Porcine Pancreatic Phospholipase A$_2$ Stimulates Secretin Release from Secretin-producing Cells*

(Received for publication, January 6, 1999)

Ta-min Chang‡, Cecilia H. Chang, David R. Wagner, and William Y. Chey

From the Konar Center for Digestive and Liver Diseases, Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

We have isolated, from canine pancreatic juice, two 14-kDa proteins with secretin-releasing activity that had N-terminal sequence homology with canine pancreatic phospholipase A$_2$ (PLA$_2$). In this study we have obtained evidence that secretin-releasing activity is an intrinsic property of pancreatic PLA$_2$. Porcine pancreatic PLA$_2$ from Sigma or Boehringer Mannheim was fractionated into several peaks by reverse phase high performance liquid chromatography. They were tested for stimulation of secretin release from murine neuroendocrine intestinal tumor cell line STC-1 and secretin cells enriched mucosal cell preparations isolated from rat upper small intestine. Each enzyme preparation was found to contain several components of secretin-releasing activity. Each bioactive fraction was purified to homogeneity by rechromatography and then subjected to mass spectral analysis and assays of PLA$_2$ and secretin-releasing activities. It was found that the fraction with highest enzymatic activity also had the highest secretin-releasing activity and the same $M_r$ as porcine pancreatic PLA$_2$. Moreover, it also had the same N-terminal amino acid sequence (up to 30 residues determined) as that of porcine pancreatic PLA$_2$, suggesting that it was identical to the enzyme. Purified porcine pancreatic PLA$_2$ also stimulated secretin release concentration-dependently from both STC-1 cells and a mucosal cell preparation enriched in secretin-containing endocrine cells isolated from rat duodenum. Abolishment of the enzymatic activity by pretreatment with bromophenacyl bromide did not affect its secretin-releasing activity. The stimulatory effect of purified pancreatic PLA$_2$ on secretin secretion from STC-1 cells was inhibited by an L-type Ca$^{2+}$ channel blocker, by down-regulation of protein kinase C or by pretreatment of the cell with pertussis toxin. It is concluded that porcine pancreatic PLA$_2$ possesses an intrinsic secretin-releasing activity that was independent of its enzymatic activity. This action is pertussis toxin-sensitive and is in part dependent on Ca$^{2+}$ influx through the L-type channel and activation of protein kinase C.

Phospholipases A$_2$ (PLA$_2$) are a family of enzymes that catalyze hydrolysis of ester bond at sn-2 position of phospholipids producing fatty acids and lysophospholipids. These enzymes exist either in extracellular secretions or intracellularly in the cell cytosol or other organelles (1–4). The cytosolic forms, including an 85-kDa enzyme and other smaller forms, are believed to function as effector enzymes in various receptor-mediated signal transduction cascades that involve the release of arachidonic acid as the second messenger. The secretory PLA$_2$s are classified into three major subtypes according to their primary structural homology with PLA$_2$s purified from snake venom (1–4). Those from the Elapidae and Hydrophiidae are grouped as type I and those from Crotalidae and Viperidae as type II. The bee venom PLA$_2$, which has no sequence homology with either types of snake PLA$_2$s, but has a similar structural organization for calcium ion binding and catalytic domains (4), is classified as Type III. Both type I and type II PLA$_2$s are 14–18-kDa proteins that are dependent on Ca$^{2+}$ in millimolar concentration for enzymatic activity. In mammalian species, both type I and type II secretory PLA$_2$s of 14 kDa (1–4) and a 60-kDa form from bovine seminal plasma (5) have been isolated. Pancreatic PLA$_2$ is a type I enzyme secreted into the pancreatic juice. Non-pancreatic PLA$_2$ isolated from other tissues and tissue fluids, including the one isolated from human synovial fluid of rheumatoid arthritis, are type II enzymes. However, it has become clear recently that type I PLA$_2$ is also present in other tissues, including kidney, small intestine, spleen, lung, and stomach (6, 7).

Along with function as an extracellular enzyme, type I PLA$_2$ has been shown to elicit receptor-mediated cellular responses, including stimulation of prostaglandin (8) and steroid hormone (9) secretion, cell proliferation (10–12), and vascular smooth muscle contraction (13). Specific receptors for secretory PLA$_2$ have been cloned (14, 15). Receptor binding and receptor-mediated action by type I PLA$_2$ is not dependent on Ca$^{2+}$ nor PLA$_2$’s enzymatic activity (16, 17). Thus, type I PLA$_2$ appears to have a cell-mediated function independent of its enzymatic activity.

We have shown previously in the rat (18) that acid-induced release of secretin from secretin cells in the upper intestinal mucosa is mediated by a lumennally active secretin-releasing factor (SRF). Similarly, canine pancreatic juice has also been shown to possess an SRF activity (19). Our attempt to isolate SRF from canine pancreatic juice has led to the purification of two SRFs of 14 kDa whose N-terminal sequences were identical to that of canine pancreatic PLA$_2$ (20). This observation suggests that pancreatic PLA$_2$ may function as a modulator of intestinal endocrine cells besides its function as a digestive enzyme. In the present report we will present evidence that pancreatic PLA$_2$ indeed possesses secretin-releasing activity by acting directly on secretin-producing cells. This action is independent of its enzymatic activity and appears to involve a receptor-mediated signal cascade.
Materials—Porcine pancreatic PLA₂ was obtained from Sigma or Boehringer Mannheim. Diltiazem, 4-[12-tetradecanoylphorbol-13-acetate (β-TPA), 3-isobutyl-1-methylxanthine, 4-bromophenacyl bromide (BBP), HPLC grade trifluoroacetic acid, and pertussis toxin were purchased from Sigma. HPLC grade water and acetonitrile were obtained from Fisher. Percoll and 1,2-his-(S-decanol)-1,2-dithio-sn-glyceryl-sn-phosphorylethanolamine were obtained from Amersham Pharmacia Biotech and Molecular Probes, Inc., Eugene, OR, respectively. Streptomyacin, penicillin, and gentamicin sulfate were obtained from Flow Laboratories, McLean, VA. All the tissue culture ware and media were purchased from Life Technologies, Inc.

**Pancreatic PLA₂ at various concentrations** or other agents in Earle's ers of STC-1 cells were incubated in the presence or absence of porcine preparation was studied as described previously (24). Briefly, monolayer gradient according to a method described previously (24).

**Purification of Porcine Pancreatic PLA₂**—Porcine pancreatic PLA₂ suspended in ammonium sulfate solution was centrifuged in an Eppendorf microcentrifuge for 2 min at 4 °C. The supernatant solution was removed, and the pellet corresponding to 2.5 mg of protein was dissolved in 16% acetonitrile, 0.1% trifluoroacetic acid and injected into a Vydac 218TP semipreparative column (7.5 × 250 mm). The column was linked to an ISCO single pump HPLC system (ISCO, Omaha, NE) consisting of a model 2360 gradient former, a model 2350 pump, and a V̇ absorbance detector that were controlled by an IBM personal computer. The column was eluted with 0.2% (v/v) acetonitrile in a balanced salt solution containing Hepes, L-glutamine, pyruvate, soybean trypsin inhibitor, and bovine serum albumin as described above for Earle's balanced salt solution. The cell suspension was pre-equilibrated with 20% ethionyl solvent B and then eluted with a gradient of 30–54% solvent B at an increment rate of 0.3%/min followed by 54–100% solvent B at 2.3%/min at a flow rate of 2.5 ml/min. The elution solvents were: solvent A, 0.1% trifluoroacetic acid and solvent B, 80% acetonitrile/0.09% trifluoroacetic acid. The elution profile was monitored by absorbance at 215 nm. The major peaks were collected and dried in vacuo in a Speed Vac (Savant Instruments, Inc., Farmingdale, NY). Each peak was rechromatographed to obtain a homogenous single peak and tested for PLA₂ enzymatic activity, secretin-releasing activity, and molecular mass determination.

**Inactivation of Enzymatic Activity of Purified PLA₂ with 4-Bromophenacyl Amide**—Two mg of purified PLA₂ were dissolved in 0.5 ml Dulbecco's phosphate-buffered saline and then incubated with 0.1 mM BBP at room temperature in the dark for 18 h. The reaction mixture was then filtered through a Sephadex G-25 column (0.9 × 25 cm) eluted in the same phosphate-buffered saline to collect the protein peak. The concentration of the BBP-treated enzyme was determined by protein assay as described below.

**Cell Culture**—STC-1 cells of a murine intestinal neuroendocrine tumor cell line that secrete secretin (21) were maintained in monolayer cultures in 24-well plates as described previously (22, 23).

**Preparation of Secretin Cell-enriched Cell Preparation from Rat Duodenal Musca**—Mucosal cells enriched in secretin-containing endocrine cells were prepared by collagenase digestion of rat duodenal mucosa followed by centrifugation in a discontinuous Percoll density gradient according to a method described previously (24).

**Studies of the Release of Secretin from STC-1 and Rat Mucosal Cell Preparation**—The release of secretin from STC-1 or rat mucosal cell preparation was measured as described previously (24). Briefly, cultures of STC-1 cells were incubated in the presence or absence of porcine pancreatic PLA₂ at various concentrations or other agents in Earle's balanced salt solution containing 10 mM Hepes, pH 7.4, 5 mM sodium pyruvate, 2 mM L-glutamine, 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin under 95% air, 5% CO₂ at 37 °C for 60 min or various time periods as specified. The plate was chilled on ice, and an aliquot of the medium was then removed for assay of secretin using a specific radioimmunoassay as described (24, 25). In some experiments the cells were preincubated with diltiazem (10 μM) for 30 min or other pertussis toxin (10 ng/ml) or β-TPA (0.1 μM) for 10 h before incubation of the cells with PLA₂ and assay of secretin release. Rat S cell-enriched preparation was suspended at 0.5–1.0 × 10⁵ cells/ml in Hanks' balanced salt solution containing Hepes, 1-glutamyl pyruvate, soybean trypsin inhibitor, and bovine serum albumin as described above for Earle's balanced salt solution. The cell suspension was incubated in the absence or presence of varying concentrations of PLA₂ or other test agents under 95% O₂, 5% CO₂ with gentle giration in a water bath at 37 °C for 30 min. The cell suspension was then centrifuged at 500 × g for 5 min at 4 °C, and an aliquot of the supernatant was removed for assay of secretin. The supernatant was rechromatographed to homogeneity by rechromatography. As examples, using the same gradient of 34–54% solvent B at a rate of 0.4%/min as shown in Fig. 2, Peak 2 was purified as a single peak at 18.1 min, Peak 3 at 20.5 min, Peak 4 at 21.6 min, and Peak 7 at 36.1 min, respectively.

**Characterization of Purified Peaks with Secretin-releasing Activity**—The purified peaks were analyzed for molecular mass, PLA₂ activity, and secretin-releasing activity in STC-1 cells. As summarized in Table I, Peaks 2 and 4 from both sources had the highest secretin-releasing- and enzymatic activities. Their molecular masses were 13,969–14,001 Da, which were the same within experimental error as 13,982 Da calculated from the amino acid sequence of porcine pancreatic PLA₂ (27). The mass spectra of Peaks 2 and 4 are shown in Fig. 3, A and B, respectively. In each spectrum, the lower m/z peak represented the molecule of PLA₂ with two net charges. The N-terminal amino acid sequences of these two peaks were determined. The results indicated that except for the blanked cycles at cystine residues, their N-terminal 10 residues were identical to that of porcine pancreatic PLA₂ (Table II). Peak 5a has a molecular mass of 13,956 Da and is high in both enzymatic and secretin-releasing activities. Peaks 3 and 3a that had molecular masses lower than porcine PLA₂ by 500 and 900 Da, respectively, were lower in both enzymatic activity and secretin-releasing activity than the above mentioned peaks. On the other hand, Peaks 7 and 8 with molecular masses of 8,213 and 17,503 Da, respectively, had very little enzymatic activity but had secretin-releasing activities comparable with those of Peaks 3 and 3a. It should be mentioned that the calculated PLA₂ activities of these two peaks were not significantly diff-
different from the variation observed in the substrate blank of the enzyme assay. In addition, the mass spectrum of Peak 8 also indicated the presence of a minor component (about 15%) with molecular mass of 17,035 Da.

All five active peaks derived from Sigma PLA₂ and all four main peaks from Boehringer Mannheim exhibited concentration-dependent stimulation of secretin release in STC-1 cells. The results of these dose-response studies are shown in Fig. 4, A and B, respectively. The results shown in Fig. 4A indicated that Peaks 2 and 4 from Sigma were more than 10 times as potent as Peaks 3, 7, and 8. Also at the same concentration of 1 μM, Peaks 2 and 4 were about two to three times as effective as the other three peaks. The half-maximal dose of Peak 2 (400 nM) was slightly higher than that of Peak 4 (100 nM). Similarly, Peaks 2 and 4 from Boehringer Mannheim were about 10–20 times as potent as Peaks 5a and 3a (Fig. 4B). Since Peak 4 was the predominant and most potent fraction to stimulate secretin release, it was used in the subsequent studies.

The effect of Peak 4 on secretin release from STC-1 cells was also time-dependent. Thus, addition of Peak 4 (0.5 μM) to STC-1 cells resulted in a continuous stimulation of secretin release for 60 min, reaching 130% increase over basal secretion at 15 min and 250% at 60 min.

Although the secretin-releasing activity was highest in the fractions with the highest PLA₂ enzymatic activity, stimulation of secretin release did not appear to depend on its enzymatic...
activity. Thus, as shown in Fig. 5, pretreatment of Peak 4 with 4-bromophenacyl bromide resulted in inhibition of its enzymatic activity by 95% (from 5.3 units/mg to 0.3 units/mg). The treated enzyme, BPB-PLA₂, at 0.1 mM stimulated secretin release by 337 ± 49% over basal, which was not significantly different from 283 ± 11% stimulated by the untreated enzyme.

The Effect of Purified PLA₂ on Secretin Release from Rat Mucosal S Cell Preparation—Purified PLA₂, Peaks 2 and 4 from Sigma also stimulated secretin release from an S cells-enriched preparation isolated from rat small intestinal mucosa. As shown in Fig. 6, the purified fraction stimulated the release of secretin from isolated rat mucosal cells concentration-dependently and was more than 10 times as active as the unfractionated PLA₂.

Cellular Mechanism of Stimulation of Secretin Release by PLA₂ in STC-1 Cells—We studied if any signal transduction pathway mediated the action of PLA₂ on secretin release. Incubation of STC-1 cells with PLA₂ did not increase cellular level of cAMP over a period of 30 min (data not shown), although the cells responded well to pituitary adenylate cyclase activating polypeptide which increased cellular cAMP level from 31.3 ± 2.1 pmol/mg of cell protein to a peak level of 94.5 ± 3.4 pmol/mg of protein at 2 min. PLA₂ also did not affect the cellular content of inositol 1,4,5-triphosphate over a period of 10 min (data not shown), although the cells responded well to bombesin which increased inositol 1,4,5-triphosphate level from 5.4 ± 1.6 pmol/mg of cell protein to 51.3 ± 9.6 and 54.9 ± 12.4 pmol/mg of cell protein at 15 and 30 s, respectively. These two neuropeptides had been shown to stimulate secretin release through the generation of the corresponding second messengers (24). However, as shown in Table III, when STC-1 cells were incubated with PLA₂ in the presence of an L-type calcium channel blocker, diltiazem (10 μM), the stimulatory effects of PLA₂ on secretin release at 50 and 500 nM were inhibited by 47%. Down-regulation of protein kinase C by pretreatment of the cells with 0.1 μM β-TPA also resulted in a significant inhibition (~50%) of PLA₂-stimulated secretin release. A sim-

**Fig. 3. Mass spectra of purified Peaks 2 and 4 of Sigma PLA₂.** A, Peak 2; B, Peak 4.
ilar inhibition was observed when incubation of STC-1 cells with PLA₂ was carried out in the presence of 1 μM staurosporine, a protein kinase C-selective inhibitor (data not shown). Moreover, the stimulatory effects of PLA₂ at these two concentrations decreased significantly (38 and 49%, respectively) after pretreatment of STC-1 cells with pertussis toxin (10 ng/ml, 10 h).

**DISCUSSION**

The result of the present study provides strong evidence that porcine pancreatic PLA₂ possesses an intrinsic secretin-releasing activity. Thus, commercially available porcine pancreatic PLA₂ contained several peaks of secretin-releasing activity. Among the active peaks, those with the highest enzymatic activity, the same molecular weight and N-terminal amino acid sequence as porcine pancreatic PLA₂ were the most potent fractions. Among the resolved peaks, Peak 4 with the highest enzymatic activity and identical Mᵣ with PLA₂ probably represented the native enzyme. Peak 2 and Peak 5a, with a slightly higher and lower Mᵣ than Peak 4, respectively, were probably genetic variants of PLA₂ with reduction of both enzymatic activity and potency of secretin-releasing activity. Peaks 3 and 3a had a significantly lower molecular weight than that of the native enzyme and about 50% enzymatic activity, and substantially decreased potency in secretin-releasing activity could be partially degraded product of PLA₂ that lost a few amino acid residues. All these peaks were found to cross-react well (≥10%) with an anti-PLA₂ serum raised against purified Peak 4 (data not shown), suggesting that they are all antigenically related to PLA₂. On the other hand, the relationship between Peak 7 and PLA₂ cannot be clearly discerned at present due to its lack of enzymatic activity and absence of amino acid sequence data. Peak 8 does not appear to be related to the enzyme as it lacked enzymatic activity, and both its major and minor components had relative molecular masses greater than that of prepro-PLA₂ (16,278.5 Da), calculated from the amino acid sequence deduced from the cDNA coding sequence of porcine PLA₂ precursor (27). Moreover, both of these peaks did not appear to contain the antigenic determinant of PLA₂, since they had very low cross-reaction with the anti-PLA₂ serum (<0.2%) that could be due to a small contamination of the enzyme.

The effect of PLA₂ on secretin release does not appear to depend on its enzymatic activity, as inactivation of the enzymatic activity with 4-bromophenacyl bromide did not diminish its secretin-releasing activity. This observation suggested that the release of secretin elicited by PLA₂ was not due to membrane damage and leakage of the hormone nor due to enzymatic release of fatty acids that are known to be stimulants of secretin release (25). The fact that we did not observe any increase in trypan blue inclusion after treatment of STC-1 cell
from rat mucosal cells, this secretory response to PLA₂ appar-
tively is a common property of secretin-producing cells rather
than a result of tumorigenic transformation. Since PLA₂ also stimulated the release of secretin
antibody-antigen complex, the secretin-releasing factor activity
of unfractionated (circles) or purified PLA₂ (squares) for 30 min at 37 °C to
determine the amount of secretin released as described under "Experimental Proce-
dures." (*) depicts significant increase in secretin release over the basal with p <
0.05 (n = 4).

TABLE III

| Treatment | n  | 50 nM | 500 nM |
|-----------|----|-------|--------|
| − TPA     | 8  | 150 ± 7 | 384 ± 21 |
| + TPA     | 8  | 74 ± 13 (51) | 196 ± 18 (49) |
| − PTX     | 8  | 186 ± 26 | 360 ± 16 |
| + PTX     | 8  | 103 ± 15 (38) | 185 ± 13 (49) |
| − Diltiazem | 4 | 121 ± 21 | 355 ± 31 |
| + Diltiazem | 4 | 64 ± 7 (49) | 188 ± 17 (47) |

* p < 0.01 versus the control.  
* p < 0.05 versus the control.

with PLA₂ (data not shown) appeared to support the former argument. Since PLA₂ also stimulated the release of secretin from rat mucosal cells, this secretory response to PLA₂ apparently is a common property of secretin-producing cells rather than a result of tumorigenic transformation.

The results of recent studies have revealed that pancreatic PLA₂ is also present in other organs, including the spleen, lung, kidney, small and large intestine, and stomach (6, 7). In addition to function as a digestive enzyme, pancreatic PLA₂ has also been shown to regulate cellular function, including cell proliferation (10–12), vascular smooth muscle contraction (13), stimulation of prostaglandin production and type II PLA₂ gene expression (8, 28), and secretion of a steroid hormone (9). Many of these actions of pancreatic PLA₂ have been shown to occur through mediation by a specific receptor independent of its enzymatic activity. Moreover, specific receptor for PLA₂ has been cloned (14, 15) and shown to bind PLA₂ independent of its enzymatic activity (16, 17). It is likely that PLA₂ stimulates secretin release through a similar receptor. In the present study we have also observed that stimulation of secretin release by PLA₂ from STC-1 cells is partially inhibited by PTX, an L-type Ca²⁺ channel blocker, and by down-regulation of protein kinase C activity or a protein kinase C inhibitor. These observations suggest that the action of PLA₂ on secretin release may be mediated in part by activation of a PTX-sensitive G protein, the L-type Ca²⁺ channel and protein kinase C. However, the relationships among these three elements of signal cascade remain to be studied.

It should be noted that the release of both secretin and cholecystokinin (CCK) from the upper small intestinal mucosa is subject to feedback inhibition by pancreatic juice (29–32). This effect has been shown to be due to inactivation of the corresponding luminal releasing factors for these hormones by pancreatic proteases. Indeed, two luminally active CCK-releasing factors, lumenal CCK-releasing factor and diazepam-binding inhibitor, have been isolated and shown to release CCK when given to the intestinal lumen (33, 34). In addition, another CCK-releasing factor, monitor peptide, which is a variant form of pancreatic Kazal-type trypsin inhibitor, has been isolated from rat pancreatic juice (35). Therefore, it is not surprising that another protein from the pancreatic juice, PLA₂, is found to have secretin-releasing activity. Indeed, we have found recently that, in conscious dogs, intraduodenal administration of fresh canine pancreatic juice in the interdigestive state resulted in a significant increase in pancreatic secretion of fluid and bicarbonate as well as plasma secretin concentration (36). Although more studies are needed to define its possible physiological role on the release of secretin and pancreatic secretion, pancreatic PLA₂ in the duodenal lumen may exert a stimulatory action on pancreatic secretion of fluid and bicarbonate as well as plasma secretin concentration (36).
cosa, it is tempting to speculate that the enzyme may also participate in a regulatory function in the release of other gut hormones.

Acknowledgment—We thank Laura Braggins for her excellent technical assistance.

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