



Functions, structures and Triton X-100 effect for the catalytic subunits of heterodimeric phospholipases A₂ from *Vipera nikolskii* venom

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ABSTRACT

Phospholipases A₂ (PLA₂s) from snake venoms have diverse pharmacological functions including neurotoxicity, and more studies are necessary to understand relevant mechanisms. Here we report the different crystal structures for two enzymatically active basic subunits (HDP-1P and HDP-2P) of heterodimeric neurotoxic PLA₂s isolated from *Vipera nikolskii* venom. Structural comparisons with similar PLA₂s clearly show some flexible regions which might be important for the catalytic function and neurotoxicity. Unexpectedly, Triton X-100 molecule bound in the hydrophobic channel of HDP-1P and HDP-2P was observed, and its binding induced conformational changes in the Ca²⁺ binding loop. Enzymatic activity measurements indicated that Triton X-100 decreased the activity of PLA₂, although with comparatively low inhibitory activity. For the first time exocytosis experiments in pancreatic β cells were used to confirm the presynaptic neurotoxicity of relevant snake PLA₂. These experiments also indicated that Triton X-100 inhibited the influence of HDP-1P on exocytosis, but the inhibition was smaller than that of MJ33, a phospholipid-analogue inhibitor of PLA₂. Our studies performed at a cellular level are in good agreement with earlier findings that enzymatic activity of the snake presynaptic PLA₂ neurotoxins is essential for effective block of nerve terminals.

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1. Introduction

Phospholipases A₂ (PLA₂s; EC 3.1.1.4) are enzymes that hydrolyze the sn-2 acyl ester bond of various phospholipids to produce free fatty acids and lysophospholipids. They are widely distributed in nature, occurring as both intracellular (cytosolic) and extracellular (secretory) forms (Six and Dennis, 2000), and play important roles in various biological processes such as phospholipid metabolism and remodeling, homeostasis of cellular membranes, host

defence and mediator production as well as signal transduction (Valentin and Lambeau, 2000). Snake venom PLA₂s are toxic and possess diverse pharmacological and toxicological functions, e.g., neurotoxicity, myotoxicity and cardiotoxicity etc. (Kini, 2003).

Some PLA₂s, found mainly in snake venoms, are presynaptic neurotoxins that block nerve-muscle communication at the neuromuscular junction in a characteristic way. These neurotoxins have been extensively investigated as promising tools for studying various aspects of nerve system function and dysfunction (Montecucco and Rossetto, 2000; Krizaj and Gubensek, 2000). Neurotoxic PLA₂s in crystal structures have been found to be heterodimeric, homodimeric or monomeric (Westerlund et al., 1992; Georgieva et al., 2004) depending on the nature of

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enzyme and crystallization conditions. However, the toxic site and the mechanism of their action at the molecular level are still largely unknown. In particular, the neurotoxicity of venom PLA₂s, which has been one of the most studied pharmacological effects, is currently thought to involve both catalytic activity and binding to target proteins on membranes. The phospholipase A₂ activity varies greatly among these neurotoxins, and its involvement in the neurotransmission blockade is still debated (Montecucco and Rossetto, 2000; Rosenberg, 1997). Some studies suggest that other factors may also contribute to neurotoxicity (Rouault et al., 2006).

The traditional way for determining presynaptic PLA₂ neurotoxicity is to study its influence on signal transduction in the muscle-nerve preparation. In this preparation neurotoxic PLA₂ usually act presynaptically disturbing neurotransmitter release (exocytosis) from nerve terminals. However, due to complexity muscle-nerve preparation is not very good model for studying molecular mechanism of neurotoxicity. For this purpose primary cell lines from central nervous system as well as other cell lines (e.g., PC12 and NCS34) were utilized (Wei et al., 2003; Rigoni et al., 2004; Praznikar et al., 2009). Considering the application of different cell lines for exocytosis experiments, we suggested that this process can be studied at cellular level in non-neuronal cells and used pancreatic islet β cells in our work. It might be possible that the exocytosis in pancreatic β cells is not identical to that in motoneurons (or neurons in general). This process probably is not the same even in different types on neural cells which are used for the investigation of molecular mechanisms of PLA₂ action. However there are some general mechanisms involved in exocytosis both in neurons and in pancreatic β cells. Thus, the secretion is a Ca²⁺-dependent process in both types of cells. The same proteins are involved in exocytosis in beta cells and neurons (Wheeler et al., 1996). Synaptotagmins, t-SNAREs syntaxin 1 and SNAP-25, the v-SNARE synaptobrevin/VAMP-2 and munc-13 are just few examples of such proteins. The mechanism of vesicle fusion with membrane is also similar in neurons and beta cells (see review of Eliasson et al., 2008 for the details). Neurotoxic PLA₂s affect not only motoneurons but also many other neurosecretory cells, which probably have some differences in exocytosis mechanism (see review of Pungercar and Krizaj, 2007). Basing on this consideration one can suggest that PLA₂ interaction with beta cells may have a mechanism similar to that of neurotoxic action of PLA₂ on motoneurons. It should be noted that cobra venom PLA₂, which is not neurotoxic, does not influence exocytosis in β cells from mouse pancreas (Juhl et al., 2003). This means that β cells can be used as a model for investigation of PLA₂ effects on motoneurons. Moreover, the kinetics of secretion from β cells is much slower (Barg et al., 2002) that allows more detailed investigation of mechanism. To study effects of neurotoxic PLA₂ HDP-1P on exocytosis, we used for the first time β cell line INS-1 (Wheeler et al., 1996) and the whole-cell patch-clamp, which allowed us to determine the capacitance of cell membrane and thus to monitor the exocytosis of neurotransmitter (Xu et al., 1997). We have found that HPD-1P inhibits exocytosis in

β cells. This result may be regarded as confirming the presynaptic toxicity of HPD-1P.

The venom of the viper *Vipera nikolskii* was chosen as a source of new PLA₂s. This black viper, which inhabits the forest steppes of southern Russia and Ukraine, recently has been established as an independent species (Bakiev et al., 2005). Two heterodimeric neurotoxic PLA₂s of class II were isolated from *V. nikolskii* venom and characterized (Ramazanov et al., 2008). Their enzymatically active subunits (HDP-1P and HDP-2P) belong to the basic Asp49 PLA₂s of the group II. Here, we report the crystal structures determined for these two new PLA₂s. Moreover, we have found Triton X-100 molecule within the hydrophobic channel of PLA₂ molecules. Biological activity measurements have shown that Triton X-100 diminishes both enzymatic activity and neurotoxicity of PLA₂. This inhibitory effect should be taken into account when using Triton X-100 for PLA₂ studies.

2. Materials and methods

2.1. Purification and crystallization of proteins

Proteins HDP-1P and HDP-2P were purified from crude *V. nikolskii* venom as described (Ramazanov et al., 2008). The crystals were obtained as described in our preliminary communication (Gao et al., 2005a). The proteins were crystallized by the hanging drop vapour-diffusion method using Linbro plates and a simplified screen designed by our group (Gao et al., 2005b).

2.2. Data collection and structure determination

Diffraction data sets for the crystals were collected at the Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences. All the diffraction data were indexed, integrated and scaled using the programs DENZO and SCALEPACK (Otwinowski and Minor, 1996). The parameters of the data-collection statistics are listed in Table 1. The initial phase problem was solved by the molecular replacement method with the program MOLREP (Vagin and Teplyakov, 1997) using the coordinates of chain B of Vipoxin (Vipoxin B, PDB code: 1jlt-B). The refinement was performed using the program CNS (Brunger et al., 1998) and the program O (Jones, 1978). The quality of the final model was analyzed using the program PROCHECK (Laskowski et al., 1993). The refinement statistics of HDP-2P, HDP-1P and HDP-1Piso are also given in Table 1.

2.3. Phospholipase A₂ activity measurements

Phospholipase A₂ activity was determined essentially as described (Radvanyi et al., 1989) using 1-palmitoyl-2-(10-pyrenyldecanoyl)-sn-glycero-3-phosphorylcholine as substrate. Substrate (2 μ M) was incubated with 1 nM HDP-1P in 50 mM Tris-HCl containing 100 mM NaCl, 1 mM EDTA, and 6 mM CaCl₂. The fluorescence measurements were performed on F-4000 Fluorescence Spectrophotometer (Hitachi, Japan). Excitation and emission wavelengths were 346 and 397 nm, respectively. To determine the influence

Table 1

The data collection and structure refinement statistics of HDP-1P, HDP-2P and HDP-1Piso. (Values in parentheses are for the last resolution shell.)

Parameters	HDP-2P	HDP-1P	HDP-1Piso
Wavelength (Å)	0.98	1.0	1.0
Space group	R32	R32	R32
Unit-cell parameters (Å)	$a = b = 76.29, c = 303.35$	$a = b = 76.28, c = 304.39$	$a = b = 115.11, c = 42.46$
No. of molecules in AU	2	2	1
No. of unique reflections	7107	17 760	11 756
No. of observed reflections	20 434	259 886	129 343
Resolution (Å)	29.0–3.0	29.0–2.2	50–1.7
Completeness of data (%)	100 (99.9)	100 (100)	98.4 (100)
I/σ (I)	17.7 (5.4)	25.1 (4.1)	41.3 (5.5)
Rmerge (%)	17.0 (54.3)	9.8 (49.7)	6.1 (44.9)
<i>Refinement statistics</i>			
Rvalue/Rfree (%)	23.4/29.3	20.4/23.9	20.0/22.6
Number of amino acids	122	122	122
Number of water molecules	35	208	131
<i>R.m.s deviations</i>			
Bond distance (Å)	0.008	0.0061	0.0058
Bond angle (deg)	1.2	1.02	1.14
<i>Ramachandran plot</i>			
Non-Gly in core regions (%)	90.6	90.6	89.6
Allowed regions (%)	8.5	8.5	10.4
Generously allowed regions (%)	0.9	0.9	0.0

of Triton X-100 on activity, the enzyme was incubated with different concentrations of the detergent for 30 min before the addition to the reaction mixture. Different batches of Triton X-100 produced by Serva (Germany), Koch-Light, Hampton Research Corp. and NPKO “Diagnostikum” (Russia) were used for measurements.

2.4. Exocytosis experiments

2.4.1. Cell culture

Pancreatic β cells (INS-1) were grown in RPMI 1640 supplemented with 10% (v/v) foetal bovine serum, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 mM 2-mercaptoethanol as described previously (Asfari et al., 1992).

2.4.2. Membrane capacitance (C_m) measurement

The C_m of INS-1 cells were measured in real time using an EPC10 amplifier (Heka Electronics, Lambrecht, Germany) in conventional whole-cell patch-clamp configuration. A sine + DC protocol was applied using the Lockin extension of the Pulse program (Heka Electronics). The cells were voltage clamped at a holding potential of -70 mV and a sine wave voltage command with amplitude of 20 mV and frequency of 1042 Hz was applied. Currents were filtered at 2.9 kHz and sampled at 25 kHz. The normal bath solution contained (in mM): 138 NaCl, 5.6 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 5 Hepes, and 3 glucose (adjusted to pH 7.4 with NaOH, 310 mOsm). The influence of HDP-1P, Triton X-100 and MJ33 on exocytosis was measured by adding them in the normal bath solution and by reacting for half an hour.

2.4.3. Ca^{2+} -uncaging and $[Ca^{2+}]_i$ measurement

Homogeneous global $[Ca^{2+}]_i$ elevation was generated by photolysis of the caged- Ca^{2+} compound, 1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane-N,N,N',N'-tetraacetic acid

(DMNP-EDTA, Molecular Probes, Carlsbad, CA, USA), with UV light sourced as previously described (Xu et al., 1997). The DMNP-EDTA containing pipette solution contained (in mM): 110 CsGlu, 5 DMNP-EDTA, 8 NaCl, 3.6 CaCl₂, 2 MgATP, 0.3 GTP, 0.2 fura-6F or 0.2 mag-fura 2, and 35 Hepes, adjusted to pH 7.2 using CsOH or HCl (osmolarity, 300 mOsm). The free Ca^{2+} concentration of the pipette solution was determined to be around 200 nM.

2.5. Protein Data Bank accession number

Coordinates and experimental structure factors of the HDP-1P crystal structure have been deposited in the RCSB Protein Data Bank with the code 2I0U.

3. Results and discussion

3.1. Preparation and crystallization of proteins

Enzymatically active proteins HDP-1P and HDP-2P used for crystallographic studies were isolated from *V. nikolskii* venom as described (Ramazanov et al., 2008). When tested on the frog neuromuscular junction, these PLA₂s affected neuromuscular transmission acting presynaptically. The sequences of the HDP-1P and HDP-2P are similar to those of basic subunits of known neurotoxic PLA₂s: vipoxin from *Vipera ammodytes meridionalis* (Mancheva et al., 1987) and vaspin from *Vipera aspis aspis* (Jan et al., 2002). Among these four proteins there are differences only in three positions, i.e. the position 74, 110, and 118 (Fig. 1). The residue numbering system proposed by Renetseder et al., 1985 is used throughout this work.

The crystals of HDP-1P and HDP-2P were obtained using crystallization conditions described in our preliminary communication (Gao et al., 2005a). A new crystal form (HDP-1Piso) of the protein HDP-1P was obtained by

10	20	30	40	50	60	70	
NLFQFAKMIN	GKLG-AFSVW	NYISYGICYG	WGGQGTTPKDA	TDRCCFVHDC	CYGRVRG---	C-----NPKL	
NLFQFAKMIN	GKLG-AFSVW	NYISYGICYG	WGGQGTTPKDA	TDRCCFVHDC	CYGRVRG---	C-----NPKL	
NLFQFAKMIN	GKLG-AFSVW	NYISYGICYG	WGGQGTTPKDA	TDRCCFVHDC	CYGRVRG---	C-----NPKL	
NLFQFAKMIN	GKLG-AFSVW	NYISYGICYG	WGGQGTTPKDA	TDRCCFVHDC	CYGRVRG---	C-----NPKL	
ALWQFNGMIK	CKIPSSPELL	DFNNYGCYCG	LGGSGTTPVDD	LDRCCQTHDN	CYKQAKKLDL	CKVLVDNPFYT	
80	90	100	110	120	130		
AIYAYSFKKG	NIVCGK-NNG	CLRDICECDR	VAANCFHQNQ	NTYNKNYKFL	SS-SRCRQTS	EQC	Q1RP79 HDP-1P
AIYAYSFKKG	NIVCGK-NNG	CLRDICECDR	VAANCFHQNK	NTYNKNYRFL	SS-SRCRQTS	EQC	Q1RP78 HDP-2P
AIYAYSFKKG	NIVCGK-NNG	CLRDICECDR	VAANCFHQNK	NTYNKNYRFL	SS-SRCRQTS	EQC	Q8JFG0 VASPIN
AIYAYSFKKG	NIVCGK-NNG	CLRDICECDR	VAANCFHQNK	NTYNKNYKFL	SS-SRCRQTS	EQC	P14420 VIPOXIN
NNYSYSCSN	EITCSSENA	CEAFICNCDR	NAAICFSKV-	-PYNKEHKNL	DK-KNC		P00593 BOVINE PANCREAS

Fig. 1. Sequence alignment of the HDP-1P and HDP-2P with vaspin and vipoxin. Gray shading indicates differing amino acid residues. Renetseder residue numbering system (Renetseder et al., 1985) based on amino acid sequence of bovine pancreatic PLA₂ is used throughout this work.

screening and optimization of conditions in a manner similar to that used for protein HDP-1P but without Triton X-100 (0.1 M HEPES-Na, pH 7.5, 0.8 M potassium-sodium tartrate, 0.2 μl 2-methyl-2,4-pentanediol (MPD) and 20 mg/ml HDP-1P).

3.2. Crystal structures of HDP-2P and HDP-1P

The crystals of HDP-1P and HDP-2P belong to the same space group *R*32 with little difference in the unit-cell dimensions (Table 1). The asymmetric units of crystal structures of HDP-1P and HDP-2P contain two PLA₂ molecules with small interface concerning solvent and water molecules. The crystals of HDP-1Piso diffracting to 1.7 Å resolution belong to the same space group *R*32, but with significantly different unit-cell dimensions ($a = b = 115.11$ and $c = 42.46$ Å) and with only one molecule in an asymmetric unit. The HDP-1Piso structure is the first structure for the catalytic subunits of heterodimeric phospholipases A₂ in monomeric state.

PLA₂s are enzymes that hydrolyze the sn-2 acyl ester bond of various phospholipids to produce free fatty acids and lysophospholipids. As substrates, various phospholipids go through hydrophobic channel to active centre containing His48 where the hydrolysis takes place. Structural comparisons of HDP-1P with HDP-2P have shown that the conformations of all residues forming hydrophobic channel and active site are similar, with the exception of the residues Cys29 and Lys69 which are located at the entrance into the channel. The conformations of Cys29 and Lys69 residues in HDP-1P allow the substrate to enter hydrophobic channel more easily than in HDP-2P and this may make PLA₂ activity of HDP-1P higher than that of HDP-2P. Indeed, the enzymatic activity of HDP-1P is 1.5-fold higher than that of HDP-2P (Makarova et al., 2006).

Structural comparisons between different PLA₂ molecules in the determined crystal structures have clearly shown that these structures are very similar and contain remarkable conformationally flexible regions such as Ca²⁺ binding loop (residues 25–35) and N- and C-terminal regions (residues 1–13 and 113–133, respectively). Moreover, comparison of HDP-1Piso structure with that of Vipoxin B (Vipoxin subunit possessing PLA₂ activity) revealed remarkable similarity in overall structure with some conformational differences in several regions, including N- and C-terminus, turns Arg56-Leu70 and

Ser76-Asn81, as well as stretch Cys84-Arg93. The conformational difference between HDP-1Piso and Vipoxin B in the N-terminal region may be explained by the fact that Vipoxin B structure is determined in heterodimeric form in which the N-terminal regions are located at the interface between two molecules (Banumathi et al., 2001). Most of the above mentioned conformational differences are located in the flexible regions, which do not take direct part in the formation of active site (Thunnissen et al., 1990). However, in spite of low importance of these regions for PLA₂ neurotoxicity (Prijetelj et al., 2008), their different conformations may result in differences in enzymatic activity and presynaptic neurotoxicity of individual PLA₂s.

3.3. Triton X-100 bound in the hydrophobic channel of PLA₂

To prepare the crystals of proteins under study, two sets of crystallization conditions were employed, one of them containing non-ionic detergent Triton X-100. This detergent is widely used for preparation of mixed micelles with lipids during PLA₂ activity measurements (Yu et al., 1990; Boegeman et al., 2004). Unexpectedly, we have found Triton X-100 molecule within the hydrophobic channel of PLA₂s in the crystal structures of HDP-2P and HDP-1P.

In PLA₂ molecules the well-formed hydrophobic channel, consisting of the residues Leu2, Phe5, Met8, Ile9, Val19, Trp31, Tyr52 and Lys69, is very important for binding substrates (or inhibitors) and directing substrates to catalytic site containing residues Gly30, His48, Asp49 and Asp99. In the solved structures of HDP-2P and HDP-1P the Triton X-100 molecule was clearly located in the centre of hydrophobic channel. Based on the electron density of the detergent in the ($F_0 - F_c$) map for HDP-1P (Fig. 2A), the atomic coordinates of Triton X-100 molecule in the crystal structure were determined. Triton X-100 molecule interacted with a number of amino acid residues within the hydrophobic channel. The residues involved in detergent binding include Leu2, Phe5, Val19, Trp20, Ile23, Tyr28, Gly30, Cys44, Cys45, His48 and Lys69 (Fig. 2B). Some Triton X-100 atoms form hydrogen bonds with amino acid residues, while others participate in hydrophobic interactions. Although the hydrophobic interactions are similar to those of other small inhibitors reported earlier (Thunnissen et al., 1990), the specific interactions between the Triton X-100 and His48, Asp49 as well as Asp99 residues within the

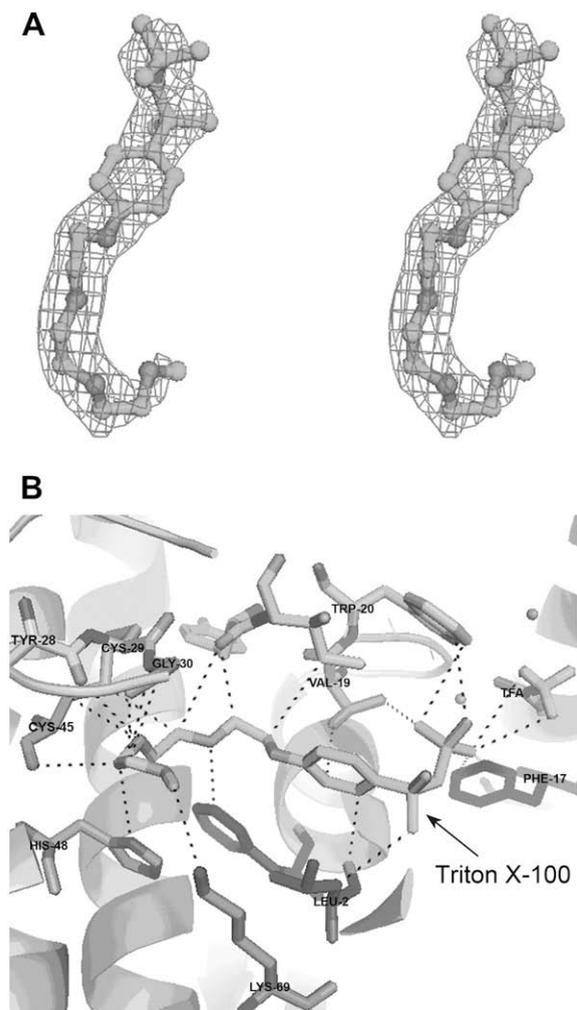


Fig. 2. The Triton X-100 bound in the hydrophobic channel of HDP-1P. (A) Stereo view of Triton X-100 electron density in the (Fo-Fc) map contoured at 3.0σ . The oxygen atoms of Triton X-100 are more dark. (B) The interactions between Triton X-100 and the amino acid residues within the hydrophobic channel. The interatomic interactions within 4.0 Å are shown as dashed lines.

catalytic site are weaker. Nevertheless, Triton X-10 molecule could perfectly fit into the hydrophobic channel of PLA₂ (Fig. 2B).

It is interesting that an MPD molecule was found in the structure of HDP-1P_{iso} within the hydrophobic channel. The residues interacting with MPD include Tyr28, Gly30, Trp31, His48, Asp49, and Lys69. When crystallizing HDP-2P with both Triton X-100 and MPD in the crystallization solution, only Triton X-100 was found within the hydrophobic channel (Fig. 2B). This means that Triton X-100 has stronger ability for binding to the active site and the hydrophobic channel of PLA₂ than MPD. The larger number of amino acid residues involved in binding of Triton X-100 as compared to MPD implies that Triton X-100 molecule binds to the enzyme much more stable than MPD molecule.

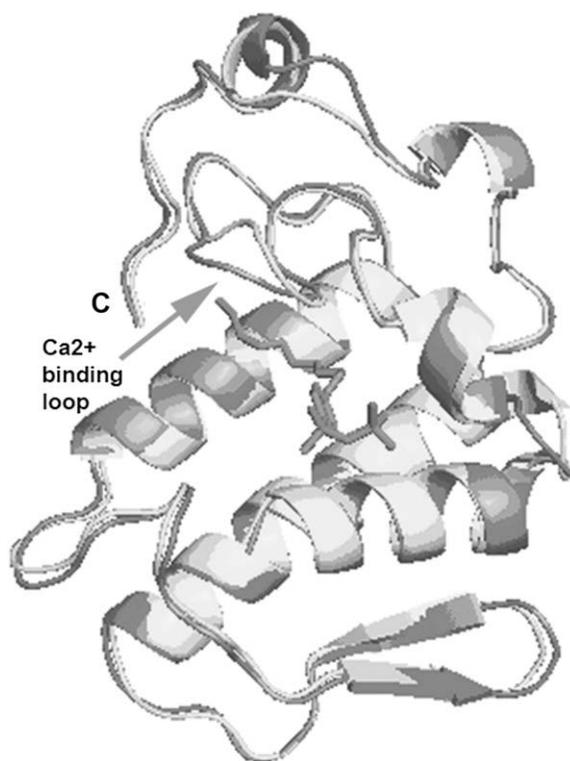


Fig. 3. The superimposed crystal structures of HDP-1P and HDP-1P_{iso} structure (more dark). Triton X-100 molecule is shown inside of HDP-1P. The arrow indicates the maximal structural difference between the two structures. C indicates the C-terminus.

The comparison of HDP-1P structures with and without Triton X-100 has indicated that binding of Triton X-100 to the hydrophobic channel did not induce any significant conformational changes except those in the flexible Ca²⁺ binding loop including residues 25–35 (as indicated by the arrow in Fig. 3) that is shifted by Triton X-100.

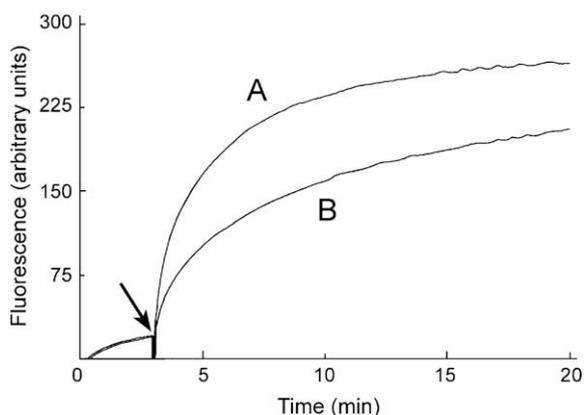


Fig. 4. The influence of Triton X-100 on the phospholipase activity of HDP-1P. Fluorescence substrate (2 μM) was incubated with 1 nM HDP-1P in the absence (A) or in the presence (B) of 150 μM Triton X-100. Direct fluorescence recording, indicating change in fluorescence intensity upon substrate hydrolysis is shown. Arrow indicates the addition of the enzyme.

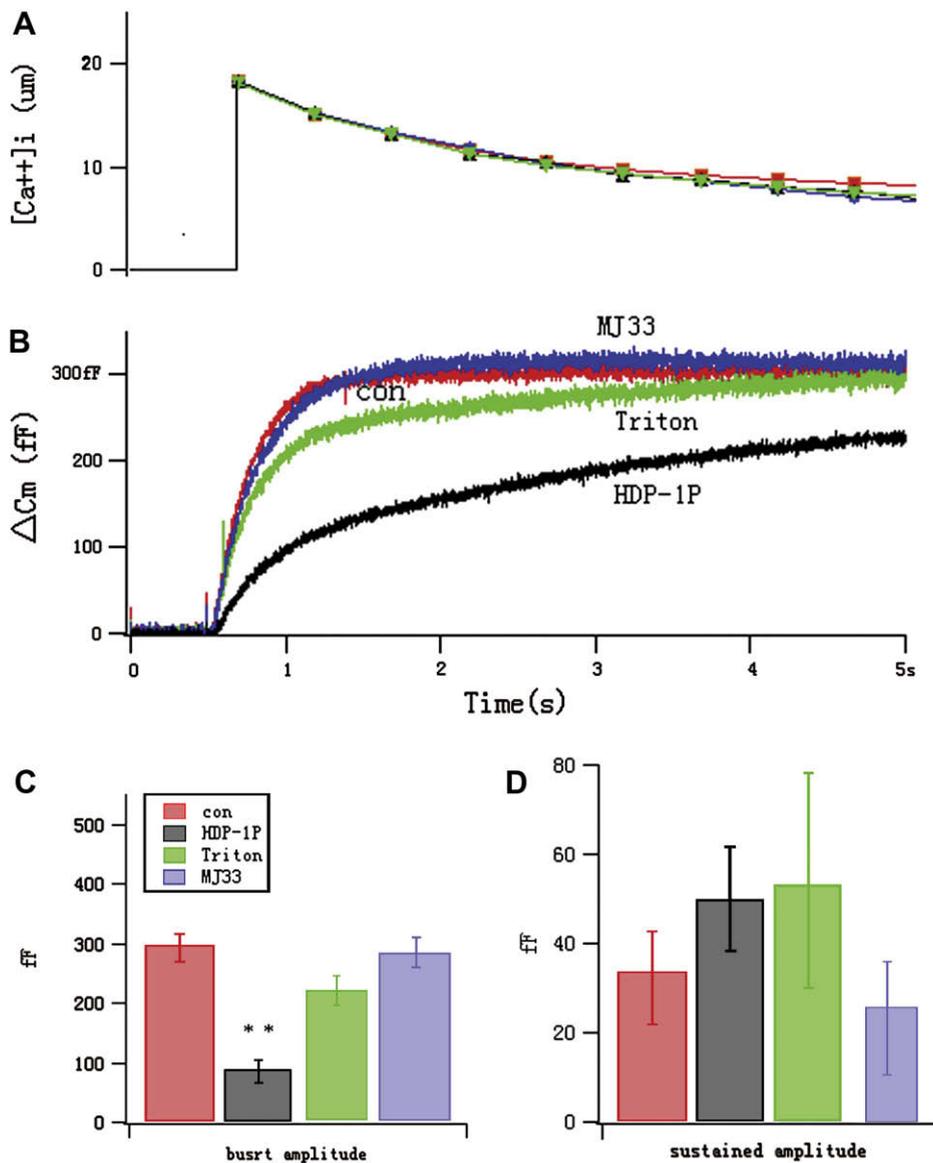


Fig. 5. Triton X-100 and MJ33 inhibition of HDP-1P influence on exocytosis in pancreatic β cells. Averaged $[Ca^{2+}]_i$ (A) and C_m responses (B) from control cells (con, $n = 20$, red trace), cells in the presence of HDP-1P (HDP-1P, $n = 11$, black trace), in the presence of both HDP-1P and MJ33 (MJ33, $n = 10$, blue trace) and in the presence of both HDP-1P and Triton X-100 (Triton, $n = 10$, green trace). Averaged amplitudes of the exocytotic burst (C) and sustained components (D) for control (red) cells, cells in the presence of HDP-1P (black), in the presence of both HDP-1P and MJ33 (blue) and in the presence of both HDP-1P and Triton X-100 (green). The C_m response was fitted by double exponentials and the amplitudes of the burst and sustained were taken as the size of the burst and sustained components, respectively. Values represent the mean \pm SEM. Asterisks denote significant differences (t -test, $**p < 0.01$) compared to control cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.4. Influence of Triton X-100 on PLA₂ activity

In the early study of group I PLA₂ from cobra venom, an apparent inhibition of phospholipase A2 activity at high concentrations (above ~ 15 mM) of Triton X-100 was described (Dennis, 1973). In later work it was shown (Roberts et al., 1977a) that Triton X-100 either in monomeric or in micellar form did not interact with the enzyme from cobra venom. On the other hand, high concentration of Triton X-100 (30 mM) protected cobra venom PLA₂ from inactivation by p -bromophenacyl bromide (Roberts et al.,

1977b). The authors suggested that this protection was due to sequestering p -bromophenacyl bromide into the apolar micellar core.

We have tested the effect of Triton X-100 on the activity of HDP-1P and found that it was influenced by the detergent (Fig. 4). For the activity determination we have used method based on change in fluorescence intensity upon release of labelled fatty acid from fluorescently labelled diacylphosphatidylcholine (Radvanyi et al., 1989). Before the addition to the substrate, the PLA₂ was incubated with Triton X-100 for 30 min. The chosen method

did not allow us to use the millimolar detergent concentrations, as they resulted in immediate increase in fluorescence irrespective of the presence of PLA₂. However, if the enzyme was incubated with 50–150 μM Triton X-100 before addition to substrate we have observed the decrease in the rate of phospholipid hydrolysis. Thus, 150 μM Triton X-100 decreased the initial rate of fluorescent substrate hydrolysis by 1.8 times (Fig. 4). The degree of this decrease strongly depended on the batch of Triton X-100 used. Such a difference in detergent activity may be explained by heterogeneity of commercial Triton X-100 preparation.

3.5. The influence of HDP-1P, Triton X-100 and MJ33 on exocytosis

Pancreatic INS-1 β cells were used to study the influence of PLA₂ on exocytosis. These cells are very convenient for exocytosis study (see Introduction) and have been earlier used for investigating the mechanism of action of different compounds (e.g., neurotoxin α-latrotoxin (Hu et al., 2006)). To avoid the complications of Ca²⁺ microdomains associated with Ca²⁺ influx and Ca²⁺ mobilization, exocytosis was elicited by flash photolysis of caged Ca²⁺. Exocytosis was monitored as an increase in the whole-cell membrane capacitance (Cm). Cm increase in β cells has been mostly ascribed to the contribution of exocytosis from large dense-core vesicles (LDCVs) (Braun et al., 2004). As shown in Fig. 5, the Cm increase in response to step-like [Ca²⁺]_i elevation (Fig. 5A) starts with an exocytotic burst component (within 500 ms after flash) followed by a linear sustained release (Fig. 5B, control).

Fig. 5B shows Cm responses (burst release 300 ± 40fF) from control cells (con, n = 20, red trace), cells in the presence of 1.45 μM HDP-1P (HDP-1P, n = 11, black trace), in the presence of 1.45 μM HDP-1P and 1 μM 1-hexadecyl-3-trifluoroethylglycero-sn-2-phosphomethanol (MJ33, n = 10, blue trace) and in the presence of 1.45 μM HDP-1P and 1 μM Triton X-100 (Triton, n = 10, green trace). We found that in pancreatic β cells HDP-1P resulted in a significant blockade of the burst (Fig. 5C) but not the sustained exocytosis (Fig. 5D). The observed effect closely resembles the first phase of neurotoxic PLA₂ action on neuromuscular preparation that is an initial depression of evoked transmitter release (e.g., Caccin et al., 2006). This result means that HDP-1P may influence exocytosis in neurons as well, which is in accord with earlier published electrophysiological data about presynaptic neurotoxicity of this enzyme (Ramazanov et al., 2008). It is interesting to note that cobra venom PLA₂, which is not neurotoxic, does not influence exocytosis in β cells from mouse pancreas (Juhl et al., 2003). Taken together these data indicate that pancreatic β cells can be used as an appropriate model for neurotoxicity measurements. The blockade of exocytotic burst by PLA₂ was abolished when HDP-1P (1.45 μM) was added to cells together with MJ33 (1 μM) or Triton X-100 (1 μM) (Fig. 5B, C). These data shows that PLA₂ influence on exocytosis is suppressed by both MJ33 and Triton X-100 suggesting that HDP-1P neurotoxicity may be suppressed by both these compounds as well.

4. Conclusion

The structures of two active basic subunits HDP-1P and HDP-2P of heterodimeric PLA₂s from *V. nikolskii* venom were determined in different crystal forms. Structural comparisons with structures of similar PLA₂s clearly show some flexible regions which might be important for the catalytic function and neurotoxicity. In two forms the non-ionic detergent Triton X-100 was found in the hydrophobic channel of PLA₂ molecule. Bound Triton X-100 induced conformational changes in the flexible Ca²⁺ binding loop that may be crucial for PLA₂ catalytic function. Enzymatic activity determination showed that Triton X-100 decreased the phospholipase A₂ activity of HDP-1P.

For the first time we have demonstrated that in pancreatic β cells the PLA₂ influence on exocytosis can be related to the neurotoxicity of PLA₂ tested. Thus, HDP-1P, a PLA₂ with presynaptic neurotoxicity, inhibited exocytosis, and this inhibition was abrogated by MJ33 and to the less extent by Triton X-100. Taken together our data show that Triton X-100 can inhibit phospholipase activity and neurotoxicity of active subunits of heterodimeric PLA₂. Although the interaction of Triton X-100 with PLA₂ is not strong, the finding reported here might be helpful for designing potent PLA₂ inhibitors of novel structural type. Furthermore, this interaction should be taken into account when this detergent is used for PLA₂ studies. Our studies performed at cellular level are in good agreement with earlier findings (Priatelj et al., 2006; Rouault et al., 2006) that enzymatic activity of the snake presynaptic PLA₂ neurotoxins is essential for effective block of nerve terminals.

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Conflict of interest

The authors declare that there is no conflict of interest.

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