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Lysophosphatidylcholine Acts as an Anti-hemostatic Molecule in the Saliva of the Blood-sucking Bug *Rhodnius prolixus**

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Blood-sucking arthropods possess a variety of anti-hemostatic factors in their salivary glands to maintain blood fluidity during feeding. In this work we demonstrate the anti-hemostatic properties of lysophosphatidylcholine (lysoPC) isolated from the salivary glands of *Rhodnius prolixus*. First, we examined salivary glands of fourth and fifth instar nymphs for their phospholipid composition. The lumen displayed an accumulation of its phospholipid content, mainly phosphatidylcholine and lysoPC, with a 6-fold increase for the latter. To determine the presence of phospholipids in the saliva, fourth instar nymphs were fed with a ^{32}P-enriched blood meal. After 28 days their saliva was collected and subjected to lipid extraction, thin-layer chromatography, and autoradiography. The results showed the presence in the saliva of the same phospholipids present in the lumen. We then examined possible biological roles of these phospholipids when compared with other known effects of lysoPC. The luminal lipid extract and purified lysoPC from the lumen and saliva were tested for inhibition of washed rabbit platelets' aggregation induced by α-thrombin and platelet-activating factor. Both the luminal lipid extract and salivary lysoPC showed an increasing inhibition of aggregation, which correlated with the response of the platelets to standard lysoPC (up to 13 μg/ml). Next, salivary lysoPC was incubated with porcine arterial endothelial cells for 24 h. After incubation, culture medium was assayed for nitric oxide and showed increased nitric oxide production, similar to control cells exposed to standard lysoPC (up to 20 μg/ml). Together these data demonstrate the presence of lysoPC in the saliva of *Rhodnius prolixus* and its potential anti-hemostatic activities.

Hematophagous arthropods rely on a wide array of anti-hemostatic substances to counteract vertebrate responses to blood loss and to obtain their blood meal successfully (1). These salivary compounds show distinct properties and are generally involved with inhibition of coagulation, platelet aggregation, and vasoconstriction (2). These molecules include peptides such as tachykinins (3), maxadilan (4), nitric oxide (NO)[1]-binding proteins such as nitrophorins (5), and prostaglandins (6). The small lesions elicited by the mouthparts of the arthropod most likely evoke platelet aggregation and vasoconstriction by the vertebrate host. Therefore, the formation of the platelet plug is specifically inhibited by collagen inhibitors, apyrases, catechol oxidases, thrombin inhibitors, NO-releasing proteins, fibrinogen receptor agonists, and a specific platelet aggregation inhibitor (7, 8). The complexity of anti-hemostatic mechanisms has recently been addressed with the use of proteomic techniques (9, 10). Hundreds of gene sequences were obtained, and most of them await future tests concerning the anti-hemostatic properties of the proteins they code for.

Lysophosphatidylcholine (lysoPC) is a component of oxidized low-density lipoprotein, which is involved in the pathogenesis of atherosclerosis and inflammation (11, 12). Recently, great research efforts have been directed to understand and characterize lysoPC effects on cell biology (13). The list is continuously growing and is quite diverse, including the induction of endothelial genes involved in early atherosclerosis, such as adhesion molecules and growth factors (14, 15), and secretion of matrix metalloproteinase (16). Indeed, the lysoPC content of atherosclerotic arteries is higher than in normal vessels, and oxidized low-density lipoprotein displays a great proportion of lysoPC (17). LysoPC is able to increase the production of NO by endothelial cells by enhancing endothelial NO synthase (e-NOS) transcription (18, 19), besides acting as an inhibitor of platelet aggregation (20). This evidence shows that lysoPC is potentially pro-atherogenic by inducing the production of several growth factors and the expression of chemoattractant genes (21). On the other hand, lysoPC also can display an important anti-atherogenic function because it increases the local synthesis of NO in the initial phases of the formation of atherosclerotic plaques, leading to vasodilation (22, 23).

To date, anti-hemostatic activities found in salivary secretions from blood-feeding arthropods are mainly of proteic nature or are gases such as NO (24). In the present study we demonstrate, for the first time, the presence of phospholipids in the saliva of a blood-sucking insect, *Rhodnius prolixus*. The dynamics of the accumulation of phospholipids in the growing salivary glands, as well as salivary lysoPC anti-hemostatic properties, are also described.

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[1] The abbreviations used are: NO, nitric oxide; e-NOS, endothelial nitric oxide synthase; lysoPC, lysophosphatidylcholine; PAEC, porcine arterial endothelial cells; PAF, platelet-activating factor; PC, phosphatidylcholine; TLC, thin-layer chromatography.

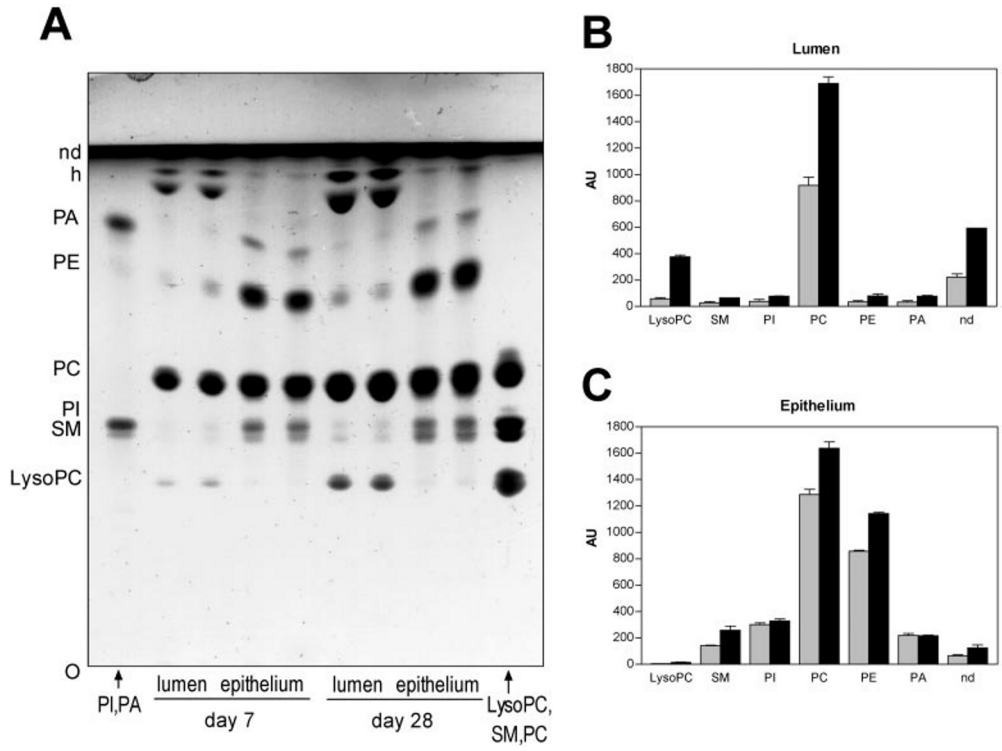

FIG. 1. **Phospholipid composition of the lumen and epithelium of salivary glands.** Fourth and fifth instar insects (days 7 and 28 post blood meal, respectively; $n = 15$) were dissected and the salivary glands removed. Glands were punctured in a drop of saline, releasing the contents of the lumen, and the epithelium was recovered. Samples were subjected to lipid extraction and TLC. *A*, TLC plate, salivary glands from fourth instar nymphs (*day 7*), and fifth instar *nymphs (day 28*), *O*, origin; *lysoPC*, lysophosphatidylcholine; *SM*, sphingomyelin; *PI*, phosphatidylinositol; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PA*, phosphatidic acid; *nd*, not determined; *h*, heme pigment. The following phospholipid standards were also applied: PI (30 μg) and PA(10 μg), LysoPC (30 μg), SM (15 μg), and PC (20 μg). *B* and *C*, TLC was scanned and subjected to densitometric analysis (*gray bars*, day 7; *black bars*, day 28; *AU*, arbitrary units).

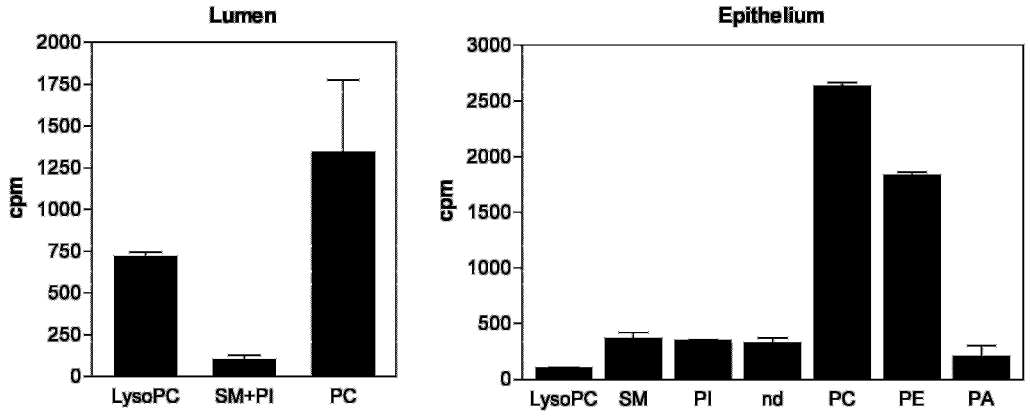

FIG. 2. ***In vivo* ^{32}P-labeling of phospholipids of the salivary glands.** Fourth instar insects were fed with a ^{32}P-enriched blood meal (<1μCi of ^{32}P per insect). After 28 days, molted insects were dissected (2 groups of 20 insects each), and the lumen and epithelium were isolated. Samples were subjected to lipid extraction followed by TLC and autoradiography. The radioactive spots were scraped from the plate and subjected to liquid scintillation counting.

EXPERIMENTAL PROCEDURES

Obtainment of Salivary Glands and Phospholipid Profile Analysis—Insects were reared and handled as described previously (25). Fourth and fifth instar nymphs of *R. prolixus* kept in our departmental colony at 28 °C, 70% humidity and fed at 28-day intervals were used in this study. Salivary glands were isolated in a drop of saline, cleaned of any adhering tissue, rinsed twice in saline, and gently punctured under a stereo-microscope by means of a fine syringe needle, while holding the gland with forceps. The emptied epithelium was removed from the drop and further rinsed, and the drop containing the luminal contents was recovered. Both samples were subjected to lipid extraction (26). The phospholipid profile of the salivary glands was examined after TLC on silica gel plates using a mixture of acetone:methanol:acetic acid:chloroform:water (15:13:12:40:8, v/v). After evaporation of the solvents the plate was immersed for 15 s in a charring solution consisting of 10% $CuSO_4$, 8% H_3PO_4 and heated to 170 °C for 5–10 min (27). The charred TLC plate was then subjected to densitometric analysis. Each phospholipid spot was identified by comparing to phospholipid standards (Sigma) run in parallel.

Salivary Gland Labeling—^{32}P-labeled salivary glands were obtained following a ^{32}P-enriched blood meal offered to fourth instar nymphs, using established procedures (28). After 28–30 days, the molted fifth instar nymphs were dissected and their salivary glands removed and analyzed as above. Following TLC, the plate was subjected to autoradiography, and the corresponding ^{32}P-phospholipids were scraped from the plate after coating with Strip-Mix (Alltech, Deerfield, IL) and subjected to liquid scintillation counting.

Saliva Obtainment—Fifth instar nymphs were allowed to repeatedly probe a Parafilm membrane of an artificial feeder filled with deionized water for 2 min. After several collection cycles the contents of the artificial feeder were collected, and the Parafilm was cut and rinsed with methanol. Samples were combined and subjected to lipid extrac-

tion. To obtain ^{32}P-labeled saliva, fourth instar nymphs were fed with a ^{32}P-enriched blood meal as described above, and saliva was collected after 28–30 days.

Lipid Extraction from TLC Plate—Purification of TLC-separated lipids was performed essentially as described by Yuan *et al.* (20), with modifications. Briefly, after staining with iodine, the target phospholipid spot was scraped into glass tubes and vortexed with 4 ml of an extraction solution consisting of methanol:iso-butanol:H_2O (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 rpm for 2 min, and the supernatant was collected. This procedure was repeated two more times, and to the combined supernatants (~12 ml) 3 ml of chloroform were added. After intense vortexing and centrifugation (3000 rpm, 8 min), the lower organic phase was recovered and dried under a stream of N_2. Purified lipids were then resuspended in phosphate-buffered saline and used in the assays.

Platelet Aggregation Assays—Washed rabbit platelets were obtained from blood anticoagulated with 5 mM EDTA. Platelets were isolated by centrifugation and washed twice according to Zingali *et al.* (29) with calcium-free Tyrode's buffer, pH 6.5, containing 0.1% glucose, 0.2% gelatin, 0.14 M NaCl, 0.3 M $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 2.7 mM KCl, and 0.2 mM EGTA. Washed platelets were resuspended in a modified Tyrode's buffer, pH 7.4, containing 2 mM $CaCl_2$ at 300,000–400,000 cells/μl. Assays were performed at 37 °C using a Chronolog Aggregometer (Havertown, PA). Aggregation in the volume of 300 μl was induced either by α-thrombin (5 μl of a 60-nM stock solution, 1 nM final concentration) or platelet-activating factor (PAF) (5 μl of a 100-nM stock solution, 1.67-nM final concentration). Molecules to be tested for inhibition of platelet aggregation were added 1 min before induction. The inhibition was calculated using the maximum peak height of each tracing, which was compared with control incubation values.

Cell Culture and NO Production—Porcine aortas were obtained in a local slaughterhouse, and endothelial cells (PAEC) were isolated after dissection of the aortic artery. Arteries were cut open longitudinally, exposing the endothelium. After being rinsed several times with ice-cold phosphate-buffered saline, a small amount of Dulbecco's modified Eagle's medium was added, the surface was gently scrubbed with a cell scraper, and cells were recovered. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (for 3–4 passages) and seeded in 6-well plates. Twenty-four hours before each experiment, serum-free fresh medium was added. Incubations were carried out for 24 h with serum-free medium (1 ml), after which the culture medium was recovered. To determine the nitrite content, 50-μl aliquots were incubated with 10 μl of a solution containing 2,3-diaminonaphtalene (DAN, Calbiochem) for 10 min in a final volume of 100 μl according to the manufacturer's instructions. Reactions were terminated with 20 μl of 2.8 M NaOH, diluted to 1 ml with deionized water, and the fluorescent nitrite adduct was measured at 365 nm excitation and 450 nm emission.

RESULTS

LysoPC Is Accumulated in Salivary Glands—After blood feeding, insects have to refill the contents of their salivary glands while preparing for a next blood meal. To characterize the phospholipid profile of *R. prolixus* salivary glands, we used fourth instar nymphs from day 7 post blood meal and nymphs from day 28 post blood meal (already molted to fifth instars). The luminal contents and the epithelium were separated and subjected to TLC. The results confirmed the presence of phospholipids in both compartments with an overall increase from day 7 to 28 (Fig. 1*A*). LysoPC content is specifically increased by ~6-fold in the lumen of the gland after insect molting, which represents the greatest increase among all lipid classes analyzed. The epithelium displayed great amounts of phosphatidylcholine (PC) and phosphatidylethanolamine, and to a lesser extent, sphingomyelin, phosphatidylinositol, phosphatidic acid, and a non-determined lipid, with trace amounts of lysoPC (Fig. 1*C*). Most importantly, the profile of luminal phospholipids was quite different from that observed for the epithelium, consisting mainly of PC and lysoPC with minor amounts of sphingomyelin, phosphatidylinositol, and a non-determined lipid (Fig. 1*B*). The percentage of each phospholipid remained roughly constant in both compartments throughout development, with the exception of luminal lysoPC (increasing from 5% on day 7 to 13% on day 28) and PC (decreasing from 73% on day 7 to 60% on day 28). Therefore, on day 28 when the glands have already been refilled and the nymphs are ready for a new blood meal, the concentration of luminal lysoPC of a single salivary gland pair would be 75 μg/ml, as estimated with a standard lysoPC curve.

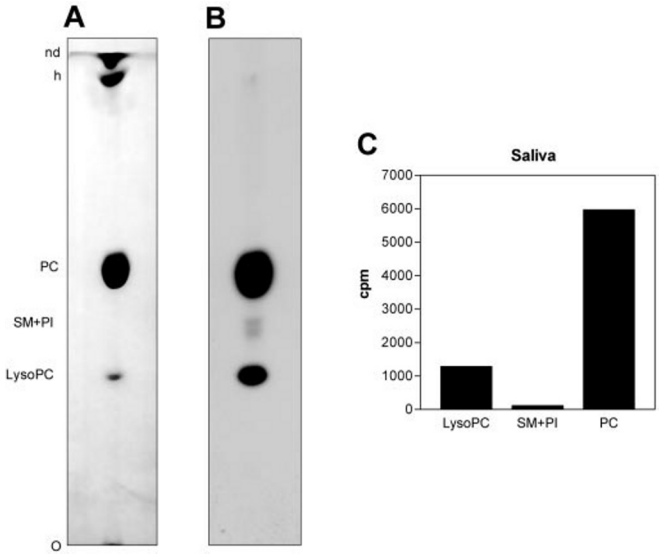

FIG. 3. **Phospholipid composition of the saliva.** Fourth instar insects (n = 200) were fed with a ^{32}P-enriched blood meal (<1μCi of ^{32}P per insect). After 28–32 days, molted insects were separated in small groups (20 each) and the saliva collected by means of an artificial feeder filled with deionized water. Samples were subjected to lipid extraction, followed by TLC (*panel A*, iodine-stained) and autoradiography (*panel B*). Radioactive spots were scraped and subjected to liquid scintillation counting (*C*).

Following a ^{32}P-enriched blood meal, we verified the incorporation of label in the phospholipids present both in the lumen and epithelium of the salivary glands (Fig. 2) with a pattern similar to that observed with non-labeled phospholipids from the 28th day post blood meal. Next, we sought to determine whether the saliva also contained these phospholipids. After collection of saliva from ^{32}P-labeled nymphs (Fig. 3) and lipid extraction followed by TLC (Fig. 3*A*), the autoradiograph confirmed the presence of labeled PC and lysoPC (Fig. 3*B*). The amount of label in each phospholipid present in the saliva was determined (Fig. 3*C*) and found to be similar to the profile previously observed for the luminal phospholipids. Therefore, these experiments linked blood feeding with the refilling of phospholipids (and other anti-hemostatic components) in the salivary glands: nymphs are able to use precursor molecules obtained from their meal to accumulate luminal phospholipids that could be used during the next feeding. The experiments also show that phospholipids, including lysoPC, are a component of *R. prolixus* saliva.

Salivary LysoPC Shows Anti-hemostatic Properties—To clarify the possible roles of this lipid component present in the saliva we focused on lysoPC, a molecule with well documented effects in important aspects of hemostasis. The first possibility analyzed was that the presence of lysoPC in saliva injected into the vessel could inhibit platelet aggregation. When washed rabbit platelets were induced to aggregate by α-thrombin and PAF, addition of increasing amounts of standard lysoPC resulted in a dose-dependent inhibition (Fig. 4*A*). This inhibition was also verified when the assay was promoted in the presence of the luminal lipid extract; when using extract from 20 pairs of salivary glands, the final concentration of lysoPC in the assay corresponds to 10 μg/ml (Fig. 4*B*). This effect suggested lysoPC as an effective component of the luminal lipids in platelet

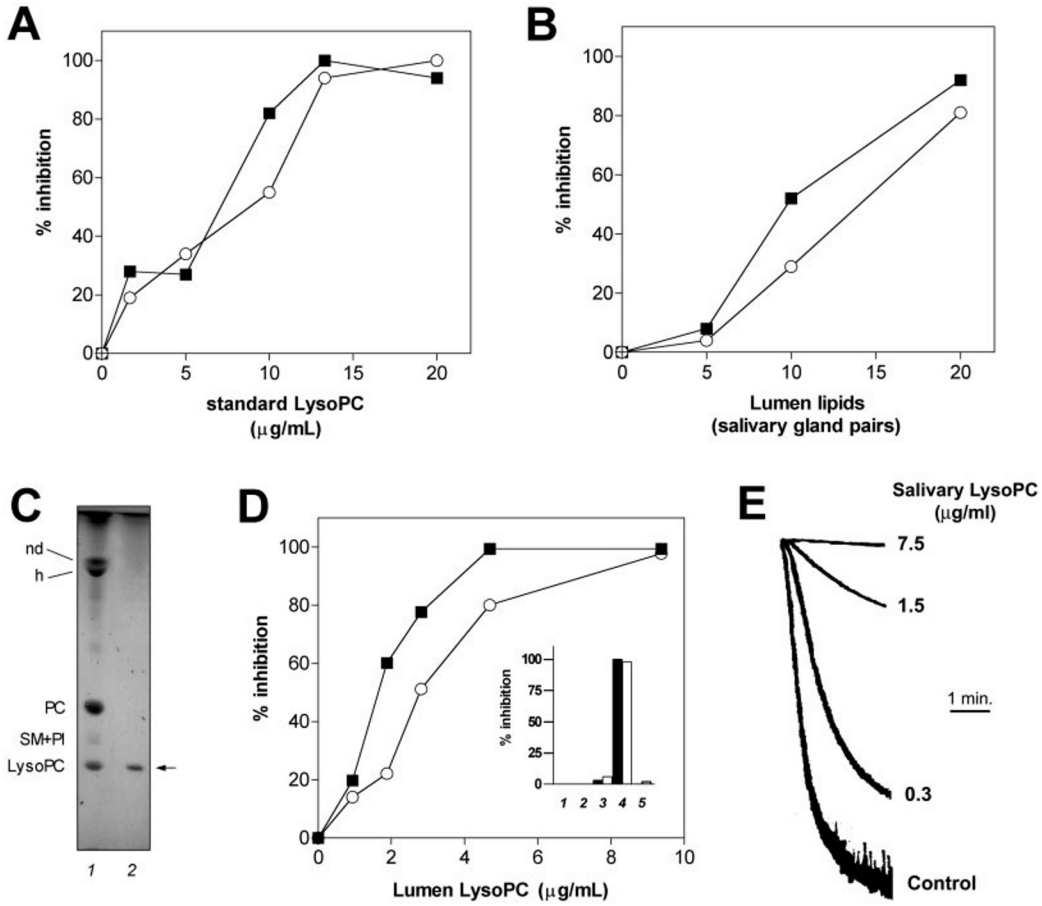

FIG. 4. **Lysophosphatidylcholine from the lumen and saliva of salivary glands inhibits platelet aggregation.** Washed rabbit platelets were isolated, and aggregation in the volume of 300 μl was induced by 1 nM α-thrombin (■) or 1.67 nM PAF (○) in the presence or absence of the inhibitors. *A*, standard lysoPC. *B*, lipids extracted from the lumen of salivary glands. *C*, TLC showing the luminal lipid extract (*1*) and purified luminal lysoPC (*2*). *D*, lysoPC purified from the lumen of salivary glands. *Inset*, controls. *1*, PC; *2*, lysophosphatidylethanolamine; and samples subjected to the lipid purification procedure (*3*, blank silica; *4*, lysoPC; and *5*, PC). *Black bars*, α-thrombin; *white bars*, PAF. *E*, inhibition of α-thrombin-induced platelet aggregation by lysoPC purified from the saliva. The standard lysoPC concentration required in all assays to achieve 100% inhibition of α-thrombin-induced platelet aggregation ranged from 8–13 μg/ml.

aggregation inhibition. When lysoPC was purified from the luminal extract (Fig. 4*C*), addition of increasing amounts of this purified lysoPC to the platelet suspension led to a similar inhibition response (Fig. 4*D*). Control assays showed that either PC or lysophosphatidylethanolamine, respectively a lysoPC parent molecule and a related lysophospholipid, could not inhibit platelet aggregation (Fig. 4*D*, *inset*). PC purified from the lumen showed no inhibition of aggregation, similar to standard PC (data not shown). By adding excess inducers (9-fold for PAF and 140-fold for α-thrombin) in the presence of lysoPC, aggregation was readily restored, confirming platelet viability (data not shown). Moreover, lysoPC purified from the saliva elicited a marked inhibition on platelet aggregation induced by α-thrombin (Fig. 4*E*). Hence the lysoPC found in the lumen of the salivary glands and secreted into the vertebrate host as saliva acts as an inhibitor of platelet aggregation.

Another important effect of lysoPC is increased NO production by endothelial cells. Using cultured PAEC we were able to test the effect of standard lysoPC or salivary lysoPC on the amount of NO end products released to the culture medium (Fig. 5). Cells incubated with increasing amounts of standard lysoPC showed an increase in NO production when compared with control cells, whereas standard PC was ineffective (Fig. 5, *inset*). When cells were exposed to the purified salivary lysoPC there was a correspondent increase in NO production similar to that observed for the standard lysoPC response; salivary PC did not elicit NO production in these cells. These data confirm that the lysoPC present in the saliva is able to increase NO production by the endothelial cells in culture.

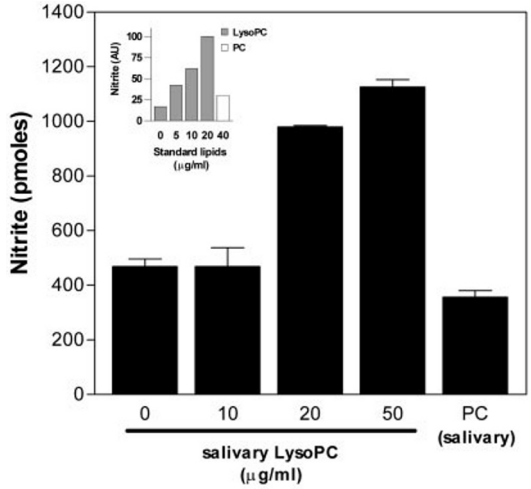

FIG. 5. **Salivary lysoPC elicits increased NO production by PAECs.** Confluent PAECs were incubated for 24 h in the presence or absence of either standard lipids (*inset*: lysoPC, *gray bars;* PC, *white bar*), purified salivary lysoPC (0–50 μg/ml), or salivary PC (50 μg/ml). The incubation medium was collected and assayed for NO_2 produced as described under "Experimental Procedures."

DISCUSSION

Hematophagous arthropods are able to effectively block the host's response that would lead to reduced blood ingestion. In *R. prolixus* the main anti-hemostatic activities can be assigned to a series of NO-releasing proteins denoted nitrophorins, which release NO when injected into the bloodstream of the host, leading to increased vasodilation and inhibition of platelet aggregation; to an apyrase, which upon cleavage of ADP also reduces platelet aggregation; and finally to an ADP-binding lipocalin termed RPAI-1 (*R. prolixus* aggregation inhibitor-1) (8). Hence the formation of the platelet plug is the target for several molecules described in *R. prolixus* saliva, as well as other arthropods.

The lipid profile of the salivary glands of blood-sucking arthropods has been examined in detail for the lone-star tick, *Amblyomma americanum* (30). In this case careful work has been undertaken in analyzing the arachidonic acid (AA) profile associated with each lipid present in the salivary glands of the tick, especially phospholipids, and in relating AA levels to the occurrence of salivary prostaglandins. One of the main aspects concerning the presence of these prostaglandins, besides immunosuppressive and vasodilatory activities, is platelet aggregation inhibition. Thus, ticks are able to produce prostaglandins from AA derived from dietary lipids, which is mobilized upon hydrolysis of phospholipids present in the epithelium of the salivary glands by phospholipase A_2 (31). However, these studies focus on the neutral lipid and phospholipid profiles of whole salivary glands, using homogenized glands as the starting material. Also, the phospholipids present in the epithelium are considered mainly as a source of AA, which is subsequently converted to prostaglandins via the cyclooxygenase pathway and secreted into the lumen. The presence of salivary prostaglandins is, to date, the only demonstration of lipid-derived anti-hemostatic molecules in arthropods. The present work is the first to consider the presence of phospholipids in the salivary glands, besides the epithelium of the glands, where the presence of phospholipids is expected because of its membranous structure. We have examined the lumen of glands for their phospholipid content because it contains the salivary secretion to be injected into the host. The method used throughout this work, consisting of extrusion of the luminal contents followed by recovery and analysis of the separated "compartments," unequivocally showed the presence of phospholipids in the lumen of the glands with a distinct composition when compared with the epithelium. This luminal phospholipid profile suggested to us that phospholipids are possibly being injected into the vertebrate bloodstream during feeding. To further demonstrate this and confirm their occurrence, we collected saliva from insects, and the analysis showed the main presence of PC and lysoPC.

The presence of lysoPC in the saliva would suggest the possibility of increasing blood-feeding effectiveness by reducing anti-hemostatic responses. Accordingly, some of the diverse cellular responses to lysoPC are related to counteracting hemostasis, mainly by inhibiting platelet aggregation (20) and increasing the amount of e-NOS in endothelial cells (32). The salivary lysoPC elicited an increased production of NO in PAEC. This effect is similar to the described response for lysoPC in other cultured endothelial cells. The role of lysoPC in the increased synthesis of e-NOS has been clarified in human umbilical vein endothelial cells and bovine arterial endothelial cells (18, 19). Treatment of endothelial cells with lysoPC resulted in an increased transcription of the e-NOS mRNA in these cells, followed by an increased amount of protein and the corresponding activity. The time span needed for this response (33, 34, 18) in the case of injected saliva would preclude a significant role of lysoPC regarding the increase in local NO levels, because the feeding of *R. prolixus* is successfully accomplished in a few minutes (35). Perhaps in ticks, which remain attached to the host on the same site for a greater period, this role of lysoPC would be more noticeable.

Among the effects evoked by lysoPC, evidence shows that it has potent platelet inhibitory effects (20) and is essential for the inhibition of aggregation by secretory phospholipase A_2 (20), which uses circulating lipoproteins as a lipid substrate source. Inhibition was verified with a number of different agonists, involving inhibition of fibrinogen binding and platelet shape change (20). The molecular mechanism of this inhibition involves a stimulatory G-protein linked to the activation of adenylyl cyclase, which results in the accumulation of cytosolic cAMP, an event that down-regulates all signaling events required for activation (36). The potent inhibition of platelet aggregation promoted by lysoPC purified from both the lumen and saliva confirms its role as an anti-hemostatic molecule. In this case, as opposed to the long-term response leading to increased NO, the inhibitory effect occurs in a more appropriate time span and seems to be a more important role for salivary lysoPC in *R. prolixus*. The actual concentration of lysoPC in the feeding site would amount to ~15 μg/ml, which is close to the range capable of full inhibition of platelet aggregation in our assays.

LysoPC has long been thought of as a pro-atherogenic molecule (17). Only recently its anti-atherogenic profile has emerged, specifically by eliciting increased NO production by endothelial cells (32), increased thrombomodulin expression (36), and reduced tissue-factor expression in human monocytes (37). Therefore, lysoPC would have a dual role in atherogenesis, with both pro- and anti-atherogenic effects. The finding of lysoPC in the saliva of *R. prolixus* further suggests an anti-hemostatic/anti-atherogenic character for this molecule, which would account for several controversies regarding the initial stages of atherosclerosis (38, 12). Finally, the data reported in the present work demonstrate the dual anti-hemostatic role of lysoPC (platelet-inhibitory and NO-inducer) in *R. prolixus* salivary secretions, including this lipid molecule as a novel anti-hemostatic compound found in the saliva of blood-sucking insects.

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