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A NEWLY CLONED PHOSPHOLIPASE A_2-ACTIVATING PROTEIN ELICITS Ca^{2+} OSCILLATIONS AND PANCREATIC AMYLASE SECRETION VIA MEDIATION OF G PROTEIN BETA/PHOSPHOLIPASE A_2/ARACHIDONIC ACID CASCADES

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Recently we have demonstrated that in rat pancreatic acini the high affinity cholecystokinin receptors are coupled to the phospholipase A2 (PLA2)/arachidonic acid (AA) cascades to mediate Ca2+ oscillations and amylase secretion. This intracellular signal transduction system is associated with an unidentified G protein(s) which is neither Gi/Go nor Gq-alpha. Using a newly cloned PLA2-activating protein (PLAP), we further examined the mechanisms by which PLA2 activates Ca2+ oscillation and pancreatic enzyme secretion. In intact acini, 0.1-1 μM PLAP evoked Ca2+ oscillations in a dose-dependent manner (delta [Ca2+]i: 18-121 nM and frequency: 2.3-5.5 cycles/10 min). PLAP elicited a 3-fold increase in monophasic amylase secretion with an EC50 of 0.1 μM. PLAP dose-dependently caused an increase in the AA metabolite 15-HETE. The PLA2 inhibitor, but not inhibitors of lipoxygenase, cytochrome P-450 and cyclooxygenase, inhibited the action of PLAP, suggesting that AA, but not AA metabolites, functions as a signal messenger. In permeabilized acini, a monoclonal antibody of G protein beta subunits inhibited the action of PLAP. Because of the structural similarity between PLAP and Gbeta protein we hypothesize that the PLA2 coupled G protein is Gbeta and it elicits Ca2+ oscillations and monophasic amylase secretion via the AA pathway.

Several lines of evidence suggest that the phospholipase A_2 (PLA_2)/arachidonic acid (AA) cascades function as an intracellular signal transducer in different cell systems [1,2]. In pancreatic acinar cells, both AA and AA metabolites have been implicated as signal messengers during stimulus-secretion coupling [3-7]. Recently we have demonstrated that in rat pancreatic acini the high affinity cholecystokinin (CCK) receptors are coupled to the PLA_2/AA pathways to mediate Ca^{2+} oscillations and monophasic amylase secretion [8]. Utilizing antimelittin antibodies, a mammalian protein that activates PLA_2 has recently been cloned [9]. This protein has been termed PLA_2-activating protein (PLAP: NH_2-Glu-Ser-Pro-Leu-Ile-Ala-Lys-Val-Leu-Thr-Thr-Glu-Pro-Pro-Ile-Ile-Thr-Pro-Val-Arg-Arg-OH). Of interest, PLAP has a sequence similarity with the G protein beta subunits [10]. Several

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recent reports have indicated functional roles of $G_{beta/gamma}$ dimers as well as G_{alpha} subunits [11]. Using PLAP, we further examined the mechanisms by which PLA_2 activates Ca^{2+} oscillations and enzyme secretion in rat pancreatic acini.

METHODS AND MATERIALS

Chemicals were purchased from the following sources: phospholipase A_2-activating protein from Bachem (Torrance, CA); fura-2 acetoxymethyl ester from Calbiochem (San Diego, CA); ONO-RS-082, ketoconazole, nordihydroguaiaretic acid (NDGA) and indomethacin from Biomol (Plymouth Meeting, PA); G protein beta subunits monoclonal antibody from UBI (Lake Placid, NY); 15-HETE and prostaglandin E2 assay systems from Amersham (Arlington Heights, IL); streptolysin O from GIBCO BRL (Grand Island, NY); creatine phosphate, creatine phosphokinase, ATP and soybean trypsin inhibitor (SBTI) from Sigma Chemical Co. (St. Louis, MO); collagenase (CLSPA) from Worthington Biochemical Corp. (Freeehold, NJ).

Isolated rat pancreatic acini were prepared by collagenase digestion of pancreas obtained from male Sprague-Dawley rats [12]. Acini were suspended in a physiological salt solution (PSS) containing 0.1% BSA, 0.1 mg/ml SBTI and (in mM): 137 NaCl, 4.7 KCl, 0.56 MgCl2, 1.28 CaCl2, 1.0 NaH2PO4 , 10 HEPES, Eagles' minimum essential amino acid neutralized with NaOH, and 2.0 L-glutamine. The PSS was adjusted to pH 7.4 and equilibrated with 100% O2.

[Ca2+]i measurements in individual pancreatic acini were determined as previously published [12]. In brief, isolated acini were incubated with 2 μM fura-2 AM at 37°C in 10 ml PSS solution for 30 min. All experiments utilized a dual excitation-wavelength (340/380 nm emitted at 505 nm) modular fluorometer system (Spex Fluorolog 2) coupled to a Nikon Diaphot inverted microscope (x 40). Isolated acini placed on a cover glass and mounted on the closed chamber were continuously superfused by an 8 chambered reservoir (1 ml/min). A fluoroescence ratio was converted to [Ca2+]i according to in vitro calibration determined with an external standard (Calcium Kit I, Molecular Probes) and 25 μM fura-2 potassium salt [13].

For a study of amylase secretion, acini were preincubated for 30 min in 40 ml PSS, washed twice (50xg for 3 min), and resuspended in 40 ml fresh PSS. Aliquots of 2 ml were then distributed into each flask and incubated with reagents for 60 min at 37°C. The incubation was terminated by centrifugation for 30 s at 4°C in a microfuge (1 mlx2 in 20 groups). Amylase released into the supernatant and that remaining in the pellet in each tube were assayed using procion yellow starch as substrate. Amylase secretion was expressed as percentage of the total content in each sample.

For measurement of the metabolites of arachidonic acid (15[S]-hydroxy-5,8,11,13-eicosatetraenoic acid/15-HETE and prostaglandin E2), a radioreceptor assay was performed using [3H]-15-HETE and [125I]-prostaglandin E2 assay systems. 1 ml aliquots of the acinar suspension ($2x10^6$ cells/PSS) were incubated with PLAP at 37°C. At each time interval, the cell suspension was centrifuged (10,000 r.p.m.) for 20 sec at 4°C in a microfuge and the supernatant removed. The pellet was immediately resuspended in 0.5 ml chilled 50 mM tris/HCl buffer (pH 7.5) containing 5 mM EDTA, 10 mM EGTA, 0.3% w/v beta-mercaptoethanol, 10 mM benzamide, 50 μg/ml phenylmethylsulphonyl fluoride, and 1% triton X-100 (extraction buffer) and frozen by liquid nitrogen; for measurements of prostaglandin E2, the pellet was suspended in 0.4 ml extraction buffer and 0.1 ml methyl oximation reagent (methoxyamine hydrochloride and sodium acetate in water:ethanol 9:1 v/v pH 5.6). The suspension was stored at -70°C overnight, thawed, and sonicated for 30 sec at 4°C. The sonicates were centrifuged at 10,000 r.p.m. for 15 min at 4°C to obtain cytosol and plasma membrane fractions in the supernatant. For measurements of 15-HETE, 100 μl supernatants (50 μg protein) were incubated with a 15-HETE antiserum (100 μl) and 0.01 μCi [3H]-15-HETE (100 μl), both of which were solubilized in 0.01 M sodium phosphate buffer (pH 7.1) containing 0.9% w/v sodium chloride, 0.0057% w/v thiomersal and 0.05% w/v gelatin, for 60 min at 37°C. The final volume per incubation was 300 μl. Bound and free labelled 15-HETE were separated by centrifugation at 10,000 r.p.m. for 5 min at 4°C with 1 ml dextran-coated charcoal in phosphate-buffered saline (plus gelatin) and the radioactivity in the supernatant was counted in a liquid scintillation counter. Protein in the supernatant in each sample (100 μl in extraction buffer) was measured using a Bio-rad protein assay system (Bio-rad, Hercules/CA). 15-HETE

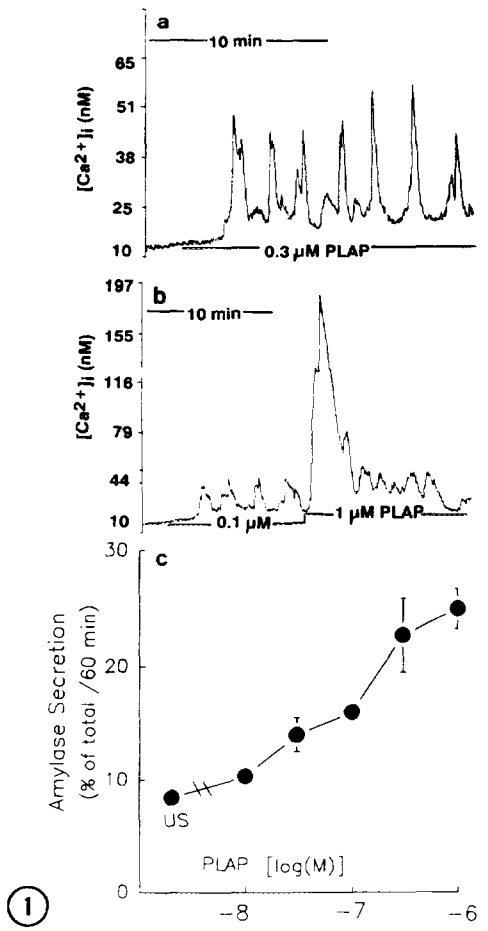
contents in the cells were expressed as ng/mg cell extract protein. For measurements of prostaglandin E2, 100 μl supernatants were incubated with a prostaglandin E2 antiserum (100 μl) and 0.015 μCi [125I] prostaglandin E2 (100 μl), both of which were solubilized in 0.05 M tris/HCl buffer (pH 7.4) including 0.9% NaCl, 0.01% triton X-100 and 0.0057% thimerosal, for 2 hrs at 25°C. Ameriex-M second antibody (250 μl) was then added in each sample and incubated for 15 min at 25°C. Bound and free labelled prostaglandin E2 were separated by centrifugation at 10,000 r.p.m. for 5 min at 4°C and the radioactivity in the pellet was counted in a gamma scintillation counter. Prostaglandin contents in the cells were expressed as ng/mg cell extract protein.

Permeabilized pancreatic acinar cells were prepared as follows. Acini suspended in fresh PSS were washed twice and resuspended with 40 ml cytosol buffer. The cytosol buffer (pH 7.2 at 37oC) contained 0.2% bovine serum albumin, 1 mM ATP, 1 mM creatine phosphate, 50 μg/ml creatine phosphokinase and (in mM) 20 NaCl, 0.5 MgSO4,100 KCl, 0.2 NaH2PO4, 0.8 Na2HPO4, 10 HEPES and 10 glucose. 100 nM free [Ca2+] was prepared in Ca2+/Mg2+/EGTA buffer (in mM: 2.55 CaSO4, 1.64 MgSO4 and 5 EGTA). 400 nM [Ca2+] was prepared using 4.03 mM CaSO4, 1.47 mM MgSO4 and 5 mM EGTA in cytosol buffer. Aliquots of streptolysin O (SLO, 1U/ml) were added to a 40 ml cell suspension (100 nM [Ca2+] cytosol buffer), and incubated for 20 min at 37°C, gassing with 100% O2. After centrifugation (50xg), cells were resuspended in 40 ml fresh SLO-free cytosol buffer which contained 400 nM [Ca2+]. Amylase secretion was determined using the same method as with intact cells.

RESULTS

PHOSPHOLIPASE A_2-ACTIVATING PROTEIN (PLAP) ELICITS Ca^{2+} OSCILLATIONS AND AMYLASE SECRETION In dispersed intact pancreatic acini, 0.1-1 μM phospholipase A_2-activating protein (PLAP) caused repetitive Ca^{2+} spiking oscillations in a concentration-dependent manner (Figs. 1a and 1b). The first Ca^{2+} spike occurred 2-4 min after PLAP stimulation. When 0.1, 0.3 and 1 μM PLAP were applied, the delta $[Ca^{2+}]_i$ amplitude (nM)(peak-basal) was 18$\pm$4 (n=3), 92$\pm$22 (n=7) and 121$\pm$25 (n=3), and the frequency (cycles/10 min) was 2.3$\pm$0.7, 5.6$\pm$0.5 and 5.5$\pm$0.9, respectively. As shown in Fig. 1c, PLAP elicited monophasic amylase secretion with an EC_{50} of 0.1 μM. There was a 3-fold increase in amylase secretion over basal when a supramaximal concentration of PLAP (1 μM) was applied.

PLAP INCREASES ARACHIDONIC ACID METABOLITE PRODUCTION 15[S]-Hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) is a metabolite of AA produced by activation of lipoxygenase and cytochrome P-450 pathways [1]. Since measurement of 15-HETE by radioimmunoassay is more quantitative than measure the AA release, we measured intracellular 15-HETE levels as an index of AA production during cell stimulation. As shown in Fig. 2a, PLAP (1 μM) caused a sustained increase in 15-HETE levels with a peak increase of 1.6-fold occurring at 2-20 min. This stimulation by PLAP was concentration-dependent with an EC_{50} of 0.03 μM (Fig. 2b). Prostaglandin E_2 (PGE_2) is also a metabolite of AA produced by activation of cyclooxygenase pathways [1]. In contrast



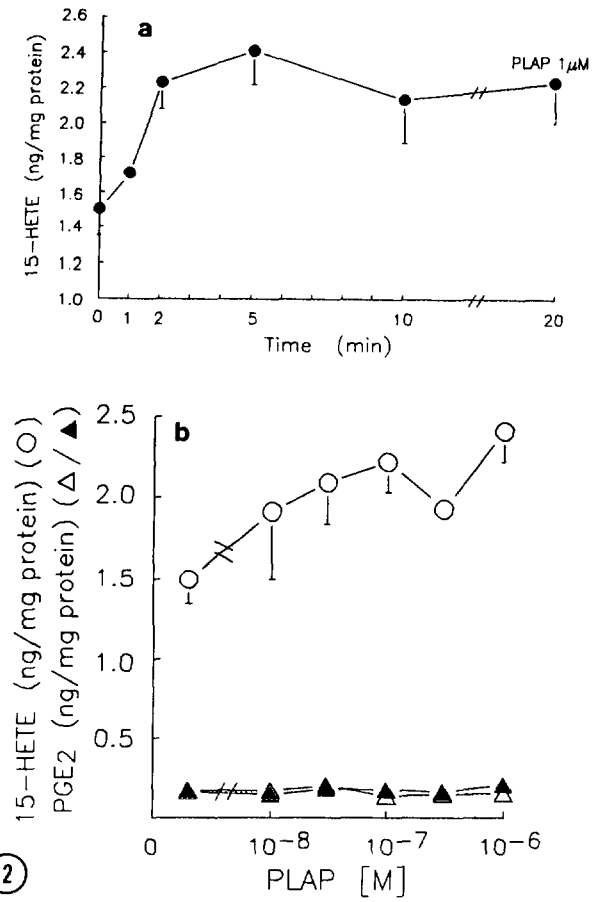


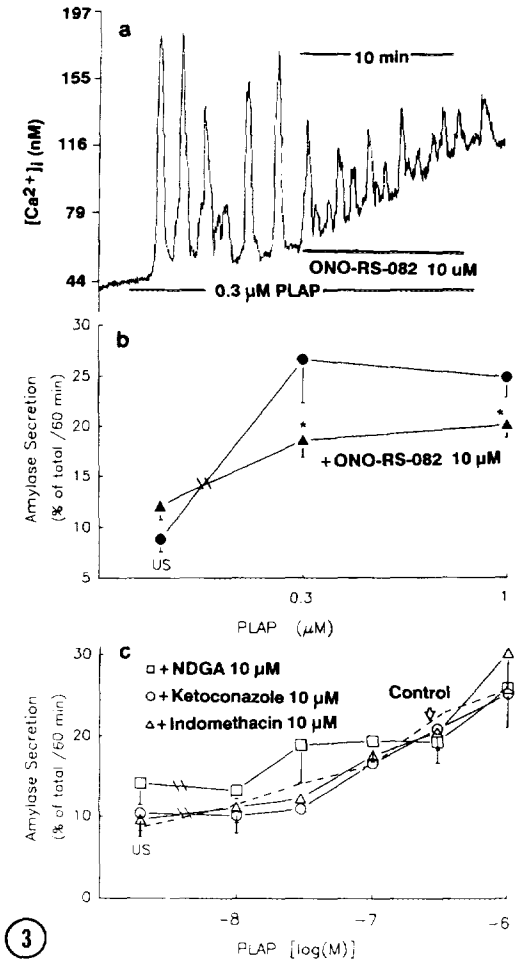
FIGURE 1. Phospholipase A2-activating protein (PLAP) elicits Ca2+ oscillations and monophasic amylase secretion in intact rat pancreatic acini. Panels a and b are representative of thirteen determinations. Panel c is a dose response study where each point represents the mean±S.E. of 9 to 13 experiments.

FIGURE 2. PLAP increases intracellular levels of 15-HETE, but not prostaglandin E2. Incubation time with PLAP was 5 min for panel b. Open and closed triangles are without and with 10 μM of indomethacin, respectively. Each point in panels a and b represents the mean±S.E. from three to six separate experiments.

to 15-HETE (basal: 1.5±0.1 ng/mg cell extract protein, n=4), PGE_2 levels in pancreatic acini were relatively low (0.17±0.04 ng/mg cell extract protein, n=4). There were no changes in PGE_2 levels during 0.01-1 μM PLAP stimulation (0.15-0.18 ng/mg cell extract protein, n=10), suggesting that AA is metabolized mainly via the lipoxygenase and cytochrome P-450 pathways in pancreatic acini (Fig. 2b).

THE PHOSPHOLIPASE A_2 INHIBITOR INHIBITS Ca^{2+} OSCILLATIONS AND AMYLASE SECRETION STIMULATED BY PLAP Since it was expected that PLAP acts via phospholipase A_2 (PLA_2)/AA pathways to mediate Ca^{2+} oscillations and amylase secretion, we next examined the effects of the PLA_2 inhibitor on the action of PLAP. We previously demonstrated that the PLA_2 inhibitor ONO-RS-082 (10 μM) caused a 70% and 75% reduction in the amplitude and frequency of Ca^{2+} oscillations, respectively, induced by the high affinity cholecystokinin (CCK) receptor agonist JMV-180 [8]. Similarly, ONO-RS-082 inhibited Ca^{2+} oscillation amplitude stimulated by PLAP (Fig. 3a). At 10 min following the application of ONO-RS-082 (10 μM) during PLAP stimulation (0.3 μM), the amplitude of Ca^{2+} oscillations decreased from 149$\pm$4 nM to 52$\pm$14 nM in the same individual acini (65% inhibition, n=3). However, the frequency evoked by PLAP (0.3 μM) was not significantly changed; 6$\pm$0.7 and 4.5$\pm$0.4 cycles/10 min without and with ONO-RS-082, respectively. ONO-RS-082 (10 μM) also significantly inhibited amylase secretion induced by 0.3-1 μM PLAP (Fig. 3b), indicating involvement of PLA_2 pathways on the action of PLAP. On the other hand, inhibitors of lipoxygenase (NDGA, 10 μM), cytochrome P-450 (ketoconazole, 10 μM) and cyclooxygenase (indomethacin, 10 μM) had no effect on the action of PLAP (Fig. 3c). This was not due to differences of effective concentrations of these inhibitors because they possess close IC_{75} levels (~10 μM). Therefore, AA itself, but not AA metabolites, may function as a signal messenger.

A MONOCLONAL ANTIBODY OF G BETA SUBUNITS INHIBITS THE ACTION OF PLAP IN PERMEABILIZED ACINI Since PLAP has a sequence similarity with the G protein beta subunits [10] and because $G_{beta/gamma}$ subunits function as signal transducers in several cells [11], we examined the effect of the antibody of G_{beta} subunits on the response to PLAP. In order to deliver the antibody into the cells, acini were permeabilized by streptolysin O. Basal $[Ca^{2+}]_i$ was increased from 100 nM to 400 nM at 30 min before cell stimulation to avoid the extracellular diffusion of Ca^{2+} following cell activation. Increasing $[Ca^{2+}]_i$ from 100 nM to 400 nM resulted in an increase in $[Ca^{2+}]$-dependent amylase secretion (from 8$\pm$2.5 to 15$\pm$2.3 % of total/30 min, n=8), suggesting that the cells were permeabilized. Application of 0.3 μM PLAP to 400 nM $[Ca^{2+}]$/cytosol buffer resulted in a further 1.82-fold increase in amylase secretion (Fig. 4). Pretreatment of permeabilized acini with a monoclonal antibody of G_{beta} subunits (250 ng/ml) for 30



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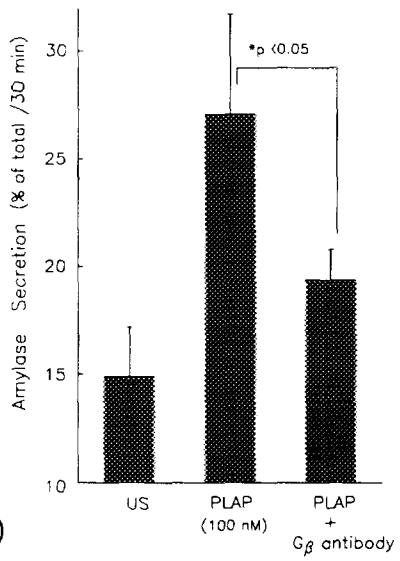

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FIGURE 3. The phospholipase A2 inhibitor inhibits Ca2+ oscillations and amylase secretion induced by PLAP. Intact acini were pretreated with inhibitors for 10 min. Panel a is representative of three determinations. Each point in panels b and c is the mean±S.E. from eight separate experiments. *P<0.05 by unpaired t tests. Abbreviation used: US; unstimulated cells.

FIGURE 4. A monoclonal antibody of G protein beta subunits inhibits amylase secretion induced by PLAP in permeabilized acini. Permeabilized acini (400 nM [Ca2+]/cytosol buffer) were pretreated without or with 250 ng/ml of the Gbeta antibody followed by a stimulation with 0.1 μM PLAP for 30 min. The Gbeta antibody inhibited the action of PLAP. *P<0.05 by unpaired t tests. Abbreviation used: US; unstimulated cells.

min significantly reduced the increase in amylase secretion stimulated by PLAP (Fig. 4). These results suggest that the ability of PLAP to activate the PLA_2 pathway may be due to its structural similarity to G_{beta} protein. Thus it is conceivable that PLA_2 coupled G protein is G_{beta} and it elicits Ca^{2+} oscillations and amylase secretion via the AA pathways.

DISCUSSION

Using dispersed rat pancreatic acini, we previously demonstrated that depending on the agonists used, cholecystokinin (CCK) receptor activation may result in the differential involvement of the phospholipase A_2 (PLA_2) and phospholipase C (PLC) pathways. Stimulation of high affinity CCK receptors by JMV-180 activates PLA_2 which is coupled to pertussis toxin-insensitive G protein and is capable of producing intracellular arachidonic acid (AA) to mediate Ca^{2+} oscillation and monophasic amylase secretion [8,14]. On the other hand, stimulation of the low affinity CCK receptors by CCK-8 activates PLC which is coupled to G_q and produces myoinositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) to mediate Ca^{2+} spiking and biphasic amylase secretion [8,14,15]. Thus a single receptor may be coupled to at least two different signal transduction systems. This is consistent with the recent findings that many receptors which are known to activate PLA_2 are also coupled to PLC in several cell types [16]. Using a recently cloned PLA_2-activating protein (PLAP), we further examined the mechanism by which PLA_2 activates Ca^{2+} oscillation and pancreatic enzyme secretion. Of interest, in intact acini PLAP elicited Ca^{2+} oscillation and monophasic amylase secretion. These actions were similar to JMV-180 but different from those induced by CCK-8, a full agonist which caused large Ca^{2+} transients and biphasic amylase secretion when supramaximal concentrations were applied [12,15,17]. Although there is no evidence that PLAP binds to specific surface receptors, the action of PLAP may be mediated by the peptide inserting into the plasma membrane, because PLAP has several hydrophobic amino acids [9]. PLAP increased intracellular levels of eicosanoid 15-HETE, but not PGE_2. While the PLA_2 inhibitor inhibited Ca^{2+} oscillation and amylase secretion induced by PLAP, the inhibitors of lipoxygenase, cytochrome P-450 and cyclooxygenase had no effect on the action of PLAP. Therefore, it appears that the major metabolic pathway during PLAP stimulation is lipoxygenase and cytochrome P-450 cascades, producing 15-HETE. However, AA itself but not AA metabolites, may function as a signal messenger to release Ca^{2+} from intracellular stores. Indeed, AA is capable of directly releasing 80% of Ca^{2+} from the canine pancreatic microsome [8]. It seems unlikely that the action of PLAP is mediated by IP_3 and DAG since the wasp venom mastoparan, a direct G protein stimulant which has a similar structure to PLAP [18], causes Ca^{2+} oscillations (in pancreatic acini), DNA synthesis and AA release (in Swiss 3T3 cells) without inositol phosphate accumulation [19,20]. The

PLA_2 activated by PLAP may be the cytosolic type (Mr 85 KDa) because of its selectivity to produce the eicosanoid [21].

In different cell types, heterotrimeric G proteins coupled to PLA_2 are either pertussis toxin sensitive or insensitive [22]. We have recently demonstrated that the PLA_2-coupled G protein in pancreatic acini is neither pertussis toxin-sensitive G_i/G_o nor $G_{q\text{-alpha}}$; its nature remains to be determined [14]. It is interesting to note that PLAP has a sequence homology with the G_{beta} subunits and has been identified as a new member of the beta-transducin (G_{beta}) superfamily [10]. The stimulation of PLA_2 activity by $G_{beta/gamma}$ subunits was first observed in the rod photoreceptor outer segment of the retina [23]. Several recent reports have demonstrated the functional roles of $G_{beta/gamma}$ dimers as well as G_{alpha} subunits [11]. We showed that the monoclonal antibody of G_{beta} subunits inhibited amylase secretion induced by PLAP in permeabilized pancreatic acini. Therefore, it is conceivable that the G protein coupled to PLA_2 may be similar or identical to G_{beta} or $G_{beta/gamma}$ in pancreatic acini. PLA_2 activated by G_{beta} subunits may phosphorylate "lipocortin" which removes an inhibitory constraint from PLA_2, thus allowing it to be activated [24]. Therefore from the data obtained with PLAP, we hypothesize that the PLA_2 coupled G protein is G_{beta} and it elicits Ca^{2+} oscillations and monophasic amylase secretion via the AA pathway.

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