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RESEARCH ARTICLE

Pancreatic and snake venom presynaptically active phospholipases A₂ inhibit nicotinic acetylcholine receptors

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Abstract

Phospholipases A₂ (PLA₂s) are enzymes found throughout the animal kingdom. They hydrolyze phospholipids in the *sn*-2 position producing lysophospholipids and unsaturated fatty acids, agents that can damage membranes. PLA₂s from snake venoms have numerous toxic effects, not all of which can be explained by phospholipid hydrolysis, and each enzyme has a specific effect. We have earlier demonstrated the capability of several snake venom PLA₂s with different enzymatic, cytotoxic, anticoagulant and antiproliferative properties, to decrease acetylcholine-induced currents in *Lymnaea stagnalis* neurons, and to compete with α-bungarotoxin for binding to nicotinic acetylcholine receptors (nAChRs) and acetylcholine binding protein. Since nAChRs are implicated in postsynaptic and presynaptic activities, in this work we probe those PLA₂s known to have strong presynaptic effects, namely β-bungarotoxin from *Bungarus multicinctus* and crotoxin from *Crotalus durissus terrificus*. We also wished to explore whether mammalian PLA₂s interact with nAChRs, and have examined non-toxic PLA₂ from porcine pancreas. It was found that porcine pancreatic PLA₂ and presynaptic β-bungarotoxin blocked currents mediated by nAChRs in *Lymnaea* neurons with IC₅₀s of 2.5 and 4.8 μM, respectively. Crotoxin competed with radioactive α-bungarotoxin for binding to *Torpedo* and human α7 nAChRs and to the acetylcholine binding protein. Pancreatic PLA₂ interacted similarly with these targets; moreover, it inhibited radioactive α-bungarotoxin binding to the water-soluble extracellular domain of human α9 nAChR, and blocked acetylcholine induced currents in human α9α10 nAChRs heterologously expressed in *Xenopus* oocytes. These and our earlier results show that all snake PLA₂s, including presynaptically active crotoxin and β-bungarotoxin, as well as mammalian pancreatic PLA₂, interact with nAChRs. The data obtained suggest that this interaction may be a general property of all PLA₂s, which should be proved by further experiments.

Introduction

Phospholipases A₂ (PLA₂s, phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) hydrolyze predominantly phospholipids with polyunsaturated fatty acid residue in the *sn*-2 position; they are therefore essential participants in lipid digestion. In addition, they are involved in a range of other cell processes including inflammation, cell proliferation and signal transduction, largely because of their phospholipolytic activity [1]. The PLA₂ superfamily includes 15 groups comprising four main types including the secreted, cytosolic, calcium-independent PLA₂s, and platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein-associated PLA₂ [2]. Group I PLA₂s are present in Elapidae snake venoms as group IA and in pancreatic juices of animals as group IB. The most obvious difference between them is the absence (group IA) or the presence (group IB) of an extra amino acid fragment, known as a pancreatic loop, next to the catalytic active site. Group II PLA₂s are present in Viperidae snake venoms and in the synovial fluids of animals. These PLA₂s, which are secreted by venomous glands of snakes, bees and other venomous animals, manifest various toxic actions. PLA₂s from snake venoms have numerous toxic effects, not all of which can be explained by phospholipid hydrolysis, and each individual enzyme may have a specific effect. Some PLA₂s are characterized by potent anticoagulant activity, for example PA11 from *Pseudechis australis* venom [3]; others manifest strong myotoxic properties, such as Lemnitoxin from *Micrurus lemniscatus* venom [4]. Among numerous PLA₂ effects, neurotoxic action is one of the most important. Neurotoxicity is due to the block of neuromuscular transmission and proceeds in several steps: an initial weak inhibition of acetylcholine (ACh) release; a more prolonged facilitation of ACh secretion; and then a progressive decline of transmission leading to irreversible arrest [5–7].

There are several hypotheses of the mechanism of PLA₂ neurotoxic action.

1. Phospholipolytic damage to the presynaptic membrane potentiates fusion of ready-to-release synaptic vesicles in the active zone of neuroexocytosis, and inhibits vesicle retrieval [6]; consequently the ACh store is depleted.
2. Interaction of PLA₂s with specific proteins: binding to these receptors facilitates a local enzyme-dependent or independent action [8]; the discovery of proteins that bind PLA₂ with high affinity in different tissues supports this hypothesis [9–12].
3. Interaction of PLA₂s with intracellular Ca²⁺ binding proteins after endocytosis or penetration through damaged membranes causing an increase in intracellular Ca²⁺ concentration, both leading to mitochondrial uncoupling [7, 13].

We have earlier reported antagonistic action of eight PLA₂s from the venoms of snakes of Viperidae and Elapidae families (PLA₂ groups IIA and IA, respectively) on nicotinic acetylcholine receptors (nAChRs) of different types [14, 15]. These enzymes, which differ in their enzymatic activities, competed with [¹²⁵I]α-bungarotoxin (α-Bgt) for binding to the muscle-type nAChRs of *Torpedo californica* electric organ, to human α7 nAChRs expressed in GH₄C₁ cell line, and to ACh-binding protein (AChBP) from *Lymnaea stagnalis*. When tested on isolated neurons of *L. stagnalis* which contain α7 similar nAChRs [16, 17], PLA₂s suppressed ACh- or cytosine-evoked currents under conditions that exclude hydrolysis of membrane phospholipids. These results indicate that binding of PLA₂s to nAChRs affects their function.

To ascertain whether all types of PLA₂s are able to interact with nAChRs, we have studied the action of three other phospholipases i.e. presynaptically active β-bungarotoxin (β-Bgt) from *Bungarus multicinctus*, crototoxin (Cro) from *Crotalus durissus terrificus* snake venom, and non-toxic mammalian PLA₂ from porcine pancreas (PP PLA₂, group IIB)—in binding assay and on *Lymnaea* neurons. β-Bgt is a heterodimeric protein in which a group IA PLA₂ and a

Kunitz type serine protease inhibitor are connected by a disulfide bond [18]. Cro is also a heterodimeric protein, and consists of a weakly toxic basic group IIA PLA₂ and crotapotin, a non-enzymatic, non-toxic acidic component [19]. Mammalian porcine pancreatic PP PLA₂ has been shown previously to induce presynaptic block of neuromuscular transmission in a mouse hemi-diaphragm preparation although it was much weaker than snake venom PLA₂s [20]. β-Bgt and Cro have been previously shown to act presynaptically in a mammalian neuromuscular junction preparation [5, 21, 22].

We found that all PLA₂s tested in this work interacted with nAChRs, with IC₅₀ values ranging from hundreds of nM to tens of μM. The data from Cro revealed the presence of two sites both in muscle-type and α7 nAChRs with affinities differing by 1–3 orders of magnitude. Thus, we conclude that presynaptically active PLA₂s interact with muscle type nAChRs located postsynaptically. Moreover, it is not only snake venom PLA₂s that are capable of binding to nAChRs, but mammalian pancreatic PLA₂ also has this ability.

Materials and methods

PLA₂ from porcine pancreas (PP PLA₂), Trizma-HCl, EGTA, HEPES, β-lactoglobulin, Pronase E, acetylcholine iodide, cytosine, choline chloride, and all chloride salts were purchased from Sigma (USA). RNase was from P-L Biochemicals, Inc. (USA), soybean trypsin inhibitor from Boehringer Mannheim GmbH (Germany), cytochrome C from Ferak Berlin. Crotoxin from *Crotalus durissus terrificus* venom was purified as previously described [23, 24]. β-Bgt was isolated from *Bungarus multicinctus* venom by procedure described in [25]. Mono-iodinated (3-[¹²⁵I]iodotyrosyl⁵⁴)-α-Bgt (~2000 Ci/mmol) was from GE Healthcare. nAChR-enriched membranes from the electric organs of *T. californica* ray were kindly provided by Prof. F. Hucho (Free University of Berlin, Germany), GH₄C₁ cells transfected with human α7 nAChR were a gift from Eli-Lilly (USA). The expressed acetylcholine binding protein (AChBP) from *L. stagnalis* was kindly provided by Prof. T. Sixma (Netherlands Cancer Institute, Amsterdam, the Netherlands); the extracellular domain (ECD) of the human neuronal α9 nAChR was expressed, enzymatically deglycosylated and purified as described [26]. Plasmid pT7TS constructs of human nAChR α9 and α10 subunits were kindly provided by Prof. D.J. Adams (University of Wollongong, Wollongong, Australia).

Electrophysiological measurements

Identified *L. stagnalis* giant neurons. Pond snails *L. stagnalis* (3–4 cm long) were collected from lakes near the Oka River (Pushchino, Moscow region) and kept in tap water at 4–6°C until use. *L. stagnalis* has the conservation status “Least Concerned” and does not require a special permission for use. The experiments were carried out on identified giant neurons (LP1,2,3, RPV2,3; according to the map of *L. stagnalis* ganglia [27]) isolated from the left and right parietal ganglia as described [17]. Neurons were internally perfused with internal solution (in mM: CsCl 95, CaCl₂ 0.3, EGTA 2, HEPES 10, pH 7.2) and voltage-clamped at –60 mV [28]. Constant flow of the external solution (in mM: NaCl 92, KCl 1.6, BaCl₂ 2, MgCl₂ 1.5, Trizma-HCl 4, pH 7.6; Ba²⁺ was used instead of Ca²⁺ to avoid phospholipolytic action of the PLA₂s on the cell membrane) was maintained, except the time of application of an agonist or neuron incubation with PLA₂s. In the experiments with proteins lacking phospholipolytic activity, the CaCl₂-containing extracellular solution was used. Acetylcholine (ACh), cytosine (Cyt) or choline were applied on the whole cell surface using 4 s pulses with intervals not less than 6 min. Agonist-induced currents were monitored and digitized with a patch-clamp amplifier A-M Systems (USA), the data acquisition was performed using Digidata1200 B interface and pClamp6 software (Axon Instruments Inc., USA). Aliquots of solutions of PLA₂s in

water were kept in the refrigerator and diluted using extracellular solution to the desired concentration immediately before use.

The effects of PLA₂s were determined by measuring the changes in peak current amplitude induced by the agonist after 5-min incubation with PLA₂ compared to the control responses before treatment and after prolonged washing. IC₅₀ values were calculated using Sigma plot 11.0 software using the Hill plot analysis.

Xenopus oocytes. Plasmid pT7TS constructs of human nAChR α 9 and α 10 subunits were linearized with *Xba*I restriction enzymes (NEB, USA). Linearized plasmid constructs were subjected to *in vitro* cRNA transcription using T7 mMessageMachine[®] transcription kit (AMBION, USA).

Mature *Xenopus laevis* female frogs used in this study were obtained commercially (NASCO, Fort Atkinson, WI, USA) and housed in a facility with 12:12 hours light:dark cycles, 18–20°C ambient temperature. Animals were fed twice a week and maintained according to supplier recommendations (https://www.enasco.com/page/xen_care). All the appropriate actions were taken to minimize discomfort to frogs. The World Health Organization's International Guiding Principles for Biomedical Research Involving Animals were followed during experiments on animals. Oocytes were prepared from mature female frogs by following the standard procedure described elsewhere [29]. Stage V-VI oocytes were defolliculated with 2 mg/mL collagenase Type I (Life Technologies, USA) at room temperature (21–24°C) for 2 h in ND96 solution composed of (in mM) 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂ and 5 HEPES at pH 7.4. Oocytes were injected with 9.2 ng of human nAChR α 9 and α 10 cRNA (in a ratio 1:1) and incubated at 18°C in Barth's solution composed of (in mM) 88 NaCl, 1.1 KCl, 2.4 NaHCO₃, 0.3 Ca(NO₃)₂, 0.4 CaCl₂, 0.8 MgSO₄ and 15 HEPES-NaOH at pH 7.6, supplemented with 40 μ g/mL gentamicin and 100 μ g/mL ampicillin for 4 days before electrophysiological recordings.

Two-electrode voltage clamp recordings at a holding potential of -60 mV were made using turbo TEC-03X amplifier (Npi electronic, Germany) and WinWCP recording software (University of Strathclyde, UK). Oocytes were briefly washed with Ba²⁺ Ringer's solution (in mM: 115 NaCl, 2.5 KCl, 1.8 BaCl₂, 10 HEPES at pH 7.2) followed by 3 applications of 25 μ M ACh. Washout with Ba²⁺ Ringer's solution was done for 5 min between ACh applications. Oocytes were incubated with PP PLA₂ for 5 min followed by its co-application with ACh. Peak current amplitudes of ACh-induced responses were measured before and after preincubation of oocytes with PP PLA₂. The ratio between these two measurements was used to assess the activity of PLA₂ on human α 9 α 10 nAChR.

Receptor binding studies

Radioligand analysis. For competition binding assays, suspensions of nAChR-rich membranes from *T. californica* ray electric organ (1.25 nM α -Bgt binding sites) in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mg/ml bovine serum albumin (BSA) (binding buffer), human α 7 nAChR transfected GH₄C₁ cells (0.4 nM α -Bgt binding sites) in binding buffer, or a solution of heterologously expressed AChBP from *L. stagnalis* (2.4 nM in binding buffer) were incubated for 3 h with various amounts of the PLA₂s, followed by an additional 5 min incubation with 0.4 nM [¹²⁵I] α -Bgt. Nonspecific binding was determined by preliminary incubation of the preparations with 20 μ M α -cobratoxin. The membrane and cell suspensions were applied to glass GF/C filters (Whatman, Little Chalfont, UK) presoaked in 0.25% polyethylenimine, and the unbound radioactivity was removed from the filter by washing (3 \times 3 ml) with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1 mg/ml BSA (washing buffer). The AChBP solutions were applied to two layers of DE-81 filters presoaked in PBS-T buffer, and washed (3 \times 3 ml)

with washing buffer. The bound radioactivity was determined using a Wizard 1470 Automatic Gamma Counter (Perkin Elmer). The binding results were analyzed using ORIGIN 7.5 (OriginLab Corporation, Northampton, MA, USA) fitting to a one-site or two-site dose-response competition curve.

For competition binding assays on the extracellular domain (ECD) of human $\alpha 9$ nAChR, PP PLA₂ in the concentration range 0.3–30 μ M was incubated for 2 h at room temperature with the ECD (final concentrations of 30 μ g/ml) in 50 μ L of a 20 mM Tris–HCl buffer, pH 8.0, containing 1 mg/ml of the bovine serum albumin (binding buffer). Then [¹²⁵I] α -Bgt was added to the reaction mixtures to a final concentration of 0.2 nM. Simultaneously 15 μ L Ni-NTA-agarose (QIAGEN) pre-washed in reaction buffer was added. After 6 min, the reaction was stopped by a rapid filtration on GF/C filters (Whatman) pre-soaked in 0.25% polyethyleneimine and the unbound radioactivity was removed from the filters by washes (3 \times 4 ml) with the 20 mM Tris–HCl buffer. Nonspecific binding was determined by preliminary incubation of the ECD with 10 μ M α -cobratoxin. The bound radioactivity was determined using Wizard 1470 Automatic Gamma Counter (Perkin Elmer). The data were analyzed using ORIGIN 7.5 as a one-site dose-response curve.

Surface plasmon resonance (SPR) experiments. SPR experiments were performed at 20°C, using a Biacore[®] 2000 system (GE Healthcare, Biacore AB). AChBP was covalently immobilized to a CM5 sensor chip at acidic pH. For binding experiments, the running and dilution buffer was composed of 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.005% Surfactant P20 (GE Healthcare, Biacore AB). The concentrations of Cro ranged from 5.5 to 46 μ g/ml, and solutions were injected at a flow rate of 30 μ l/min. Background signals were obtained by injection of samples to a blank-immobilized flow cell and these signals were subtracted from the sample signals. At the end of each run, a 10 s injection of 10 mM Gly/HCl pH 1.5 was performed to restore the complete binding capacity of the AChBP coupled to the CM5 sensor chip. The kinetic constants, k_a (association rate constant), and k_d (dissociation rate constant), for the interaction between Cro and AChBP were calculated using Biacore BIAEVALUATION 3.1 software (Biacore AB). The curves were fitted according to the simple two-component model of interaction. The apparent dissociation constant (K_D^{app}) was obtained as the ratio of k_d and k_a ($K_D^{app} = k_d/k_a$).

Results

Electrophysiological experiments

Suppression of acetylcholine- or cytosine-induced currents in *L. stagnalis* neurons.

Under the experimental conditions used, ACh and cytosine elicited inward currents in identified *L. stagnalis* neurons LP1,2,3 and RPV2,3 due to an increase in chloride permeability [30]. Previously this conductance was shown to be mediated by two subtypes of nAChRs with low and high affinity for α -conotoxin ImI (ImI) and reversed relative affinities for ACh [15, 17]. A further distinction between two subtypes is in the kinetics of receptor desensitization in response to ACh. The nAChRs with a higher sensitivity to ImI and faster desensitization, in spite of possessing chloride ion conductance, are more similar to vertebrate $\alpha 7$ nAChRs; furthermore cytosine is a full agonist at this subtype whereas it is a weak partial agonist at other nAChR subtype. In most experiments, we used cytosine or choline instead of ACh because of their more selective actions on $\alpha 7$ nAChRs. To exclude a possible contribution of phospholipolytic activity, Ca²⁺ was replaced with Ba²⁺ in the extracellular solution.

It was found that 5 min treatment of a neuron with PP PLA₂ or β -Bgt resulted in a decrease of ACh- or cytosine-induced currents (Fig 1A). Peak response suppression was dependent on PLA₂ concentration (Fig 1B) and reversed slowly after PLA₂ wash out. IC₅₀ values for PP PLA₂

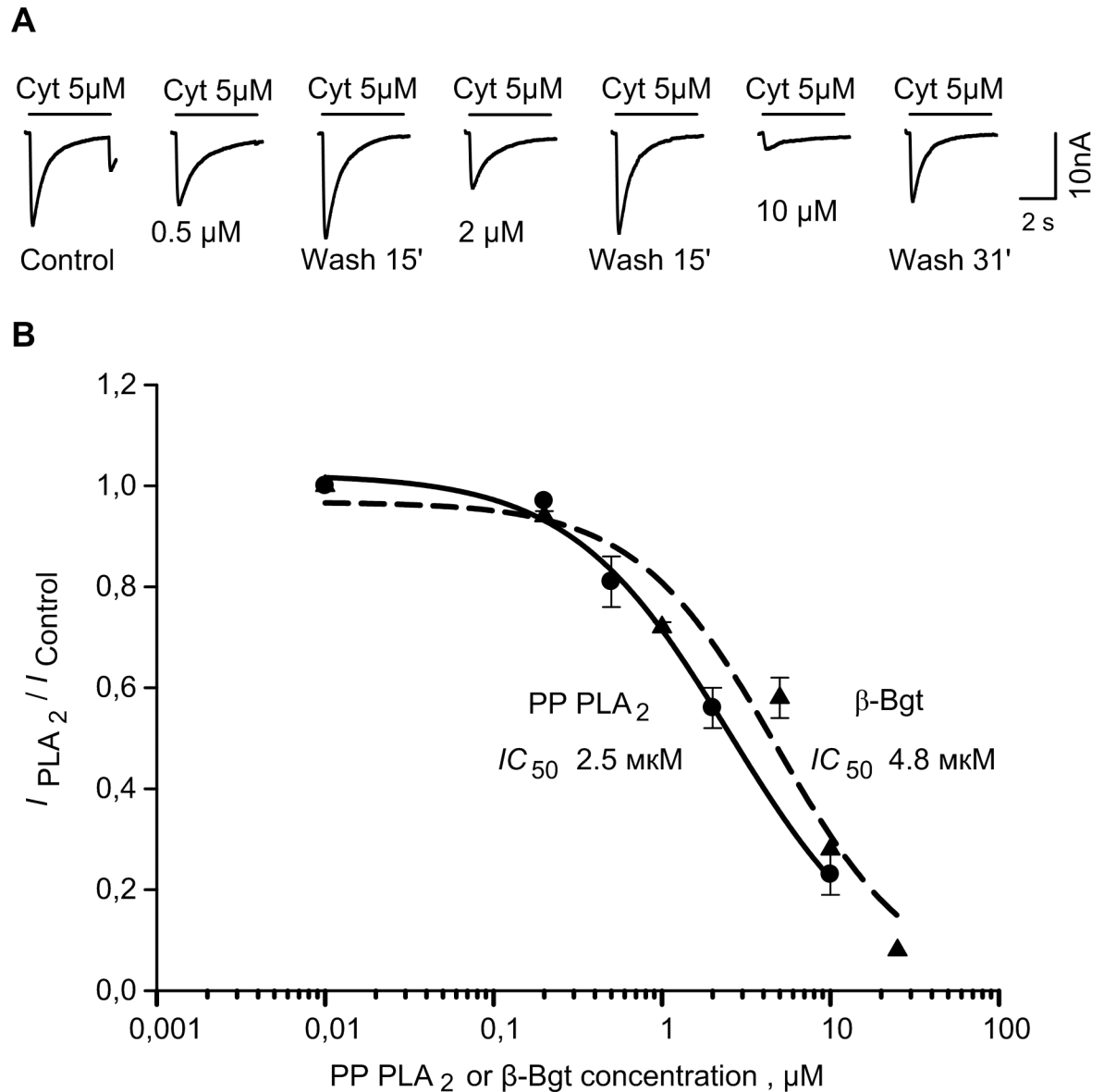


Fig 1. Inhibition of acetylcholine or cytosine-elicited current in *L. stagnalis* neurons by PP PLA₂ and β -Bgt. (A) Representative recordings from a neuron in control, after a 5-min incubation with PP PLA₂ at three different concentrations, and after PP PLA₂ wash out. (B) Dependence of ACh- or cytosine-evoked current suppression on PP PLA₂ (n = 7) and β -Bgt (n = 4) concentration.

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and β -Bgt inhibition of cytosine-induced current were 2.5 ± 0.4 (n = 7) and 4.8 ± 1.6 (n = 4) μ M, respectively.

For PP PLA₂ the type of antagonism was determined. For this purpose, a set of cytosine or ACh concentrations including saturating ones were applied to a neuron before and after treatment with PP PLA₂ at 3 μ M (a concentration slightly higher than its IC_{50}). As can be seen in Fig 2A and 2B, the curves of the current dependence on agonist concentration shifted rightward after incubation of the neurons with PP PLA₂ solution. EC_{50} values for cytosine were 2.9 in control and 3.3 μ M after PP PLA₂ treatment (n = 4) and for ACh—4.0 and 3.2 μ M (n = 1), respectively. However, the maximal responses to both cytosine and ACh were reduced by 40–

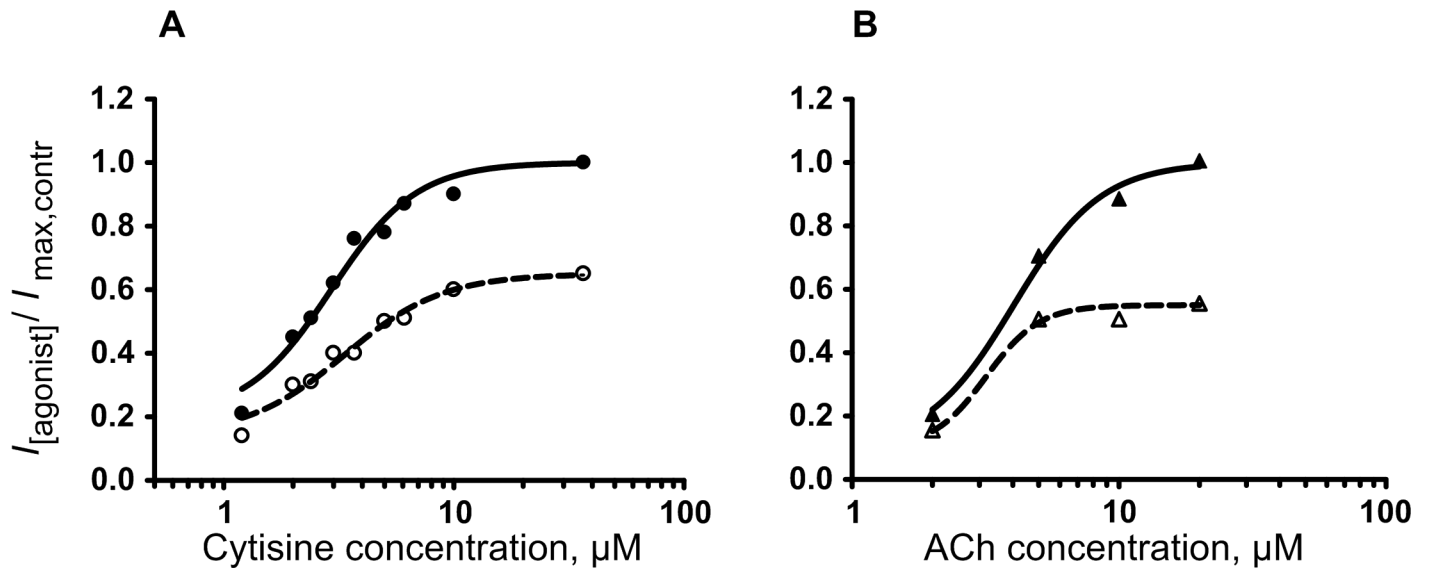


Fig 2. Determination of antagonism type for PP PLA₂. Dependence of cytosine (A) or acetylcholine (B) induced currents on agonist concentration in control (closed circles and triangles) and after 5 min treatment with PP PLA₂ at 3 μM (open symbols), (n = 5 and 1, respectively).

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50% (Fig 2). These data indicate non-competitive antagonism and support our previous results obtained with enzymatically inactive Vur-S49 from *Vipera ursinii renardi* venom [15].

We also explored the ability of the proteins unrelated to PLA₂ to interact with nAChRs. Ribonuclease A (RNase), β-lactoglobulin and soybean trypsin inhibitor were tested on *Lymnaea* neurons. We found that RNase decreased the peak of the cytosine-induced current but the effect (IC₅₀ > 50 μM, n = 3) was more than an order of magnitude weaker than that for PP PLA₂ or β-Bgt. A decrease in the response to choline caused by β-lactoglobulin or soybean trypsin inhibitor at concentrations of 10 and 50 μM was not more than 8 and 12%, respectively (n = 7 and 7), and did not depend on the concentration of these proteins (data not shown).

Suppression of acetylcholine-induced current mediated by human α9α10 nAChR heterologously expressed in *Xenopus* oocytes. The activity of PP PLA₂ was tested in electrophysiological experiments on human α9α10 nAChR heterologously expressed in *Xenopus* oocytes. It was found that a 5 min treatment of oocytes with PP PLA₂ resulted in a decrease of the ACh-induced current (Fig 3A). Peak response suppression was dependent on PP PLA₂ concentration (Fig 3A) and the IC₅₀ value was 0.19 ± 0.03 μM (n = 3).

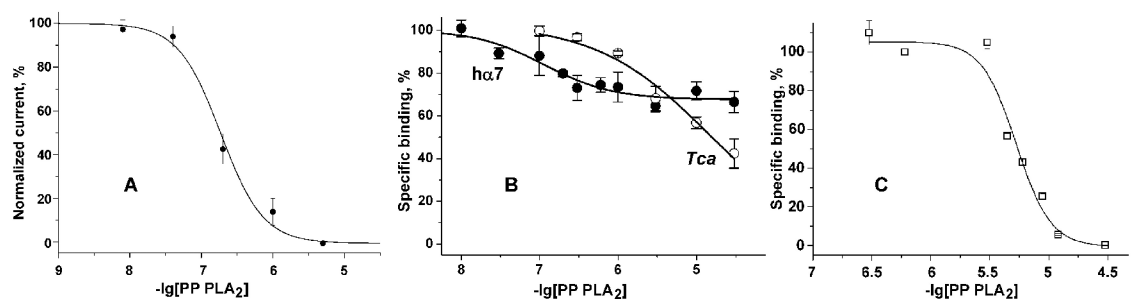


Fig 3. Inhibition experiments with PP PLA₂. (A) Dose-response curve of PP PLA₂ inhibitory action on the ACh-evoked (25 μM ACh) ionic currents mediated by human α9α10 nAChR heterologously expressed in *Xenopus* oocytes. (B) Inhibition by PP PLA₂ of the initial rate of specific [¹²⁵I]-α-Bgt binding to *T. californica* and human ha7 nAChRs expressed in GH₄C₁ cells. Only 30% of binding sites in ha7 nAChRs could be protected from [¹²⁵I]-α-Bgt binding. (C) Inhibition by PP PLA₂ of specific [¹²⁵I]-α-Bgt binding to ECD of human α9 nAChR. IC₅₀ 5.5 μM.

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Competition of PLA₂s with [¹²⁵I]α-bungarotoxin in binding assay

The capability of PLA₂s to interact with nAChRs was studied using muscle-type nAChRs of *T. californica* electric organ and human neuronal α7 nAChRs (hα7 nAChRs) heterologously expressed in cells of GH₄C₁ line. ACh-binding protein (AChBP) from *L. stagnalis*, a structural analog of the extracellular ligand-binding domain of all nAChR subtypes, and the extracellular domain (ECD) of human α9 nAChR were also used for this purpose. The affinities of PLA₂s for nAChRs, AChBP and ECD were evaluated using a radioligand competition binding assay with [¹²⁵I]-labeled α-Bgt. The data obtained showed that all these PLA₂s inhibited the initial rate of [¹²⁵I]-labeled α-Bgt binding, although their potencies differed.

At *Torpedo* nAChR, PP PLA₂ inhibited [¹²⁵I]-α-Bgt binding with low efficiency (IC₅₀ about 15 μM) with tend of complete inhibition at greater than 100 μM (Fig 3B). However, the inhibition of α-Bgt binding to hα7 nAChRs by PP PLA₂ reached a plateau at approximately 70% of binding sites, the affinity to 30% of binding sites being fairly high (IC₅₀ = 120 nM) (Fig 3B). Comparison of these results with the data on suppression of the cytosine-evoked current in *L. stagnalis* neurons indicates that both the affinities of PP PLA₂ for hα7 and α7-similar nAChRs in *Lymnaea* neurons and the degree of inhibition of these receptors differed greatly. The interaction of PP PLA₂ with ECD of human α9 nAChR showed one binding site with IC₅₀ of 5.5 μM (Fig 3C).

Cro completely inhibited [¹²⁵I]-α-Bgt binding to all three targets (Fig 4). The experimental points for *Torpedo* and hα7 nAChRs were best approximated by a two-site model, with the difference in affinities between the two sites being about an order of magnitude in *Torpedo* nAChRs (30 and 260 nM) and more than 3 orders of magnitude in hα7 nAChRs (4.9 nM and 15 μM). For both the high affinity binding sites accounted for 20–30% of all binding sites. The interaction of Cro with AChBP showed one binding site with an IC₅₀ of 640 nM (Fig 4C).

To observe the direct binding of Cro to AChBP, SPR measurements were performed (Fig 5). SPR recordings demonstrated that Cro interacted with immobilized AChBP and formed a stable complex. Quantitative analysis allowed the determination of the apparent dissociation constant (K_D^{app}) for this interaction which was 120 nM.

Several other proteins were checked for inhibition of α-Bgt binding. The proteins chosen have molecular masses close either to monomer PLA₂ (RNase, cytochrome C) or to heterodimer (soybean trypsin inhibitor). Soybean trypsin inhibitor and β-lactoglobulin were inactive at concentration up to 60 μM. These data coincide with the results on *Lymnaea* neurons. RNase inhibited α-Bgt binding to *Torpedo* nAChRs fairly well (IC₅₀ 5 μM) and cytochrome C was slightly more potent (IC₅₀ 1.12 μM) (Fig 6). However, these proteins even at high concentrations had only marginal effects on function of nAChRs in *Lymnaea* neurons.

Discussion

Snake venom PLA₂s are multi-functional proteins evolved to affect multiple biological targets in prey organisms. They possess different toxic activities including presynaptic neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, and haemolytic activity. Usually each individual enzyme manifests its own specific effect, although other weaker activities can also be observed. For example, the presynaptically acting neurotoxin Cro has analgesic actions, and also immunomodulatory and anti-inflammatory effects [19], with no direct correlation between these activities and the catalytic activity of Cro. The PLA₂ effects could be manifested through direct binding to membrane-bound receptors, and several binding proteins and glycoproteins, the so-called M- and N-receptors, which are tissue specific and bind certain PLA₂s, have been discovered [9–12]. These receptors were shown to have high affinity binding sites for PLA₂s with IC₅₀ ranging from several pM to about 100 nM, i.e. significantly higher than the affinity of

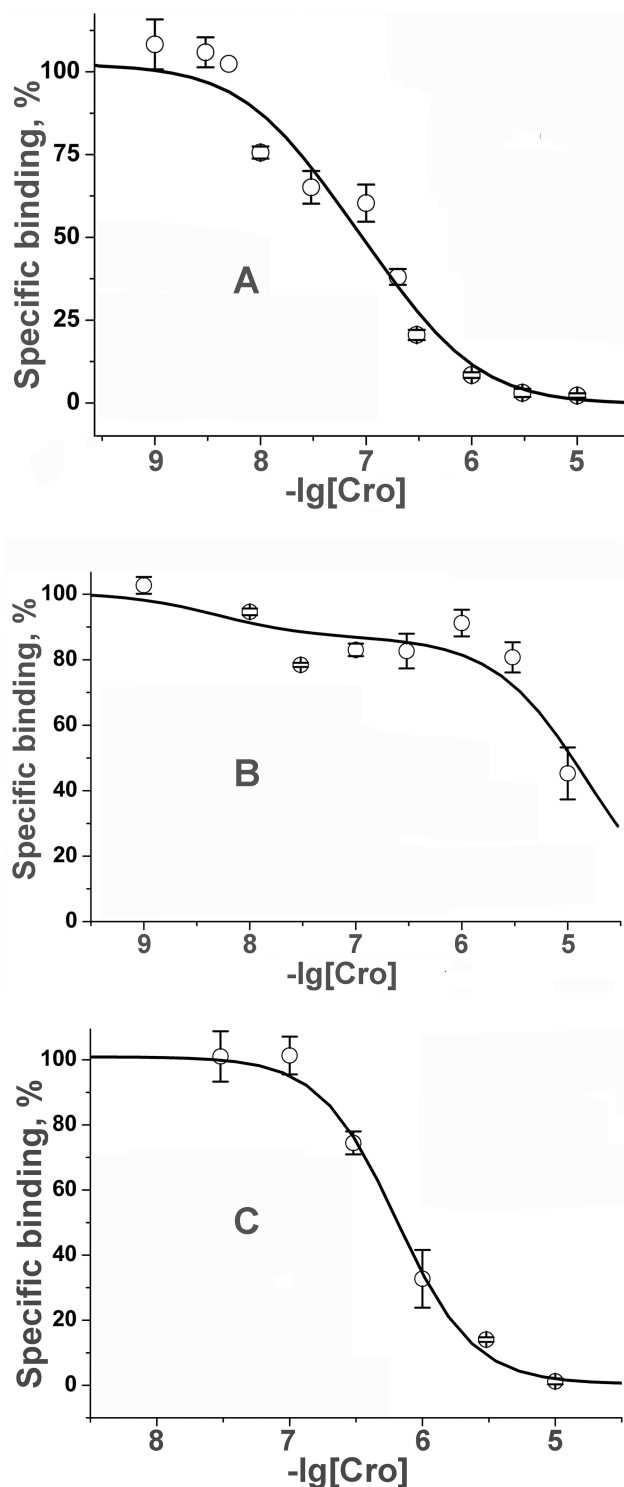


Fig 4. Interaction of Cro with nAChR of *T. californica* electric organ, human neuronal $\alpha 7$ nAChR and AChBP. (A) Inhibition of the initial rate of specific [¹²⁵I]- α -Bgt binding to *T. californica* nAChRs by Cro. Points were fit to a 2-site model with affinity for Cro of 30 nM and 260 nM. (B) Inhibition of the initial rate of specific [¹²⁵I]- α -Bgt binding to human $\alpha 7$ nAChRs by Cro. Two binding sites with affinity for Cro differing more than 3 orders of magnitude were revealed. (C) Inhibition of the initial rate of specific [¹²⁵I]- α -Bgt binding to acetylcholine-binding protein from *L. staganlis* by Cro.

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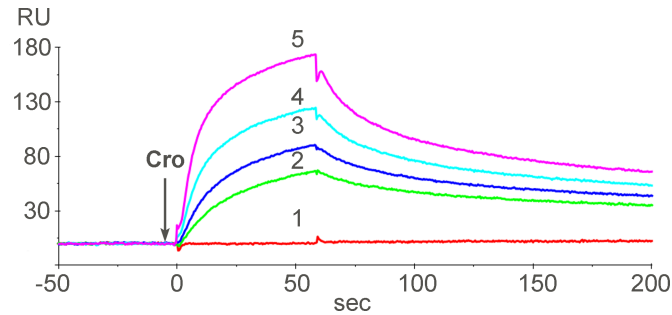


Fig 5. SPR recordings of Cro interaction with AChBP from *L. stagnalis*. An arrow indicates injection of the analyte. Line 1 corresponds to injection of buffer solution. Curve 2–5 correspond to injections of solutions with Cro at concentrations of 5.5, 11.5, 23 and 46 $\mu\text{g/ml}$, respectively.

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PLA₂s for phospholipids [8]). One receptor protein isolated from porcine cerebral cortex bound not only neurotoxic PLA₂s from snake venoms but also non-toxic PP PLA₂ with similar affinities [12]. High affinity binding of mouse non-toxic IIA and IB PLA₂s to M-type receptors in mouse colon has also been reported [31]. These data indicate that PLA₂s possess the capacity to interact with receptor proteins.

In our previous paper, we reported on the ability of PLA₂s from two families of snakes to antagonize an ACh-elicited current in *L. stagnalis* neurons containing $\alpha 7$ -like receptors, and to compete with α -Bgt binding to recombinant human $\alpha 7$ nAChRs, *T. californica* nAChRs and AChBP from *L. stagnalis* [15]. Using Ca²⁺-free solution and experiments with a natural non-enzymatic analog of PLA₂ from *V. ursinii renardi* venom (Vur-S49) allowed us to explore the type of interaction with nAChRs of different types. Although PLA₂s completely inhibited α -Bgt binding to nAChRs and AChBP, characteristic changes of current-agonist concentration curves after neuron treatment with Vur-S49 indicated a non-competitive interaction.

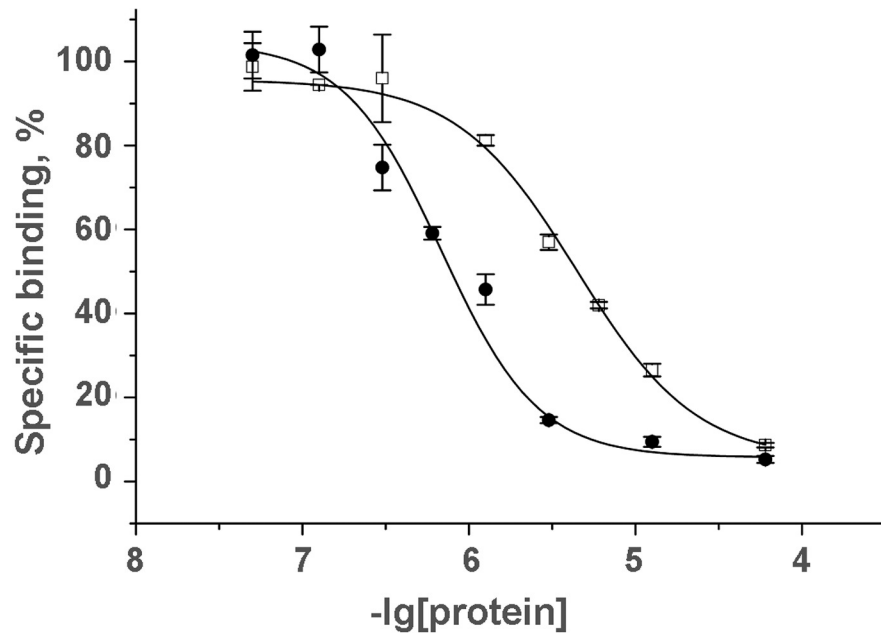


Fig 6. Interaction of non-venom proteins with *T. californica* nAChR. Inhibition of the initial rate of specific [¹²⁵I]- α -Bgt binding to *T. californica* nAChR by RNase (squares) and cytochrome C (circles).

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In this paper, we present additional evidence for interaction with nAChRs of two dimeric presynaptic PLA₂ toxins, i.e. Cro from *C. durissus terrificus* and β-Bgt from *B. multicinctus*, as well as non-toxic mammalian PLA₂ from porcine pancreas. PP PLA₂ and β-Bgt suppressed current responses of neurons to agonists with IC₅₀ values of 2.5 and 4.8 μM, respectively (Fig 1). These values are in the same range as those obtained earlier for other PLA₂s (0.4–10 μM [15]) which are monomeric enzymes. In heterodimeric β-Bgt the PLA₂ subunit is connected to a Kunitz type inhibitor subunit by a disulfide bond, but this does not interfere with β-Bgt binding to nAChR. PP PLA₂ suppressed the current responses of heterologously expressed human α9α10 nAChR to ACh with an IC₅₀ value of 0.19 μM (Fig 3A). This is the highest affinity observed in electrophysiological experiments on current suppression by PLA₂s.

In the binding assay, Cro and PP PLA₂ competed with α-Bgt for binding to nAChR and AChBP. Interestingly, PP PLA₂ at α7 nAChR competed with α-Bgt for binding to only about 30% of the total binding sites. The inhibition reached a plateau at about 3 μM and remained at the same level up to 30 μM (Fig 3B). The affinity for this 30% of sites was fairly high, the IC₅₀ value being 150 nM. It should be noted that PP PLA₂ completely inhibited the acetylcholine-induced current in *Lymnaea* neurons in a noncompetitive manner, albeit with a higher IC₅₀ value (2.5 μM). The nAChR α7 subtype contains five α-Bgt binding sites [32], but α-Bgt binding to only one site is enough to block ion currents [33]. The bound α-Bgt locks the agonist-binding site in an inactive conformation and the dominant mechanism of antagonism is non-competitive, originating from conformational arrest of the binding sites [33]. Given these data, we suggest that at α7 nAChR, PP PLA₂ competes with α-Bgt and binds to only one (or possibly two) binding site(s). However, PP PLA₂ completely inhibits receptor ionic conductance in *Lymnaea* neurons. Therefore, binding of PP PLA₂ to one or two sites could be sufficient to block *Lymnaea* receptor similar to the inhibition of α7 nAChR by α-Bgt.

PP PLA₂ inhibited α-Bgt binding to the ECD of the human α9 nAChR with an IC₅₀ value of 5.5 μM (Fig 3C), which is higher than that (0.19 μM) observed for inhibition of the ACh-induced current in oocytes expressing human α9α10 nAChR. Thus, for α9 nAChR we have observed receptor inhibition both in the ECD binding experiments and in electrophysiological experiments on whole receptors. It should be noted that signaling via α9α10 nAChRs is involved in the expression of pain [34] and inhibition of this receptor prevents neuropathic pain [35]. Consistent with these data, type IIA PLA₂ has been localized by immunohistochemistry to the spinal trigeminal and facial motor nuclei and dorsal- and ventral-horns of the spinal cord [36], implying an important role of CNS sPLA₂ in nociceptive transmission. It has also been shown that the treatment of mice with bee venom PLA₂ might prevent oxaliplatin-induced neuropathic pain [37]. Given our data shows an interaction of PP PLA₂ with α9α10 nAChR, we suggest that the PLA₂ interaction with this receptor may be involved in the pain transmission pathway.

Interaction of Cro with muscle-type nAChRs has been previously studied [38, 39]. The toxin or phospholipolytically active basic component blocked depolarization and the Na⁺ permeability increase induced by carbamylcholine in membrane preparations from electric organs of *Electrophorus electricus* and *T. marmorata*. Although Cro reduced the initial velocity of labeled α-toxin from *Naja nigricollis* binding to postsynaptic membranes by about 30%, the authors concluded that Cro did not interfere with binding of α-toxin to nAChRs. These data along with Cro evoked enhancement of affinity to agonist was considered as a sign of non-competitive interaction of Cro with nAChR, leading to stabilization of the desensitized state [38]. In support of this, Cro decreased depolarization of the guinea-pig end-plate and the frequency of miniature end-plate postsynaptic potentials [39].

In this work, we found that Cro could compete with α-Bgt for binding to nAChRs. It is interesting to note that two binding sites with different affinities for Cro were revealed in both

Torpedo and $\alpha 7$ nAChRs: IC₅₀ were of 30 and 260 nM for the first and 4.9 nM and 15 μ M for the second (Fig 4). In *Torpedo* nAChR the ratio of low and high affinity binding sites was 1:1, corresponding to the presence of two agonist/competitive antagonist binding sites. Non-equivalence in the affinity of two binding sites of *Torpedo* nAChR was earlier shown for d-tubocurarine and α -conotoxins [40, 41]. In $\alpha 7$ nAChR the high affinity binding sites represented about 20% of total sites. This finding could be explained by the assumption that the high affinity Cro binding to one site out of five ones resulted in some changes in the receptor which were responsible for a decrease in affinity to Cro in the other four sites.

In experiments with water soluble AChBP, Cro binding to one binding site was observed (Figs 4C and 5). The binding to water soluble protein observed both in competition with radioactive α -Bgt and direct SPR experiment allowed complete exclusion of membrane effects in its interaction.

Inhibition of α -Bgt binding to *Torpedo* nAChRs found in this study is consistent with Cro inhibition of responses to carbamylcholine observed in membrane preparations from electric organs and in guinea-pig diaphragm, although competitive binding of Cro and α -Bgt was not reported [38]. Our finding of muscle type nAChR inhibition by Cro demonstrates the postsynaptic activity of this toxin, although the block of $\alpha 7$ nAChR by Cro may contribute to its presynaptic activity as the participation of $\alpha 7$ nAChR in acetylcholine release in mouse motor synapses was suggested [42].

According to our electrophysiological data, antagonism of PLA₂s (Fig 2 here and Fig 4B in [15]) of nAChRs was non-competitive. This fact seems contradictory to the ability of all PLA₂s to inhibit α -Bgt binding (Figs 3 and 4 here and Fig 6 in [15]). The reason of the discrepancy might be structural differences between $\alpha 7$ similar *Lymnaea* nAChRs and *Torpedo* or human $\alpha 7$ receptors. For another explanation of the PLA₂ competition with α -Bgt, one should consider the existing model of α -neurotoxin-nAChR interaction: it is accepted that the tip of the toxin central loop is inserted into the receptor at the interfaces between two subunits and the toxin molecule is placed almost equatorially to the extracellular domain of the nAChR. Recently, a possible participation of the membrane in which nAChR is embedded in neurotoxin-receptor interaction was suggested [43, 44]. It was found that a snake venom neurotoxin can bind membrane and this binding can be considered as a first interaction step facilitating the receptor recognition by the toxin. Thus, it can be suggested that any disturbance of toxin interaction with the membrane can constrain its binding to the receptor. At the extreme, this may result in inhibition of toxin binding to the receptor. Interestingly, in competition experiments the interaction of Cro with the water soluble AChBP (Fig 4C) was weaker than with the membrane-bound nAChRs which could be due to participation of membrane in toxin-receptor interaction. Finally, competition with α -Bgt might be explained by PLA₂ binding to nAChR in close vicinity to the agonist/competitive antagonist binding site that leads to steric hindrance of α -Bgt binding.

Here we found that PLA₂s interact with nAChRs with different affinities, with IC₅₀ ranging from tens of nM to tens of μ M. The fairly low affinities in the micromolar range raise the question about specificity of interaction, and thus four proteins lacking phospholipolytic activity—RNase, β -lactoglobulin, soybean trypsin inhibitor, and cytochrome C—were tested for their ability to interact with nAChRs. Unexpectedly, RNase and cytochrome C could compete with α -Bgt for binding to *Torpedo* nAChR with IC₅₀s of 1.12 and 5 μ M, respectively (Fig 6), while soybean trypsin inhibitor and β -lactoglobulin did not compete with α -Bgt at concentrations up to 60 μ M. However, all these proteins were practically inactive in functional tests on *Lymnaea* neurons: RNase only slightly suppressed nAChR-mediated current with an IC₅₀ greater than 50 μ M, while soybean trypsin inhibitor and β -lactoglobulin were ineffective at this concentration. It is well documented that both RNase [45] and cytochrome C [46] interact with

cellular membranes, and in the view of the above consideration they might interfere with the α -Bgt binding to the membrane-associated nAChR. Indeed, such inhibition was observed in competition experiments with radioactive α -Bgt (Fig 6). However, the addition of RNase had only marginal effect on the current elicited by agonist in *Lymnaea* neurons. The competition of the PLA₂s with α -Bgt for binding to membranes could also explain the higher enzyme affinities observed in radioligand experiments as compared to electrophysiological data obtained in this and previous [15] work. PLA₂s studied here not only competed with radioactive α -Bgt for binding to nAChRs but also blocked acetylcholine elicited ion currents.

In summary, we have revealed the ability of two toxic heterodimeric snake PLA₂s and non-toxic PP PLA₂ to interact with different types of nAChRs. These data indicate that the interaction with nAChR may be a general property of all PLA₂s and defines a novel activity that can be attributed to these proteins.

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