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 venom 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 A\textsubscript{2} (PLA\textsubscript{2}s, 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 PLA\textsubscript{2} superfamily includes 15 groups comprising four main types including the secreted, cytosolic, calcium-independent PLA\textsubscript{2}s, and platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein-associated PLA\textsubscript{2} [2].

Group I PLA\textsubscript{2}s 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 PLA\textsubscript{2}s are present in Viperidae snake venoms and in the synovial fluids of animals. These PLA\textsubscript{2}s, which are secreted by venomous glands of snakes, bees and other venomous animals, manifest various toxic actions. PLA\textsubscript{2}s 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 PLA\textsubscript{2}s 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 PLA\textsubscript{2} 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 PLA\textsubscript{2} 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 PLA\textsubscript{2}s with specific proteins: binding to these receptors facilitates a local enzyme-dependent or independent action [8]; the discovery of proteins that bind PLA\textsubscript{2} with high affinity in different tissues supports this hypothesis [9–12].

3. Interaction of PLA\textsubscript{2}s with intracellular Ca\textsuperscript{2+} binding proteins after endocytosis or penetration through damaged membranes causing an increase in intracellular Ca\textsuperscript{2+} concentration, both leading to mitochondrial uncoupling [7, 13].

We have earlier reported antagonistic action of eight PLA\textsubscript{2}s from the venoms of snakes of Viperidae and Elapidae families (PLA\textsubscript{2} 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 \textsuperscript{[125]}I\alpha-bungarotoxin (\alpha-Bgt) for binding to the muscle-type nAChRs of Torpedo californica electric organ, to human \alpha\textsubscript{7} nAChRs expressed in GH\textsubscript{4}C\textsubscript{1} cell line, and to ACh-binding protein (AChBP) from Lymnaea stagnalis. When tested on isolated neurons of L. stagnalis which contain \alpha\textsubscript{7} similar nAChRs [16, 17], PLA\textsubscript{2}s suppressed ACh- or cytisine-evoked currents under conditions that exclude hydrolysis of membrane phospholipids. These results indicate that binding of PLA\textsubscript{2}s to nAChRs affects their function.

To ascertain whether all types of PLA\textsubscript{2}s are able to interact with nAChRs, we have studied the action of three other phospholipases i.e. presynaptically active \beta-bungarotoxin (\beta-Bgt) from Bungarus multicinctus, crototoxin (Cro) from Crotalus durissus terrificus snake venom, and non-toxic mammalian PLA\textsubscript{2} from porcine pancreas (PP PLA\textsubscript{2}, group IIB)—in binding assay and on Lymnaea neurons. \beta-Bgt is a heterodimeric protein in which a group IA PLA\textsubscript{2} 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, cytisine, 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-[¹²⁵]I)[iodotyrosyl][⁵⁴]-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), cytisine (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 PLA₂₅ 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 α9 and α10 subunits were linearized with XbaI restriction enzymes (NEB, USA). Linearized plasmid constructs were subjected to *in vitro* cRNA transcription using T7 mMessageMachine transcription kit (AMBIION, 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 α9 and α10 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 µg/mL gentamicin and 100 µg/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 µM 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 α9α10 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 α7 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 PLA₂₅, 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 µM α-cobratoxin. The membrane and cell suspensions were applied to glass GF/C filters (Whatman, Little Chalfont, UK) presoaked in 0.25% polyethelyenimine, and the unbound radioactivity was removed from the filter by washing (3 × 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 × 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 α9 nAChR, PP PLA2 in the concentration range 0.3–30 μM was incubated for 2 h at room temperature with the ECD (final concentrations of 30 μg/ml) in 50 μL 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 μL 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 × 4 ml) with the 20 mM Tris–HCl buffer. Nonspecific binding was determined by preliminary incubation of the ECD with 10 μM 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 μg/ml, and solutions were injected at a flow rate of 30 μl/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 AhBP coupled to the CM5 sensor chip. The kinetic constants, kₐ (association rate constant), and k₅₇ (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 k₅₇ and kₐ (K_{D,app} = k₅₇/kₐ).

Results
Electrophysiological experiments

Suppression of acetylcholine- or cytisine-induced currents in L. stagnalis neurons.
Under the experimental conditions used, ACh and cytisine 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 α7 nAChRs; furthermore cytisine is a full agonist at this subtype whereas it is a weak partial agonist at other nAChR subtype. In most experiments, we used cytisine or choline instead of ACh because of their more selective actions on α7 nAChRs. To exclude a possible contribution of phospholipolytic activity, Ca^{2+} was replaced with Ba^{2+} 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 cytisine-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
and β-Bgt inhibition of cytisine-induced current were 2.5 ± 0.4 (n = 7) and 4.8 ± 1.6 (n = 4) μM, respectively.

For PP PLA₂ the type of antagonism was determined. For this purpose, a set of cytisine or ACh concentrations including saturating ones were applied to a neuron before and after treatment with PP PLA₂ at 3 μM (a concentration slightly higher than its IC₅₀). 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. EC₅₀ values for cytisine were 2.9 in control and 3.3 μM after PP PLA₂ treatment (n = 4) and for ACh—4.0 and 3.2 μM (n = 1), respectively. However, the maximal responses to both cytisine and ACh were reduced by 40–
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 cytisine-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 2. Determination of antagonism type for PP PLA2. Dependence of cytisine (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).](https://doi.org/10.1371/journal.pone.0186206.g002)

![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.](https://doi.org/10.1371/journal.pone.0186206.g003)
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

**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 α7 nAChR and AChBP. (A) Inhibition of the initial rate of specific $[^{125}I]$-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 $[^{125}I]$-α-Bgt binding to human α7 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 $[^{125}I]$-α-Bgt binding to acetylcholine-binding protein from *L. staganalis* by Cro.

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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 PLA2 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 α7-like receptors, and to compete with α-Bgt binding to recombinant human α7 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 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 μg/ml, respectively.](https://doi.org/10.1371/journal.pone.0186206.g005)

![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 RNAase (squares) and cytochrome C (circles).](https://doi.org/10.1371/journal.pone.0186206.g006)
In this paper, we present additional evidence for interaction with nAChRs of two dimeric presynaptic PLA$_2$ toxins, i.e. Cro from $C.\,duriuss\,terricicus$ and $\beta$-Bgt from $B.\,multicinctus$, as well as non-toxic mammalian PLA$_2$ from porcine pancreas. PP PLA$_2$ and $\beta$-Bgt suppressed current responses of neurons to agonists with IC$_{50}$ values of 2.5 and 4.8 $\mu M$, respectively (Fig 1). These values are in the same range as those obtained earlier for other PLA$_{2s}$ (0.4–10 $\mu M$ [15]) which are monomeric enzymes. In heterodimeric $\beta$-Bgt the PLA$_2$ subunit is connected to a Kunitz type inhibitor subunit by a disulfide bond, but this does not interfere with $\beta$-Bgt binding to nAChR. PP PLA$_2$ suppressed the current responses of heterologously expressed human $\alpha_9\alpha_{10}$ nAChR to ACh with an IC$_{50}$ value of 0.19 $\mu M$ (Fig 3A). This is the highest affinity observed in electrophysiological experiments on current suppression by PLA$_{2s}$.

In the binding assay, Cro and PP PLA$_2$ competed with $\alpha$-Bgt for binding to nAChR and AChBP. Interestingly, PP PLA$_2$ at $\alpha_7$ nAChR competed with $\alpha$-Bgt for binding to only about 30% of the total binding sites. The inhibition reached a plateau at about 3 $\mu M$ and remained at the same level up to 30 $\mu M$ (Fig 3B). The affinity for this 30% of sites was fairly high, the IC$_{50}$ value being 150 nM. It should be noted that PP PLA$_2$ completely inhibited the acetylcholine-induced current in $Lymnaea$ neurons in a noncompetitive manner, albeit with a higher IC$_{50}$ value (2.5 $\mu M$). The nAChR $\alpha_7$ subtype contains five $\alpha$-Bgt binding sites [32], but $\alpha$-Bgt binding to only one site is enough to block ion currents [33]. The bound $\alpha$-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 $\alpha_7$ nAChR, PP PLA$_2$ competes with $\alpha$-Bgt and binds to only one (or possibly two) binding site(s). However, PP PLA$_2$ completely inhibits receptor ionic conductance in $Lymnaea$ neurons. Therefore, binding of PP PLA$_2$ to one or two sites could be sufficient to block $Lymnaea$ receptor similar to the inhibition of $\alpha_7$ nAChR by $\alpha$-Bgt.

PP PLA$_2$ inhibited $\alpha$-Bgt binding to the ECD of the human $\alpha_9$ nAChR with an IC$_{50}$ value of 5.5 $\mu M$ (Fig 3C), which is higher than that (0.19 $\mu M$) observed for inhibition of the ACh-induced current in oocytes expressing human $\alpha_9\alpha_{10}$ nAChR. Thus, for $\alpha_9$ 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 $\alpha_9\alpha_{10}$ nAChRs is involved in the expression of pain [34] and inhibition of this receptor prevents neuropathic pain [35]. Consistent with these data, type IIA PLA$_2$ 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 sPLA$_2$ in nociceptive transmission. It has also been shown that the treatment of mice with bee venom PLA$_2$ might prevent oxaliplatin-induced neuropathic pain [37]. Given our data shows an interaction of PP PLA$_2$ with $\alpha_9\alpha_{10}$ nAChR, we suggest that the PLA$_2$ 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 $\alpha$-toxin from $Naja\,nigrilis$ binding to postsynaptic membranes by about 30%, the authors concluded that Cro did not interfere with binding of $\alpha$-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 $\alpha$-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 hα7 nAChRs: IC$_{50}$ were of 30 and 260 nM for the first and 4.9 nM and 15 μM 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 hα7 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 hα7 nAChR by Cro may contribute to its presynaptic activity as the participation of α7 nAChR in acetylcholine release in mouse motor synapses was suggested [42].

According to our electrophysiological data, antagonism of PLA$_2$ (Fig 2 here and Fig 4B in [15]) of nAChRs was non-competitive. This fact seems contradictory to the ability of all PLA$_2$s 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 α7 similar Lymnaea nAChRs and Torpedo or human α7 receptors. For another explanation of the PLA$_2$ 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 PLA$_2$ 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 PLA$_2$s interact with nAChRs with different affinities, with IC$_{50}$ ranging from tens of nM to tens of μM. 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 IC$_{50}$ of 1.12 and 5 μM, respectively (Fig 6), while soybean trypsin inhibitor and β-lactoglobulin did not compete with α-Bgt at concentrations up to 60 μM. However, all these proteins were practically inactive in functional tests on Lymnaea neurons: RNase only slightly suppressed nAChR-mediated current with an IC$_{50}$ greater than 50 μM, 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 \( \alpha \)-Bgt binding to the membrane-associated nAChR. Indeed, such inhibition was observed in competition experiments with radioactive \( \alpha \)-Bgt (Fig 6). However, the addition of RNAse had only marginal effect on the current elicited by agonist in \textit{Lymnaea} neurons. The competition of the PLA\(_2\)s with \( \alpha \)-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. PLA\(_2\)s studied here not only competed with radioactive \( \alpha \)-Bgt for binding to nAChRs but also blocked acetylcholine elicited ion currents.

In summary, we have revealed the ability of two toxic heterodimeric snake PLA\(_2\)s and non-toxic PP PLA\(_2\) to interact with different types of nAChRs. These data indicate that the interaction with nAChR may be a general property of all PLA\(_2\)s and defines a novel activity that can be attributed to these proteins.

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