Regulation of mammalian phospholipase D2: interaction with and stimulation by G_{M2} activator

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We have previously reported that a heat-stable activator for ganglioside metabolism, G_{M2} activator, potently stimulates ADP-ribosylation factor (ARF)-dependent phospholipase D (PLD) activity (presumably PLD1) in an *in vitro* system [Nakamura, Akisue, Jinnai, Hitomi, Sarkar, Miwa, Okada, Yoshida, Kuroda, Kikkawa and Nishizuka (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 12249–12253]. However, little is known about the regulation of PLD2. In the present studies we have investigated the regulation of PLD2 by G_{M2} activator and various other regulators including ARF. PLD2 was potently stimulated *in vitro* by G_{M2} activator in a time- and dose-dependent manner. Neither ARF nor protein kinase C caused any significant changes in PLD2 activity. Importantly, PLD2 responsiveness to ARF was greatly enhanced by G_{M2} activator, suggesting a possible role for G_{M2} activator as a coupling factor. G_{M2} activator was also demonstrated to physically associate with PLD2 in a stoichiometric manner. Further, PMA stimulation of COS-7 cells overexpressing both G_{M2} activator and PLD2 resulted in a marked increase in the association of the two molecules. Interestingly, ARF association with PLD2 was greatly increased by G_{M2} activator. Moreover, G_{M2} activator enhanced PMA-induced PLD activity in a synergistic manner with ARF in streptolysin-*O*-permeabilized, cytosol-depleted HL-60 cells, suggesting that G_{M2} activator may regulate PLD in a concerted manner with other factors, including ARF, inside the cells.

Key words: ADP-ribosylation factor, phospholipase D1, protein kinase C.

INTRODUCTION

The hydrolysis of phosphatidylcholine (PtdCho) by phospholipase D (PLD) is thought to generate an important lipid mediator, phosphatidic acid [1]. Phosphatidic acid may be metabolized to other messenger molecules, such as 1,2-diacylglycerol [2], and to lysophosphatidic acid [3]. Although the precise physiological relevance of PLD remains to be elucidated, receptor-stimulated PLD activity has been implicated in a broad range of physiological responses including secretion, superoxide generation, proliferation, differentiation and immune responses (for reviews, see [1,4]).

Two mammalian PLDs, PLD1 and PLD2, have been cloned in the past decade [5–7]. PLD1 is activated *in vitro* by two small G-proteins, ADP-ribosylation factor (ARF) [8,9] and RhoA [10,11], as well as by protein kinase C (PKC) [12,13] and by the lipid PtdIns(4,5)P_2 [8,14]. ARF-dependent PLD from haematopoietic cells is also activated by a 50 kDa soluble protein [15,16] although its biochemical features remain unclear. In contrast to PLD1 the regulation of PLD2 is largely unknown. PLD2 is believed to be constitutively active and requires only PtdIns(4,5)P_2 for its activity as a lipid cofactor [6].

It has recently been reported from this laboratory that a heat-stable protein activator for ganglioside metabolism, G_{M2} activator, potently activates the ARF-dependent PLD (presumably PLD1) partially purified from rat kidney [17]. G_{M2} activator stimulated the kidney PLD synergistically with ARF. The aim of the present studies was to clarify the regulation of the less-characterized isoenzyme PLD2 through studies on activation by and physical association with G_{M2} activator. The data demonstrate clearly that a purified recombinant G_{M2} activator strongly stimulates PLD2 *in vitro*. Importantly, the responsiveness of PLD2 to ARF is greatly enhanced by G_{M2} activator. Physiological relevance of G_{M2} activator as a PLD regulator is also discussed.

EXPERIMENTAL

Materials

1,2-Di[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]-PtdCho, 115 mCi/mmol) was purchased from DuPont-New England Nuclear. 1-[1-^{14}C]Palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine ([^{14}C]lysoPtdCho, 54.0 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Phosphatidylethanol and phosphatidylbutanol for TLC standards were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Plasmalogen-rich phosphatidylethanolamine (PtdEtn; 60 % plasmalogen) was from Serdary Research Laboratories (Englewood Cliffs, NJ, U.S.A.). PtdIns-(4,5)P_2, anti-FLAG M2 affinity gel, anti-FLAG antibody and FLAG peptide were from Sigma. PKCα monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). ARF monoclonal antibody was kindly provided by Dr R. A. Kahn (Emory University, Atlanta, GA, U.S.A.). Guanosine 5′-[γ-thio]triphosphate (GTP[S]) was from Boehringer Mannheim. Reduced streptolysin-*O* (SLO) was from Murex Diagnostics (Dartford, Kent, U.K.). PMA was a product of LC services (Woburn, MA, U.S.A.). Ni^{2+}-nitrilotriacetate agarose was from

Abbreviations used: PtdCho, phosphatidylcholine; [^{14}C]PtdCho, 1,2-di[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; [^{14}C]lysoPtdCho, 1-[1-^{14}C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine; PtdEtn, phosphatidylethanolamine; PLD, phospholipase D; ARF, ADP-ribosylation factor; PKC, protein kinase C; SLO, streptolysin-*O*; GTP[S], guanosine 5′-[γ-thio]triphosphate; 5′-RACE, 5′ rapid amplification of cDNA ends.

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The nucleotide sequence reported here has been submitted to the DDBJ/EMBL/GenBank® Nucleotide Sequence Databases under the accession number AB051391.

Pharmingen (San Diego, CA, U.S.A.). Other chemicals were of analytical grade.

Cell culture

Sf9 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). They were maintained at 27 °C in SF-900 II SFM medium (Life Technologies, Rockville, MD, U.S.A.) supplemented with 10% fetal calf serum (Flow Laboratories) containing 20 μg/ml gentamycin. HL-60 cells were maintained at a cell density between 0.1 and 1.0×10^6 cells/ml as a suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. COS-7 cells or HEK-293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Preparation of recombinant baculovirus and adenovirus

The cDNA for rat G_{M2} activator was isolated by reverse transcriptase PCR from total RNAs of rat kidney. Primers consisted of the sense oligonucleotide 5′-TGGGATCCCCGGT-GGCTTCTCCTGGGATAA-3′ (coding for the N-terminal peptide of the mature mouse G_{M2} activator) and the antisense oligonucleotide 5′-ATGAATTCTTCATTCTGTGGTGGCTG-CTGCC-3′ (corresponding to nucleotides three bases downstream from the stop codon of mouse G_{M2} activator). The 5′ terminal sequence including the initiation codon of rat G_{M2} activator was determined by 5′ rapid amplification of cDNA ends (5′-RACE). This reaction was carried out with the rat G_{M2} activator-specific primer 5′-GAGAAGTGAGGGGAATGCT-GG-3′ and the anchor primer 5′-GGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG-3′ (where I is inosine) and the nested amplification of the first PCR product was performed with the inner primer specific to rat G_{M2} activator 5′-CGGAAT-TCTGAGGCTTTTGATCACTGCAGG-3′ and the universal amplification primer 5′-GGCCACGCGTCGACTAGTAC-3′ by 5′-RACE using the Rapid Amplification of cDNA Ends Kit, version 2.0, according to the manufacturer's protocol (Life Technologies).

The rat PLD1 cDNA was a generous gift from Dr J. H. Exton (Howard Hughes Medical Institute, Vanderbilt University, Nashville, TN, U.S.A.). The rat PLD2 cDNA was isolated by reverse transcriptase PCR from total RNAs of rat brain using rPLD2 gene fragments (accession number D88672). The full-length cDNA encoding either PLD1 or PLD2 was cloned into the baculovirus transfer vector pAcHLT (Pharmingen, San Diego, CA, U.S.A.) with a hexahistidine epitope tag to make N-terminally hexahistidine-tagged PLD1 or PLD2. All constructs were verified by DNA sequencing. This transfer plasmid and linearized BaculoGold DNA were co-transfected into Sf9 cells to get recombinant baculovirus by baculovirus expression vector system according to the manufacturer's instructions.

For the adenoviral gene construct full-length cDNA for rat PLD2 was subcloned into the pCMV5 encoding a FLAG-epitope tag that becomes expressed in frame at the N-termini of the PLDs. The cDNA for rat FLAG-epitope-tagged PLD2 or rat G_{M2} activator was ligated into the cosmid cassette pAxCAwt at the *Swa*I site. These cosmid cassettes and the DNA–terminal-protein complex of Ad5-dlX (the name of the parent adenovirus, which has an E3 deletion) were co-transfected into HEK-293 cells to obtain recombinant adenovirus.

Purification of recombinant proteins

Sf9 cells (1×10^8 cells/50 ml) were infected with recombinant baculovirus encoding either (His_6)-PLD1 or (His_6)-PLD2 at 27 °C for 48 h. At the end of the infection period the cells were detached, centrifuged at 500 ***g*** for 5 min to a pellet, washed once with PBS and resuspended in ice-cold insect cell-lysis buffer (Pharmingen) containing 1 mM dithiothreitol. The (His_6)-PLD1 and (His_6)-PLD2 were purified from the lysate by affinity chromatography using Ni^{2+}-nitrilotriacetate agarose as described previously [18]. The final preparations of both enzymes were free of ARF, PKCα and G_{M2} activator as judged by immunoblot analyses.

Recombinant G_{M2} activator was purified from *Escherichia coli* expressing rat G_{M2} activator using a protocol similar to the one used for the isolation of rat kidney G_{M2} activator [17].

Recombinant N-myristoylated human ARF1 was prepared from *E. coli* expressing recombinant human ARF1 and human myristoyltransferase (kind gifts from Dr R. A. Kahn) as described previously [19].

Cell-free PLD assay

PLD activity was determined with PtdIns(4,5)P_2-containing mixed lipid vesicles essentially as described by Brown et al. [8]. Under the standard assay conditions the reaction mixture (100 μl) contained 5 μM [^{14}C]PtdCho (55000 d.p.m./nmol), 80 μM PtdEtn, 7 μM PtdIns(4,5)P_2, 20 mM Hepes/NaOH, pH 7.4, 2% ethanol, 1 mM $MgCl_2$, 50 nM purified recombinant G_{M2} activator, purified recombinant PLD2 and various activators as specified in the Figure legends. After 20 min of incubation at 37 °C reactions were stopped by the addition of 1 ml of ice-cold chloroform/methanol/HCl (1:1:0.006, by vol.). Lipids were extracted and analysed as described previously [20].

PLD assay in SLO-permeabilized HL-60 cells

HL-60 cells were metabolically labelled with [^{14}C]lysoPtdCho (0.5 μCi/1×10^7 cells) for 2 h at 37 °C. The labelled cells (10^7 cells/ml) were washed by centrifugation for 10 min at 500 ***g*** and resuspended in buffer (20 mM Hepes/NaOH, pH 7.0, 137 mM NaCl and 2.7 mM KCl). The cells were then incubated for 30 min with 0.4 i.u./ml SLO at 37 °C. After SLO treatment, the cells were washed by centrifugation and resuspended in the same buffer. The membrane-permeabilized and cytosol-depleted cells were reconstituted with various activators for PLD assay. Reaction mixture contained (100 μl) 3 mM magnesium acetate, 1 mM $CaCl_2$, 3 mM EGTA, 0.5 mM ATP, 100 μM GTP[S], 0.3% butanol, cytosol-depleted HL-60 cells (5×10^5 cells) and various combinations of activators as specified in the Figure legends. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 1 ml of ice-cold chloroform/methanol/HCl (1:1:0.006, by vol.). Lipids were extracted and analysed as described previously [20].

Immunoprecipitation and immunoblot analyses

COS-7 cells plated in 6 cm dishes were simultaneously infected with adenovirus carrying rat G_{M2} activator (25 plaque-forming units/cell) or adenovirus carrying FLAG-tagged PLD2 gene (25 pfu/cell) as indicated in the Figure legends. Two days after infection cells were stimulated with either 100 nM PMA or 0.1% DMSO for 30 min at 37 °C. Cells were centrifuged for 10 min at 1000 ***g*** and resuspended in an ice-cold lysis buffer (20 mM Hepes/NaOH, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin and 0.25 M sucrose). Cells were lysed by five freeze–thaw cycles followed by sonication. The cell lysates (0.5 ml) were clarified by centrifugation for 15 min at 10 000 ***g*** and incubated for 3 h with anti-FLAG M2 affinity gel (30 μl) with constant agitation. The gels were then washed three times with the lysis buffer without sucrose. The immunoprecipitated proteins were eluted with a FLAG peptide (100 μg/ml) and subjected to SDS/PAGE using 12.5 % gels [21] followed by immunoblot analysis [22]. In some experiments (see Figure 5 below) FLAG-tagged PLD2 immunoprecipitated with anti-FLAG M2 affinity gels was incubated with the heat-treated supernatant fractions (100 μg of protein each) prepared from rat kidney [23], purified recombinant G_{M2} activator (3 μg) or ARF (3 μg) in the presence of 5 μM PtdCho, 80 μM PtdEtn, 7 μM PtdIns(4,5)P_2 and 1 mM $MgCl_2$ for 2 h. The immunoprecipitated proteins were eluted from the gels and analysed as above.

Other procedures

Conventional PKC (a mixture of PKCα, PKCβI, PKCβII and PKCγ) purified from rat brain as described in [24] was a kind gift from Dr U. Kikkawa (Biosignal Research Center, Kobe University, Kobe, Japan). G_{M2} activator was purified from rat kidney as reported previously [17]. Rabbit polyclonal antibody against the oligopeptide Ser-Ser-Phe-Ser-Trp-Asp-Asn-Cys-Asp-Glu-Gly-Lys-Asp-Pro, which is a part of the deduced N-terminal region of human G_{M2} activator (amino acid residues 1–14) was prepared as described previously [22]. This antibody to G_{M2} activator was reactive with rat and mouse G_{M2} activator but not human G_{M2} activator for unknown reasons. Protein was quantified by the method of Bradford [25].

RESULTS

Recombinant rat G_{M2} activator expressed in *E. coli* is functionally active as a PLD activator

The original findings that G_{M2} activator purified from rat kidney stimulates ARF-dependent PLD (presumably PLD1) [17] prompted us to study the regulation of the less-characterized PLD isoenzyme PLD2 by G_{M2} activator. First, recombinant rat G_{M2} activator was prepared and assayed for PLD1 activation to confirm the previous observation [17]. The deduced amino acid sequence of rat G_{M2} activator was 92 % identical to the corresponding sequence of a mouse homologue (Figure 1). G_{M2} activator was expressed in *E. coli* expression system and purified. G_{M2} activator at the final stage of purification was more than

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       1                                                50
rGM2A  GGFSWDNCDEGKDPAVIKSLTLQPDPIVVPGDVIVSAEGKTSIPLTSPQK
       ||||||||||||||||||||| ||||||||||| || ||||| ||| |||
mGM2A  GGFSWDNCDEGKDPAVIKSLTIQPDPIVVPGDVVVSLEGKTSVPLTAPQK

       51                                              100
rGM2A  VELTVEKEVAGFWVKIPCVEQLGSCTYENVCDLIDQYIPPGETCPEPLHT
       ||||||||||||||||||||||||| ||| ||||| |||||| |||||||
mGM2A  VELTVEKEVAGFWVKIPCVEQLGSCSYENICDLIDEYIPPGESCPEPLHT

       101                                             150
rGM2A  YGLPCHCPFKEGTYSLPSSNFTVPDLELPSWLSTGNYRIQSILSSGGKRL
       ||||||||||||||||| ||||||||||||||||||||||||||||||||
mGM2A  YGLPCHCPFKEGTYSLPTSNFTVPDLELPSWLSTGNYRIQSILSSGGKRL

       151        162
rGM2A  ACIKIAASLKGR
        |||||||||||
mGM2A  GCIKIAASLKGR
```

Figure 1 Alignment of amino acid sequences of rat and mouse G_{M2} activators

The deduced amino acid sequence of rat G_{M2} activator ($rG_{M2}A$) was compared with that of the mouse protein ($mG_{M2}A$). Vertical bars indicate identical amino acids.

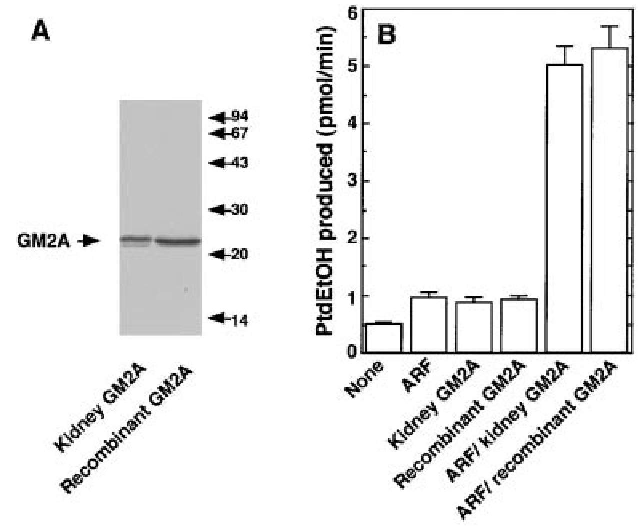

Figure 2 Comparison of PLD1 activation by G_{M2} activator purified from rat kidney or from *E. coli* expressing recombinant rat G_{M2} activator

(**A**) The purified kidney and recombinant G_{M2} activator were analysed by SDS/PAGE using 12.5% polyacrylamide gels followed by silver staining. GM2A, G_{M2} activator. The positions of molecular-size markers are indicated in kDa. (**B**) The purified kidney or recombinant G_{M2} activator was also measured for PLD1 activation with 100 μM GTP[S] in the presence or absence of 100 nM ARF as indicated under the conditions described earlier [33]. Data presented are means $\pm$ S.E.M. ($n = 3$). Similar results were obtained in two separate experiments. PtdEtOH, phosphatidylethanol.

90 % pure, as judged by silver staining (Figure 2A). The recombinant G_{M2} activator was slightly smaller in its molecular size than the one purified from kidney. This may be due to the lack of post-translational modifications such as glycosylation and phosphorylation in the *E. coli* expression system. The purified recombinant G_{M2} activator was assayed for PLD1 activation and compared with the one purified from rat kidney for the potency of PLD activation. The recombinant G_{M2} activator protein activated PLD1 synergistically with ARF with a potency similar to that of G_{M2} activator protein from kidney (Figure 2B). This indicates that post-translational modifications of G_{M2} activator, if there are any, are not essential for its role in the activation of PLD. This also confirms an earlier observation by Klima et al. [26] who studied the enhancement of enzymic conversion of G_{M2} into G_{M3} ganglioside by G_{M2} activator and found that recombinant human G_{M2} activator expressed and purified from *E. coli* was functionally fully active.

Stimulation of PLD2 by the recombinant G_{M2} activator

When recombinant G_{M2} activator was assayed for PLD2 activation using the conditions developed by Brown et al. [8] but in the absence of GTP[S], it caused a strong stimulation of PLD2 ($\sim$ 8-fold) in a time- and dose-dependent manner (Figure 3). The reactions proceeded linearly for 20 min in the absence and presence of G_{M2} activator (Figure 3A). PLD2 was stimulated by G_{M2} activator in a saturable manner and maximally stimulated (7–9-fold) with 17 nM G_{M2} activator (Figure 3B). These conditions also helped to rule out the possibility of any action of G-protein(s), which were unlikely to be contaminants in the samples anyway.

Previously G_{M2} activator has been shown to stimulate PLD1 synergistically with ARF ([17] and Figure 2). Reciprocal actions of G_{M2} activator and ARF on PLD2 activation were studied

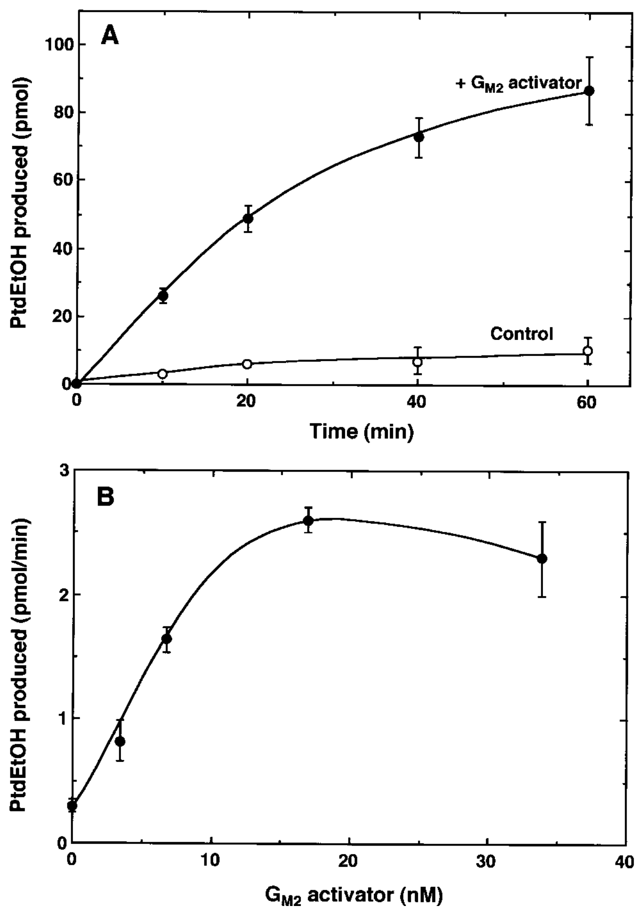

Figure 3 Characterization of PLD2 activation by a purified recombinant G_{M2} activator

(**A**) PLD2 activity was measured for various time intervals as indicated in the absence (○) or presence (●) of 17 nM G_{M2} activator. (**B**) PLD2 activity was also measured as a function of G_{M2} activator concentration as indicated. Data presented are means ± S.E.M. ($n = 3$). Similar results were obtained in two separate experiments. PtdEtOH, phosphatidylethanol.

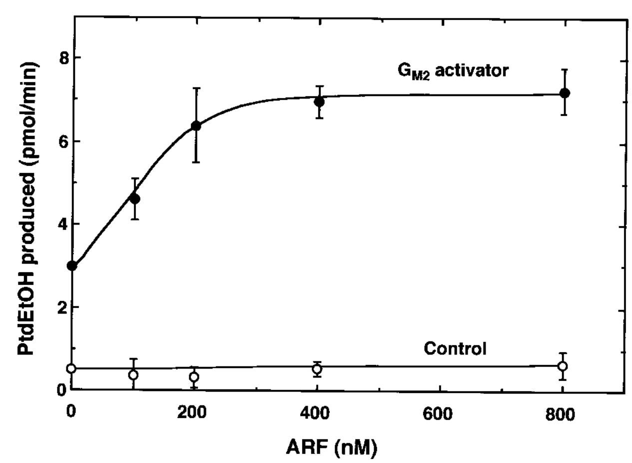

Figure 4 Alteration of ARF responsiveness of PLD2 by G_{M2} activator

PLD2 was assayed with 100 μM GTP[S], various concentrations of ARF as indicated, without (○) or with (●) 17 nM purified recombinant G_{M2} activator. Data presented are means ± S.E.M. ($n = 3$). Similar results were obtained in two separate experiments. PtdEtOH, phosphatidylethanol.

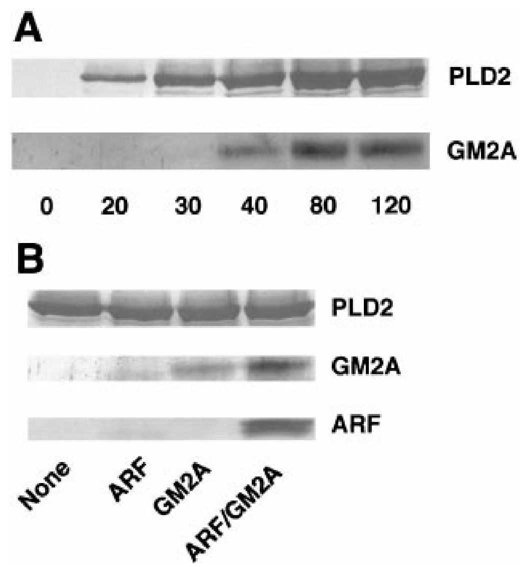

Figure 5 Physical association of G_{M2} activator with FLAG-epitope-tagged PLD2

FLAG-tagged PLD2 expressed in COS-7 cells was immunoprecipitated with anti-FLAG M2 affinity gels. (**A**) various amounts of immunoprecipitates as indicated in terms of gel volume (μl) were further incubated with the heat-treated cytosolic fractions from rat kidney. Gel volume was normalized with equivalent untreated gels. (**B**) Immunoprecipitates (40 μl each) were incubated with various combinations of purified activators. The molecules associated with the immunoprecipitates were eluted with a FLAG peptide and subjected to SDS/PAGE followed by immunoblot analysis. The upper half of the blot was probed with an anti-FLAG antibody while the blots around the 23 kDa and 21 kDa positions were probed with anti-G_{M2} activator and anti-ARF antibody, respectively. GM2A, G_{M2} activator.

next. When G_{M2} activator was added to the reaction, it altered ARF responsiveness of PLD2; in the presence of G_{M2} activator ARF caused a dose-dependent stimulation of PLD2 with a maximal stimulation of 2.3-fold (Figure 4), whereas ARF caused almost no stimulation in the absence of G_{M2} activator, which is consistent with previous reports [6,7].

Association of G_{M2} activator with PLD2

To elucidate further the mechanism of stimulation of PLD2 by G_{M2} activator, the interaction between the two molecules was investigated. As G_{M2} activator is known to be the most abundant in kidney [27], supernatant fractions from rat kidney homogenates were used as a source of G_{M2} activator in the interaction study. Various amounts of anti-FLAG M2 affinity-gel-purified FLAG-epitope-tagged PLD2 were incubated with a fixed amount of heat-treated supernatant fractions from rat kidney. After washing the gels, molecules associated with FLAG-tagged PLD2 were eluted by a FLAG peptide and analysed by immunoblot analysis. G_{M2} activator was co-immunoprecipitated with FLAG-tagged PLD2. The amount of G_{M2} activator proportionally increased as FLAG-tagged PLD2 increased (Figure 5A). No G_{M2} activator was detected when FLAG-tagged PLD2 was absent. The stoichiometry of association of the two molecules was estimated to be about 1 : 1 as judged by the silver staining pattern of the gels after SDS/PAGE in parallel experiments (results not shown). As ARF stimulation of PLD2 activity was strongly enhanced by G_{M2} activator (Figure 4), the effect of G_{M2} activator on the association between PLD2 and ARF was studied next. ARF itself had weak interaction with PLD2 (Figure 5B).

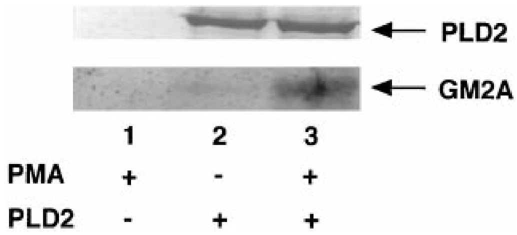

Figure 6 PMA-induced association of G_{M2} activator with FLAG-tagged PLD2

COS-7 cells were infected simultaneously with adenovirus carrying G_{M2} activator and without or with adenovirus carrying FLAG-tagged PLD2 as indicated. Two days after infection cells were stimulated with either 100 nM PMA or vehicle (0.1% DMSO) for 30 min at 37 °C. Cells were lysed and immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitates were eluted with a FLAG peptide and subjected to SDS/PAGE followed by immunoblot analysis. The upper half of the blot was probed with an anti-FLAG antibody whereas the lower half was probed with an anti-G_{M2} activator antibody. GM2A, G_{M2} activator.

However, in the presence of G_{M2} activator association of ARF with PLD2 was greatly enhanced. Interestingly, G_{M2} activator binding to PLD2 was also increased by ARF, suggesting positive co-operativity of physical association among these proteins.

PMA-induced association of G_{M2} activator with PLD2 in COS-7 cells

To elucidate further the mechanism of PLD2 activation by G_{M2} activator, the interaction between the two molecules before and after cell stimulation by PMA was investigated. By an adenoviral gene-transfer technique G_{M2} activator and FLAG-tagged PLD2 were overexpressed simultaneously in COS-7 cells. Cells were lysed and immunoprecipitated with anti-FLAG M2 affinity gels before and after cell stimulation by PMA. The molecules specifically associated with FLAG–PLD2 were eluted by a FLAG peptide followed by immunoblot analyses using anti-G_{M2} activator antibody (Figure 6). PMA stimulation caused a dramatic increase in the association of G_{M2} activator with PLD2 (Figure 6, compare lanes 2 and 3). When only G_{M2} activator was expressed in the cells it could not be detected (Figure 6, lane 1). We failed to detect obvious enhancement of PMA-induced PLD activity in COS-7 cells by the expression of G_{M2} activator with an adenoviral gene-transfer technique (results not shown).

Activation of PLD by G_{M2} activator in a cytosol-depleted HL-60 cell system

Assuming that G_{M2} activator is a physiologically relevant regulator of PLD, a possible explanation for our inability to enhance PMA-induced PLD activation by the exogenous expression of G_{M2} activator is the existence of endogenous G_{M2} activator in COS-7 cells, which are of kidney origin, kidney being the richest source of G_{M2} activator protein expression [28]. To test this hypothesis we used an SLO-permeabilized HL-60 cell system for studying the regulation of PLD by G_{M2} activator, as this system has proved to be useful for probing potential activators of PLD [9]. HL-60 cells were first metabolically labelled with [^{14}C]-lysoPtdCho, then permeabilized and cytosol-depleted by treatment with SLO as described in the Experimental section. This treatment caused no obvious changes in the content of PKCα in HL-60 cells as judged by immunoblot analyses (results not shown). The cytosol-depleted HL-60 cells were then reconstituted with various combinations of activators for PLD assay. When SLO-treated cells were stimulated by PMA, the response to

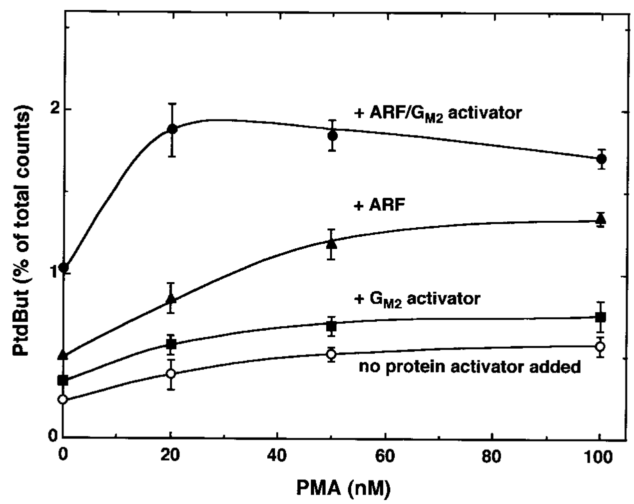

Figure 7 Enhancement of PMA-induced PLD activity by recombinant G_{M2} activator in SLO-permeabilized HL-60 cells

HL-60 cells were metabolically labelled with [^{14}C]lysoPtdCho and treated with SLO as described in the Experimental section. The membrane-permeabilized and cytosol-depleted cells were reconstituted with various combinations of activators for PLD assay as indicated (G_{M2} activator, 50 nM; ARF, 100 nM). Data presented are means ± S.E.M. ($n = 3$). Similar results were obtained in two separate experiments. PtdBut, phosphatidylbutanol.

PMA was strongly diminished due to the loss of activators (Figure 7, ○). ARF partially restored the PMA responsiveness (Figure 7, ▲). G_{M2} activator caused a small enhancement of PMA-induced PLD stimulation (Figure 7, ■). When both ARF and G_{M2} activator were included in the reaction, PMA response was greatly increased (Figure 7, ●). The effect of G_{M2} activator on PLD activation was more evident at a lower stimulatory concentration (20 nM) of PMA. When cells were reconstituted and stimulated simultaneously with SLO treatment without cytosol depletion, the G_{M2} activator effect was small (results not shown).

DISCUSSION

PLD2 has so far been considered a constitutively active enzyme by virtue of the reason that it was already "fully" active under the conditions of the "PtdIns(4,5)P_2- and PtdEtn-containing mixed lipid vesicle method" [6,7] and thus may be regulated negatively. In fact several proteins including α-synuclein, β-synuclein [29] and fodrin [30] have been shown to inhibit PLD2 *in vitro*. Recent analysis of PLD2 revealed that membrane-associated PLD2 prepared from insect cells expressing PLD2 was 1.5–2-fold activated by ARF [31]. More recently, removal of most of the non-core N-terminal region comprising amino acids 1–308 resulted in a protein with much lower basal activity that was stimulated up to 13-fold by ARF [32]. These results raise the possibility that PLD2 possesses the potential capacity of ARF regulation and may acquire ARF responsiveness through an interaction with a 'coupling protein'. In the studies described here we have demonstrated that G_{M2} activator alone potently ($\approx$ 8-fold) stimulates PLD2 *in vitro*. This differs from PLD1 activation by G_{M2} activator in that G_{M2} activator alone had a minimal effect on PLD1 activity and required other factors like ARF for its maximal effect ([17] and Figure 2). Importantly, PLD2 acquired potent ARF-responsiveness in the presence of G_{M2} activator. The PLD2–G_{M2} activator complex may cause structural changes in PLD which then allow the enzyme to be activated by ARF. This hypothesis was strongly supported by the present findings that ARF binding to PLD2 was markedly enhanced by G_{M2} activator (Figure 5B). The present results potentially support the above 'coupling hypothesis' although it has not yet been fully demonstrated that G_{M2} activator is a physiologically relevant regulator of PLD in mammalian cells.

In the case of the enhancement of β-hexosaminidase A-catalysed conversion of G_{M2} into G_{M3} ganglioside by G_{M2} activator, the mechanism of action has been proposed to involve a substrate modification by the activator [27]. G_{M2} activator forms a water-soluble complex with G_{M2} ganglioside which becomes a favourable substrate for β-hexosaminidase A. The mechanism of PLD stimulation by G_{M2} activator may involve a protein–protein interaction between the two entities (Figure 5). The demonstration of PMA-stimulated increase in the association of FLAG-tagged PLD2 with G_{M2} activator (Figure 6) supports the idea of the physiological relevance of the two molecules interacting. PMA-induced modifications such as phosphorylation by PKC of either PLD2 or G_{M2} activator may account for the increased association of two molecules. However, PKC had little effect on PLD2 activity irrespective of the addition of any combinations of other activators, including ARF and G_{M2} activator (results not shown).

The main question as to how G_{M2} activator can become available to PLD is still unanswered because G_{M2} activator is believed to be localized mainly in lysosomes [27], whereas PLDs may be on the cytoplasmic surfaces of plasma membranes or membranes of intracellular organelles. It is intriguing that the mouse G_{M2} activator transcript, like the human protein, was detected in some non-neuronal tissues at much higher levels than in brain, the primary site of G_{M2} ganglioside catabolism, suggesting that G_{M2} activator may have additional functions [28]. Further studies on agonist-induced topological changes in the distribution of both PLD and G_{M2} activator in intact cells may be essential to elucidate physiological relevance of G_{M2} activator in the regulation of mammalian PLD.

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