting PLD. The identification of ARF as an activator of PLD allowed us to use the previously-characterised inhibitory ARF(2–17) peptide as a possible inhibitor of PLD and exocytosis.

We report here that ARF1(2–17) inhibits ARF1-reconstituted PLD activity in HL60 cells depleted of their endogenous cytosol. In addition, the peptide also inhibits both exocytosis and PLD activity stimulated by GTP-γ-S when added to the cells simultaneously with the permeabilizing agent, streptolysin O. Since the ARF peptide is amphipathic, 2 forms of mastoparan which are also amphipathic were used as controls. Both mastoparans were found to inhibit exocytosis and PLD activity similarly to ARF1(2-17). To further check the specificity of the peptides, their effect on G-protein-regulated phospholipase Cβ (PLC β) was also monitored. As well as inhibiting PLD activity and exocytosis, inhibition of PLC β was also observed indicating that ARF1(2–17) may not act as specifically as previously thought.

2. Materials and methods

2.1. Materials

Culture media (RPMI 1640 and Medium 199) were purchased from Flow laboratories. Foetal Calf Serum was from Imperial Laboratories. Streptolysin O was obtained from Wellcome Diagnostics. Guanosine 5′-[γ-thio]triphosphate (GTP-γ-S) and ATP were purchased from Boehringer-Mannheim. [methyl-^{3}H]Choline chloride and myo-[2-^{3}H]inositol were obtained from Amersham. Mastoparan-*Polistes jadwagae* (M-8528) and Mastoparan-*Vespula lewisii* (M-5280) (purity of both mastoparans: minimum 97% by HPLC) and all other chemicals were from Sigma. ARF1 was prepared from bovine brain cytosol as described previously [2], PI-TP was purified from bovine brain cytosol [11] and PLC-β1 was purified from bovine brain membranes [12].

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ARF(2-17) was synthesised on an applied Biosystems 430A using Fmoc chemistry. Purity was determined by HPLC, automated amino terminal sequencing and by time of flight mass analysis using a FinniganMAT Lasermat.

2.2. Culturing and labelling of HL60 cells

HL60 cells were maintained in culture as previously described [13]. Cells were either labelled with [methyl-^{3}H]choline chloride for 48 h if PLD activity was being monitored [14] or myo-[2-^{3}H]-inositol for 48 h for measuring PLC activity [13].

2.3. Reconstitution of PLD with ARF1 in cytosol-depleted cells

[methyl-^{3}H]choline-labelled HL60 cells (50 ml, 10^6 cells/ml) were centrifuged at 450 × *g* for 5 min and then washed twice in 50 ml of 20 mM PIPES, 137 mM NaCl and 3 mM KCl (pH 6.8) (referred to as PIPES buffer). Cells were then resuspended in 4.5 ml of PIPES buffer and incubated at 37°C for 20 min to equilibriate. The cells were permeabilized with 0.6 i.u./ml streptolysin O at pCa 7 for 30 min so that cytosolic proteins would leak out. The permeabilized cells were washed in PIPES buffer and centrifuged at 2,000 × *g* for 5 min at 4°C. Cells were then resuspended in the required amount of PIPES buffer supplemented with 2 × concentrated assay cocktail (final concentrations in assay – 1 mM MgATP, 2 mM $MgCl_2$, Ca^{2+} buffered with 3 mM EGTA (pCa 5). The cells were then added to tubes containing ARF1, GTP-γ-S and ARF1(2–17) as indicated in a total assay volume of 50 μl. The reaction was initiated by transferring the tubes from ice to 37°C and was incubated for 30 min. The reaction was then quenched with chloroform/methanol (1:1) and processed for choline release as previously described [14].

2.4. Assay for GTP-γ-S-stimulated PLD activity and exocytosis

Prelabelled HL60 cells (50 ml, 10^6 cells/ml) were centrifuged at 450 × *g* for 5 min and then washed twice in 50 ml of PIPES buffer. Cells were then resuspended in 4.5 ml of PIPES buffer and incubated at 37°C for 20 min to equilibrate. The cells were then transferred to tubes containing assay cocktail (final concentrations in assay – 0.6 i.u/ml streptolysin O; 1 mM MgATP; 2 mM $MgCl_2$; Ca^{2+} buffered with 3 mM EGTA (pCa 5)) and ± peptides and 10 μM GTP-γ-S in total volume of 100 μl. The samples were incubated for 10 min at 37°C and then transferred to ice followed by centrifugation at 2,000 × *g* for 5 min at 4°C. 50 μl of the supernatant was then removed and assayed for exocytosis of hexosaminidase as previously described [10]. The remainder of the incubation mixture was quenched and processed for choline release [14].

2.5. Reconstitution of GTP-γ-S-stimulated PLC activity with PI-TP and PLC-β1 in cytosol-depleted cells

[^{3}H]Inositol labelled HL60 cells were used; they were handled as for the PLD reconstitution assay except that during permeabilization, 2 mM MgATP was also present and the assay cocktail consisted of 2 mM MgATP, 2 mM 2,3-diphosphoglycerate, 10 mM LiCl, 2 mM $MgCl_2$ and Ca^{2+} buffered with 3 mM EGTA (pCa 6) (final concentrations). Total assay volume was 55 μl. The assay was incubated for 20 min at 37°C. The reaction was quenched with 1 ml of ice-cold 0.9% NaCl. The cells were then sedimented at 2,000 × *g* for 5 min at 4°C and the supernatant was removed and used for the assay of released inositol phosphates [15].

2.6. Assay for GTP-γ-S-stimulated PLC activity

GTP-γ-S-stmulated PLC activity was monitored as for PLD activity except that the assay cocktail consisted of 0.6 i.u/ml, 2 mM MgATP, 2 mM $MgCl_2$, 10 mM LiCl, 2 mM 2,3-diphosphoglycerate and pCa 6, final concentrations. After 10 min incubation at 37°C the reaction was quenched and assayed for inositol phosphates [15].

2.7. Presentation of results

All assays were done in duplicate. Each set of experiments were performed at least twice under identical conditions and most observations were verified on 3–4 occasions with slight modifications in the protocol. The data shown are from representative experiments.

3. Results

Streptolysin O allows the efflux of cytosolic proteins from HL60 cells within 5 min [13]. GTP-γ-S-stimulated PLD becomes refractory when HL60 cells are permeabilized first with streptolysin O and subsequently triggered with GTP-γ-S. GTP-γ-S-stimulated PLD activity can be reconstituted with exogenously-added ARF1 to the cytosol-depleted HL60 cells [2]. We examined the effect of the N-terminal ARF1(2–17) peptide on this ARF1-reconstituted PLD activity. Cytosol-depleted HL60 cells were incubated in the presence and absence of ARF1 and GTP-γ-S with varying concentrations of ARF1(2–17) (Fig. 1). As reported previously, ARF1 reconstituted GTP-γ-S-dependent PLD activity but ARF1 or GTP-γ-S alone were insufficient. As the concentration of peptide was increased there was a corresponding inhibition of ARF1-dependent PLD activity with maximum inhibition occurring at 30 μM. The small endogenous response observed with GTP-γ-S alone was also inhibited by ARF1(2–17) peptide.

When GTP-γ-S *plus* Ca^{2+} (pCa 5) is added to HL60 cells simultaneously with streptolysin O, a robust activation of PLD is seen [16]. This activation is presumed to be utilising endogenous ARF proteins and therefore should be susceptible to inhibition by the ARF1(2–17) peptide. Fig. 2A illustrates that PLD activation is inhibited with increasing concentrations of ARF1(2–17). It effectively blocked PLD activity with a half maximal inhibition at 10 μM and maximal inhibition at 30 μM. These values are similar to those reported for its effects on intra-golgi transport [7], ER to cis-golgi [5] endosome-endosome fusion [6] and ARF reconstitution of PLD activity (see Fig. 1).

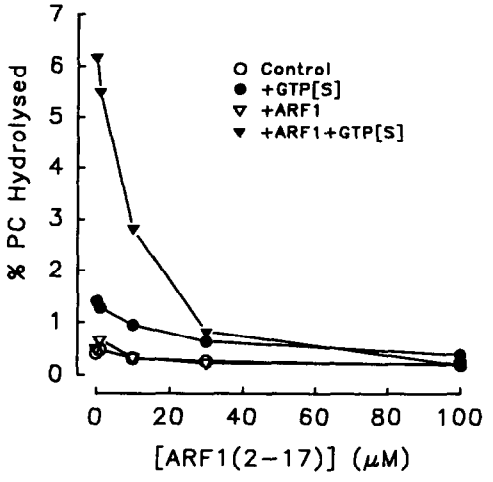

Fig. 1. Inhibition of ARF1-reconstituted PLD activity in cytosol-depleted HL60 cells by the ARF1(2–17) peptide. [^{3}H]choline-labelled cells were permeabilized for 30 min to deplete the cytosolic proteins. The washed cells were incubated with GTP-γ-S (10 μM) and 625 ng ARF1 (assay volume, 50 μl) as indicated for 30 min. [^{3}H]Choline release in the supernatant was monitored and is expressed as a percentage of radioactivity incorporated into the choline-labelled lipids. (○), no additions; (●), 10 μM GTP-γ-S; (▽), 625 ng ARF1; (▼), 10 μM GTP-γ-S + 625 ng ARF1.

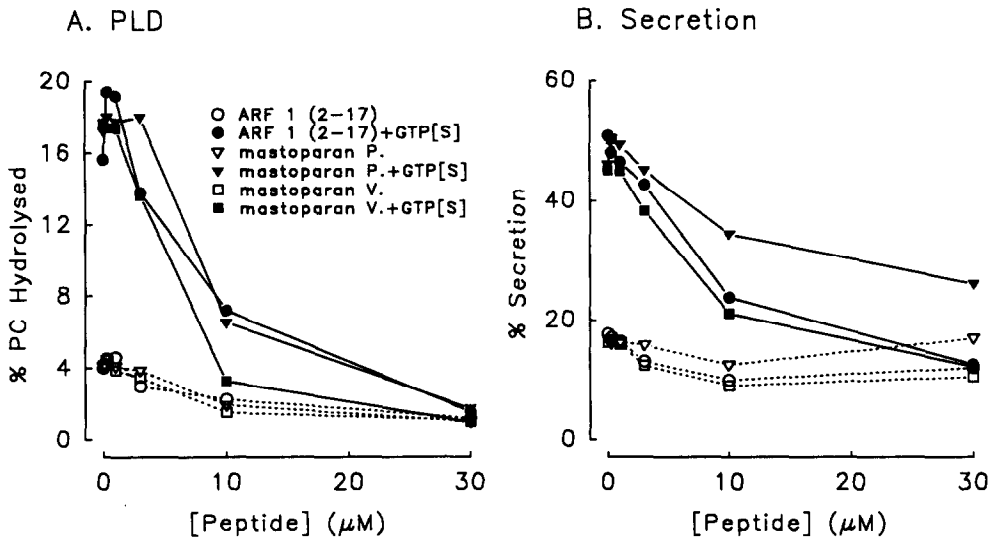

Fig. 2. Effect of ARF1(2–17) and mastoparan *Politses jadwagae* and mastoparan *Vespula lewisii* on PLD activity and exocytosis stimulated with GTP-γ-S in HL60 cells. HL60 cells were incubated with streptolysin O in the presence of GTP-γ-S (10 μM) and peptides as indicated. (A) Release of [^{3}H]choline and (B) exocytosis of hexosaminidase was monitored from the same cell preparation. Open symbols, no GTP-γ-S; closed symbols, 10 μM GTP-γ-S. (○, ●), ARF1(2–17), (▽, ▼), mastoparan *Politses jadwagae*; (□, ■) mastoparan *Vespula lewisii*.

A correlation between PLD activity and exocytosis has previously been observed [10] and the well documented evidence for the role of ARF in vesicular transport [5–7,17] led us to investigate the effect of ARF1(2–17) on exocytosis in HL60 cells. GTP-γ-S *plus* Ca^{2+} (pCa 5) when added with streptolysin O stimulates exocytosis [13]. The secretory response (measured in the same experiment as PLD activity) was also inhibited by ARF1(2–17) (Fig. 2B); both responses were inhibited half-maximally at 10 μM.

Exocytosis from HL60 cells can be triggered by multiple effectors. Sub-maximal exocytosis from permeabilized cells can be triggered by (a) Ca^{2+} alone (pCa 5) which requires MgATP (b) GTP-γ-S alone (pCa 7) which requires MgATP (c) Ca^{2+} *plus* GTP-γ-S (which does not require MgATP). Maximal exocytosis is observed with Ca^{2+} *plus* GTP-γ-S in the presence of MgATP (Table 1 and [10]). All these different components of MgATP-dependent and independent exocytosis are inhibited by the presence of 30 μM ARF1(2–17) peptide (Table 1).

ARF1(2–17) is an amphipathic peptide and has been shown to adopt an α-helical structure in hydrophobic environments [7]. It appears that it may be the conformation of the peptide that is more important rather than its specific amino acid sequence as it has been shown that the N-terminal 16 residues from both hARF1 and hARF4 inhibit ER to Golgi transport. These two peptides only share 50% sequence homology but both adopt α-helical conformations [5]. Another amphipathic peptide of similar size (viz 14 amino acids) which also forms an α-helix is mastoparan [18,19]. We therefore used two mastoparan peptides as a control to establish the specificity of ARF1(2–17) peptide. Two forms of mastoparan were used – Mastoparan *Vespula lewisii* (referred to as mastoparan V.) and mastoparan *Polistes jadwagae* (referred to as mastoparan P.). A comparison of the amino acid composition of mastoparans with the ARF1(2–17) is shown in Table 2.

Like ARF1(2–17), both mastoparans inhibited GTP-γ-S-stimulated PLD activity (Fig. 2A) and exocytosis (Fig. 2B). Although the secretory response was inhibited by all the 3 peptides, mastoparan P. was not quite as effective as the others at inhibiting GTP-γ-S-stimulated exocytosis (Fig. 2B). This was not investigated further.

To analyse the specificity of the ARF1(2–17) peptide, we examined the effect of ARF(2–17) and mastoparan V. on GTP-γ-S-stimulated PLC activity. This pathway is well-defined; G-proteins of the G_q family and βγ-subunits derived from pertussis toxin-sensitive G-proteins are responsible for activating PLC-β isoforms [20–22]. In

Table 1
Inhibition of exocytosis by ARF(2–17)

	% Exocytosis			
	−MgATP		+MgATP	
	Control	+ARF(2–17)	Control	+ARF(2–17)
pCa 7	5	5	6	8
pCa 7 + GTP-γ-S	6	5	16	8
pCa 5	5	5	12	8
pCa 5 + GTP-γ-S	26	6	50	11

HL60 cells were permeabilized with streptolysin O in the presence of Ca^{2+} buffers, GTP-γ-S (10 μM), MgATP (1 mM) and ARF (2–17) peptide (30 μM) as indicated. After 10 min at 37°C the cells were transferred to ice and centrifuged. The supernatant was analysed for the release of hexosaminidase.

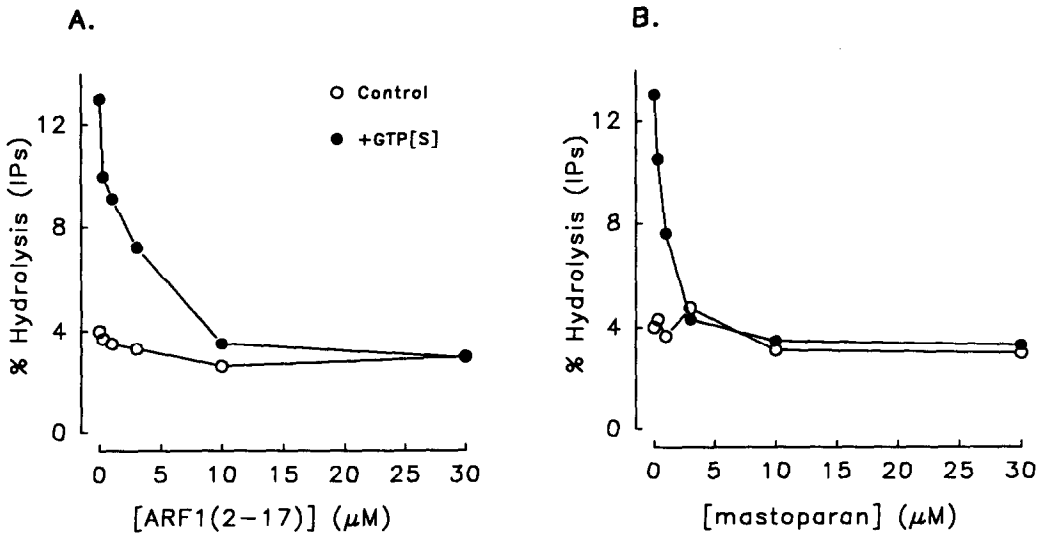

Fig. 3. (A) ARF1(2–17) and (B) Mastoparan *Vespula lewisii* inhibit GTP-γ-S-stimulated phospholipase Cβ activity in HL60 cells. [^{3}H]inositol-labelled cells were incubated with streptolysin O in the presence of GTP-γ-S (10 μM) and peptides as indicated for 10 min. [^{3}H]inositol phosphates released in the supernatant were monitored and are expressed as a percentage of radioactivity incorporated into the inositol-labelled lipids. (○), no GTP-γ-S; (●), 10 μM GTP-γ-S.

addition, a cytosolic protein, phosphatidylinositol transfer protein (PI-TP) is also required [11]. There is no known requirement for ARF proteins in this signalling pathway. Addition of GTP-γ-S in the presence of streptolysin O stimulates PLC β activity [13] and this is inhibited by ARF1(2–17) (Fig. 3A) and by mastoparan V. (Fig. 3B). Both peptides inhibited GTP-γ-S-stimulated PLC β activity with maximum inhibition occurring at 10 μM; this is lower than the concentrations required for maximum inhibition of exocytosis and PLD activity.

GTP-γ-S-stimulated PLC activity is refractory in cytosol-depleted HL60 cells and can be reconstituted by the addition of PI-TP [11] or PLC-β1 [15]. GTP-γ-S-stimulated PLC β activity was reconstituted in cytosol-depleted cells with PI-TP or PLCβ1 added alone or added together. ARF1(2–17) (30 μM) effectively abolished the reconstitution under all conditions (Fig. 4).

4. Discussion

N-terminal ARF peptides have been extensively used as specific inhibitors of ARF function to elucidate the role(s) of ARF in vesicular trafficking [5–7] and regulated exocytosis [17]. In this study we have used the N-terminal 16 residues of ARF1 (ARF1(2–17)) to investigate the role of ARF in both the PLD and exocytotic pathways in HL60 cells.

The reconstitution of GTP-γ-S-mediated PLD activity has been shown to be dependent on the presence of ARF proteins [2,3], therefore it was not surprising that ARF1(2–17) inhibited ARF1-reconstituted PLD activity (Fig. 1). The inhibition of both PLD activity and exocytosis in permeabilized cells by ARF1(2–17) (Fig. 2) indicated that ARF-regulated PLD may be directly involved in exocytosis in these cells.

It is thought that the conformation ARF1(2–17) adopts is important for inhibition of ARF function. ARF1(2–17) forms an α-helix in a hydrophobic environment [7]. The amino acid composition is important in that the equally spaced hydrophobic residues (Ileu-Phe-X-X-Leu-Phe-X-X-Leu-Phe) result in the formation of the α-helix with all the charged residues aligned on the same face of the helix [7]. Mastoparan peptides isolated from wasp venom are also amphipathic and form an α-helix with the charged residues aligned on one face [18,23] as does ARF1(2–17). Two forms of mastoparan were used, mastoparan V. and mastoparan P. By comparison of the peptide sequences in Table 2 it can be seen that mastoparan V. has regularly spaced hydrophobic residues (Leu-X-X-Leu-X-X-Leu) like ARF1(2–17). The hydrophobic residues in mastoparan P. are not so regularly spaced. Mastoparan V. therefore is likely to adopt an α-helical conformation that is very similar to ARF1(2–17), whereas that adopted by mastoparan P. may not be quite so similar.

When these peptides were introduced into permeabilized cells, they inhibited the GTP-γ-S-stimulated PLD activity (Fig. 2A) and exocytosis (Fig. 2B). It was noted that mastoparan P. was not as effective at inhibiting

Table 2
Comparison of the peptide sequences used in this study

ARF1 (2–17)	GN**I**F**A**N**L**F**K**G**L**F**G**KKE
Mastoparan V.	IN**L**K**A**L**A**A**L**A**K**K**I**L
Mastoparan P.	V**D**W**K**K**I**G**Q**H**I**L**S**V

Letters that are in bold and underlined indicate hydrophobic residues.

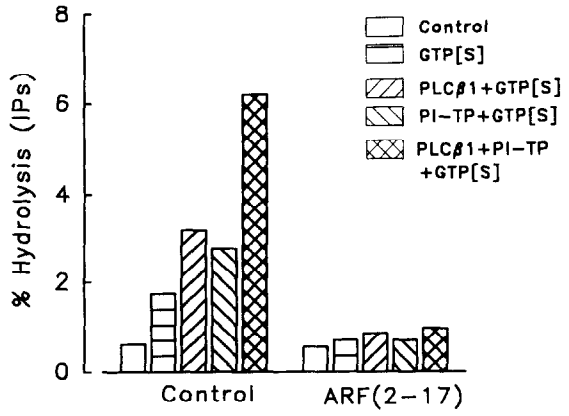

Fig. 4. ARF1(2–17) inhibits reconstituted G-protein-stimulated phospholipase Cβ activity in cytosol-depleted HL60 cells. [^{3}H]Inositol-labelled cells were permeabilized for 30 min to deplete the cytosolic proteins. The washed cells were incubated with GTP-γ-S (10 μM), ARF(2–17) (30 μM final), PI-TP (675 ng) and PLC-β1 (375 ng) as indicated in an assay volume of 55 μl for 20 min. [^{3}H]Inositol phosphates released in the supernatant were monitored and are expressed as a percentage of radioactivity incorporated into the inositol-labelled lipids.

exocytosis as PLD (Fig. 2). The observation that ARF1(2–17) and mastoparans were both inhibitory for PLD activity and exocytosis suggests that the conformation adopted by these peptides is more important than their amino acid composition.

Mastoparan has been shown to promote nucleotide exchange by G-proteins [19,24], and to stimulate arachidonic acid release and DNA synthesis in quiescent Swiss 3T3 cells, although it was cytotoxic at higher concentrations (30 μM) [25]. It has also been shown to stimulate PLC activity in intact differentiated HL60 cells but to inhibit the same response in streptolysin O-permeabilized cells [26]. Mastoparan has also been shown to disrupt membranes by increasing their permeability [27,28]. Although ARF1(2–17) has not been examined so extensively, it is very likely that, like the mastoparans, ARF1(2–17) may equally affect many biologically unrelated responses.

To further emphasize the non-specificity of ARF1(2–17), we examined its effects on GTP-γ-S-stimulated PLC activity. This activity was reconstituted with purified proteins (PI-TP and PLC-β1) in the cytosol-depleted HL60 cells under conditions where the majority of ARF proteins have leaked out. Inhibition of this reconstituted PLC-β activity was also inhibited by ARF1(2–17) (Fig. 4). Addition of ARF1 does not reconstitute GTP-γ-S-stimulated PLC β activity (data not shown). There is no evidence for ARF requirement in the G-protein-stimulated PLC-β pathway, i.e. it is an ARF-independent pathway. Inhibition by ARF1(2–17) of this pathway seriously questions the use of these peptides as a means of inhibiting ARF function. In conclusion, the data presented in this paper indicate that results obtained using ARF1(2–17) should be interpreted with caution due to its lack of specificity.

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