Phospholipase A_2 activity of pancreatic secretory factor, a new secretagogue isolated from the venom of *Heloderma suspectum*

Jean-Paul Dehaye, Jacques Winand, Patrick Michel, Piotr Poloczek, Catherine Damien, Marie-Claire Vandermeers-Piret, André Vandermeers and Jean Christophe*

Department of Biochemistry and Nutrition, Medical School, Université libre de Bruxelles, Bld. de Waterloo 115, B-1000 Brussels, Belgium

Received 21 May 1984

Pancreatic secretory factor (PSF), an efficient pancreatic secretagogue recently isolated from the venom of *Heloderma suspectum*, is shown to exert phospholipase A_2 activity towards phosphatidylcholine. This activity is strictly dependent on calcium (apparent K_a 40 nM) and has an optimum pH around 9. At pH 7.4 and in the presence of calcium, PSF retains 40% of its phospholipase A_2 activity. These results are compared to the calcium dependency of the secretory effect of PSF on rat pancreatic acini. Taken collectively, the present data on PSF suggest that a similar endogenous phospholipase A_2 activity might be involved in the late steps of stimulus-secretion coupling in the exocrine pancreas.

Rat pancreatic acini *Amylase secretion* *Pancreatic secretory factor* *Gila Monster venom* *Phospholipase A_2*

1. INTRODUCTION

The venom from the lizard *Heloderma suspectum* stimulates the release of amylase and the production of cyclic AMP by guinea pig [1] and rat [2] pancreatic acini. We demonstrated recently that these two effects are unrelated in rat pancreatic acini: a VIP-like component is indeed responsible for the cyclic AMP production while the secretory effect is due to a 17.5-kDa protein that neither modifies cyclic AMP concentration nor alters calcium movements. We named this protein pancreatic secretory factor (PSF) [3–5]. We report here that PSF is a calcium-dependent phospholipase A_2 (pH optimum 9.0) whose secretory action might suggest a similar contribution of endogenous phospholipase A_2 activity to stimulus-secretion coupling in the rat exocrine pancreas.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. Materials

L-3-Phosphatidylcholine from egg yolk, phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) from *Naja naja* venom and all phospholipid standards were from Sigma (St. Louis, MO). 1-Palmitoleyl-2-[1-^{14}C]oleoyl-L-3-phosphatidylcholine was from Amersham (Bucks) Chaps [3-{(3-cholamidopropyl)dimethylammonio}-1-pro-panesulfonate] was purchased from Polysciences (Warrington, PA). C-terminal octapeptide of cholecystokinin (CCK-8) was a gift from Dr J. Lucania (Squibb Institute for Medical Research, Princeton, NJ) and synthetic secretin a gift from Dr W. König (Hoechst Aktiengesellschaft, Frankfurt/M.). PSF from Gila Monster venom was isolated as in [3].

2.2. Methods

2.2.1. Phospholipase A_2 activity

L-3-Phosphatidylcholine from egg yolk at a

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final concentration of 0.5 mg/ml was sonicated for 5 s in 100 mM Tris–Mops buffer (pH 8.5), in the presence of 1.2 mg/ml Chaps, 5 mM $CaCl_2$ and 110 000 cpm 1-palmitoleyl-2-[1-^{14}C]oleoyl-L-3-phosphatidylcholine. Pure PSF [3] and phospholipase A_2 from *N. naja* venom (PLA_2) were added in a volume of 10 μl. The final incubation volume was 0.5 ml. After enzyme addition, the tubes were mixed for 30 s and incubated with continuous shaking at room temperature for 10 min. The reaction was stopped by adding 2 ml $CHCl_3/CH_3OH/HCl$ (200:100:3). After centrifugation at 4°C for 5 min at 2500 × *g*, the upper phase was discarded and the lower phase was washed with 0.5 ml of 1% HCl and 1 ml $CHCl_3/CH_3OH/HCl$ (200:100:3). After centrifugation, the lower phase was evaporated to dryness at 40°C under a stream of N_2. The lipids were dissolved in 200 μl $CHCl_3/CH_3OH/HCl$ (200:100:3). A 10 μl aliquot was layered on a silica gel plate (Kieselgel 60F-254, 0.2 mm thick, Merck, Darmstadt) and eluted with $CHCl_3/CH_3OH/NH_3$ (105:45:7.5). Lipids were visualized by iodine vapour and identified with reference to standards. The plate was cut and fragments were placed into scintillation glass vials containing 1 ml CH_3OH. After mixing, 8 ml Aquasolve 2 (New England Nuclear, Dreieich) were added and the vials were counted in an LS-8000 Beckman liquid spectrometer.

2.2.2. Amylase secretion

Rat pancreatic acini were isolated and incubated as in [6]. After washing, the acini were incubated for 30 min in the standard incubation medium, in the presence of 0.5 mM Ca^{2+} or 0.1 mM EGTA. Amylase released in the medium was assayed as in [7].

3. RESULTS AND DISCUSSION

As shown in table 1, PSF was able to release [1-^{14}C]oleic acid from the glycerol 2-position of a labelled lecithin. This activity was comparable to that of a commercial phospholipase A_2 isolated from the venom of *N. naja*. The optimum pH of PSF activity was about 9.0, this activity being reduced by 60% at pH 7.4 (fig.1A). This activity was highly dependent on the presence of calcium, the enzyme being inactive at Ca^{2+} concentrations below 1 nM and maximally active at Ca^{2+} concentrations around 1 μM (fig.1B). The apparent half-maximal effective Ca^{2+} concentration of 40 nm reflected a true K_a value [8] as it did not vary when PSF concentration was increased from 1 to 100 nM (not shown). Similarly, the secretory effect of PSF on rat pancreatic acini depended entirely on the presence of extracellular calcium (table 2). This effect was completely abolished when the acini were incubated for 30 min in the presence of 0.1 mM EGTA (instead of 0.5 mM Ca^{2+}), whereas the secretory effects of optimal concentrations of carbamylcholine, CCK-8 and secretin were only halved.

PSF is thus an extracellular phospholipase A_2 extracted from the venom of the lizard *H. suspectum* that is thermostable, lipophilic and acidic and can be added to the list of similar enzymes present in the venom of snakes, bees and scorpions [9]. Its optimum pH is around 9 and its activity strictly calcium-dependent, with a relatively low concentration requirement for the cation. The secretory effect of PSF on rat pancreatic acini is also dependent on extracellular calcium and is not accompanied by changes in cyclic AMP concentration or calcium efflux.[2,5].

Table 1

Phospholipase A_2 activity of purified pancreatic secretory factor from Gila Monster venom

Enzyme	Radioactivity distribution (%) in		
	L-3-Phosphatidylcholine	Fatty acid	1-Acyllysophosphatidylcholine
Control	96.2 ± 0.5	1.6 ± 0.2	2.2 ± 0.54
20 nM PSF	72.8 ± 1.4	23.8 ± 1.2	3.4 ± 0.3
PLA_2 (4 units/ml)	72.9 ± 2.6	25.3 ± 1.5	1.8 ± 0.4

Phospholipase activity was measured as described in section 2. Results are expressed as percentage of total radioactivity found in the 3 major classes of lipids, namely, L-3-phosphatidylcholine, lysophosphatidylcholine and free fatty acids, and are means of 3 experiments ± SE

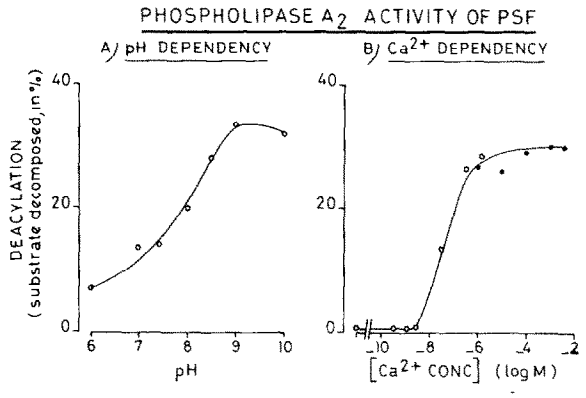

Fig.1. (A) The phospholipase A_2 assay was carried out as described in section 2, except for the pH which varied between 6 and 10. The assay was performed for 10 min in the presence of 5 mM $CaCl_2$ and 20 nM PSF. This experiment is representative of 3 experiments. (B) Phospholipase A_2 activity was measured for 10 min at pH 9 as described in section 2, except for the concentration of free calcium which varied from 0 to 1 mM. Calcium–EGTA buffers (○) allowed calcium concentrations up to 1 μM. Dilutions from a 100 mM $CaCl_2$ stock solution were used for concentrations ranging from 1 to 5 mM (●). This experiment is representative of four experiments.

The calcium dependency of the two effects of PSF and the fact that its phospholipase A_2 activity extends to pH 7.4 in the presence of calcium (fig.1A) suggest that the release of amylase depends on pancreatic phospholipids. The hypersecretion does not merely reflect the escape of hydrolases from leaking cell membranes since: (i) this effect is fully reversible when PSF is removed from the medium [5]; (ii) PSF does not provoke the leakage of lactate dehydrogenase from rat pancreatic acini [5]; (iii) PSF does not modify the sucrose space of pancreatic acini (not shown); (iv) PSF does not hemolyse washed human red blood cells incubated for 30 min with 130 mM NaCl containing 1 mM $CaCl_2$ and buffered to pH 7.4 by 20 mM Hepes (not shown). Thus, like other acidic phospholipases A_2, PSF may well be less toxic than basic phospholipases A_2 [10,11]. Taken together, our data are consistent with the hypothesis that a limited pool only of pancreatic phospholipids is available to hydrolysis by PSF [12].

Table 2

Effects of Ca^{2+} and EGTA on amylase secretion from rat pancreatic acini stimulated by various secretagogues

Secretagogue	0.5 mM Ca^{2+}	0.1 mM EGTA
Control	2.0 ± 0.4	1.8 ± 0.4
30 nM PSF	9.3 ± 1.3	1.7 ± 0.7
5 μM carbamylcholine	14.9 ± 3.2	8.1 ± 0.8
3 nM CCK-8	13.1 ± 3.8	7.6 ± 1.9
3 μM secretin	7.4 ± 2.8	4.4 ± 1.4

Results are expressed as percentage of total amylase content released in the medium during the incubation and are means of 3 experiments ± SE

Up to now, phospholipase C has attracted greater interest than phospholipase A_2 because of its involvement in the calcium-independent degradation of (poly)phosphatidylinositols that may well be a mediator of membrane calcium gating in glands stimulated by natural secretagogues. This model appears to apply to receptors for muscarinic drugs, cholecystokinin, and bombesin that are present at the blood front of rat pancreatic acinar cells and control protein secretion through Ca^{2+} mobilization [13–18]. It does not apply, however, to secretin receptors that do not couple secretion to Ca^{2+} mobilization: the secretagogue secretin does not provoke a phosphatidylinositol response [17] but can, like the ionophore A23187, bypass the phosphatidylinositol cycle. Recently, two indirect lines of evidence indicated that intracellular phospholipase A_2 activity might contribute to stimulus-secretion coupling in the exocrine pancreas: (i) the rat pancreas contains variously repressed forms of phospholipase A_2 that may coexist as soluble and membrane-associated entities (as in several mammalian tissues) and as a zymogen-bound form [9,10,19,20]; (ii) carbamylcholine and ionomycin (a calcium ionophore) increase deacylation-reacylation processes at position 2 of phospholipids in rat pancreatic acini [21]. We would like to suggest that the interaction between phospholipases C and A_2 [21,22] may be vizualized in terms of subsequent events: the action of the first enzyme on (poly)phosphatidylinositols is close to receptors at the basal front of acinar cells whereas Ca^{2+}-dependent activation of intracellular phospholipase A_2 would be closer to the secretory pole [19] and might be a late event leading to the release of hydrolases in response to all natural secretagogues, including secretin. A similar contribution of phospholipase A_2 has been suggested in pancreatic β cells, adrenal

gland [24], anterior pituitary releasing PRL [25], mast cells [26] and neurons [27]. In the rat exocrine pancreas such an involvement of phospholipase A_2 might conceivably set two mechanisms into play: (i) arachidonic acid released in acinar cells might be converted into prostaglandins and hydroxy acids, via the cyclooxygenase and lipoxygenase pathways, respectively [28]. However, the metabolic significance of such an 'arachidonic acid cascade' is unlikely for two reasons: (i) exogenous arachidonic acid, when used at high concentration, induces amylase secretion from the rat pancreas (even in the presence of indomethacin) but exogenous PGE_2 and PGF_2 are unable to exert this effect (review [29]). In addition 5,8,11,14-eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid (two inhibitors of the cyclooxygenase and lipoxygenase pathways) inhibit the synthesis of arachidonic acid derivatives in response to the calcium ionophore ionomycin without altering amylase secretion [30]; (ii) our data are more compatible with a second hypothesis: lysophospholipids are fusogens of cellular membranes [31] whose cytotoxic action may be prevented by rapid reacylation via rat pancreatic acyl-CoA:lysophospholipid acyltransferase activity [32]. The present data obviously warrant further study of several parameters including the localisation and activation of various phospholipases as well as the reacylation and flip-flow capacity of (lyso)phospholipids at different fronts of the pancreatic acinar cell.

ACKNOWLEDGEMENTS

This work was aided by grant 3.4504.81 from the Fonds de la Recherche Scientifique Médicale (Belgium), grant 2 ROI-AM 17010-8 from the National Institutes of Health and a grant from the Ministère de la Politique Scientifique (Belgium).

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