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Cytosolic PLA_2 in Zymogen Granule Fusion and Amylase Release: Inhibition of GTP-induced Fusion by Arachidonyl Trifluoromethyl Ketone Points to $cPLA_2$ in G-Protein-mediated Secretory Vesicle Fusion

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Previously we reported that the G-protein $G\alpha_{i3}$ localized in pancreatic zymogen granule (ZG) membrane participates in vesicular fusion at the cell plasma membrane (PM). In the present study, the presence of cytosolic phosholipase A_2 ($cPLA_2$) in rat ZGs was demonstrated and its potential role in G-protein-mediated ZG–PM fusion was investigated. *In vitro* fusion assays utilizing both enzymatic and fluorimetric techniques demonstrate that ZGs fuse with PM with a greater potency in the presence of GTP. Arachidonyl trifluoromethyl ketone (AACOCF3) at 40 μM reduces GTP-induced ZG–PM fusion by 25–50%. Anti-$cPLA_2$ antibody reduces ZG–PM fusion in a dose-dependent manner and a 50% reduction of the fusion takes place in the range of 0.48–0.64 ratios of $cPLA_2$ antibody to ZG proteins. PLAP, a $cPLA_2$ activator synthetic peptide increases ZG–PM fusion in a limited dose-dependent manner and tends to inhibit at higher concentrations. Exogenous arachidonic acid inhibits GTP-induced ZG–PM fusion in a dose-dependent manner. Furthermore, a non-hydrolysable GTP analogue, Gpp(NH)p, reduces PLAP effect in ZG–PM fusion; and the net effect of Gpp(NH)p and PLAP differs significantly from the net effect of GTP and PLAP on ZG–PM fusion suggesting that $cPLA_2$ is involved in G-protein-mediated secretory vesicle fusion.

Key words: phospholipase A2, phospholipase A2 inhibitor, G-protein, membrane fusion, pancreas, zymogen granule.

Abbreviations: ZG, zymogen granule; PM, plasma membrane; GTP, guanosine 5′-triphoshate; Gpp(NH)p, guanosine 5′-[β, γ-imido]triphosphate; Gα, G protein alpha subunit; HELSS, haloenol lactone suicide substrate; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; AACOCF3, arachidonyl trifluoromethyl ketone; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; $sPLA_2$, secretory PLA_2; PLAP, PLA_2 activator synthetic peptide, ESPLIAKVLTTEPPIITPVR, that spans a region of homology between natural PLAP and melittin.

Phospholipase A_2 (PLA_2) has been reported to be involved in membrane phospholipid remodelling, membrane homeostasis, arachidonate release and signal transduction (*1–6*). PLA_2 inhibitors block intra-golgi protein transport and conversely, the inhibition of protein transport correlates with that of the PLA_2 activity (*7*). Snake PLA_2 activity induces exocytosis of synaptic vesicles (*8–9*). PLA_2-mediated fusion of neutrophil-derived membranes is augmented by phosphatic acid (*10*). PLA_2-stimulation leads to exocytosis in PC-12 cells (*11*). Secretory PLA_2 stimulates exocytosis and neurotransmitter release in PC-12 cells and cultured rat hypocampal neurons (*12*). Cytosolic PLA_2 activator synthetic peptide (PLAP) increases arachidonic acid release that is inhibitable by a cytosolic PLA_2-specific inhibitor AACOCF3 (*13*). AACOCF3 also inhibits glucose-induced insulin secretion from isolated rat islets (*14*). It has been reported that vesicle fusion in the endocytic pathway requires a PLA_2 activity (*15*). Increment of lyso PE in pancreatic acinar AR42J cells is linked to regulated exocytosis (*16*). Inhibitor of PLA_2 blocks CCK-8 and carbacol-stimulated amylase release from rat pancreatic acini in a dose-dependent manner (*17*). Immunohistochemical studies show that PLA_2 is localized in the apical ZG portion of pancreatic acinar cells (*18*) and in acute pancreatitis in human (*19*). PLA_2 activity is known to alter the granule membrane and has a controlling role in electrolyte transport during stimulated secretion (*20*). Exogenous arachidonic acid increases catecholamine secretion from bovine adrenal chromaffin cells (*21–22*). On the other hand, inhibitors of PLA_2 were reported to inhibit the release of arachidonic acid and catecholamine secretion (*23*). GTP-binding proteins play a major role in the control of intracellular membrane trafficking (*24*), participate in secretory vesicle swelling (*25*) and membrane fusion (*26*). Zymogen gramule (ZG) membrane-associated PLA_2 and acyl transferase may be involved in arachidonic acid turnover in exocrine pancreas and, perhaps, in membrane fusion events associated with exocytosis (*27*).

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Thus, despite enormous interest in elucidating the role of PLA_2 in membrane fusion, there is still a paucity of information for clear understanding as to the role of pancreatic ZG-associated $cPLA_2$ in intact ZG–Pasma membrane (PM) fusion. Previously, we reported the presence of $G\alpha_{i3}$ in ZG and its participation in ZG–PM fusion (*26*). Since $G\alpha_{i3}$ and $cPLA_2$ are present in pancreatic ZG, we investigated the possible role of ZG-associated $cPLA_2$ in ZG–PM fusion and its regulation by ZG-associated G-proteins. In the present study, we demonstrate that $cPLA_2$ in pancreatic ZG participates in GTP-induced fusion with the PM and that may point to $cPLA_2$ in G-protein-mediated secretory vesicle fusion.

EXPERIMENTAL PROCEDURES

Materials—PLAP and $cPLA_2$ antibody were obtained from Santa Cruz Biotechnology, CA, USA. AACOCF3, haloenol lactone suicide substrate (HELSS) and lipid-soluble fluorescent probe octadecylrhodamine (R18) were obtained from Molecular Probes. Percoll, GTP, DTT, aprotinin, phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor and arachidonic acid were from Sigma. All other chemicals were obtained from commercial sources and were of reagent grade.

Isolation of Pancreatic Plasma Membrane—Rat pancreatic PM fractions were prepared by modification of the method of Rosenzweig *et al*. (*28*). Male Sprague–Dawley rats weighing 70–100 *g* were euthanized by ether inhalation. Pancreata were removed and placed in ice-cold phosphate-buffered saline (PBS), pH 7.5. Adipose tissue was removed and the pancreatic tissue was diced into 0.5 mm^3 using a razor blade in a few drops of homogenization buffer A (1.25 M sucrose, 0.01% trypsin inhibitor and 25 mM HEPES, pH 6.5). The chopped tissue was homogenized in 15% (w/v) ice-cold homogenization buffer A using four strokes at maximum speed of a motor-driven pestle (Wheaton overhead stirrer). One and a half millilitre of the homogenate was layered over a 125 µl cushion of 2 M sucrose and 500 µl of 0.3 M sucrose was layered onto the homogenate in Beckman ultra centrifuge tubes. After centrifugation at 145,000 *g* for 1 h using a Sorvall AH-650 rotor, bands between 1.2 and 0.3 M sucrose interface were collected and protein concentration estimated, prior to aliquotting and storage at −20°C. For each experiment, an aliquot of PM was thawed on ice and used in ZG–PM fusion assays.

Isolation of Zymogen Granules—ZGs were purified by modification of a published procedure (*29*). Rat pancreas or pancreatic lobule were chopped into 0.5 mm^3 pieces and suspended in 15% (w/v) ice-cold homogenization buffer B (0.3 M sucrose, 0.01% soybean trypsin inhibitor and 25 mM HEPES, pH 6.5). The suspension was homogenized in a Teflon-glass homogenizer using two strokes at maximum speed of a motor-driven pestle (Wheaton overhead stirrer). The homogenate was then centrifuged for 5 min at 300 *g* at 4°C using a Sorvall SS34 rotor to obtain supernatant. One volume of the supernatant fraction was mixed with two volumes of a Percoll–Sucrose–HEPES buffer C (0.3 M sucrose, 86% percoll, 0.01% soybean trypsin inhibitor and 25 mM HEPES, pH 6.5) and centrifuged for 30 min at 16,000 *g* at 4°C using a Sorvall SS34 rotor. ZGs were obtained as a loose white pellet at the bottom of the tube. ZGs were suspended in 100 mM MES buffer, pH 6.5, and the protein concentration of the suspension was assayed by Bradford method (*30*). Volume of the ZGs preparation was adjusted to obtain a protein concentration of 200–400 µg/ml and used.

Preparation of Zymogen Granule Membranes—Zymogen granules were isolated as mentioned in the previous section. All solutions used contained a cocktail of protease inhibitors, including aprotinin (3 µg/ml) and 0.5 mM PMSF. To prepare granule membranes, the intact granules were lysed at 4°C in 150 mM NaHCO3, 50 mM KCl, 2 mM EDTA, pH 8.5 containing aprotinin (3 µg/ml) and 0.5 mM PMSF. The granule membranes were recovered by centrifugation (140,000 *g*) at 4°C for 30 min.

PAGE and Immunoblot Analysis—ZG proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblotting. The membrane was blocked by 5% skim milk in PBS for 1 h and subsequently incubated with diluted PLA_2 antibody in PBS-0.1%Tween-20 (1:1000) for 1 h. After three washes in PBS-0.1%Tween-20 for 10 min each, the membrane was incubated with diluted peroxidase conjugated donkey anti-rabbit antibody for 30 min. The nitrocellulose membrane was washed three times in PBS-Tween (5 min each wash) and treated with enhanced chemiluminescence solution prior to exposure to X-ray film and developed.

Enzymatic ZG–PM Fusion Assay—In vitro enzymatic ZG–PM fusion was performed following our published method (*26*). Basically, ZGs and PM were incubated in the presence or absence of other reagents in an assay volume of 200 µl of 100 mM MES, pH 6.5, containing 25 mM KCl. The reaction was carried out at 37°C for 10 min in a shaking water bath. After incubation, the reaction tubes were placed in ice-cold water to stop the reaction. Granules were pelleted by centrifugation for 2 min at 8000 r.p.m. using an eppendorf centrifuge (5415C) and one half of the reaction volume (clear supernatant) was aliquoted in a separate eppendorf tube. Both supernatant and pellet portions were assayed for amylase following the method of Bernfield (*31*) using potato starch as the substrate. Briefly, 100 µl of appropriately diluted samples with amylase assay buffer D (10 mM NaH_2PO_4/10 mM Na_2HPO_4/20 mM NaCl) were mixed with the same volume of 1% starch solution in a borosilicate glass tube, and incubated for 10 min at 30°C in a shaking water bath. After cooling the tubes for 10 min in an ice-cooled water bath, 400 µl of colouring reagent E (44 mM dinitrosalicylic acid/200 mM KOH/20 mM Na–K tartrate) was added to the reaction mixture, and the tubes were incubated at boiling temperature for 25 min. The tubes were cooled in an ice-cold water bath and 1.4 ml distilled water was added to the tube, prior to the measurement of the optical density at a wavelength of 530 nm using a DU 530 life science uv/vis spectrophotometer. Results are expressed as a percentage of the total amylase. Each sample was assayed in triplicate. Values are mean ± SEM.

Fluorimetric ZG–PM Fusion Assay—ZGs suspension in sucrose/MES buffer, pH 6.5 with a protein concentration of ~5 mg/ml, were labelled with the lipid-soluble fluorescent probe octadecylrhodamine (R18) by incubation at 25°C for 3 min. Labelled ZGs were recovered by centrifugation at 8000 rpm for 3 min using an eppendorf centrifuge (5415C) and resuspended in the original volume of buffer. For ZG–PM fusion assay, the ZGs were incubated with unlabelled PM, in a temperature-regulated cuvette with constant stirring in a Hitachi F-2000 fluorescence spectrophotometer. Membrane fusion results in redistribution of R18, which is detected as an increase in fluorescence (*26*). An excitation wavelength of 560 nm and emission at 590 nm was used to detect the R18 fluorescence intensity. Labelled ZGs (5 μl) were incubated in absence or presence of specific inhibitor/activator/reagent in 500 μl of 280 mM sucrose/5 mM MES buffer, pH 6.5, at 37°C for 240 sec or until a steady baseline was obtained. Plasma membranes (5 μg protein equivalent) were then added, and the fluorescence signal was monitored for 360 s. In each experiment, the fluorescence signal was expressed as a percentage change from baseline.

Statistical Analysis—Data are expressed as means ± SEM. The *P*-values were calculated by Student's t-tests and a *P*-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In the present study, we report that ZG-associated cytosolic PLA$_2$ participates in G protein-mediated ZG–PM fusion. To determine the association of PLA$_2$ with ZG, we purified ZGs from exocrine pancreas of rat by using percoll gradient and carried out SDS-gel electrophoresis and immunoblot analysis of the ZG proteins. We detected 85-kDa cPLA$_2$ as the major immunoreactive band and two minor bands of apparent molecular weights of 75 kDa and 40 kDa (Fig. 1). The densitometric analyses of the immunoreactive bands indicate that 75 kDa and 40 kDa minor bands represent only 19 and 12% of the total immunoreactivity, respectively (data not shown). Our zymogen granules are highly purified as we used 86% percoll gradient centrifugation (*26*), instead of 50% percoll gradient centrifugation that proved to purify zymogen granules to a near homogeneity and free of subcellular organelles—including mitochondria and endoplasmic reticulum as analysed by transmission electron microscopy (*32*). It is possible that these two minor immunoreactive bands may be the degradation products of the 85 kDa PLA$_2$ by the action of contaminant protease activity in the preparation. Previously, a 14 kDa PLA$_2$ was found in both bovine and rat pancreases (*33, 34*), and purified from pancreatic juice (*35*) and rat pancreatic tissue by heat treatment of the homogenate and the use of cation-exchange chromatography on a CM-Sepharose column (*34*). In addition to these 14 kDa PLA$_2$ enzymes, several high molecular weight PLA$_2$ have been described, including the canine myocardinal 40 kDa PLA$_2$ (*36*), the murine macrophage cell line P388D1 80 kDa PLA$_2$ (*37*), bovine brain 100 kDa PLA$_2$ (*38*) and an 80 kDa PLA$_2$ from CHO cells (*39*).

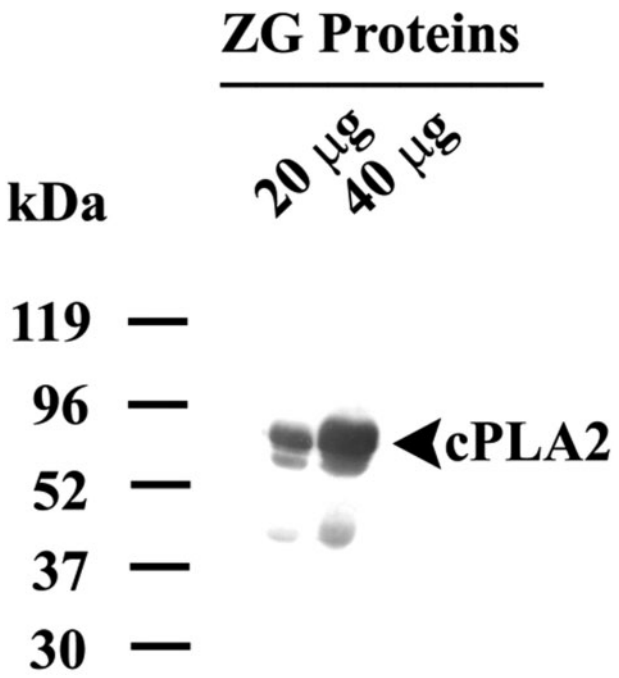

Fig. 1. **PLA2 is associated with zymogen granules in pancreatic cells.** cPLA$_2$ immunoreactivity in zymogen granules isolated from rat pancreas. Twenty and 40 μg each of rat pancreatic zymogen granule proteins were resolved using 12.5% SDS-PAGE. The resolved proteins in each fraction were electrotransferred to nitrocellulose and immunoblotted using cPLA$_2$-specific antibody. An 85 kDa, major cPLA$_2$ immunoreactive band was detected and 75 kDa as well as 40 kDa as minor PLA$_2$ immunoreactive bands. The western blot shown is representative of three similar experiments.

Molecular cloning and expression of 100 kDa PLA$_2$ from human U937 cells showed no homologous region to 14 kDa PLA$_2$ (*40, 41*). Mast cells contain 14, 30 and 85 kDa PLA$_2$ (*42*). As multiple sizes of PLA$_2$ have been reported from the same cell line (*42*), we do not rule out the presence of PLA$_2$ isoforms in pancreatic ZG. Several studies suggest that 80–100 kDa cPLA$_2$ plays a primary role in remodelling membrane phosholipids (*2, 41*). Isolation, purification and physicochemical characterization of cPLA$_2$ from pancreatic ZG will greatly facilitate clarification of this issue.

The presence of cPLA$_2$ in ZG led us to examine its possible presence in the membrane fraction of isolated ZGs. A portion of the lysate of purified ZG was separated into supernatant and membrane fractions by high speed centrifugation. The volume of the membrane fraction was adjusted to that of the volume of the supernatant. Equal volume of lysate, supernatant and the membrane was resolved by SDS-PAGE and immunoblotted using cPLA$_2$-specific antibody. cPLA$_2$ is found to be distributed in both supernatant and membrane fractions (Fig. 2). Although the amount of ZG membrane cPLA$_2$ is less than that of the supernatant, the presence of cPLA$_2$ in ZG membrane may suggest its possible role in membrane modulation as several studies confirmed that 80–100 kDa PLA$_2$ plays a primary role in remodelling membrane phospholipids (*2, 41*). The presence of cPLA$_2$ in ZG

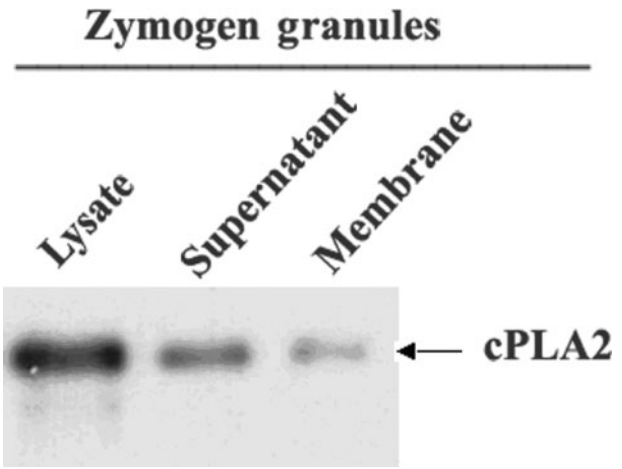

Fig. 2. **ZG-associated $cPLA_2$ is distributed both in supernatant as well as in membrane fractions.** $cPLA_2$ immunoreactivity in zymogen granules isolated from rat pancreas. A portion of the lysate of purified zymogen granules was centrifuged at 140,000*g* for 30 min, the supernatant was separated, and the appropriate buffer was added to the membrane fraction to adjust the volume to that of the supernatant. Equal volume of the lysate, supernatant and the membrane were resolved in 12.5% SDS-PAGE. The resolved proteins in each fraction were electrotransferred to nitrocellulose and immunoblotted using $cPLA_2$-specific antibody.

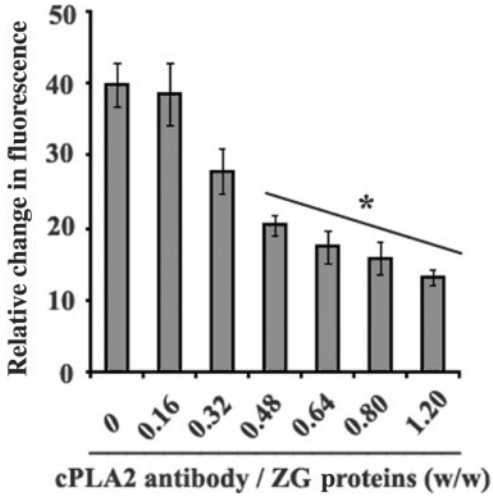

Fig. 3. **$cPLA_2$-specific antibody inhibits ZG–PM fusion.** $cPLA_2$-specific antibody dose-dependently inhibits ZG–PM fusion as assayed by fluorimetric method. The fusion is expressed as the relative change of R18 dye fluorescence from the base line. Values are the mean ± SEM from three experiments. $^*P < 0.01$ compared with control (with no $cPLA_2$ antibody).

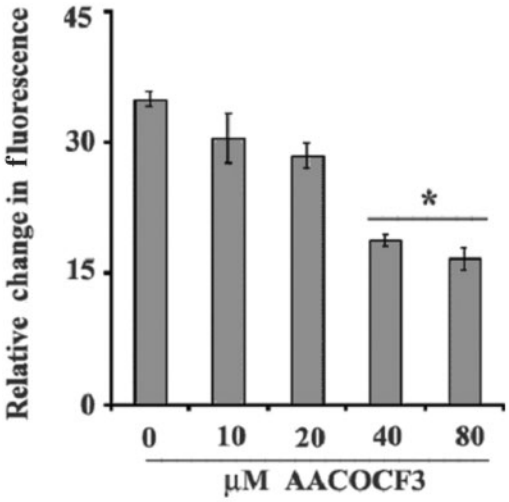

Fig. 4. **AACOCF3 inhibits fluorescence dequenching in ZG–PM fusion.** AACOCF3 inhibits ZG–PM fusion dose-dependently as assayed by fluorimetric method. The inhibition of fusion is expressed as the relative change of R18 dye fluorescence from the control base line. Values are the mean ± SEM from three experiments. $^*P < 0.01$ compared with control (with no inhibitor).

membrane also suggest its easy accessibility to other reagents that the ZG is exposed to. Thus, the presence of $cPLA_2$ in ZG led us to examine its possible involvement in the fusion of isolated ZGs with the PM preparation.

Having found the presence of $cPLA_2$ in ZG membrane, we examined its possible involvement in ZG–PM fusion. We carried out isolated ZG and plasma membrane fusion in the absence and in the presence of $cPLA_2$ antibody, employing our fluorimetric ZG–PM fusion assays, as reported earlier (*26*). We labelled isolated ZGs with rhodamine (R18) dye and challenge them to fuse with PM preparations. Upon fusion of the labelled ZG with the unlabelled PM, the energy transfer decreases proportionally; this is registered as an increase in fluorescence, called dequenching. We determined the effect of $cPLA_2$ antibody on fluorescence dequenching of ZG–PM fusion. With increasing concentrations, $cPLA_2$ antibody gradually decreases fluorescence dequenching as compared with the dequenching of the control fluorescence of ZG–PM fusion. A 50% reduction of fluorescence dequenching takes place in the range of 0.48–0.64 ratios of $cPLA_2$ antibody and ZG proteins (Figure 3). This data demonstrates that $cPLA_2$-specific antibody can effectively interrupt the participation of ZG membrane-associated $cPLA_2$ in ZG–PM fusion, and thus postulates a role of $cPLA_2$ in ZG–PM fusion.

To further corroborate the involvement of ZG-associated $cPLA_2$ in ZG–PM fusion, we set out to use $cPLA_2$-specific inhibitor AACOCF3 (*43*), in our ZG–PM fusion assays. We carried out isolated ZG and plasma membrane fusion in the presence and absence of AACOCF3 using fluorescence dequenching assay. Fluorescence dequenching in ZG–PM fusion gradually decreases as a function of increasing concentration of AACOCF3 (Fig. 4). About 40 μM AACOCF3 decreased fluorescence dequenching by ~50% whilst 80 μM AACOCF3 decreased fluorescence dequenching by 65% in ZG–PM fusion (Fig. 4). Again, this AACOCF3 dose-dependent inhibition of ZG–PM fusion suggests the involvement of $cPLA_2$ in the ZG–PM fusion process.

Having found an inhibitory effect of AACOCF3 on fluorescence dequenching of ZG–PM fusion, we wanted to investigate wheather AACOCF3 would have a similar inhibitory effect on amylase release in ZG–PM fusion.

To further confirm the earlier results from fluorimetric ZG–PM fusion assays, we employed an established enzymatic ZG–PM fusion assays as reported earlier (*26*). Previously, we reported the presence of a GTP-binding protein Gα_{i3} in ZG and its participation in ZG–PM fusion (*26*). To investigate possible correlation between fluorescence dequenching and amylase release in ZG–PM fusion, we assayed the effect of a fixed concentration (40 μM) of AACOCF3 in ZG–PM fusion in the absence and presence of 20 μM GTP, using both amylase release and R18 fluorescence dye dequenching assays. Our data shows that 20 μM GTP increases ZG–PM fusion by 20–30% over the control (Fig. 5) as measured by both amylase release and R18 dye fluorescence dequenching assays. This stimulatory effect of GTP in ZG–PM fusion is significant and consistent with our earlier findings (*26*). Both the assays showed almost similar extent of GTP-stimulated ZG–PM fusion and the differences in their magnitudes are not significantly different between the two assay systems. On the other hand, 40 μM AACOCF3 reduces ZG–PM fusion by 25–50% over the control (Fig. 5), as measured by both amylase release and R18 dye fluorescence dequenching assays. This inhibitory effect of AACOCF3 on ZG–PM fusion is significant. Although, the extent of inhibitory effect of AACOCF3 on ZG–PM fusion as measured by both methods apparently varies, the differences in their magnitudes are not significantly different. With respect to the assay systems, a similar inhibitory trend of AACOCF3 has also been shown on GTP-induced ZG–PM fusion, as measured by both amylase release and R18 dye fluorescence dequenching assays (Fig. 5). Overall, the data obtained from fluorescence dequenching in ZG–PM fusion are comparable and proportional to that of the data obtained from amylase release in ZG–PM fusion assays and thereby, validates both of our fusion assays as reported earlier (*26*).

Since we have not investigated whether secretory PLA$_2$ (sPLA$_2$) and/or calcium-independent PLA$_2$ (iPLA$_2$) are also present in the ZG membrane, it was essential to determine the involvement of possible ZG-associated iPLA$_2$ and sPLA$_2$ in ZG–PM fusion. As several investigators have reported that HELSS and DTT selectively inhibit iPLA$_2$ (*44*) and sPLA$_2$ (*45*), respectively, we carried out isolated vesicles and plasma membrane fusion in the presence and absence of i/sPLA$_2$ inhibitors HELSS or DTT. We assessed the effects of HELSS, DTT and AACOCF3 in GTP-induced ZG–PM fusion using our fluorimetric ZG–PM fusion assay. The iPLA$_2$ inhibitor HELSS and sPLA$_2$ inhibitor DTT did not reduce the fluorescence dequenching of GTP-induced ZG–PM fusion (Fig. 6), while the cPLA$_2$ inhibitor, AACOCF3 significantly reduced the fluorescence dequenching of GTP-induced ZG–PM fusion reaction (Fig. 6). The results demonstrate that neither iPLA$_2$ nor sPLA$_2$ in ZG, if any, participates in GTP-induced ZG–PM fusion.

Having found that the PLA$_2$ inhibitor, AACOCF3, inhibits ZG–PM fusion, it was essential to investigate whether a PLA$_2$ agonist or activator would have an opposite effect on ZG–PM fusion. It has been reported that the PLAP increases arachidonic acid release that is inhibitable by a cPLA$_2$-specific inhibitor AACOCF3 (*13*).

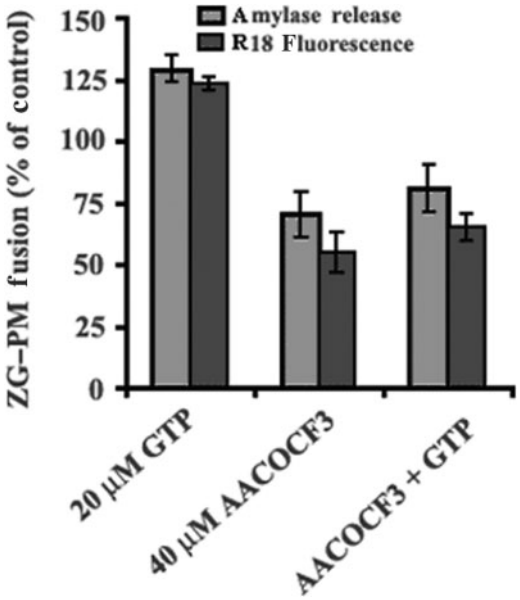

Fig. 5. **GTP stimulates whilst AACOCF3 inhibits ZG–PM fusion.** GTP stimulates whilst AACOCF3 inhibits ZG–PM fusion as measured by amylase release and R18 fluorescence dye dequenching assays. The results are expressed as the percentage of the control fusion (with no added GTP or AACOCF3). Values are the mean ± SEM from four to six experiments.

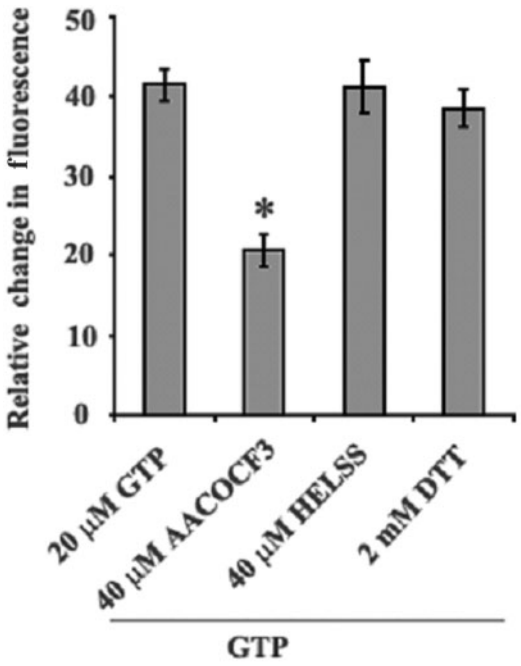

Fig. 6. **HELSS and DTT do not inhibit GTP-induced ZG–PM fusion.** The effects of HELSS, DTT, and AACOCF3 on GTP-induced ZG–PM fusion were assayed by fluorimetric method. The fusion is expressed as the relative change of R18 dye fluorescence from the base line. Values are the mean ± SEM from three to five experiments. *$P < 0.05$ compared with control (with no inhibitor).

To investigate further the involvement of ZG-associated $cPLA_2$ in ZG–PM fusion, we carried out ZG–PM fusion experiment in the presence or absence of PLAP. PLAP dose-dependent experiment on ZG–PM fusion revealed an initial rise in fusion followed by a decrease in fusion at higher concentration (160 μg/ml) of the activator (Fig. 7A). This result suggests that the $cPLA_2$ participates in ZG–PM fusion and may also regulate ZG–PM fusion through its feed back inhibition. Because, $cPLA_2$ is known to hydrolyse membrane phospholipid into free arachidonic acid and lysophosphotidylcholine, we hypothesize that a limited hydrolysis of membrane phospholipids by $cPLA_2$ is required for fusion whereas excessive hydrolytic activity of $cPLA_2$ may be an antagonist to ZG–PM fusion. This prompted us to determine whether arachidonic acid, a product generated from phospholipid hydrolysis by $cPLA_2$, would have any effect on ZG–PM fusion.

To investigate further the involvement of ZG-associated $cPLA_2$ in ZG–PM fusion, we performed a fusion experiment in the presence or absence of exogenous arachidonic acid. The presence of exogenous arachidonic acid in the fusion reaction at 320 μM did not influence the ZG–PM fusion significantly; however, at 640–960 μM arachidonic acid exhibited a significant inhibitory effect on ZG–PM fusion (Fig. 7B). The concentration-dependent inhibitory effect of added arachidonic acid on ZG–PM fusion suggests a role of arachidonic acid, a product that is usually generated from membrane phospholipid hydrolysis by $cPLA_2$ in ZG–PM fusion. The inhibitory effect of exogenous arachidonic acid on ZG–PM fusion is consistent with the effect of the $cPLA_2$ activator, PLAP, on ZG–PM fusion in the context of excess $cPLA_2$ activity. It is not known how the excess arachidonic acid could perturb ZG–PM fusion other than by the feedback inhibitory effect on $cPLA_2$.

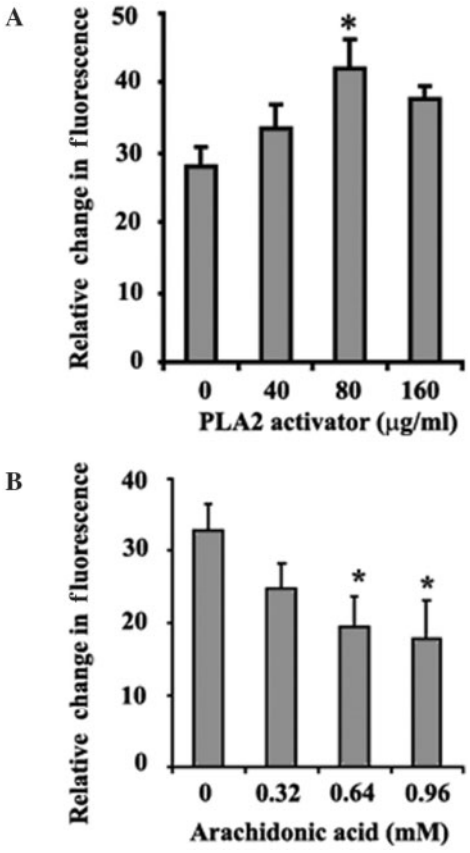

Fig. 7. **PLA_2 activator induces and exogenous arachidonic acid inhibits ZG–PM fusion.** (A), PLA_2 activator induced ZG–PM fusion as assayed by fluorimetric method. The fusion is expressed as the relative change of R18 dye fluorescence from the base line. Values are the mean ± SEM from three to five experiments. $^*P < 0.05$ compared with control (with no PLA_2 activator). (B), exogenous arachidonic acid inhibits GTP-induced ZG–PM fusion in a dose-dependent manner. The fusion is expressed as the relative change of R18 dye fluorescence. Values represent mean ± SEM from three to five experiments. $^*P < 0.05$ compared with control (with no arachidonic acid).

Next, we set out to determine if $cPLA_2$-mediated ZG–PM fusion is indirectly regulated by ZG-associated GTP-binding protein (*26*). We used a fixed concentration (20 μM) of GTP in the absence or presence of the $cPLA_2$ inhibitor AACOCF3 (Figure 5). As shown in Fig. 5 and Table 1, 20 μM GTP potentiates ZG–PM fusion by 29% as measured by amylase release. On the other hand, 40 μM AACOCF3 inhibits ZG–PM fusion by 30% under the same assay conditions. These data show that the stimulatory effect of 20 μM GTP would have been counter-balanced by the inhibitory effect of 40 μM AACOCF3 only if their respective target ZG-associated proteins did not have direct or indirect influence on each other in ZG–PM fusion reactions. In contrast to this theoretical possibility, as shown in Table 1, when GTP and AACOCF3 were present together in the fusion reaction, the overall ZG–PM fusion was reduced by 19%. These data may suggest a likely cross-talk between ZG-associated $cPLA_2$ and GTP-binding protein (*26*) in ZG–PM fusion.

To investigate further, we performed ZG–PM fusion in the presence of a non-hydrolysable GTP analogue, Gpp(NH)p. Although GTP-induced ZG–PM fusion by ~30% over the control level, Gpp(NH)p had little or no effect on ZG–PM fusion, which is consistent with our earlier finding suggesting the requirement of GTP hydrolysis in ZG–PM fusion (*26*). We also assayed the effect of $cPLA_2$ inhibitor or activator on ZG–PM fusion in the presence of Gpp(NH)p. As shown in Figure 8, the presence of Gpp(NH)p in the ZG–PM fusion enhanced AACOCF3 effect slightly but reduced PLAP effect significantly. This is expected based on the lack of additional supply of GTP that is required for the ZG–PM fusion to go on. The effect of AACOCF3 on ZG–PM fusion was significantly affected by the presence of neither GTP nor Gpp(NH)p. However, the net effect of AACOCF3 and Gpp(NH)p together significantly differed from that of the net effect of AACOCF3 and GTP together on ZG–PM fusion. Similarly, the effects of PLAP and Gpp(NH)p together significantly differed from the effects of PLAP and GTP together on ZG–PM fusion. These data suggest the requirement of GTP for ZG–PM fusion and participation of $cPLA_2$ in G-protein-mediated ZG–PM fusion.

Taken together, results from our studies suggest that $cPLA_2$ mediates ZG–PM fusion through limited

Table 1. **Effects of GTP and AACOCF3 on amylase release in ZG–PM fusion**[a].

Reagents	Fusion (% of control)	% Inhibition	Remarks
20 μM GTP	129 ± 5.7	−29[b]	Stimulates fusion
40 μM AACOCF3	70 ± 9.2	+30[c]	Inhibits fusion
GTP + AACOCF3	81 ± 9.4	+19[d]	$d > (b + c)$ implies weak recovery by added GTP

[a] The data from Fig. 5 are expressed as a percentage of the control and shown in simplified tabular form for ease of comparison. [b]Stimulatory effect of GTP is shown as a negative inhibitory value for ease of comparison.[c]Inhibitory effect of AACOCF3 is shown as a positive inhibitory value. [d]Net inhibitory effect of GTP plus AACOCF3 is shown as a positive inhibitory value.

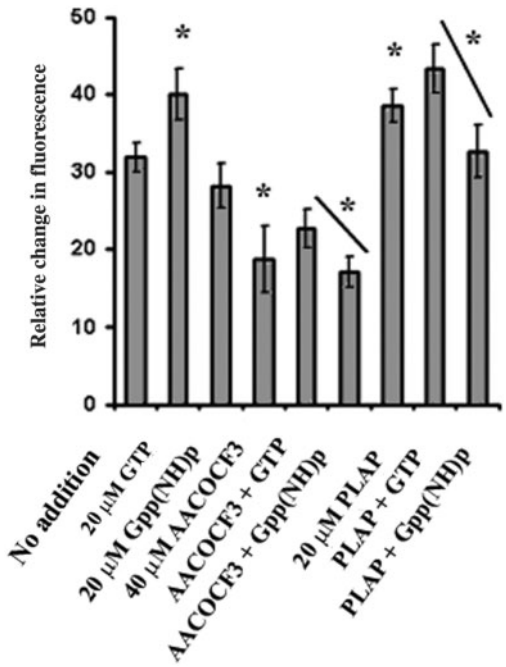

Fig. 8. **PLA$_2$ participates in G-protein mediated ZG–PM fusion.** The involvement of PLA$_2$ in G-protein-mediated ZG–PM fusion is further demonstrated using GTP and non-hydrolyzable GTP analog Gpp(NH)p in combination with cPLA$_2$-specific inhibitor AACOCF3 and activator PLAP as assayed by fluorimetric method. The fusion is expressed as the relative change of R18 dye fluorescence from the base line. Values are the mean ± SEM from three to five experiments. $^*P < 0.05$ compared with control (with no additive).

hydrolysis of phospholipids at the ZG membrane. Excess hydrolysis of phospholipids by ZG-associated cPLA$_2$ has an inhibitory effect on ZG–PM fusion. Since GTP induces ZG–PM fusion through a $G\alpha_{i3}$ protein at the ZG membrane (*26*), we hypothesize that G-proteins are involved in the control cPLA$_2$ activity, which in turn may hydrolyse membrane phospholipids to facilitate ZG–PM fusion.

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