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Paralytic activity of lysophosphatidylcholine from saliva of the waterbug *Belostoma anurum*

Lívia Silva-Cardoso[1,2], Paola Caccin[3], Anna Magnabosco[3], Maria Patrón[3], Mariane Targino[1], André Fuly[4], Giselle A. Oliveira[1], Marcos H. Pereira[2,5], Maria das Graças T. do Carmo[6], Amanda S. Souza[6], Mário A. C. Silva-Neto[1,2], Cesare Montecucco[3] and Georgia C. Atella[1,2,*]

[1]Instituto de Bioquímica Médica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, PO Box 68041, Cidade Universitária, Ilha do Fundão, Av. Bauhínia 400, Rio de Janeiro, CEP 21941-590, RJ, Brazil, [2]Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, [3]Dipartimento di Scienze Biomediche, Università di Padova, Padova, Italy, [4]Universidade Federal Fluminense, UFF, Niterói, Rio de Janeiro, Brazil, [5]Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Minas Gerais, Brazil and [6]Instituto de Nutrição, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Brazil

*Author for correspondence (atella@bioqmed.ufrj.br)

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SUMMARY

Lysophosphatidylcholine (LPC) is a major bioactive lipid that is enzymatically generated by phospholipase A_2 (PLA_2). Previously, we showed that LPC is present in the saliva of the blood-sucking hemipteran *Rhodnius prolixus* and modulates cell-signaling pathways involved in vascular biology, which aids blood feeding. Here, we show that the saliva of the predator insect *Belostoma anurum* contains a large number of lipids with LPC accounting for 25% of the total phospholipids. A PLA_2 enzyme likely to be involved in LPC generation was characterized. The activity of this enzyme is 5-fold higher in *Belostoma* saliva than in other studied hemipterans, suggesting a close association with the predator feeding habits of this insect. *Belostoma* employs extra-oral digestion, which allows for ingestion of larger prey than itself, including small vertebrates such as amphibians and fish. Therefore, prey immobilization during digestion is essential, and we show here that *Belostoma* saliva and *B. anurum* saliva purified LPC have paralytic activity in zebrafish. This is the first evidence that lysophospholipids might play an important role in prey immobilization, in addition to contributing to blood feeding, and might have been an evolutionary acquisition that occurred long before the appearance of hematophagy in this animal group.

Key words: *Belostoma*, extra-oral digestion, lysophosphatidylcholine, paralysis, phospholipase A2, saliva.

INTRODUCTION

Predatory habits are widespread in the *Heteroptera*; over half of these taxon families feed on living invertebrate prey (Edwards, 1961). Foraging choices are usually based on predator evaluation of the amount of food to be ingested and the time and energy costs. Extra-oral digestion (EOD) is ecologically relevant, as it allows small predators, such as insects, to feed on large prey that cannot be swallowed or ingested whole. In arthropods, EOD is used in at least 192 out of 236 orders (Cohen, 1995). Type I EOD involves the chemical liquefaction of prey within the prey's body. Type II EOD involves the chemical digestion of the prey within the sphere of the predator's mouthparts but outside of its mouth. In both cases, natural selection has shaped the saliva composition in order to include molecules that allow both prey paralysis and digestion. More efficient prey paralysis allows the ingestion of larger and therefore more nutrient-rich prey. Thus, pharmacological or enzyme-induced submission of the prey in the very first moments of a predator's attack is of high evolutionary advantage.

The heteropteran family *Belostomatidae* includes giant water bugs, which are predators that use EOD. Predatory heteropterans use EOD as a feeding strategy by injecting digestive enzymes into intact prey (Cohen 1995; Cohen and Wheeler, 1998). These predators inject potent hydrolytic saliva and suck the predigested and liquefied prey tissues while preserving the cuticle (Cohen, 1993; Cohen, 1995). Accordingly, the saliva of predator insects that use EOD contains a complex enzyme cocktail that is capable of liquefying the prey, which ensures feeding success. These molecules include digestive enzymes such as lipases, phospholipases, trypsin, α-chymotrypsin and glucosidases (Cohen, 1995; Swart et al., 2006). Hematophagous arthropod saliva has distinct biological effects, including the inhibition of coagulation, platelet aggregation and vasoconstriction (Ribeiro and Francischetti, 2003). This inhibition counteracts the platelet aggregation and vasoconstriction triggered in the vertebrate prey by the arthropod bite. Up to now, the anti-hemostatic activities found in salivary secretions from blood-feeding arthropods were thought to be mainly due to proteins or gases, such as nitric oxide (NO) (Champagne, 2005). The complexity of salivary molecules has recently been studied by proteomic techniques in different insect models (Ribeiro et al., 2004; Anderson et al., 2006; Andersen et al., 2007; Assumpção et al., 2008; Mans et al., 2008). Hundreds of protein sequences were obtained and need to be tested for anti-hemostatic properties.

Lysophosphatidylcholine (LPC) is a product of the hydrolysis of phosphatidylcholine by phospholipase A_2 (PLA_2). Recently, several laboratories have attempted to characterize the effect of LPC on vascular biology (Xing et al., 2008; Olofsson et al., 2008; Han et al., 2008; Bassa et al., 2007; Matsumoto et al., 2007). We have previously demonstrated the presence of LPC in saliva of the blood-sucking arthropod *Rhodnius prolixus* (Golodne et al.,

2003), and have described the dual anti-hemostatic activity of LPC that inhibits platelet aggregation and induces NO production in endothelial cells. *Belostoma* saliva enables different feeding strategies when compared with hematophagous hemipterans from the *Reduviidae* family. After catching prey, *Belostoma* inserts its piercing mouthparts to inject saliva and liquefy the prey tissues. Accordingly, *Belostoma* saliva must contain an array of digestive enzymes. Their saliva is white and viscous, indicating that it may contain an unusual amount of lipids. In the present study, we demonstrate for the first time the presence of lipids and bioactive lysophospholipids in the saliva of a heteropteran predator, *B. anurum*. We show that these compounds are toxic to neurons in culture, can block neurally evoked twitch contractions, and can paralyze and kill zebrafish (*Dario rerio*).

MATERIALS AND METHODS

Belostoma anurum saliva

Adult specimens of *Belostoma anurum* were collected from Picinguaba (São Paulo, Brazil). The belostomatids were kept, under laboratory conditions, in tanks with 200 ml of non-chlorinated tap water and provided with synthetic floats. *Aedes aegypti* fifth instar larvae and *Biomphalaria glabrata* served as food. The saliva was obtained from the proboscis by means of electrical stimulation using an isolated pulse stimulator (Model 2100 A-M Systems, Sequim, WA, USA) at the insertion of the first pair of legs to the thorax (50–100 V, 3–4 Hz, and square pulses of 3 ms duration), as previously described (Dan et al., 1993).

Lipid analysis

Samples of *B. anurum* saliva were subjected to lipid extraction (Bligh and Dyer, 1959). The organic phase was removed and dried under nitrogen, and the total amount of lipids was determined gravimetrically. Specifically, samples were transferred to a test tube pre-weighed five times, placed on a hot plate and dried using a stream of nitrogen gas. The weight difference before and after extraction and drying was assumed to be due to lipids. Extracted lipids were analyzed by thin-layer chromatography (TLC) using a mixture of acetone:methanol:acetic acid:chloroform:water (15:13:12:40:8 v/v) for determination of the phospholipid profile (Horwitz and Perlman, 1987). Fatty acid separation was achieved by the solvent system hexane-ethyl ether acetic acid (60:40:1 v/v) (Kawooya and Law, 1988). After evaporation of the solvents, the plates were usually immersed for 15 s in a charring solution consisting of 10% $CuSO_4$ and 8% H_3PO_4, and heated at 170°C for 5–10 min (Ruiz and Ochoa, 1997). The charred TLC plated was then subjected to densitometric analysis. Each phospholipid spot was identified by comparison with phospholipid standards (Sigma) run in parallel.

LPC and FA purification

Purification of TLC-separated lipids was performed as described by Yuan et al. (Yuan et al., 1996), with modifications. Briefly, after staining with iodine, the target phospholipid spot was scraped into a glass tube and vortexed with 4 ml of an extraction solution consisting of methanol:iso-butanol:water (45.8:11.5:42.7 v/v). Tubes were heated to 55°C in a dry bath incubator for 20 min, centrifuged at 3000 ***g*** for 5 min, and the supernatant collected. This procedure was repeated two more times, and chloroform (3 ml) was added to the combined supernatants (~12 ml). After intense vortexing and centrifugation (3000 ***g***, 10 min), the lower organic phase was recovered and dried under a stream of N_2. *B. anurum* LPC from saliva is henceforth designated BaLPC.

Fatty acid composition

Fatty acids purified from TLC were subjected to saponification and methylation as described (Lepage and Roy, 1986), and the fatty acid methyl esters were separated (SP2560 column, Supelco, Bellefonte, PA, USA) and quantified by gas-liquid chromatography with an ionizable flame detector (Perkin Elmer, Wellesley, MA, USA), using hydrogen as the carrier gas. Injection and detection temperatures were 260°C and 280°C, respectively. Run temperature started at 135°C and increased up to 195°C with a run time of 45 min (Albuquerque et al., 2006).

Phospholipase A_2 activity

Protein in the saliva was determined by the method of Lowry (Lowry, 1951). PLA_2 activity was assayed by measuring the release of free fatty acid from the fluorescent phospholipid substrate *N*-{[6-(2,4-dinitrophenyl) amino] hexanoyl}-2 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl) 1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (PED6) (Molecular Probes, Invitrogen, CA, USA) (Farber et al., 1999). In standard assays, *B. anurum* saliva (5-25 $\mu g\,\mu l^{-1}$) was incubated at 37°C with 100 $mmol\,l^{-1}$ Tris, 5 $mmol\,l^{-1}$ $CaCl_2$, 100 $mmol\,l^{-1}$ NaCl, pH 8.5, with 500 ng of PED6 in a final volume of 100 µl. After incubation, the lipids were extracted and analyzed by TLC for neutral lipids. The TLC plate was scanned in a Storm 860 laser scanner (Amersham Biosciences, Uppsala, Sweden). Relative fatty acid fluorescence at the end of the reactions was quantified by Image Quant software. PLA_2 activity was also measured according to Rodrigues et al. (Rodrigues et al., 2007), using NBD-fluorescent phospholipid 1-acyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (acyl 06:0 NBD PC) (Avanti Polar Lipids, Alabaster, AL, USA) as substrate. Ten micrograms of salivary proteins were assayed for PLA_2 activity in a final volume of 3.0 ml in a Hitachi F450 Fluorescence Spectrometer (Hitachi, Tokyo, Japan) at room temperature in appropriate cuvettes. Excitation and emission wavelengths were adjusted to 460 and 534 nm, respectively. The standard reaction medium contained 50 $mmol\,l^{-1}$ TBS, 8.3 $mmol\,l^{-1}$ $CaCl_2$, pH 7.5. A 2-min time scan was first taken with all components except the substrate. Then, enzymatic activity was started by adding substrate and the initial time scan was subtracted from the second scan before analysis of the data.

Lipid preparation

Extracted lipids were dissolved in small volumes of $CHCl_3$:CH_3OH (chlorofom: methanol; 3:1, v/v), dried to a thin film under a gentle nitrogen flow and vacuum pumped for at least 2 h to remove residual traces of organic solvents. The dried lipid film was suspended in 10 $mmol\,l^{-1}$ Hepes-Na, 150 $mmol\,l^{-1}$ NaCl, pH 7.4, extensively vortexed and then ultrasonically dispersed in a bath sonicator at 37–40°C until optical clarity was obtained. All suspensions were kept at 37–40°C until use.

Effect of *B. anurum* saliva purified LPC on neurons in culture

Primary rat spinal motor neurons (SCMNs) were isolated from Sprague–Dawley (embryonic day 14) rat embryos and cultured according to previously described protocols (Bohnert and Schiavo, 2005). SCMNs were plated onto 24-mm coverslips in six-well plates. After 5-8 days in culture, the coverslips were washed with E4 medium (120 $mmol\,l^{-1}$ NaCl, 3 $mmol\,l^{-1}$ KCl, 2 $mmol\,l^{-1}$ $MgSO_4$, 2 $mmol\,l^{-1}$ $CaCl_2$, 10 $mmol\,l^{-1}$ glucose, and 10 $mmol\,l^{-1}$ Hepes, pH 7.4), mounted on a thermostatted chamber (Medical Systems Corporation, Fort Lauderdale, FL, USA) and placed on

the stage of a Leica ADMIRE3 inverted microscope (Leica, Germany). Cells were exposed to the BaLPC and images were obtained through a 63× objective with a differential interference contrast system (DIC) and captured by a LeicaDC500 CCD camera. Data were collected using FW4000 software and analyzed using ImageJ (NIH, USA).

Effect of *B. anurum* saliva purified LPC on the mouse phrenic nerve-hemidiaphragm preparation

Mouse phrenic nerve hemidiaphragms were isolated from CD-1 mice weighing about 20–30 g, as previously described by Bulbring (Bulbring, 1946), and then oxygenated (95% O_2, 5% CO_2) and placed in physiological buffer (139 mmol l^{-1}, NaCl, 12 mmol l^{-1} $NaHCO_3$, 4 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} KH_2PO_4 and 11 mmol l^{-1} glucose, pH 7.4). Two innervated hemidiaphragm preparations were isolated from each animal.

The phrenic nerve was stimulated via two ring platinum electrodes with supra-threshold stimuli of 10 V amplitude and 0.1 ms pulse duration, with a frequency of 0.1 Hz (Stimulator 6002, Harvard Apparatus, Holliston, MA, USA). Muscle contraction was monitored with an isometric transducer (Harvard Apparatus). Muscles were stretched to the optimal length for twitch tension and the muscle twitch allowed to stabilize for at least 20 min at 37°C. The concentration of magnesium ions ($[Mg^{2+}]$) was increased to 10 mmol l^{-1} to reduce the twitch response by approximately 50%, and BaLPC (150 μM final concentration) was added to the tissue bath at 37°C during continuous nerve stimulation. The time course of neurally evoked twitch contraction was monitored, and the data were analyzed by the LabView-based computer program (National Instruments, Austin, TX, USA). The amplitude of the twitch was calculated as the difference between basal muscle tension and the mean of the peak value measured after stimulation.

Effect of *B. anurum* saliva purified LPC and saliva on the zebrafish

Two-year-old zebrafish were bred and grown under standard conditions (Kimmel et al., 1995). For injections, 4% methylene blue (Sigma) was added to LPC (40 mmol l^{-1}) or saliva suspension. Groups of fish (10 animals per sample) were anesthetized with 0.05 mg ml^{-1} Tricaine (3-aminobenzoic acid ethylester; Sigma) by standard methods (Westerfield, 1995) and injected into dorsal muscles on one side at the posterior part of the trunk (Miller and Neely, 2004; Anichtchik et al., 2004). Fifty microliters of the BaLPC were injected with a 3/10-cc U-100 ultrafine insulin syringe with a 0.5-inch-long 29-gauge needle (Catalog No. BD-309301; VWR) into each animal, as previously described. Our estimates indicate that a final concentration of 150 μmol l^{-1} LPC is achieved in injected fish. Samples were visually inspected up to two hours after injection. All injected animals showed normal mobility immediately after the injections, but developed paralysis within minutes. Control animals injected with buffer and methylene blue did not show any alteration of mobility.

RESULTS

The saliva of *Belostomatidae* is extremely effective at paralyzing and liquefying prey, which are often larger than the insect itself, thus allowing extra-oral digestion and ingestion. We have examined *B. anurum* saliva composition collected by electrical stimulation and Table 1 shows that it contained about 88% and 12% of lipid and protein, respectively. Neutral lipid classes accounted for 94% of the total lipids.

Table 1. *Belostoma anurum* saliva composition

Belostoma anurum saliva	Percentage
Lipids	88
Proteins	12
Lipids:	
Natural lipids	94
Phospholipids	6

B. anurum saliva was collected by electrical stimulation in physiological saline (0.15 mol l^{-1}). The saliva was centrifuged and the supernatant subjected to lipid extraction. Lipids were quantified gravimetrically and protein content was measured according to Lowry et al. (Lowry et al., 1951). *B. anurum* saliva lipid and protein composition is expressed as a percentage of the total weight.

To carry out analysis of the lipid fraction, *B. anurum* saliva was subjected to lipid extraction followed by TLC. Sixty percent of neutral lipids were fatty acids (FAs), and the remaining molecules consisted of cholesterol and esterified cholesterol, triglycerides, and di- and monoacylglycerol (Fig. 1A,B). FAs were then analyzed by gas chromatography. The major components were palmitic and oleic acids, each comprising about 26% of the total lipid fraction (Table 2). Phospholipids were found to be 6% of the total lipids from saliva (Table 1). Phosphatidylcholine (PC) and the product of PLA_2 hydrolysis, LPC, are the major phospholipids in *Belostoma* saliva, as they comprise around 60% and 30%, respectively, of the

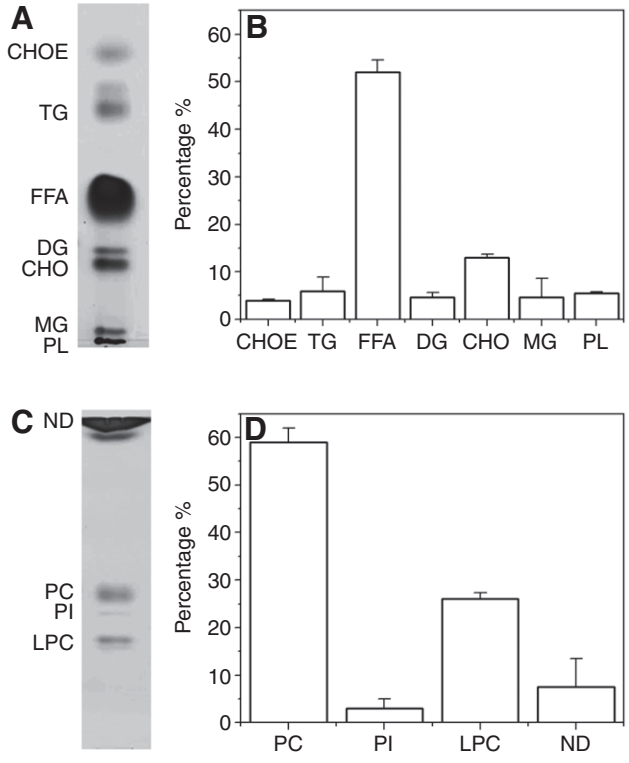

Fig. 1. Lipid profile of *Belostoma anurum* saliva. The saliva collected was centrifuged and the supernatant subjected to lipid extraction. Neutral lipids (A,B) and phospholipids (C,D) were analyzed separately by thin-layer chromatography (TLC) followed by iodine staining. Lipid spots were analyzed by densitometry. CHO, cholesterol; CHOE, cholesterol ester; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerides; FFA, free fatty acid; PL, phospholipids; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; ND, non-determined.

Table 2. *Belostoma anurum* saliva fatty acid composition

Fatty acid	*Belostoma* saliva	Common name
C 12:0	1.065	Lauric
C 14:0	10.06	Miristic
C 14:1	0.33	Miristoleic
C 15:0	0.14	Pentadecanoic
C 16:0	26.875	Palmitic
C 17:0	8.04	Heptadecanoic
C 18:0	4.68	Estearic
C 18:1 n-9 *trans*	0.93	Oleic
C 18:1 n-9 *cis*	26.48	Oleic
C 18:1 n-9 isomer	0.31	Oleic
C 18:2 n-6 *trans*	0.04	Linoleic
C 18:2 n-6 *cis*	12.265	Linoleic
C 20:1 n-9	0.06	Eicosanoic
C 18:3 n-3	1.385	Linolenic
C21:0	0.155	Heneicosanoic
C20:4 n-6 AA	3.035	Aracdonic
C20:5 n-3 EPA	0.085	Eicosapentaenoic
C24:1 n-9	0.085	Tetracosapentaenoic
C22:5 n-3	0.175	Docosapentaenoic
C22:6 n-3	0.055	Docosahexaenoic

The saliva was centrifuged, and the supernatant subjected to lipid extraction followed by thin-layer chromatography. The fatty acids spots were scraped and analyzed by gas chromatography.

Table 3. Effect of intramuscular injections on the zebrafish *Danio rerio*

	N	Time to paralysis (min)	Time to death (min)
LPC (40 mmol l^{-1})	20	5±3	30±10
Saliva	7	5±2	30±10
Control	10	–	–

Zebrafish were injected under the skin with 50 µl of *Belostoma anurum* saliva or *B. anurum* saliva-purified lysophosphatidylcholine (40 mmol l^{-1}) and the effects observed for 1 h. *N* is number of samples for each type of injection.

total amount of phospholipids (Fig. 1C,D). This finding suggested the presence of an enzyme that catalyses the hydrolysis of PC in the *sn*-2 position (PLA_2), producing LPC and FA. Accordingly, we used NBD-PC or PED6 as substrates for PLA_2. Indeed, PLA_2 activity was detected in the *B. anurum* saliva, and this activity was linear up to 500 s (Fig. 2A) and proportional to the amount of saliva added (Fig. 2B).

The vast majority of insects of the sub-order Heteroptera are phytophagous, but some groups, such as the *Triatominae*, are hematophagous and are commonly known as kissing bugs. This diversity in food habits is likely to shape distinct salivary compositions in order to maximize the possibility of successful feeding by insects (Schuh and Slater, 1996). We compared the PLA_2 activity present in the saliva of hemipterans with different feeding habits, including the phytophagous *Oncopeltus fasciatus*, the hematophagous *Rhodnius prolixus*, and *B. anurum*, a predator. Saliva was collected and submitted for protein quantification using 10 µg of total protein from hemipteran saliva samples. We found that PLA_2 activity in the saliva of *B. anurum* was 5-fold higher than that in the saliva of the two other hemipterans (Fig. 3). PLA_2 was reported in other insects as an enzyme involved in the digestion and emulsification of dietary lipids, which facilitates their further enzymatic digestion (Bowman et al., 1997; Stanley, 2006).

Here, we tested the possibility that the LPC generated by the saliva PLA_2 of *B. anurum* is involved in prey capture, similar to what has been described for some snake PLA_2 neurotoxins (Rigoni et al., 2005). Following prey capture and during feeding, *B. anurum* continuously pumps saliva into the body of its prey. This can be observed owing to the transparency of the insect exoskeleton (data not shown). In order to successfully feed, the insect predator must ensure that the prey is completely immobilized. Lipids are the main component of the saliva of *B. anurum*, and it was recently found that LPC blocks neurally evoked twitch contractions, and affects nerve terminals of both vertebrates and invertebrates (Rigoni et al., 2005; Caccin et al., 2006; Caccin et al., 2009; Megighian et al., 2007). The ability of lipids from *Belostoma* saliva to block neurally evoked twitch contractions was measured using a well-established model of the neuromuscular junction (NMJ), the mouse phrenic nerve–hemidiaphragm preparation. The addition of BaLPC caused a complete blockade of twitch contractions (Fig. 4A). Similar findings were obtained with whole saliva (not shown). The blockade was fully reversed by the addition of bovine serum albumin (BSA, 0.2%), which binds hydrophobic substances (Varshney et al., 2010; Simard et al., 2006; Melia et al., 2006) (not shown). This finding is in agreement with a lipid-derived block of neurotransmission, and excluded a specific effect of the sample used. Snake presynaptic PLA_2 neurotoxins and a mixture of LPC plus oleic acid are able to induce a characteristic swelling of synaptic boutons in cultured

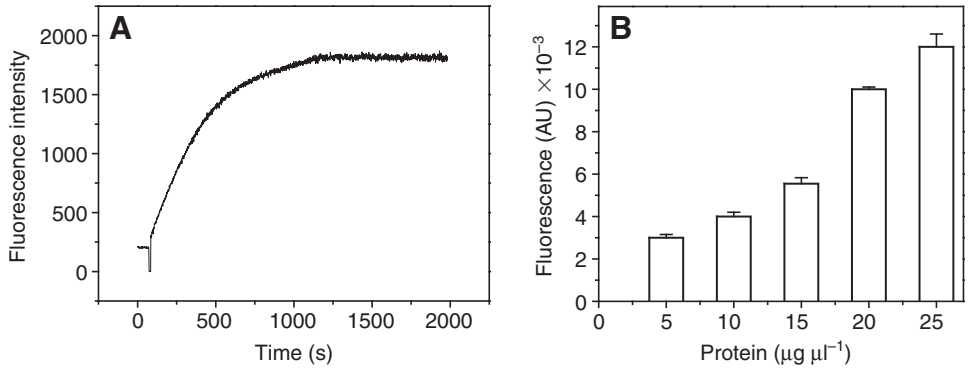

Fig. 2. Time-course and saliva protein-concentration dependence of phospholipase A_2 (PLA_2) activity. (A) Time-course of PLA_2 activity of *B. anurum* saliva. Enzymatic activity was measured using 10 µg of total *B. anurum* saliva protein and NBD-PC as substrate in a fluorescence spectrometer. (B) Dose dependence of PLA_2 activity after 120 min of incubation of the saliva protein content. *B. anurum* saliva was incubated in reaction media with 500 ng PED6 and the products of the reaction were separated by TLC. The TLC plate was scanned using a Storm 860 laser scanner and fluorescence of fatty acids were determined by Image Quant and plotted.

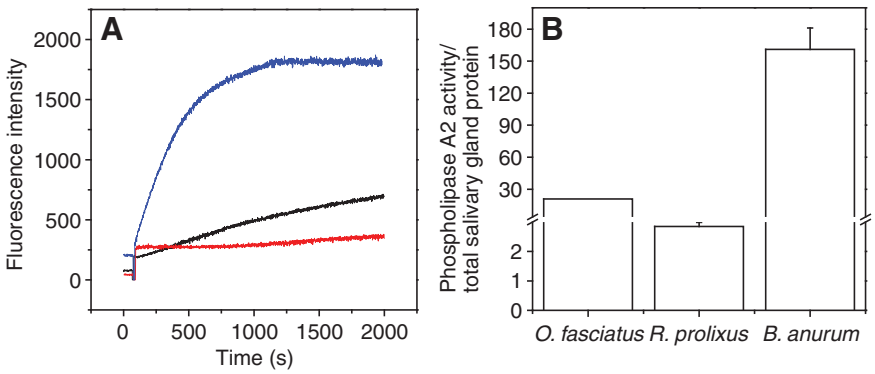

Fig. 3. Comparative PLA_2 activity of three insects with different alimentary habits. *Oncopeltus fasciatus* and *Rhodnius prolixus* were dissected and salivary were glands removed. Glands were punctured in a drop of saline to release the content of the lumen. The saliva of *B. anurum* was collected by electrical stimulation. (A) Samples were assayed for PLA_2 activity using NBD-PC as a substrate (10 μg of proteins from each sample): *R. prolixus* (red line); *O. fasciatus* (black line); *B. anurum* (blue line). (B) Fluorescence intensity in 1500 s was divided by total protein content of saliva (25 μg, *O. fasciatus*; 100 μg, *R. prolixus*; 10 μg, *B. anurum*).

neurons with depletion of synaptic vesicles (Rigoni et al., 2005; Bonanomi et al., 2005). BaLPC caused nerve terminal bulging (Fig. 4B), and a similar effect was caused by the saliva from *B. anurum* (not shown). The presence of FAs in saliva enhanced the paralyzing effect of LPC. It has previously been shown that FA and LPC act synergistically to induce a reduction in the number of synaptic vesicles and an enlargement of the nerve terminals (Rigoni et al., 2005).

To test the paralyzing effect of *Belostoma* saliva and LPC on animals, we chose zebrafish, which are of a similar size to prey fish present in the river waters where *Belostoma* is active. Intramuscular injection of 50 μl of *Belostoma* saliva or of a BaLPC suspension (40 mmol l^{-1}) caused paralysis followed by death (Table 3). Paralysis usually developed within 5 min of injection and death followed, usually 30 min post-injection. Taken together, these results indicate that BaLPC is very effective in inducing paralysis and death in fish, and support the hypothesis that it is involved in the process of capture and immobilization of prey by *Belostoma*.

DISCUSSION

LPC produced by the hydrolysis of PC catalyzed by PLA_2 mediates a variety of physiological and pathological effects (Matsumoto et al., 2007; Kougias et al., 2006). Among other activities, LPC is known to be a powerful modulator of endothelium-dependent arterial relaxation (Kugiyama et al., 1990). We showed that it is present in the saliva of a blood-sucking insect, *R. prolixus*, one of the main vectors of Chagas disease in South America (Golodne et al., 2003). In addition, we showed that it might act as an antihemostatic molecule during blood feeding, by blocking platelet aggregation and inducing NO production in cultured endothelial cells. *R. prolixus* saliva LPC is injected into human skin during hematophagy, and we have recently shown that LPC plays a role in the mechanism of vertebrate-host *Trypanosoma cruzi* infection, as it increases the association between the injected parasite and target macrophages, and inhibits macrophage and dendritic cell activation, which could attenuate *T. cruzi*-induced immune responses (Mesquita et al., 2008).

In the present study, we show that lipids are abundant in the saliva of an arthropod with predation habits (Table 1), and that LPC is the second-most abundant phospholipid present in this secretion after PC (Fig. 1C,D). Accordingly, we detected strong PLA_2 activity in *Belostoma* saliva. The hydrolysis of PC is only partial, and this is most probably because of a product-inhibition enzymatic effect. A comparison of the PLA_2 activity in the saliva of insects with different feeding habits shows that *B. anurum* exhibits the strongest enzymatic generation of LPC.

The major finding of the present paper is that LPC is functional in the predator lifestyle of *B. anurum* owing to its strong neuroparalytic activity, as shown *in vitro* and *in vivo*. We show here that both saliva and isolated BaLPC inhibit neurally evoked twitches of the mouse hemidiaphragm and cause bulging of nerve terminals. In addition, we have reported here that zebrafish are efficiently immobilized and killed when injected with sufficient amounts of lysophospholipid. These results indicate that the *B. anurum* saliva purified LPC might act similarly to snake LPC, as this causes a similar bulging of nerve terminals. Immobilization is a prerequisite for the subsequent injection of the saliva into the prey to be digested before ingestion. Previous studies focused on antihemostatic and digestive properties of salivary components of arthropods engaged in Type I EOD (Swart et al., 2006). Here, we have focused on a previously overlooked aspect of major importance in feeding success. The results obtained allow us to suggest that BaLPC might be used in prey capture by predator insects and other animals.

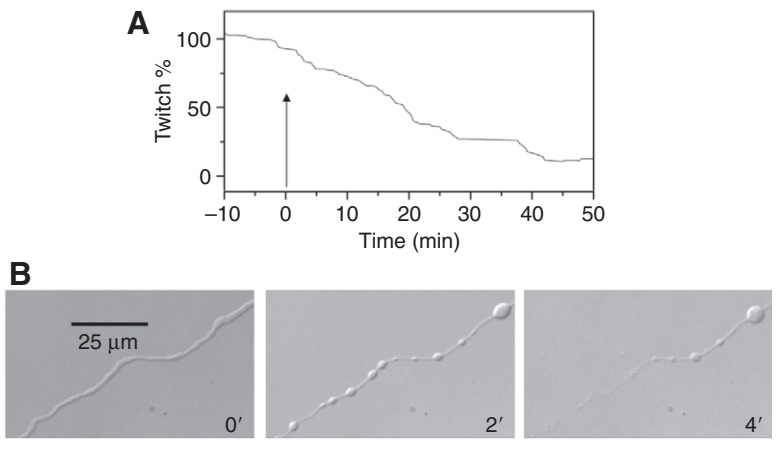

Fig. 4. Effects of *B. anurum* saliva-purified LPC in neuromuscular junction and motor neurons. (A) Effect of BaLPC (bath concentration 150 μM, arrow indicates its addition) on the nerve-stimulated contraction of mouse phrenic nerve hemidiaphragm. (B) Representative images of rat spinal cord motor neuron terminals before (left panel), and 2 and 4 min after addition of BaLPC (150 μM) (centre and right panel, respectively). Bar: 25 μm.

LIST OF ABBREVIATIONS

BaLPC	*Belostoma anurum* saliva-purified LPC
EOD	extra oral digestion
FA	fatty acid
LPC	lysophosphatidylcholine
NBD-PC	1-acyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine
NMJ	neuromuscular junction
NO	nitric oxide
oxLDL	low density lipoprotein, oxidated
PC	phosphatidylcholine
PED6	*N*-{[6-(2,4-dinitrophenyl) amino] hexanoyl}-2 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl) 1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine
PGE2	prostaglandin E2
PLA_2	phospholipase A_2
SCMNs	primary rat spinal motor neurons
TLC	thin layer chromatography

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