ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 6, pp. 678-684. © Pleiades Publishing, Inc., 2006.
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Influence of Phospholipases A_2 from Snake Venoms on Survival and Neurite Outgrowth in Pheochromocytoma Cell Line PC12

Ya. V. Makarova, A. V. Osipov, V. I. Tsetlin, and Yu. N. Utkin*

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (495) 335-5733; E-mail: utkin@ibch.ru

Received November 3, 2005
Revision received February 8, 2006

Abstract—To determine whether the ability to induce neurite outgrowth in rat pheochromocytoma cell line PC12 is characteristic of phospholipases of different types, we have studied the influence of phospholipase A_2 (PLA2) from cobra *Naja kaouthia* venom and two PLA2s from viper *Vipera nikolskii* venom on PC12 cells. Phospholipases from the viper venom are heterodimers in which only one of the subunits is enzymatically active, while PLA2 from the cobra venom is a monomer. It was found that all three PLA2s induce neurite outgrowth in PC12. The PLA2 from cobra venom exhibits this effect at higher concentrations as compared to the viper enzymes. We have not observed such an activity for isolated subunits of viper PLA2s, since the enzymatically active subunits have very high cytotoxicity, while the other subunits are not active at all. However, co-incubation of active and inactive subunits before addition to the cells leads to a marked decrease in cytotoxicity and to restoration of the neurite-inducing activity. It has also been shown that all enzymatically active PLA2s are cytotoxic, the PLA2 from cobra venom being the least active. Thus, for the first time we have shown that PLA2s from snake venoms can induce neurite outgrowth in PC12 cells.

DOI: 10.1134/S0006297906060125

Key words: phospholipase A_2, cytotoxicity, snake venom, PC12 cells, neurite outgrowth

Phospholipases A_2 (PLA2s) constitute a large and heterogeneous family of enzymes, hydrolyzing the ester bond at the S_n2 position in glycerophospholipids with the formation of lysophospholipids and free fatty acids [1]. Secretory Ca^{2+}-dependent PLA2s [2] are components of the pancreatic juice produced by acinar cells in mammals; PLA2s are also found in fungi and bacterial filaments as well as in animal venoms (and in snake venoms in particular). PLA2s are one of the main toxic components of snake venoms, and their biological properties are well studied [3]. As a rule, venom PLA2s are toxic and display various pharmacological properties including neuro-, myo-, and cardiotoxic actions. In particular, they cause depolarization of muscle fibers [4] and can polarize nerve terminal membrane [5]. Mammalian secretory PLA2s play an important role in physiological as well as in pathological processes. Thus, they are involved in the formation of signal molecules, in particular, arachidonic acid, the key precursor of eicosanoids (prostaglandins, leukotrienes, thromboxanes) [6], influence the contraction of smooth muscles, and participate in inflammatory processes. The secretory PLA2s are involved in the regulation of different cellular functions, including the migration of endothelial cells [7] and stimulation of neurite outgrowth [8]. The effects caused by these enzymes result from direct phospholipid hydrolysis and disruption of membrane structure, or, in a majority of cases, from indirect involvement of hydrolysis products in signal transduction pathways [6].

There are several different classifications of PLA2s. In the currently acknowledged classification, secretory and intracellular enzymes are divided into 11 groups [9]. According to this classification, group I contains the enzymes isolated from mammalian pancreas, as well as from venom of *Elapinae* and *Hydrophiinae* snake subfamilies, whereas the enzymes from *Viperinae* and *Crotalinae* snake subfamilies belong to group II. According to their tertiary structures, PLA2s can be divided in four classes [10]. Class I includes single chain enzymes with a molecular weight within 13-15 kD. PLA2 from *N. kaouthia* cobra venom, which was studied in this work, belongs to this class. Class II includes the enzymes consisting of two

Abbreviations: PLA2) phospholipase A_2; HDP) heterodimeric phospholipase.

* To whom correspondence should be addressed.

non-covalently bound homologous (but not identical) subunits, at least one of which has enzymatic activity. PLA2 from *V. nikolskii* viper venom, which was also studied in this work, belongs to the second class. Therefore, the enzymes from two different classes are used in the present work.

As pointed out above, PLA2s display a wide spectrum of biological activities, and this spectrum is being broadened. Thus, it was recently shown that PLA2s from bacteria, fungi, and bee venom induce neurite outgrowth in rat pheochromocytoma PC12 cell line [11]. PC12 cells, an adrenal chromaffin cell line, are a suitable model for studying the mechanism of neuronal differentiation. Neurite outgrowth can be considered as a marker for cell differentiation [12]. It should be noted that the initiation of a differentiation process can be used for suppression of uncontrolled tumor growth, and thus compounds triggering this process can be regarded as cancerostatics. There is a wide array of compounds initiating nerve cell differentiation. The most well known are the factors from the neurotrophin family (NGF, BDNF, NT3, etc.) [13]; however, besides these proteins there are a number of compounds (in particular, some neurotransmitters [14]), which can initiate this important process. In the work cited above [11] a conclusion based on the study of supernatants from cell cultures expressing different PLA2s was made that mammalian PLA2s belonging to subgroups V and X induce neurite outgrowth in PC12 cells, whereas the enzymes from subfamilies IB and IIA did not produce such an effect. To determine whether the other PLA2 enzymes from groups I and II are able to induce neurite outgrowth, we studied the influence of PLA2s isolated from cobra *N. kaouthia* (PLA2 of group IA) and viper *V. nikolskii* (PLA2 of group IIA) on rat pheochromocytoma PC12 cells.

MATERIALS AND METHODS

Isolation of phospholipases. Isolation of phospholipases A_2 from viper *V. nikolskii* venom was performed according to a previously described protocol [15]. For isolation of PLA2 from cobra *N. kaouthia* venom, 1 g of dried venom was fractionated by gel filtration on a Sephadex G-50 superfine column (150 × 4.5 cm) in 0.1 M ammonium acetate buffer (pH 6.2). The volume of the collected fractions was 9 ml. The basic toxic fraction obtained after gel filtration was separated on a HEMA BIO 100 CM cation-exchange column (Tessek, Czech Republic) using a gradient of ammonium acetate buffer (pH 7.5) from 5 mM to 1 M during 100 min at a flow rate of 1.4 ml/min. Purified phospholipase A_2 was obtained by reverse-phase HPLC using a Vydac C18 column (4.6 × 250 mm) (Vydac, USA) and acetonitrile gradient from 15 to 45% (in water containing 0.1% trifluoroacetic acid) during 30 min. The flow rate was 1 ml/min.

Determination of phospholipase activity. The enzymatic activity of the preparations was determined as described in [16] using 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (Molecular Probes, Holland) as a substrate.

Identification of isolated proteins. N-Terminal amino acid sequences of pyridylethylated proteins and the products of their enzymatic hydrolysis were determined by the Edman method using a 473A automated sequencer (Applied Biosystems, USA).

Molecular weights of the isolated compounds, their derivatives, and peptide fragments were determined using Vision 2000 (Thermo BioAnalysis Corp., USA) and BRUKER REFLEX III (Bruker, Germany) time-of-flight MALDI mass spectrometers operating in reflection mode. The matrix was 2,5-dihydroxybenzoic acid.

Determination of cytotoxicity. Rat pheochromocytoma PC12 cells were cultivated at 37°C and 5% CO_2 in RPMI 1640 medium (Sigma, USA) containing 15% fetal bovine serum (HyClone) and 2 mM glutamine. For the investigation of phospholipase action, the cells were applied to a 96-well plate (Corning, USA) with a density of $(5\text{-}10)\cdot10^4$ cells per well (60-70% of monolayer). The cells were incubated in the medium containing 1% serum for 24 h, then the studied proteins were added at different concentrations, and incubation was continued. The number of living cells after additional 24 h of incubation was determined by staining with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2H-tetrazolium bromide (MTT). MTT was added to a final concentration of 0.5 mg/ml and incubated for 1-1.5 h. Formed crystals of formazan were dissolved in dimethylsulfoxide and the optical density was measured on a Multiscan plate reader at wavelength of 540 nm. Statistical data processing was performed using Statistica 6.0 software. The data are presented as mean ± standard deviation. Significance of differences was estimated using Student's *t*-criterion.

Determination of neurite outgrowth. The cells were cultivated and incubated with proteins as described for the determination of cytotoxicity. After 24 h, the morphological changes observed during the neurite outgrowth were detected visually and filmed. The cells were considered differentiated if the length of a neurite exceeded the length of the cell body. For quantification of neuritogenic activity the percentage of differentiated cells was calculated in relation to the total number of observed cells.

Mass spectrometric determination of cell membrane lipids. The cells were plated in a 6-well plate with a density of $3\cdot10^5$ cells per well and cultivated for 24 h in the presence of different concentrations of heterodimeric phospholipases HDP-1 and HDP-2. Incubation without PLA2 was used as a control. The cells were detached from the plate by versene solution, pelleted by centrifugation, washed three times with phosphate buffered saline (PBS; 5 mM sodium phosphate, pH 7.2, 145 mM NaCl), then

pelleted again and resuspended in 100 μl of PBS. The lipids were extracted by a mixture of chloroform and methanol (2 : 1) as described in [17]. After removal of the solvent, the residue was dissolved in 20 μl of chloroform–methanol mixture (2 : 1) and analyzed by MALDI mass spectrometry as described above.

RESULTS AND DISCUSSION

We recently established [15] that venom from *V. nikolskii* viper contains two different heterodimeric phospholipases (HDP-1 and HDP-2), comprising enzymatically active and inactive subunits. Reverse-phase HPLC of each of the phospholipases resulted in purified subunits, where only one of them had phospholipase activity. Enzymatically active subunits obtained from HDP-1 and HDP-2 will be further referred to as HDP-1P and HDP-2P, whereas inactive subunits are referred to as HDP-1I and HDP-2I, respectively. Phospholipase activity of isolated HDP-1P (1.25 mmol/min per μmol protein) and HDP-2P (0.81 mmol/min per μmol protein) subunits determined by using a fluorescent substrate according to [16] was higher than that of the original heterodimeric HDP-1 (0.56 mmol/min per μmol protein) and HDP-2 (0.31 mmol/min per μmol protein), respectively. Phospholipase activity was not detected for HDP-1I and HDP-2I. By chromatographic behavior and amino acid sequences our proteins are very similar to neurotoxic heterodimeric PLA2 vipoxin from viper *V. ammodytes* venom [18] and vaspin from *V. aspis* venom [19]. Crotoxin from rattle snake *Crotalus durissus terrificus* venom [20] and neurotoxic PLA2 from Taiwan viper *V. russeli formosensis* venom [21] are also very close by structure and properties to these enzymes. All these proteins belong to group IIA.

The protein isolated by us from cobra *N. kaouthia* venom corresponded by its N-terminal amino acid sequence to acidic PLA2 CM-2 isolated from the same venom previously [22]. This enzyme belongs to group IA.

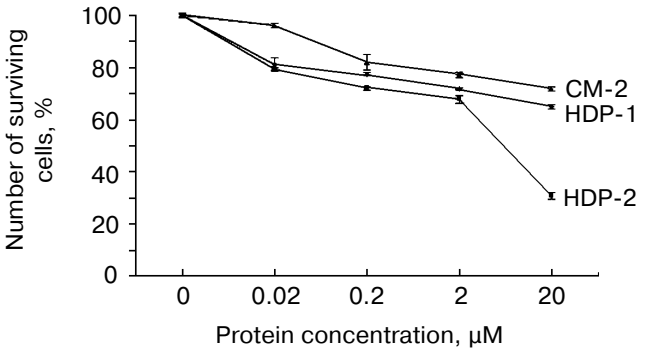

Fig. 1. Dependence of the number of viable PC12 cells on the concentration of snake venom PLA2. The cells were incubated with different concentrations of PLA2 during 24 h and then the number of living cells was determined by MTT staining.

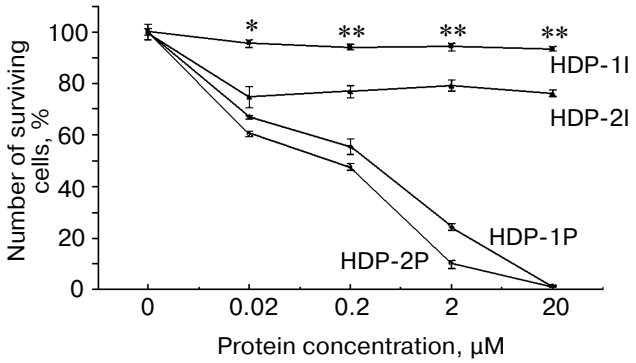

Fig. 2. Dependence of the number of viable PC12 cells on the concentration of isolated subunits of PLA2 from *V. nikolskii*. The cells were incubated with different concentrations of the subunits during 24 h and then the number of living cells was determined by staining with MTT. The cells incubated without PLA2 were used as a control ($p < 0.01$, * $p < 0.06$, and ** $p < 0.02$ compared to control, $n = 12$).

To investigate the influence of the obtained PLA2s on PC12 cell culture, the cells were incubated with the proteins for 24 h, and the morphological changes and cell viability were determined. PLA2 concentrations ranged from 20 nM to 20 μM.

Cytotoxicity. One of the main biological effects of PLA2 from snake venom is cell death. To the highest extent cytotoxicity is manifested upon action of myotoxic enzymes on skeletal muscle cells, PLA2 toxicity towards other cell types usually being lower [23]. When studying the influence of heterodimeric PLA2s from *V. nikolskii* on viability of PC12 cells, it was determined that, as expected, the number of viable cells decreased with the increase in HDP-1 and HDP-2 concentrations in the medium (Fig. 1), where the higher cytotoxicity was displayed by phospholipase HDP-2 (for all studied concentrations the differences were significant with $p < 0.01$). Cytotoxicity of heterodimeric phospholipases from viper *V. nikolskii* appeared to be higher than that of cobra *N. kaouthia* PLA2 (for all studied concentrations the differences were significant with $p < 0.01$) (Fig. 1).

The study of individual components forming heterodimeric PLA2 from *V. nikolskii* demonstrated that HDP-1I and HDP-2I were not cytotoxic. Enzymatically active HDP-1P and HDP-2P subunits (Fig. 2) with higher enzymatic activity (1.25 and 0.81 mmol/min per μmol of protein, respectively) displayed higher cytotoxicity compared to the original proteins HDP-1 (0.56 mmol/min per μmol) and HDP-2 (0.31 mmol/min per μmol) (Table 1). Simultaneous preincubation of HDP-1I and HDP-2I with HDP-1P and HDP-2P, respectively, decreased the toxic effect of the latter proteins (Table 1).

Table 1. Suppression of cytotoxic activity of HDP-1P and HDP-2P by HDP-1I and HDP-2I after co-incubation during 1 h at room temperature

Protein	Number of surviving cell relative to control, %*
HDP-1	68.68 ± 1.02**
HDP-1I	97.19 ± 0.77
HDP-1P	1.33 ± 0.22**
HDP-1I + HDP-1P	72.89 ± 0.67**
HDP-2	40.42 ± 0.89**
HDP-2I	98.3 ± 0.7
HDP-2P	2.21 ± 0.12**
HDP-2I + HDP-2P	78.75 ± 1.99**

* Proteins at the concentration of 20 μM were incubated with cells during 24 h and the number of living cells was determined using MTT.

** $p < 0.01$ compared to control. The difference in average activity values upon comparison of activity of the reconstructed complexes with that of individual subunits (HDP-1P – HDP-1I + HDP-1P; HDP-1I – HDP-1I + HDP-1P; HDP-2P – HDP-2I + HDP-2P; HDP-2I – HDP-2I + HDP-2P) is statistically significant ($p < 0.05$).

At the same time, according to ion-exchange chromatography data (Fig. 3), interactions between individual subunits result in the reassembly of heterodimeric HDP-1 and HDP-2, which have lower toxicity compared to the enzymatically active subunits (HDP-1P and HDP-2P, respectively). Our results about manifestation of cytotoxicity only by enzymatically active subunits are in agreement with other data obtained for phospholipases from other sources [24, 25]. For example, the cytotoxic effect of crotoxin, a heterodimeric phospholipase from *Crotalus durissus terrificus* venom, was exhibited only upon dissociation of heterodimer and formation of enzymatically active subunit B [26].

Therefore, the cytotoxic effect of PLA2 on PC12 cells is, obviously, related to enzymatic activity and caused by disruption of the cell membrane integrity. As for the mechanism of cytotoxic action, depending on the cell type, type of phospholipase, and even its concentration [27], the cell death can be either necrotic or apoptotic [28].

Neurite outgrowth. As mentioned in the introduction, secretory proteins from fungi and bacteria with phospholipase activity as well as PLA2 from bee venom (group III) and mouse embryo brain (groups V and X) belonging to different groups of secretory phospholipases, induce neurite outgrowth [11] in PC12 cells to different extents. The maximal effect after 48 h incubation of cells with PLA2 was observed at the concentration of 10 nM in the case of bee venom enzyme; 0.1 μM for fungal enzyme p15, and 1 μM for enzyme Scp15 from *Streptomyces coelicolor* [12]. It is important to note that by its tertiary structure all these proteins belong to monomeric PLA2. The studies performed by us have shown that heterodimeric PLA2s from viper venom in the concentration range from 0.2 to 20 μM also induce neurite outgrowth in PC12 cells (Fig. 4). Decrease in number of differentiated cells at HDP-1 concentrations higher than 0.2 μM (Table 2) can be explained by lower stability of these cells against cytotoxic action of the enzyme. Analogous dependence of neuritogenesis (with maximum) on concentration was observed in the case of PLA2 from bee venom [11]. Such an effect was not found for HDP-2.

Induction of neurite outgrowth by PLA2 from cobra venom was reproducible only at concentrations higher than 2 μM (Table 2). It should be noted that neurite outgrowth was observed both upon incubation of cells with the native proteins (heterodimeric HDP-1 and HDP-2)

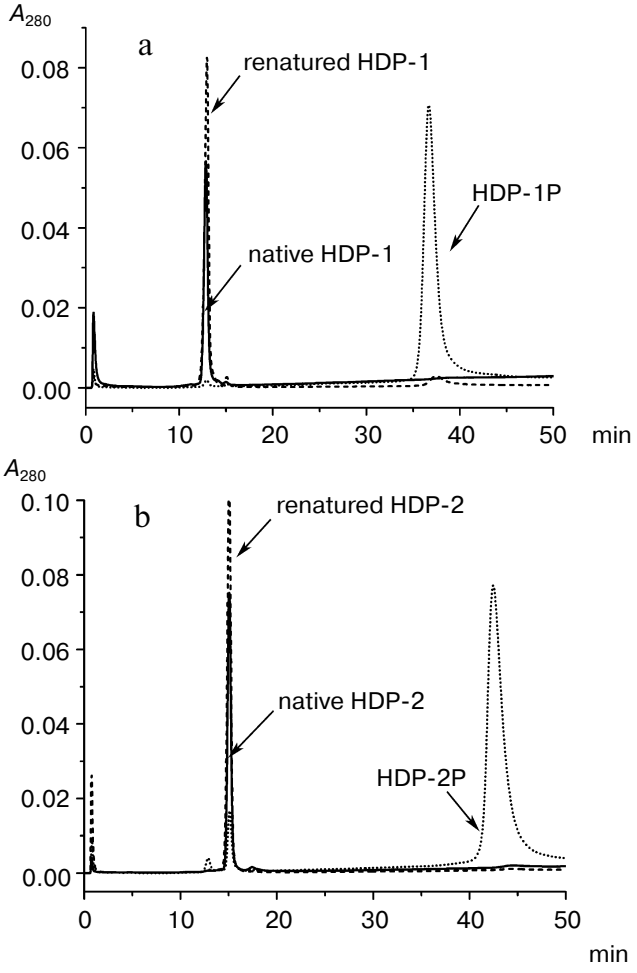

Fig. 3. a) Ion-exchange chromatography of the native (solid line) and reconstructed (dashed line) HDP-1, as well as isolated HDP-1P subunit (dotted line) on a HEMA BIO 1000 CM column (3 × 30 mm) in a gradient of ammonium acetate buffer (pH 7.5) from 60 mM to 1 M for 47 min at the flow rate of 0.3 ml/min. HDP-1I is not retained on the column under these conditions. b) HDP-2 and HDP-2P. The legend and separation conditions are identical to those for HDP-1 and HDP-1P.

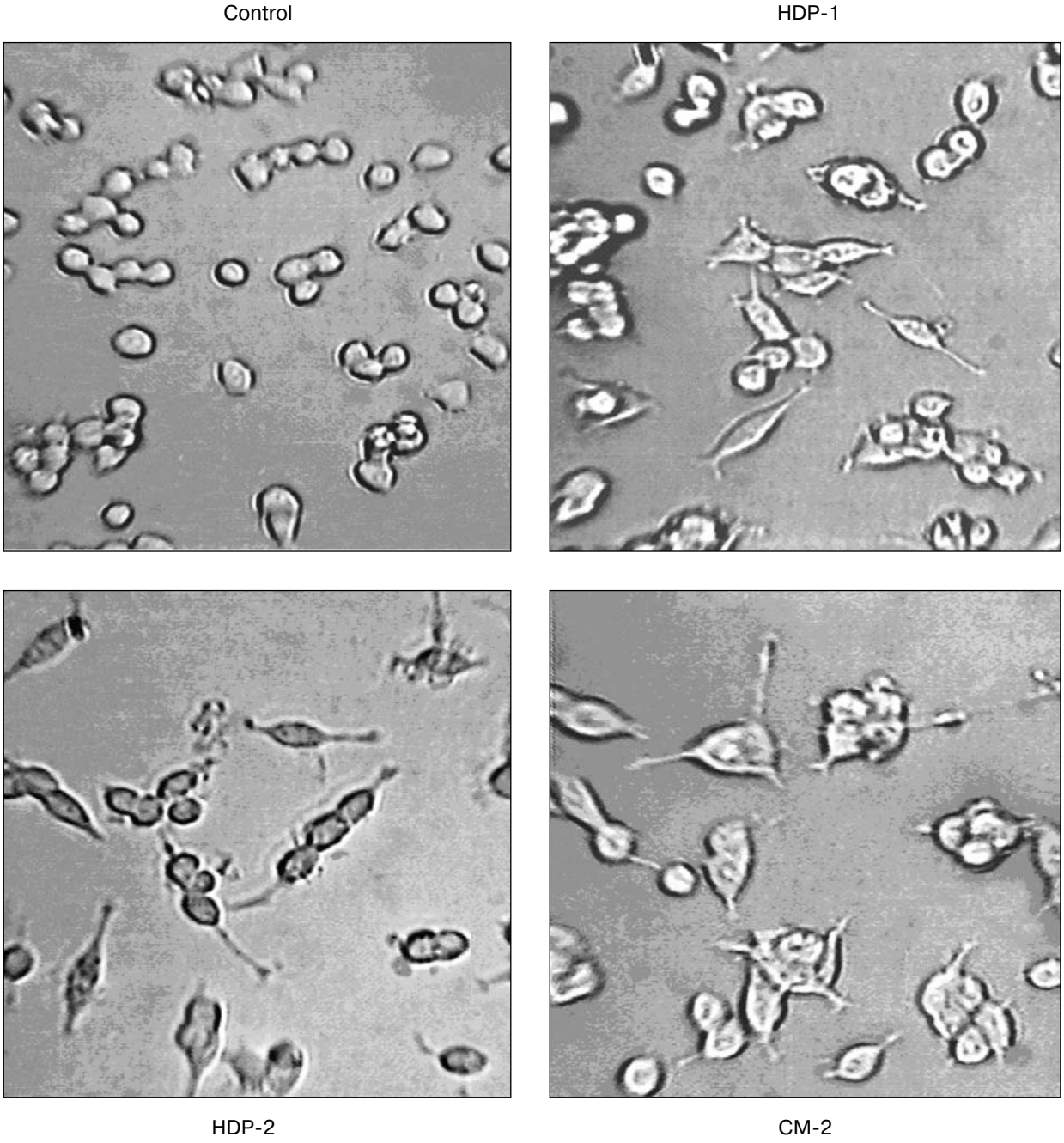

Fig. 4. Neurite outgrowth in PC12 cells after incubation with PLA2 from snake venoms during 24 h. PLA2 concentration: HDP-1 and CM-2, 20 μM; HDP-2, 2 μM.

and upon proteins reassembled after co-incubation of the isolated subunits. As for the possible mechanism for this effect, data described in the literature [12, 29] demonstrate that PLA2 ability to stimulate neurite outgrowth correlates with its enzymatic activity. This conclusion is in a good agreement with our data on induction of neurite outgrowth by enzymatically active proteins. Neuritogenic activity was not detected for isolated subunits of PLA2 from viper venom, since enzymatically active subunits have high cytotoxicity, whereas other subunits are not active at all. Recently it was established [29, 30] that neurite outgrowth in PC12 is induced by lysophosphatidylcholine formed as a result of degradation of cell membrane phospholipids by PLA2, and different ability of PLA2s of various types to stimulate neurite outgrowth is directly correlated to its ability to release lysophosphatidylcholine from the cell membrane. It is possible that the ability of PLA2 from snake venom to induce neu-

Table 2. Dependence of neuritogenic activity of PLA2 on concentration

PLA2	Protein concentration, μM	Number of cells with neurites longer than the cell body, %*
HDP-1	20	3.7 ± 1.9
	2	11.6 ± 1.1**
	0.2	13.2 ± 1.4**
	0.02	1.1 ± 0.6
HDP-2	20	27 ± 2.1**
	2	13 ± 1.4**
	0.2	2.6 ± 0.2**
	0.02	1.2 ± 0.3
CM-2	20	9.1 ± 0.9**
	2	3.4 ± 0.5**
	0.2	1.3 ± 0.5
	0.02	1 ± 0.5
Control		0.7 ± 0.4

* Cells were incubated with the proteins during 24 h and then counted.
** $p < 0.01$ compared to control. The differences between HDP-1 and HDP-2, HDP-1 and CM-2, HDP-2 and CM-2 at the concentration of 20 μM are statistically significant ($p < 0.05$).

rite outgrowth at higher concentrations compared to enzymes from other groups is associated with lower enzymatic activity of snake proteins towards phospholipids of the PC12 cell membrane. However, in the cited publications there are no data on specific activity of PLA2s inducing neurite outgrowth. Since measured specific activity for this enzyme depends dramatically on both the nature of the substrate and the method used, these values should be compared with care. Nevertheless, the data available from the literature [16] indicate that the activity of PLA2 from bee venom exceeds that of crotoxin (PLA2 from *Crotalus durissus terrificus* venom) by 2-3 orders of magnitude in the reaction of hydrolysis of fluorescent phosphatidylcholine analogs. As mentioned above, HDP-1 and HDP-2 are structurally similar to crotoxin, and the phospholipase activities of HDP-1 (0.56 mmol/min per μmol of protein) and HDP-2 (0.31 mmol/min per μmol of protein) determined using fluorescent phosphatidylcholine analogs are very similar to that of crotoxin (0.69 mmol/min per μmol of protein) determined using the same substrate [16]. Basing on these data, one can suggest that activities of HDP-1 and HDP-2 in the hydrolysis of phosphatidylcholine from PC12 cell membrane will also be lower than that of PLA2 from bee venom by several orders of magnitude. This assumption is in good agreement with the discovered ability of HDP-1 and HDP-2 to induce neurite outgrowth at concentration higher than that found for PLA2 from bee venom.

To determine whether phosphatidylcholine hydrolysis occurs at the concentrations of HDP-1 and HDP-2 that induce neurite outgrowth, we analyzed phospholipids from PC12 cells by mass spectrometry. Figure 5 presents the data obtained without HDP-1 (a) as well as after cell incubation with the enzyme at the concentration of 0.02 μM (b) and 0.2 μM (c). It should be noted that neurites are virtually absent at the enzyme concentration of 0.02 μM, whereas at the enzyme concentration

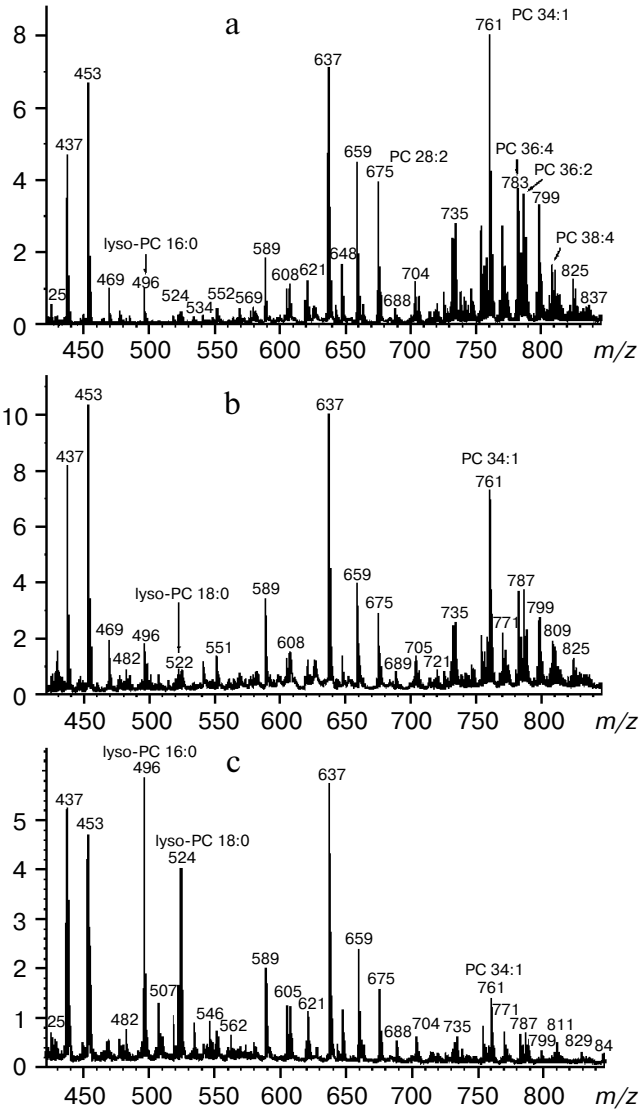

Fig. 5. Analysis of phospholipids from PC12 cell membrane by MALDI mass spectrometry: a) control without cell treatment with PLA2; b, c) after cell incubation with HDP-1 at the concentration of 0.02 and 0.2 μM, respectively. Phosphatidylcholine (PC) and lysophosphatidylcholine (lyso-PC) subclasses are marked. The ordinate axis is signal intensity in relative units.

of 0.2 μM differentiated cells account for 13% (Table 2). As seen from Fig. 5, the increase in HDP-1 concentration results in dramatic decrease in signal intensity within the mass range from 720 to 830 daltons, corresponding to different phosphatidylcholine sub-classes, and in parallel increase in intensity of signals within the mass range from 480 to 550 daltons, corresponding to different sub-classes of lysophosphatidylcholine. The intensity increases most of all for the signals corresponding to sub-classes C16:0 (m/z 496) and C18:0 (m/z 524) which were shown to induce neurite outgrowth [29, 30]. A similar phenomenon is observed for HDP-2 (data not shown). Therefore, the induction of neurite outgrowth in PC12 cells by snake PLA2 is, obviously, driven by the same mechanism as for other PLA2 groups.

The described mechanism of neuritogenesis is different from that for nerve growth factor, which stimulates neurite outgrowth by interaction with specific nerve cell receptors [13]. Moreover, PLA2 increases the action of nerve growth factor, and, probably, can be involved in PC12 cell differentiation [29]. It should be noted that PLA2 can induce differentiation not only in nerve cells. Thus, the available data indicate that PLA2 promotes differentiation in HL-60 cells upon the action of diacylglycerol and phorbol ester [31] and stimulates maturation of dendritic cells [32].

Hence, PLA2s from snake venoms are able to induce neurite outgrowth in PC12 cells, similar to other secretory phospholipases mentioned above. This effect was found by us for the first time in PLA2s belonging to groups I and II, including heterodimeric phospholipases A_2. Our data indicate that neuritogenic activity and cytotoxicity of phospholipases are not directly linked to each other. Therefore, the existence of PLA2 with low cytotoxicity and high differentiating activity is possible. Since phospholipases are contained in venoms in sufficiently large quantities (for instance, the total content of HDP-1 and HDP-2 in *V. nikolskii* venom is higher than 20%), and their purification is relatively simple, they can be used as an alternative to nerve growth factor for stimulation of cell differentiation. However it should be kept in mind that at high concentrations snake phospholipases display cytotoxic properties.

This work was supported by the Russian Foundation for Basic Research (project Nos. 03-04-48496 and 02-04-39014) and partly by a grant of the President of Russian Federation for young scientists (MK-1779.2005.4).

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