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Enhancement and inhibition of snake venom phosphodiesterase activity by lysophospholipids

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Abstract Lysophospholipids are liberated during venomous action. In this study we demonstrated that lysophosphatidyl choline (LPC) of various acyl chains enhances considerably the activity of snake venom phosphodiesterase (PDE). Lysophosphatidic acid (LPA) and its cyclic form (cLPA), on the other hand, were found to inhibit this enzyme in a non-competitive (LPA) or competitive (cLPA) manner. Both of these activities may contribute to the progression and subsidence of the poisoning profile. PDE from cellular origin was not substantially affected by any of the above lysophospholipids.

Key words: Phosphodiesterase; Cyclic lysophosphatidic acid; Snake venom; Cyclic AMP

1. Introduction

Phosphodiesterases (EC 3.1.4) are the enzymes which catalyze the hydrolysis of phosphodiesters to phosphomonoesters. They exist both intracellularly and extracellularly in a wide variety of tissues and organisms [1–4]. Intracellular phosphodiesterases (PDEs) play a role in signal transduction by regulating the cellular concentrations of cyclic nucleotides [5]. Extracellular PDEs known as exonucleases, exist in venoms and their route in envenomation is mostly by attacking nucleic acids [6] through removal of mononucleotide units from the polynucleotide chain in a stepwise fashion [7,8]. They also possess the endonucleolytic activity towards single-stranded DNA [9] and as such they have been used for sequencing oligonucleotides and polynucleotides [10–13]. Many selective inhibitors of each isoenzyme of intracellular PDEs have been developed, which have many advantages in clinical use [14–16]. However, for venom PDE only polyclonal anti-venom antibodies and heparin have been reported as inhibitors [17–20]. In this report we describe the effect of various lysophospholipids on the activity of snake venom PDE, as well as on intracellular cAMP specific PDE.

2. Materials and methods

2.1. Materials

Bis-*p*-nitrophenyl phosphate sodium salt (Bis-pNPP), 3-isobutyl-1-methyl xanthine (IBMX), cAMP, DL-α-glycerophosphate disodium salt hexahydrate, β-glycerophosphate disodium salt, and the snake venom PDE were purchased from Sigma Chemicals (St. Louis, MO). 1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate (lysophosphatidic acid, LPA) monosodium salt, 1-oleolyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine and 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphocholine were obtained from Avanti Polar Lipids (Albaster, AL). Tritium labelled cAMP was procured from American Radiolabelled Chemicals, Inc. (St. Louis, MO) and Lumax was purchased from Lumac-LSC (Groningen, The Netherlands). Cyclic palmitoyl lysophosphatidic acid (cLPA) was synthesized by dehydration of the acid form of LPA with dicyclohexylcarbodiimide in dimethylsulfoxide. The obtained product was analytically pure. A full account on the synthesis of cLPA will be published elsewhere. All other chemicals and solvents used were of analytical grade.

2.2. Cells and cell lysates

NIH 3T3 cells were grown in tissue culture at 37°C under 5% CO_2 atmosphere in the presence of 1640 Eagle medium and 10% bovine calf serum. Cells were washed thoroughly with phosphate buffered saline (PBS) and then lysed by freeze-thawing.

2.3. Assay of snake venom phosphodiesterase

The activity of snake venom PDE was assayed in 1 ml of 50 mM Tris-HCl buffer, pH 8.4 containing 5 mM bis-pNPP and snake venom PDE (10 μg). The reaction was arrested by the addition of 50 μl of 0.5 N NaOH, at the end of 1 h incubation at 37°C. The *p*-nitrophenol liberated was monitored at 405 nm in a UV double beam spectrophotometer.

2.4. Assay of cyclic AMP degradation

cAMP degradation activity of the cell lysate was monitored by radiolabelled ^{3}H-cAMP as described [21]. ^{3}H-cAMP (0.5 million counts) was incubated with snake venom PDE (10 μg) and cell lysate (50 μg protein) for 1 h at 37°C in a reaction mixture of final volume 200 μl containing 50 mM Tris-HCl buffer, pH 7.4, and 0.1 mM cAMP. The reaction was stopped by adding 0.1 ml of a solution containing 2% SDS, 1 mM cAMP and 45 mM ATP, followed by boiling for 5 min. Then 0.9 ml of double distilled water was added and mixed well. The reaction mixture was then passed through a Dowex AG50 WX4 and neutral chromatographic alumina WN-3 columns (0.4×15 cm) respectively. Finally, the radiolabelled ^{3}H-cAMP was eluted from the alumina column by using 4.0 ml of imidazole-HCl buffer (0.1 M, pH 7.5) and 4.0 ml of scintillation fluid was added and mixed well. The radioactivity was measured in a Beckman-LS5000TD model β-counter. At the same time control reaction also was run as mentioned above but without the enzyme. The enzyme activity was presented in terms of percentage of ^{3}H-cAMP degradation with respect to control.

3. Results

3.1. Activation of snake venom PDE by lysophospholipids

Phospholipase A_2 (PLA_2) is a major component in snake venom. It acts predominantly on phosphatidyl choline (PC) to liberate lysoPC (LPC) which in turn disintegrate cell membranes. Our preliminary tests indicated that LPC of various acyl chains can activate snake venom PDE in a dose dependent manner. In the presence of 50 μM of LPC compounds the activation approached its maximal level and was in the range of 75–85%. LPC can be further degraded to lysophosphatidic acid (LPA) [22,23] by lysophospholipase D in the rat [24] and human [25] sera. The cyclic form of LPA (cLPA) [26,27] appears as an intermediate in this reaction [25–27]. The effects on snake venom PDE of different LPCs, as well as LPA and

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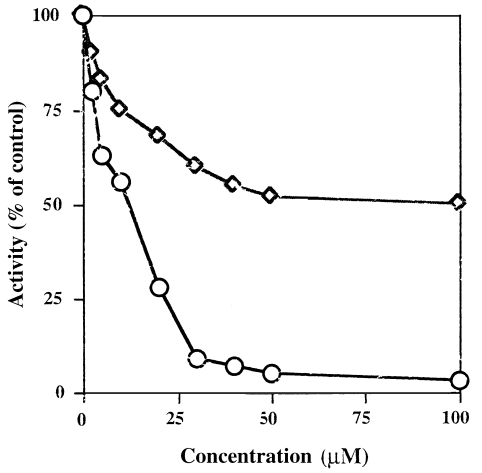

Fig. 1. Inhibition of snake venom PDE by increasing concentration of cLPA (circles) and LPA (diamonds).

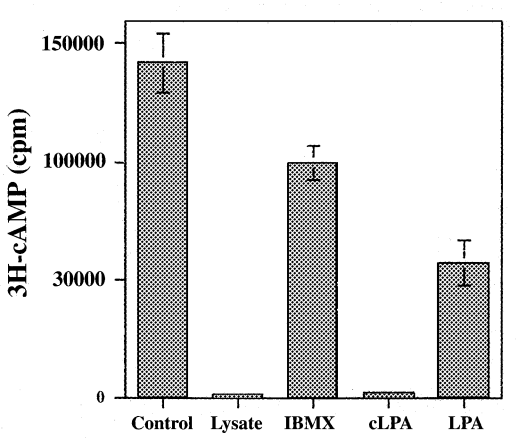

Fig. 3. Level of ^{3}H-cAMP after incubation with NIH 3T3 cell lysate in the absence and presence of IBMX, LPA and cLPA. The presented values are means of triplicates. For experimental details see text.

cLPA, are summarized in Table 1. All the LPC compounds were found to increase the enzyme activity. Among them 1-oleolyl-2-hydroxy-*sn*-glycero-3-phosphocholine showed highest activation while 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphocholine exhibited the lowest activation.

3.2. Inhibition of snake venom PDE by cLPA and LPA

In contrast to LPC, at 50 μM cLPA imposed almost complete inhibition of the enzyme activity while its open form LPA showed approximately 50% inhibition at 50 μM. Fig. 1 presents the effect of different concentrations of cLPA and LPA on venom PDE. As shown, the effect of LPA levelled off at approximately 50% inhibition while the inhibition by cLPA increased steadily and approached complete inhibition. It could be therefore assumed that the inhibitory effect of LPA is non-competitive while that of cLPA could be a competitive one. The inhibitory action of cLPA on the venom PDE activity was therefore analyzed according to the classical Michaelis-Menten mechanism. Fig. 2 shows the effect of 50 μM cLPA on PDE activity in $1/V$ vs. $1/S$ plot. As shown, the two lines intersect on the $1/V$ axis (at $1/V_{max}$), which indicates

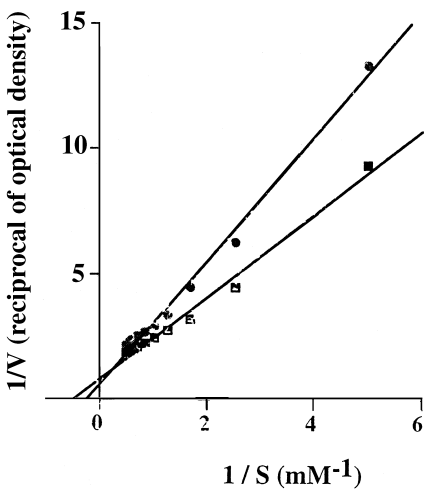

Fig. 2. Lineweaver-Burk plot (reciprocal of enzyme V vs. reciprocal of substrate concentration S) of snake venom PDE activity in the absence (squares) and presence (circles) of 50 μM palmitoyl cLPA.

that the inhibition is competitive. The inhibitory effects of LPA and cLPA on the snake venom PDE prompted us to test a series of related compounds at the 50–100 μM range. This series included α-glycerophosphate, β-glycerophosphate, 1,2-cyclic glycerophosphate, palmitic acid, monopalmitoyl glycerol and phosphatidic acid. None of these compounds displayed any significant inhibitory action on this enzyme (data not shown), strongly suggesting that the observed effects of LPA and cLPA are specific.

3.3. Effect of snake venom PDE on cAMP degradation

Snake venom PDE did not degrade cAMP (data not shown). The difference in substrate specificity between the venom and the intracellular PDEs was further confirmed by the effect of IBMX, and theophylline, the conventional inhibitors of cAMP-PDEs [28,29] on snake venom PDE. None of these inhibitors displayed inhibition activity on the venom PDE. Furthermore, the cell lysate PDE did not act on bis-pNPP.

3.4. Effect of phospholipids on cAMP specific phosphodiesterase from NIH 3T3 cells

The effect of the lysophospholipids tested with venom PDE (see above) was further checked on cellular PDE using NIH 3T3 cells. As expected, the cell lysate contained PDE specific to cAMP, as shown in Fig. 3. IBMX, a known inhibitor of cAMP PDE [22] was used as a reference (see Fig. 3). Of all the lysophospholipids which effected the venom PDE activity, only LPA exhibited inhibition activity but only at concentration above 1 mM (see Fig. 3). The inhibition of cAMP degradation by LPA was reached within a few minutes (data not shown) and it remained thereafter unchanged as expected from a simple effect of finding.

4. Discussion

The results presented in this paper clearly indicate that venom PDE is markedly different from cellular PDE with respect to substrates and inhibitors. The snake venom contains the enzymes phospholipase A_2 (PLA_2) and PDE as major venomous components [30,31]. The prime substrate for PLA_2 is

Table 1
Effect of lysophospholipids on snake venom phosphodiesterase activity

Lysophospholipid (50 μM)	Activity (%)
1-oleolyl-2-hydroxy-*sn*-glycerol-3-phosphocholine	164
1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine	162
1-caproyl-2-hydroxy-*sn*-glycero-3-phosphocholine	142
1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate	50
1-palmitoyl 2,3-cyclic-*sn*-glycerophosphate	5

Lysophospholipids (50 μl of 1 mM) were added to the enzyme reaction mixture (950 μl) prior to venom PDE enzyme (10 μg/ml) and incubated for 1 h at 37°C. The enzyme activity was determined as described in Section 2.3 and in the absence of lysophospholipid was taken as 100% activity. Data represent the means of three separate enzyme assays. For each of the presented values, S.D. $< \pm 10\%$.

phosphatidyl choline (PC) which upon cleavage is converted to lysoPC. In this study, we demonstrated that lysoPC of various acyl chains acts as an activator of snake venom PDE (see Table 1). This finding raises the possibility that upon venom inception PLA_2 and PDE act in tandem, i.e. the liberate lysoPC, which causes cell lysis acts further to activate the PDE in its deleterious DNA and RNA degradation. Phospholipase D, which is present in various tissues [32] including the plasma [24,25], can in principle convert lysoPC to the cLPA cyclic form of lysophosphatidic acid [26]. We have recently observed such an activity in human plasma [25]. This unique phospholipid [26] was shown here to inhibit the activity of snake venom phosphodiesterase in a classical competitive fashion (see Fig. 2). In this respect it might be proposed that the cleavage of lysoPC by phospholipase D is one of the mechanisms responsible for subsiding the venomous action. The open form of cLPA, i.e. LPA which is also formed in this enzymatic reaction has also an inhibitory effect on the venom PDE in a non-competitive nature which can only attenuate the activity of this enzyme by approximately 50% (see Table 1 and Fig. 1). Unlike the case of the venom enzyme, LPA showed inhibitory action on this enzyme only at concentrations in the millimolar range (see Fig. 3), which are much higher than those expected to be found in biological systems. Furthermore, the various LPC compounds had no effect on the cell lysate PDE.

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