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The Relationship Between Calcium and the Metabolism of Plasma Membrane Phospholipids in Hemolysis Induced by Brown Spider Venom Phospholipase-D Toxin

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ABSTRACT

Brown spider venom phospholipase-D belongs to a family of toxins characterized as potent bioactive agents. These toxins have been involved in numerous aspects of cell pathophysiology including inflammatory response, platelet aggregation, endothelial cell hyperactivation, renal disorders, and hemolysis. The molecular mechanism by which these toxins cause hemolysis is under investigation; literature data have suggested that enzyme catalysis is necessary for the biological activities triggered by the toxin. However, the way by which phospholipase-D activity is directly related with human hemolysis has not been determined. To evaluate how brown spider venom phospholipase-D activity causes hemolysis, we examined the impact of recombinant phospholipase-D on human red blood cells. Using six different purified recombinant phospholipase-D molecules obtained from a cDNA venom gland library, we demonstrated that there is a correlation of hemolytic effect and phospholipase-D activity. Studying recombinant phospholipase-D, a potent hemolytic and phospholipase-D recombinant toxin (LiRecDT1), we determined that the toxin degrades synthetic sphingomyelin (SM), *lyso*phosphatidylcholine (LPC), and *lyso*-platelet-activating factor. Additionally, we determined that the toxin degrades phospholipids in a detergent extract of human erythrocytes, as well as phospholipids from ghosts of human red blood cells. The products of the degradation of synthetic SM and LPC following recombinant phospholipase-D treatments caused hemolysis of human erythrocytes. This hemolysis, dependent on products of metabolism of phospholipids, is also dependent on calcium ion concentration because the percentage of hemolysis increased with an increase in the dose of calcium in the medium. Recombinant phospholipase-D treatment of human erythrocytes stimulated an influx of calcium into the cells that was detected by a calcium-sensitive fluorescent probe (Fluo-4). This calcium influx was shown to be channel-mediated rather than leak-promoted because the influx was inhibited by L-type calcium channel inhibitors but not by a T-type calcium channel blocker, sodium channel inhibitor or a specific inhibitor of calcium activated potassium channels. Finally, this inhibition of hemolysis following recombinant phospholipase-D treatment occurred in a concentration-dependent manner in the presence of L-type calcium channel blockers such as nifedipine and verapamil. The data provided herein, suggest that the brown spider venom phospholipase-D-induced hemolysis of human erythrocytes is dependent on the metabolism of membrane phospholipids, such as SM and LPC, generating bioactive products that stimulate a calcium influx into red blood cells mediated by the L-type channel. J. Cell. Biochem. 112: 2529–2540, 2011.

KEY WORDS: PHOSPHOLIPASE-D; HEMOLYSIS; CALCIUM; LIPID METABOLITES; BROWN SPIDER; VENOM

The dermonecrotic toxin (phospholipase-D) present in the brown spider (genus *Loxosceles*) venom is a potent active molecule, with several biological effects on hemostasis and the inflammatory response. This molecule is part of a family of powerful toxins containing several members with related activities [da Silva et al., 2004; Kalapothakis et al., 2007]. Members of this toxin family trigger platelet aggregation, cause hemolysis, increase vessel permeability, induce a potent inflammatory response, are related

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with nephropathy, and cause dermonecrosis after accidents [Chaim et al., 2006; Appel et al., 2008; Kusma et al., 2008; Chaves-Moreira et al., 2009].

Through proteomic approaches, and using crude venom of different *Loxosceles* species, the existence of 11 distinct toxins (*Loxosceles gaucho* venom) belonging to the family of phospholipases-D was reported [Machado et al., 2005], with three isoforms of phospholipases-D (*Loxosceles bonetti* venom) [Ramos-Cerrillo et al., 2004] and three toxin isoforms in the venom of *Loxosceles reclusa* [dos Santos et al., 2009]. Using molecular biology techniques, six different phospholipase-D isoforms were cloned and expressed from a cDNA library of the venom gland of *L. intermedia* [Chaim et al., 2006; da Silveira et al., 2006, 2007a, b; Appel et al., 2008]. Additionally, from the venom glands of other *Loxosceles* species, the existence of other phospholipase-D isoforms was found, which increases the number of toxin members and strengthens the idea that they are a family of toxins that act synergistically [Binford et al., 2005; Kalapothakis et al., 2007].

The first studies regarding the biochemistry of the brown spider venom dermonecrotic toxin characterized this molecule as a sphingomyelinase-D, based on its activity to hydrolyze sphingomyelin (SM) into choline and C1P [Futrell, 1992]; however, based on lipid hydrolysis analysis following toxin treatments, the term phospholipase-D was postulated because members of this toxin family hydrolyzes sphingolipids and *lyso*glycerophospholipids, generating C1P or *lyso*phosphatidic acid (LPA) [Lee and Lynch, 2005].

Some lipid metabolites that originate from cell membranes such as ceramide, C1P, sphingosine, sphingosine-1-phosphate, phosphatidic acid, and LPA are bioactive molecules essential for cell functions such as signal transduction, cell cycle arrest, apoptosis, plasma membrane viability, cell differentiation, and others [Ohanian and Ohanian, 2001; Anliker and Chun, 2004; Mitsutake and Igarashi, 2007].

Several case reports have described intravascular hemolysis following brown spider accidents [Williams et al., 1995; de Souza et al., 2008; McDade et al., 2010]. This hemolytic activity is confirmed by in vitro laboratory tests that pointed to *Loxosceles* venom phospholipase-D as a hemolytic agent [Ribeiro et al., 2007; Chaves-Moreira et al., 2009; Tambourgi et al., 2010]. The mechanism by which brown spider venom phospholipase-D causes hemolysis is currently under investigation. The degradation of membrane phospholipids by brown spider phospholipase-D triggered the synthesis of inflammatory molecules such as arachidonic acid and Prostaglandin E2 (PGE2) [Chalfant and Spiegel, 2005]. Phospholipase-related hemolysins from several pathogens have been shown to be involved in membrane fluidity and in the stimulation of calcium ion cytosolic activity [Lang et al., 2010; Oda et al., 2010]. Molecules such as PGE2, C1P, and LPA have been reported to increase intracellular calcium ion in erythrocytes in vitro [Yang et al., 2000; Chalfant and Spiegel, 2005; Kaestner et al., 2006].

Recently, we compared the hemolytic activities of one brown spider venom recombinant active phospholipase-D [Chaim et al., 2006] and this same molecule with a site-directed mutation H12A in the catalytic domain [Kusma et al., 2008]. The results showed hemolytic activity for active phospholipase-D and an absence of hemolytic activity for the mutated toxin, suggesting the direct involvement of the catalysis in hemolysis [Chaves-Moreira et al., 2009]. Herein, using this same model of recombinant toxins, we present additional data that support a direct molecular mechanism dependent on the catalytic activity of phospholipase-D for hemolysis and the relationship between membrane phospholipids toxin-induced metabolism and calcium.

MATERIALS AND METHODS

REAGENTS

Polyclonal antibodies to phospholipase-D toxin were produced in rabbits as previously described [Luciano et al., 2004; Chaim et al., 2006]. Hyperimmune IgGs were purified from serum using a mixture of Protein-A and Protein-G Sepharose beads (GE Healthcare Life Sciences, Uppsala, Sweden) as recommended by the manufacturer. All experimental protocols using animals were performed according to the "Principles of laboratory animal care" (NIH Publication no 85–23, revised 1985), "Brazilian Federal Laws," and the ethical committee agreement number 245 of the Federal University of Paraná. Verapamil, nifedipine, clotrimazole, flunarizine, procaine, ruthenium red, EDTA, $CaCl_2$, sucrose and alkaline phosphatase-conjugated anti-rabbit IgG were purchased from Sigma (St. Louis, MO). Whole venom from *L. intermedia* was extracted from spiders captured from the wild as described by Feitosa et al. [1998].

RECOMBINANT PROTEIN CLONING AND EXPRESSION

The venom gland cDNA library was previously compiled [Chaim et al., 2006; da Silveira et al., 2006]. The GenBank data deposition information for *L. intermedia* cloned LiRecDT1 cDNA is DQ218155, for LiRecDT2 cDNA, DQ266399, LiRecDT3 cDNA, DQ267927, LiRecDT4 cDNA, DQ431848, LiRecDT5 cDNA, DQ431849, and LiRecDT6 cDNA, EF474482.

The cDNA corresponding to the mature phospholipase-D LiRecDT1 protein was amplified by PCR. The forward primer used was 30Rec sense (50-CTCGAGGCAGGTAATCGTCGGCCTATA-30) and was designed to contain an *Xho*I restriction site plus the sequence that is related to the first seven amino acids of the mature protein. The reverse primer was 30Rec antisense (50-CGGGATCCT-TATTTCTTGAATGTCACCCA-30), which contains a *Bam*HI restriction site and a stop codon. The PCR product was cloned into a pGEM-T vector (Promega, Madison). The pGEM-T vector containing the cDNA, which encoded the mature protein, was then digested with *Xho*I and *Bam*HI restriction enzymes. The excised insert was gel purified using a QIAquick Gel 74 Extraction Kit (Qiagen, Valencia) and subcloned into a pET-14b vector (Novagen, Madison) digested with the same enzymes.

The cDNA corresponding to the other isoforms of dermonecrotic toxin "phospholipase-D" proteins were obtained according to da Silveira et al. [2006] for LiRecDT2 and LiRecDT3, according to da Silveira et al. [2007b] for LiRecDT4 and LiRecDT5, according to Appel et al. [2008] for LiRecDT6.

The mutated toxin LiRecDT1 H12A was obtained by a Megaprimer PCR method, which was performed with rounds of PCR to introduce a site-directed mutagenesis in the LiRecDT1 sequence [Kusma et al., 2008]. Briefly, the first round introduced the site-directed mutation into the first histidine amino acid residue of

the catalytic domain using the reverse primer P1H12A (50-ATTTACCATGGCCCCCATGATC-30), which was designed to contain the codon substitute for alanine plus the sequence related to the other original amino acids of the mature protein.

All recombinant constructs were expressed as fusion proteins, with a 6× His-Tag at the N terminus and a 13 amino acid linker (including a thrombin site) between the 6× His-Tag and the mature protein (N-terminal amino acid sequence before the mature protein: MGSSHHHHHHSSGLVPRGSHMLE). pET-14b/*L. intermedia* cDNA constructs were transformed into One Shot *Escherichia coli* BL21(DE3)pLysS competent cells (Invitrogen, Carlsbad) and plated on LB agar plates containing 100 mg/ml ampicillin and 34 mg/ml chloramphenicol. A single colony was inoculated in 50 ml LB broth (100 mg/ml ampicillin and 34 mg/ml chloramphenicol) and grown overnight at 37°C. A 10 ml portion of this overnight culture was grown in 1 L of LB broth/ampicillin/chloramphenicol at 37°C until the OD at 550 nm reached 0.5. IPTG (isopropyl β-D-thiogalactoside) was added to make a final concentration of 0.05 mM, and the culture was induced by incubation for an additional 3.5 h at 30°C (with vigorous shaking). Cells were harvested by centrifugation (4,000*g*, 7 min), and the pellet was frozen at −20°C overnight.

RECOMBINANT PROTEIN PURIFICATION

Cell suspensions were thawed and additionally disrupted by six cycles of 10 s of sonication at low intensity. Lysed materials were centrifuged (20,000*g*, 20 min), and the supernatants were incubated with 1 ml Ni^{2+}-NTA agarose beads for 1 h at 4°C with gentle agitation. The suspensions were loaded into a column, and the packed gel was exhaustively washed with the appropriate buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 20 mM imidazole) until the OD at 280 nm reached 0.01. Recombinant proteins were eluted with 10 ml of elution buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 250 mM imidazole), and 1 ml fractions were collected and analyzed by 12.5% SDS–PAGE under reducing conditions. Fractions were pooled and dialyzed against phosphate buffered saline (PBS).

ERYTHROCYTES

Human erythrocytes were obtained from healthy donors. The blood collection procedure was authorized by the ethical committee agreement of the Federal University of Paraná.

PREPARATION OF ERYTHROCYTES

After blood was harvested with acidic EDTA Na^+ 5% and a 21-gauge needle (BD Plastipak, Franklin Lakes) on the day of each experiment, the platelet-rich plasma and buffy coat were removed by aspiration after centrifugation at 200*g* for 15 min. Packed erythrocytes were washed three times with tris buffer sucrose (TBS) for hemolysis assays (250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 280 mOsm/kg H_2O). Washed erythrocytes were resuspended in the appropriate buffer at a concentration of 5×10^8 cells/ml.

DETERMINATION OF HEMOLYTIC ACTIVITY

The hemolysis assay was performed as described by Harris and Phoenix [1997]. Washed red blood cells (10^8 cells) were added to each Eppendorf tube containing the appropriate concentration of venom/toxin in TBS buffer (25 μg/ml) without or with calcium (in a range of 0, 0.001, 0.01, 0.1, 1, or 10 mM), according to the experiments described in the Figure legends. For this assay, samples were performed in pentaplicate along with negative (in the presence of the TBS only; in a final volume of 400 μl) and positive (red blood cells in distilled water and 0.1% (v/v) Triton X-100) controls. After 12 h (for hemolysis assays in the presence of calcium in the medium) or 24 h (for hemolysis assays in absence of calcium) of incubation with gentle agitation, controls, and samples were centrifuged at 4°C (3 min at 200*g*), and the absorbance of the supernatants were read immediately at 550 nm (ELISA ELX 800 Auto Reader, Meridian Diagnostics, Inc.). Absorbance values were converted to percent hemolysis using the absorbance values of the positive control as 100% lysis. Alternatively, toxin-induced hemolysis was evaluated, as reported above, but at time periods of 5, 15, 30, 60, 120, and 240 min following toxin treatment and in presence of 1 mM of calcium. The absorbance of supernatants was read at 550 nm, and the percentage of hemolysis was determined using the absorbance values induced by distilled water as 100% of hemolysis (positive control). The results represent an average of five experiments ± SEM, $^{***}P \leq 0.0001$.

PHOSPHOLIPASE ACTIVITY ASSAY

Phospholipase activity was measured using the Amplex Red Assay Kit (Molecular Probes, Eugene). In this assay, Phospholipase-D activity is monitored using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2 [Appel et al., 2008]. First, recombinant phospholipase-D (LiRecDT1) hydrolyzes SM to yield ceramide-phosphate and choline. Then choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with the Amplex reagent in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin. All recombinant toxins (10 μg each, in three trials) were added to the Amplex Red reagent mixture. The reaction tubes were incubated at 37°C for 30 min, and fluorescence was measured in a *fluorimeter* Tecan Infinite® M200 (Tecan, Männedorf, Switzerland) using excitation at 540 nm with emission detection at 570 nm. The same method was used to test the hydrolysis ability of other phospholipids such as C16 *Lyso* PAF (1-*O*-hexadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine), Egg SM (Sphingomyelin Egg, Chicken), 16:0 *Lyso* PC (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) and 16:0-18:0 PC (1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine); however, the SM in the kit was changed by other phospholipids and the choline generated was measured. All phospholipids were acquired from Avanti Polar Lipids, Inc. Alabaster, AL. Alternatively, to evaluate whether calcium directly controls the catalytic activity of recombinant phospholipase-D, phospholipase activity was measured identically as described above, but in the presence of calcium at concentrations of 0.1 and 10 mM.

CHOLINE RELEASE DETECTION FROM ERYTHROCYTE GHOST AND ERYTHROCYTE GHOST EXTRACT

Ghosts of erythrocyte membranes were obtained from approximately 5×10^8 cells. Erythrocytes were lysed and washed in hypoosmotic buffer (NaH_2PO_4 5 mM, PMSF 2 mM, and pH 8.0). The lysed

cells were collected by centrifugation (12,000 *g*, 10 min, 4°C), and this procedure was repeated four times. The supernatant was aspirated and the lysed cells were white; therefore, they were practically hemoglobin-free.

The erythrocyte ghosts were resuspended in 1 ml of cold extraction buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 0.5%). After gently homogenizing for 10 min at 4°C, cells were centrifuged at 20,000 × *g* for 20 min at 4°C, and supernatants were collected for later use. Ghosts and extracts (100 μl) were utilized as a substrate for Whole Venom and LiRecDT1 and LiRecDT1 H12A (100 μg) in a total final volume of 250 μl for 1 h at 37°C and gently mixed using a rotational shaker in the BOD incubator. The treated tubes were then added to a 250 μl reaction mixture adapted from the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes) containing choline oxidase (4 U), alkaline phosphatase (80 U), horseradish peroxidase (20 U), and Amplex Red reagent (100 μM), excluding the SM substrate. After incubation in a water bath for 1 h at 37°C, fluorescence development was measured in a *fluorimeter* Tecan Infinite® M200 (Tecan, Männedorf, Switzerland) using excitation at 540 nm with emission detection at 570 nm.

DEGRADATION OF SPHINGOMYELIN AND *LYSO*PHOSPHATIDYLCHOLINE

First, 100 mM Egg SM (Sphingomyelin Egg, Chicken) and 100 mM 16:0 *Lyso* PC (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) were dissolved in chloroform. Next, 5 mM SM and 5 mM LPC were diluted in TBS Buffer with 0.005% Triton X-100. Then materials were incubated with 25 μg/ml LiRecDT1 for 24 h with gentle agitation at 37°C, and controls (lipids without toxin treatment) and samples were centrifuged at 4°C (3 min at 200*g*). The supernatants were incubated with 5 μl Ni^{2+}-NTA agarose beads for 12 h at 4°C with gentle agitation. Finally, the samples were centrifuged at 4°C (3 min at 200*g*), and fractions were collected and analyzed by 12.5% SDS–PAGE and Coomassie Blue Dye under reduced conditions. For immunoblotting, proteins were transferred to nitrocellulose filters and immunostained using purified IgGs that react with dermonecrotic toxin LiRecDT1. The molecular mass markers were acquired from Sigma.

MEASUREMENT OF Ca^{2+} INFLUX INTO ERYTHROCYTES CONTAINING FLUO-4

Human erythrocytes (1×10^8 cells/ml) were prepared in Ringer Solution (122.5 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM HEPES, 11 mM glucose, 1 mM NaH_2PO_4, pH 7.4, 300 mOsm/kg H_2O), containing 2.5 mM $CaCl_2$ and performed according with Kaestner et al. [2006] and Haase et al. [2009]. Erythrocytes were loaded with Fluo-4 AM (5μM) in buffer with Pluronic F-127 (0.01%) for 30 min at 37°C. This indicator has a high affinity binding for Ca^{2+} ($K_d = 345$ nM) and a very large fluorescence intensity increase in response to Ca^{2+} binding (>100-fold). Subsequently, cells were washed twice with Ringer Solution and equilibrated for de-esterification for 30 min at room temperature. Cells were incubated with 25 μg/ml of recombinant phospholipase-D (LiRecDT1) and 25 μg/ml of mutated phospholipase-D (LiRecDT1 H12A) for 5, 15, 30, 45, 60, and 120 min. Additionally, 10 μM of the *ionophore* A23187 was used as a positive control. After the reaction, the cells were transferred into a 96-well plate at a density of 1×10^6 cells per well in a total volume of 100 μl. The resulting fluorescence was recorded on a *fluorimeter* Tecan Infinite M200 (Tecan, Männedorf, Switzerland) using an excitation wavelength of 485 nm, and the emission was measured at 535 nm.

Moreover, Fluo-4 dye-loaded human erythrocytes were settled onto poly-L-lysine coated coverslips, and calcium images were performed using a Zeiss Axio Observer.Z1 inverted microscope (Carl Zeiss, Germany). The Fluo-4 AM was excited at 488 nm with emission using the LP 505 nm filter (green channel). Single images were obtained using the 63× oil lens for differential interface contrast (DIC) and fluorescence intensity with monochromatic camera (AxioCam HRm, Carl Zeiss). Finally, AxioVision LE software was used for image processing and for morphometric measures in the Zeiss image format (ZVI).

EFFECT OF CATIONIC CHANNEL BLOCKERS ON THE TOXIN-INDUCED HEMOLYSIS

To understand the toxin-stimulated Ca^{2+} uptake pathway, we investigated the effect of cationic channel blockers on toxin-induced Ca^{2+} entry and hemolysis. We performed the assay according with Ochi et al. [2003]. Cells were treated with 25 μM of Flunarizine (T-type Ca^{2+} channel blocker), 25 μM of Verapamil, 25 μM Nifedipine (L-type Ca^{2+} channel blockers), 25 μM of Clotrimazole (Gardos-type Ca^{2+}-activated K^+ channel blocker), 25 μM of Procaine (Na^+ channel blocker), or 25 μM of ruthenium red (unspecific Ca^{2+} channel blocker). Then samples were incubated with 25 μg/ml of LiRecDT1 and 100 μM calcium in TBS Buffer for 12 h. The hemolytic activity was evaluated according to the previously mentioned method. Additionally, two calcium channel blockers (nifedipine and verapamil) were investigated upon toxin-induced hemolysis as described above, except they were used at serial concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 mM and toxin-induced hemolysis detected as previously reported.

RESULTS

DIFFERENT MEMBERS OF PHOSPHOLIPASE-D-FAMILY TOXINS OF BROWN SPIDER VENOM CAUSE DISTINCT LEVELS OF HEMOLYSIS

Previously, we provided experimental data reporting direct hemolysis of human erythrocytes following treatment with a phospholipase-D toxin-family member (LiRecDT1) expressed as a recombinant toxin from a cDNA library of the *Loxosceles intermedia* venom gland [Chaim et al., 2006; Chaves-Moreira et al., 2009]. We cloned and expressed six different isoforms of brown spider venom phospholipase-D; therefore, we studied their hemolytic activity on human erythrocytes. Additionally, we studied LiRecDT1H12A, a recombinant phospholipase-D isoform, with a mutation by substitution of a residue of histidine by alanine at the catalytic site [Chaim et al., 2006; Chaves-Moreira et al., 2009]. Figure 1A depicts hemolysis of human red blood cells incubated with six different isoforms of recombinant phospholipase-D, and a mutated isoform expressed and purified under the same experimental conditions, named LiRecDT1, LiRecDT2, LiRecDT3, LiRecDT4, LiRecDT5, LiRecDT6, and LiRecDT1H12A. As observed, the treatment of erythrocytes with different toxin isoforms under the same experimental conditions caused different percentages of

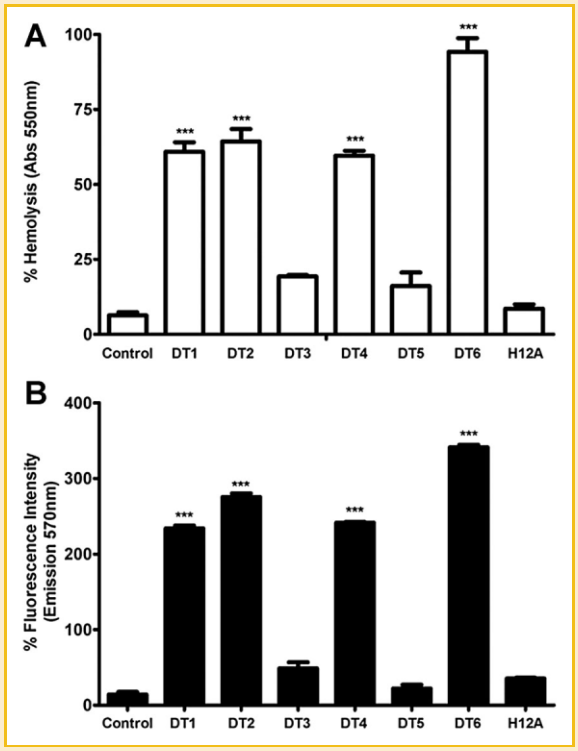

Fig. 1. Different members of phospholipase-D family toxins of brown spider venom cause distinct levels of hemolysis and have different phospholipase-D activity. A: Human erythrocytes were exposed to 25 μg/ml of different purified recombinant phospholipase-D toxins named LiRecDT1 (DT1), LiRecDT2 (DT2), LiRecDT3 (DT3), LiRecDT4 (DT4), LiRecDT5 (DT5), LiRecDT6 (DT6), and LiRecDT1H12A (H12A) for 24 h at 37°C and were evaluated for hemolysis. As a control, erythrocytes were incubated in the absence of toxins but under the same laboratory conditions. The results show an average of five experiments ± SEM. $^{***}P \leq 0.0001$. B: Alternatively, phospholipase-D activity of purified recombinant toxins was evaluated using a fluorimetric method with the Amplex Red Assay Kit at 37°C for 30 min as shown in the Materials and Methods section. The control was a reaction in the absence of any toxin. The product of a reaction was determined at 540 nm (absorption) with emission detection at 570 nm. Reactions used 10 μg of each toxin in triplicate. Values given are the average ± SEM, $^{***}P \leq 0.0001$.

hemolysis. Additionally, Figure 1B shows that these same toxin isoforms have different phospholipase-D activity. The results suggest a correlation between hemolysis induced by toxins and their phospholipase-D activity (catalysis).

THE RECOMBINANT PHOSPHOLIPASE-D ISOFORM LIRECDT1 CAUSES DEGRADATION OF SYNTHETIC PHOSPHOLIPIDS AND PHOSPHOLIPIDS OF THE CYTOPLASMIC MEMBRANE OF HUMAN ERYTHROCYTES

To determine the specificity and which kind of phospholipid(s) is degraded by LiRecDT1, different synthetic phospholipids such as sphingomyelin (SM) a sphingophospholipid, and *lyso*phosphatidylcholine (LPC), *lyso*-platelet activating factor (LPAF), and phosphatidylcholine (PC), glycerophospholipids, were treated with LiRecDT1 under the same experimental conditions, and choline generation was evaluated using a fluorimetric method. As shown in Figure 2A, recombinant toxin was able to degrade SM, LPC, and LPAF but did

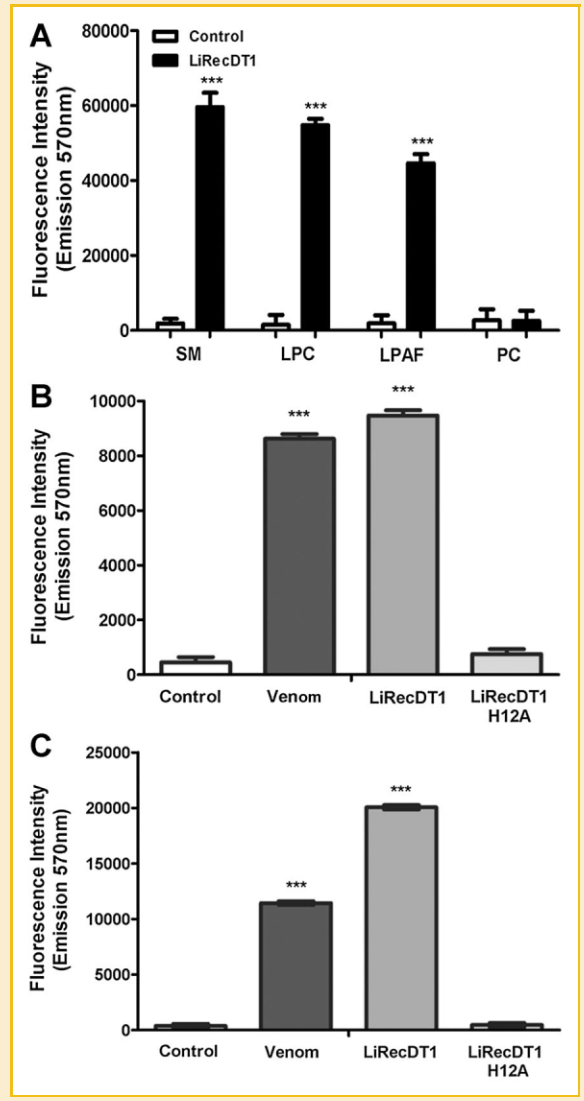

Fig. 2. The recombinant phospholipase-D LiRecDT1 degrades synthetic phospholipids and phospholipids of the plasma membrane of human erythrocytes. A: The ability of recombinant phospholipase-D to degrade phospholipids was evaluated through a fluorimetric method using an Amplex Red Assay Kit, substituting a phospholipid substrate by synthetic phospholipids such as sphingomyelin (SM), *lyso*phosphatidylcholine (LPC), *lyso*-platelet activating factor (LPAF), and phosphatidylcholine (PC) and verifying the production of choline after phospholipids/toxin exposure at 37°C for 30 min (see Materials and Methods for details). As a negative control, phospholipids were incubated in the absence of toxin but under the same laboratory conditions. The results show an average of five experiments ± SEM. $^{***}P \leq 0.0001$. As shown, recombinant toxin was able to degrade SM, LPC, and LPAF, under the conditions evaluated. Additionally, ghosts of human red blood cells (B) or detergent extract of human erythrocytes (C) were treated with crude venom, recombinant phospholipase-D LiRecDT1, recombinant toxin LiRecDT1H12A (H12A), and in the absence of toxins but under the same laboratory conditions as a negative control. The generation of choline was analyzed by a fluorimetric assay under identical conditions as described above. The results show an average of five experiments ± SEM. $^{***}P \leq 0.0001$. As depicted, both crude venom and LiRecDT1 degraded phospholipids of ghost and detergent extract of human erythrocytes, compared to the negative control and mutated toxin that did not generate choline.

not act on PC under the conditions used. Additionally, to ascertain whether this recombinant phospholipase-D could change the levels of phospholipids present in the cytoplasmic membrane of erythrocytes, ghosts of human red blood cells (Fig. 2B) or detergent extract of human erythrocytes (Fig. 2C) were treated with crude venom, LiRecDT1, and LiRecDT1H12A, and the generation of choline was analyzed by a fluorimetric assay. As depicted in figures, production of choline was observed following crude venom and LiRecDT1 treatments but not after LiRecDT1H12A in both situations, supporting the accessibility and activity of recombinant phospholipase-D on membrane erythrocyte phospholipids.

THE PRODUCTS OF DEGRADATION OF SPHINGOMYELIN AND *LYSO*PHOSPHATIDYLCHOLINE FOLLOWING TREATMENT WITH RECOMBINANT PHOSPHOLIPASE-D CAUSE HEMOLYSIS OF HUMAN BLOOD CELLS

With the goal of confirming the relationship between the metabolism of phospholipids and hemolytic activity induced by recombinant phospholipase-D, we next treated synthetic SM, or LPC, or both phospholipids together (Fig. 3A) with recombinant phospholipase-D. The products made were submitted to affinity chromatography on Ni^{2+}-agarose to remove His-tag phospholipase-D. The efficiency of chromatography was confirmed by electrophoresis visualized by Coomassie Blue dye and by immunoblotting using antibodies to the recombinant toxin (Fig. 3B,C). After chromatography, the products (flow through) were sequentially incubated with human erythrocytes. As shown, when red blood cells were incubated with the product of the degradation of SM, LPC, or both the products of degradation together, lyses of cells occurred, suggesting that the products of metabolism of phospholipids following recombinant phospholipase-D treatment can induce hemolysis.

RECOMBINANT PHOSPHOLIPASE-D-INDUCED HEMOLYSIS INCREASES IN A CALCIUM-DEPENDENT MANNER

Several literature reports have described a role for calcium in hemolysis that is induced by products of metabolism of phospholipids [Yang et al., 2000; Ochi et al., 2003]. With the objective to verify the involvement of calcium on recombinant phospholipase-D-induced hemolysis, we next analyzed the hemolytic activity of recombinant phospholipase-D on washed human red blood cells with increasing concentrations of calcium. Human erythrocytes were incubated in synthetic medium containing increased doses of calcium (0–10 mM) and the same concentration of LiRecDT1, and the percentage of hemolysis was determined (Fig. 4A). As shown, the percentage of hemolysis increased with an increase in the dose of calcium. Additionally, we repeated hemolytic assays using other isoforms of recombinant toxins such as LiRecDT2, LiRecDT3, LiRecDT4, LiRecDT5, LiRecDT6, and LiRecDT1H12A, and we compared hemolysis in the absence or in the presence of 1 mM of calcium. As depicted in Figure 4B, an increase in the hemolytic activity of all toxin isoforms in the presence of calcium was observed. These data suggested the possibility that the phospholipase-D-induced metabolism of phospholipids constituents of membrane may play a role in calcium influx or that calcium may directly control the activity of enzyme.

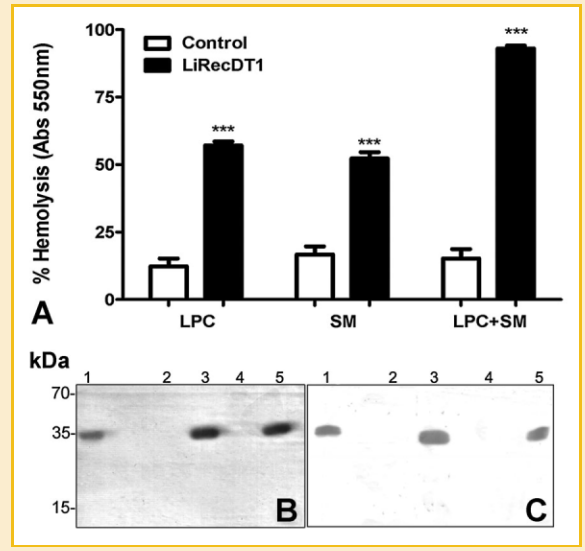

Fig. 3. The products of degradation of sphingomyelin and *lyso*phosphatidylcholine following treatment with recombinant phospholipase-D caused hemolysis of human blood cells (A). Synthetic sphingomyelin (SM) and *lyso*phosphatidylcholine (LPC) were incubated with purified recombinant toxin LiRecDT1 at the concentration of 25 μg/ml, for 24 h at 37°C. The products created following phospholipid treatment with toxin were submitted to affinity chromatography on Ni^{2+}-agarose to remove phospholipase-D. Human erythrocytes were incubated for 24 h at 37°C with the product of degradation of sphingomyelin after toxin treatment (SM), the product of degradation of *lyso*phosphatidylcholine after toxin exposure (LPC), or both products of degradation of phospholipids together (SM + LPC) following Ni^{2+}-chromatography (flow through). As a negative control, erythrocytes were incubated with purified synthetic phospholipids before toxin treatment. B,C: Shows SDS–PAGE under reducing conditions of products of degradation of phospholipids after chromatography that were visualized by Coomassie Blue dye or immunoblotting using an antibody specific to recombinant toxin, respectively. Molecular protein masses are shown on the left of figures. Lanes 1 contain purified toxin, lanes 2 depict flow through of Ni^{2+}-chromatography of sphingomyelin-toxin treated mixture. Lanes 3 show eluate of Ni^{2+}-chromatography of sphingomyelin-toxin treated mixture. Lanes 4 contain flow through of Ni^{2+}-chromatography of *lyso*phosphatidylcholine-toxin treated mixture. Lanes 5 show eluate of Ni^{2+}-chromatography of *lyso*phosphatidylcholine-toxin treated mixture. As shown, no toxin was observed in the products of degradation of phospholipids after Ni^{2+}-chromatography.

CALCIUM DOES NOT CHANGE THE CATALYTIC ACTIVITY OF RECOMBINANT PHOSPHOLIPASE-D. RECOMBINANT PHOSPHOLIPASE-D CAUSES AN INFLUX OF CALCIUM IN HUMAN ERYTHROCYTES

To better understand the relationship between the molecular pathways regulating the metabolism of phospholipids induced by phospholipase-D and calcium ions, we analyzed whether calcium directly controls the catalytic activity of recombinant phospholipase-D, measuring the phospholipase-D activity in the presence of different concentrations of calcium, and we used SM as a substrate for a fluorimetric assay. As depicted in Figure 5A, no change in phospholipase-D activity was witnessed when the toxin was incubated with different calcium concentrations, suggesting that calcium does not directly control the catalytic activity of the enzyme because lower and higher calcium concentrations induced phospholipase-D activity in a similar manner. To ascertain the

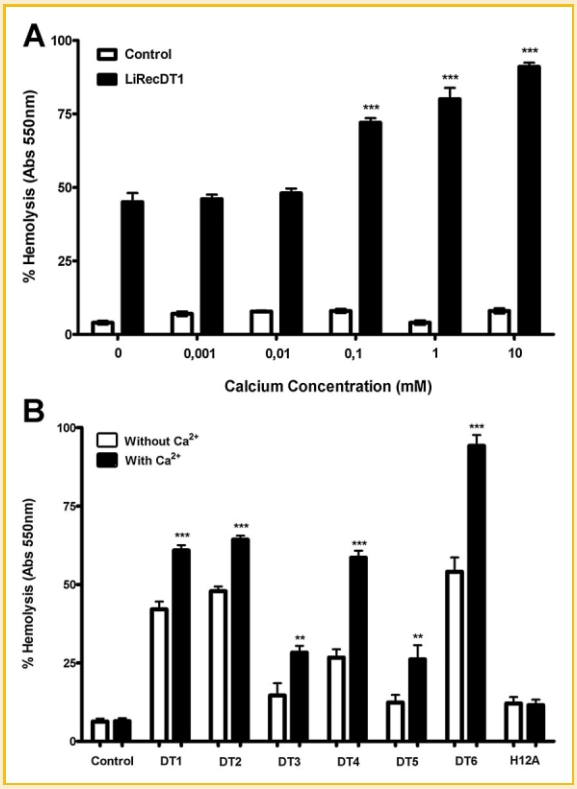

Fig. 4. Recombinant phospholipase-D toxin-induced hemolysis increases in a calcium-dependent manner. A: Human erythrocytes were incubated with 25 μg/ml of purified recombinant toxin (LiRecDT1) or in the absence of toxin (negative control) for 12 h at 37°C and with increasing concentrations of calcium (0–10 mM). The absorbance of supernatants was read at 550 nm, and the percentage of hemolysis was determined using the absorbance values induced by distilled water as 100% of hemolysis. The results represent an average of five experiments ± SEM. $^{***}P \leq 0.0001$. B: Additionally, we compared the hemolysis caused by all different isoforms of phospholipases-D (LiRec**DT1**, LiRec**DT2**, LiRec**DT3**, LiRec**DT4**, LiRec**DT5**, LiRec**DT6**, and LiRec**DT1-H12A**) in the absence or presence of 10 mM of calcium. Hemolysis was determined under identical conditions as described above. As a control, hemolysis was evaluated by incubating cells with toxins using a buffer without calcium. The results represent average of five experiments ± SEM. $^{***}P \leq 0.0001$. As observed, toxin-induced hemolysis occurs in a calcium concentration-dependent manner for LiRecDT1, and for all toxin isoforms studied, the presence of calcium increased the percentage of hemolysis, except for the toxin with modification at the catalytic site H12A.

relationship between phospholipase-D activity, the metabolism of erythrocyte phospholipids and calcium and to evaluate whether toxin treatment induces a calcium influx into erythrocytes, we examined calcium influx into erythrocytes following recombinant phospholipase-D treatment in the presence of Fluo-4, a cell permeable and calcium-sensitive fluorophore. As shown in Figure 5B, phospholipase-D treatment (LiRecDT1) but not LiRecDT1H12A (toxin mutated at the catalytic site) caused an increase of fluorescence in erythrocytes in a time-dependent manner. Additionally, fluo-4 loaded erythrocytes were treated with recombinant phospholipase-D in different time intervals, and the cells were observed using an inverted microscope for DIC and fluorescence intensity (see details in Materials and Methods section). An increase in fluorescence and calcium up take was observed relative to the time following toxin treatment (Fig. 5C), strengthening the idea that toxin exposure induced a calcium influx into the cells and a relationship exists among toxin-induced metabolism of phospholipids (catalysis), calcium, and hemolysis. This finding is corroborated by toxin-induced hemolysis of human erythrocytes incubated with the same concentration of recombinant phospholipase-D as used for the calcium uptake experiment (Fig. 5C) at different time intervals. As shown (Fig. 5D), hemolysis occurred in a time-dependent manner, but it has initiated only 1 h following toxin exposure. At this point, the calcium concentration inside the cells was sufficiently high to induce hemolysis, which was not as a consequence of membrane leakage.

RECOMBINANT PHOSPHOLIPASE-D INDUCED HEMOLYSIS DEPENDS ON A CALCIUM CHANNEL

Additionally, we investigated whether recombinant toxin induces calcium influx into human erythrocytes using calcium channel inhibitors. Washed human erythrocytes were incubated with recombinant phospholipase-D (LiRecDT1) in the presence of calcium and different calcium channel inhibitors such as ruthenium red (unspecific calcium channels inhibitor), nifedipine and verapamil (selective calcium channel L-type inhibitors), clotrimazole (specific inhibitor of Ca^{2+}-activated K^{+} channel), flunarizine (selective calcium channel T-type inhibitor), and procaine (sodium channel inhibitor, as negative control). As shown in Figure 6A, only ruthenium red, nifedipine, and verapamil inhibited toxin-induced hemolysis, suggesting that calcium influx and the participation of calcium channel L-type play a role in Brown spider phospholipase-D-induced hemolysis. Additionally, to strengthen the idea that calcium channel L-type plays a role in hemolysis, we examined hemolysis induced by recombinant phospholipase-D by incubating washed erythrocytes treated with phospholipase-D in the presence of calcium and various concentrations of nifedipine and verapamil (L-type Ca^{2+} channel blockers). As shown in Figure 6B, nifedipine and verapamil caused the inhibition of hemolysis occurred in a concentration-dependent manner, corroborating the evidence of a calcium influx dependent on a L-type channel. As observed, nifedipine blocked hemolysis more efficiently compared to verapamil, which strengthened the hypothesis of calcium influx playing a role in hemolysis stimulated by brown spider phospholipase-D.

DISCUSSION

The mechanism by which the venom exerts its hemolytic effect is currently under investigation. Blood cell lysis induced by *Loxosceles* spider venom has been demonstrated based on the clinical features observed in accident victims, which include hematuria, hemoglobinuria, proteinuria, elevated creatine kinase levels, and shock [Williams et al., 1995; Bey et al., 1997; Lung and Mallory, 2000; França et al., 2003]. Additionally, a great number of case reports have described hemolysis following brown spider accidents [Murray and Seger, 1994; Williams et al., 1995; de Souza et al., 2008].

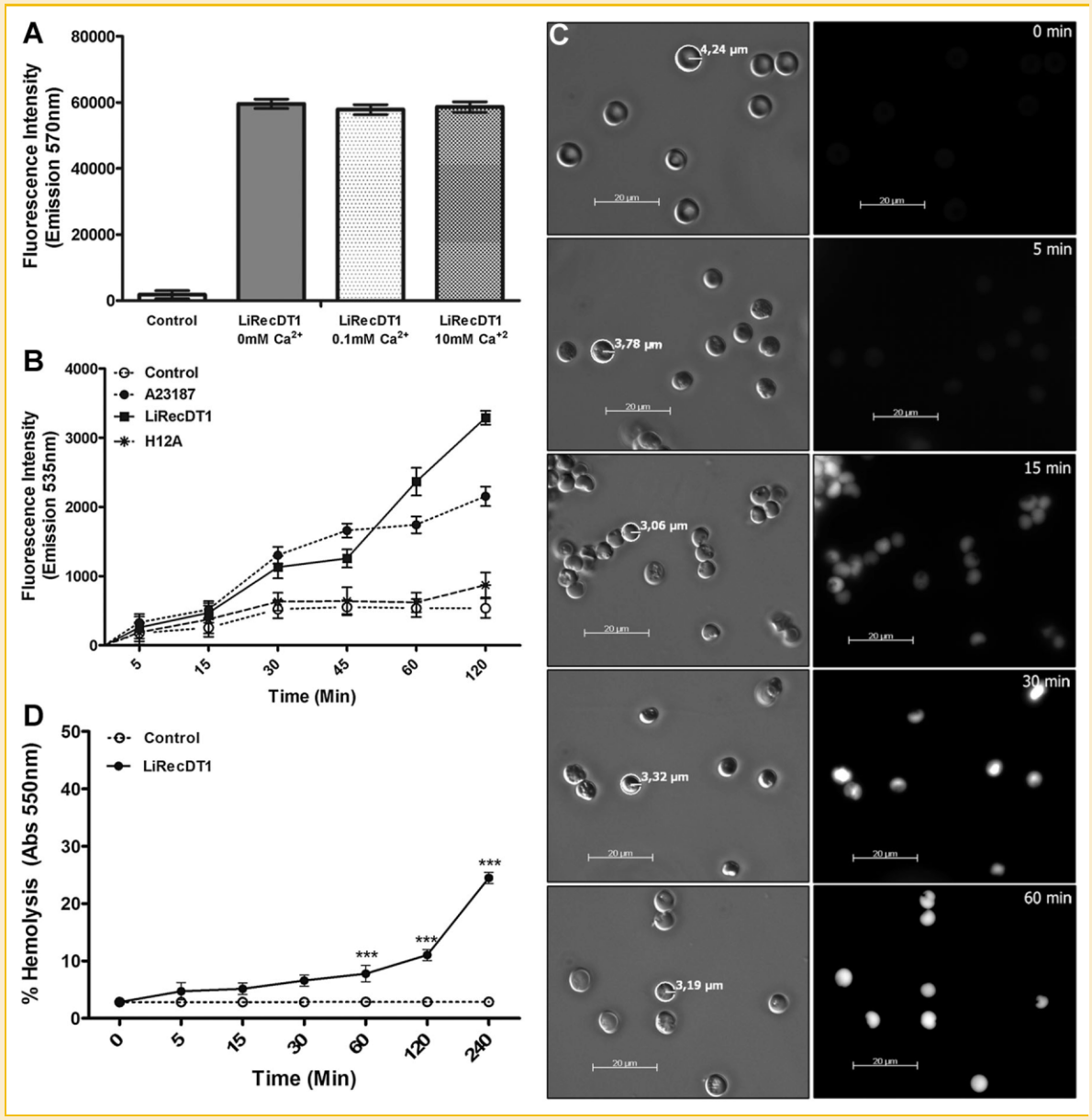

Fig. 5. Comparative phospholipase-D activity of purified recombinant toxin (LiRecDT1) in the absence and in the presence of different calcium concentrations. Human erythrocyte treatment with recombinant phospholipase-D causes an influx of calcium (A). Initially, phospholipase-D activity of LiRecDT1 was evaluated in the absence or in the presence of 0.1 and 10 mM of calcium with the Amplex Red fluorimetric assay at 37°C for 30 min. The product of the reaction was determined at 540 nm (absorption) with emission detection at 570 nm. As a negative control, PBS was used. As observed, variation in the calcium concentration did not change phospholipase-D activity (B). Next, we analyzed the effect of phospholipase-D treatment on calcium uptake into human erythrocytes. Human erythrocytes in the presence of Fluo-4 were incubated with LiRecDT1 in a calcium containing buffer, and fluorescence of Fluo-4 was measured after various periods. As a negative control, erythrocytes were incubated under the same laboratory conditions but in the absence of toxin treatment. As a positive control, erythrocytes were incubated with a calcium ionophore A23187. Comparatively, LiRecDT1H12A with mutation on the catalytic site was also used. As detected, there was an increase in the uptake of calcium in a time-dependent manner following LiRecDT1 exposure, but no calcium uptake was observed after LiRecDT1H12A treatment (C). Additionally, human erythrocytes were exposed to 25 μg/ml of recombinant phospholipase-D (LiRecDT1) for different times (5, 15, 30, and 60 min) and then observed by transmission microscopy with DIC and fluorescence imaging of fluor-4-loaded cells. The control represents erythrocytes in the absence of toxin but fluor-4-loaded. As shown, toxin treatments induced an increase of calcium inside cells in a time-dependent manner. Scale bars are shown at the (D). Erythrocytes were incubated with 25 μg/ml of LiRecDT1 over different time periods.

The brown spider venom phospholipase-D (also named dermonecrotic toxin) is the best-known toxin found in different *Loxosceles* species venom. Currently, a family of related molecules characterized as phospholipases-D is thought to exist [Kalapothakis et al., 2007]. The family-members of phospholipase-D toxins contribute to the major deleterious effects of brown spider bites, including hemolysis [Forrester et al., 1978], platelet aggregation [Kurpiewski et al., 1981], blood vessel hyperpermeability [da Silveira et al., 2006], nephrotoxicity

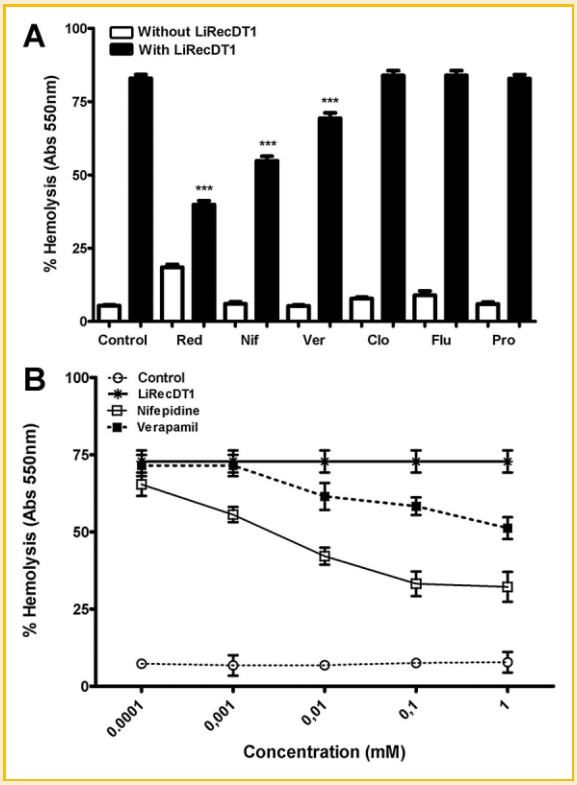

Fig. 6. Recombinant phospholipase-D-induced hemolysis depends on a Calcium channel. A: To study the influence of calcium channel blockers upon toxin-induced hemolysis, human erythrocytes were incubated with or without phospholipase-D toxin (LiRecDT1) in the presence of various ion channel blockers such as ruthenium red (Red) (unspecific calcium channels inhibitor), nifedipine (Nif) and verapamil (Ver) (selective calcium channel L-type inhibitors), clotrimazole (Clo) (specific inhibitor of Ca^{2+}-activated K^{+} channel), flunarizine (Flu) (selective calcium channel T-type inhibitor), and procaine (Pro) (sodium channel inhibitor). As a control for positive hemolysis, erythrocytes were incubated with recombinant toxin but in the absence of ion channel inhibitors. Hemolysis was determined as previously described above. The results represent an average of five experiments ± SEM. $^{***}P \leq 0.0001$ (B). Additionally, we examined hemolysis induced by recombinant phospholipase-D (LiRecDT1), incubating washed erythrocytes treated by phospholipase-D, in the presence of calcium and various concentrations of nifedipine and verapamil. As observed, both calcium channel inhibitors blocked toxin-induced hemolysis in a dose-dependent way. The results represent the average of five experiments ± SEM.

and mouse lethality [Chaim et al., 2006; Kusma et al., 2008], and typical dermonecrotic lesions [Futrell, 1992; da Silva et al., 2004].

Evidence for the participation of phospholipase-D family members playing a role in hemolysis following brown spider bites comes from experimental data using native and/or recombinant toxins [Futrell, 1992; da Silva et al., 2004; Ribeiro et al., 2007]. It was described that crude venom toxins or purified native phospholipase-D toxin bind directly to the membranes of erythrocytes and lyses these cells, and it was postulated that such as interaction with the membranes of erythrocytes is through SM [Futrell, 1992]. Recently, by studying recombinant phospholipase-D (LiRecDT1) [Chaim et al., 2006] compared to a mutated isoform LiRecDT1 H12A [Kusma et al., 2008] that had a substitution of a residue of histidine at position 12 by a residue of alanine at the catalytic site of the toxin [Murakami et al., 2006], we were able to identify a direct hemolytic effect for LiRecDT1 toxin. We described that this effect is dependent on catalysis because the mutated isoform was depleted of hemolytic activity [Chaves-Moreira et al., 2009]. By studying human erythrocytes treated with recombinant phospholipase-D and immunostained with antibodies against this toxin or red blood cells treated with a recombinant fusion toxin GFP-LiRecDT1 and analyzed by confocal microscopy, specific binding was reported of recombinant phospholipase-D to the membrane of erythrocytes [Chaves-Moreira et al., 2009].

Herein, we studied recombinant isoforms of the phospholipase-D toxin from the *L. intermedia* venom gland and searched for a relationship among hemolysis of human erythrocytes, phospholipase-D catalysis, phospholipids metabolism, and calcium. Initially, by analyzing six different isoforms of recombinant phospholipase-D, our experimental results support a differential activity of hemolysis according to the isoform of the toxin used. Additionally, there is a correlation between phospholipase-D activity (analyzed by degradation of SM treated by distinct isoforms of toxin and a fluorimetric assay) and the percentage of hemolysis because isoforms of the toxin with high hemolytic effect have high phospholipase-D activity. These results are in agreement with literature data, which have reported that phospholipase catalysis plays a role in hemolysis [Ochi et al., 2004; Oda et al., 2008; Chaves-Moreira et al., 2009].

To prove that phospholipase-D catalysis and the degradation of membrane phospholipids play a role in the lysis of human red blood cells, we additionally showed that a recombinant phospholipase-D (LiRecDT1) isoform was able to degrade SM, LPC, and *lyso*-platelet activating factor, some of which are very important membrane constituents of human erythrocytes [Virtanen et al., 1998]. Additionally, we described that detergent extract of human erythrocytes and erythrocyte ghosts treated with LiRecDT1 generated the production of choline, detected by a fluorimetric assay. Based on these results, it appears that the toxin does stimulate the hydrolysis of important synthetic phospholipid constituents of membrane erythrocytes, and additionally, has the accessibility and activity of both erythrocyte membrane detergent extract and erythrocyte ghost phospholipids (proved by the generation of choline). These observations, together with literature data [Futrell, 1992; Chaves-Moreira et al., 2009], indicate that recombinant phospholipase-D can interact with erythrocyte membrane constituents, has hydrolytic activity toward purified phospholipids, and can directly metabolize phospholipids structurally organized on membrane erythrocytes, or extracted from erythrocyte membranes. The absence of a nucleus in human erythrocytes precludes the cell from synthesizing new proteins in response to extracellular stimuli; therefore, together with the lack of organelles such as the endoplasmic reticulum and Golgi apparatus that precludes the cell to regenerate the plasma membrane, we speculated that the toxin-induced membrane phospholipid metabolism and generated products are directly related to red blood cell lysis.

It is well known that the enzymatic activity of phospholipase-D on SM generates choline plus ceramide1-phosphate, and phospholipase-D catalysis on LPC produces choline plus LPA [Anliker and

Chun, 2004; Moolenaar et al., 2004; Lee and Lynch, 2005]. Both ceramide1-phosphate and its metabolites such as ceramide, sphingosine and sphingosine 1-phosphate, and LPA are potent bioactive molecules related with biological functions including cell survival, mammalian inflammatory response, cellular differentiation, cellular senescence, apoptosis, cell proliferation, platelet aggregation, and hemolysis [Yang et al., 2000; Ohanian and Ohanian, 2001; Chalfant and Spiegel, 2005]. After reporting that recombinant phospholipase-D was able to hydrolyze synthetic and erythrocyte membrane phospholipids, we next showed that the products of metabolism of synthetic SM and LPC following treatments with recombinant phospholipase-D, after removing the phospholipase of mixture, and when incubated with washed human erythrocytes caused hemolysis of cells. The product of degradation of SM following toxin incubation caused lysis of cells, and the product of hydrolysis of LPC also lysed cells. Additionally, interestingly, when both degradation products were mixed, the percentage of hemolysis increased. These observations indicate that the products generated by toxin-activated metabolism of SM and metabolism of LPC plays a role in hemolysis of human cells. Therefore, it appears that the toxin stimulates the production of ceramide 1-phosphate from SM (a lipid constituent of erythrocyte membrane), generates LPA from LPC (another lipid constituent of erythrocyte membrane), and these bioactive lipid metabolites lead to hemolysis of human erythrocytes as reported in several examples in the literature [Oda et al., 2008; Oda et al., 2010; Ohanian and Ohanian, 2001].

Additionally, we detected that the percentage of hemolysis induced by the treatment of washed erythrocytes with recombinant phospholipase-D occurs in a calcium-dependent concentration manner because hemolysis increases following toxin exposure, and it also increases depending on calcium doses used in the medium. Therefore, this result showed that the activation of phospholipid metabolism and hemolysis after toxin exposure stimulated calcium influx related to lyses of cells, which is in agreement with literature data that report that other toxin-induced hemolysis such as *Clostridium perfringens* alpha-toxin is dependent on calcium uptake [Ochi et al., 2003]. Moreover, an influx of calcium ions inside erythrocytes following phospholipase-D treatment is supported by experiments using Fluo-4, a cell permeable and calcium-sensitive fluorophore, which showed an increase in fluorescence after toxin treatment. Finally, the above mentioned results, which demonstrated that calcium is required in brown spider phospholipase-D-induced hemolysis, are in agreement with the data described by Yang et al. [2000], who reported that LPA (a product generated following toxin treatment of cells) induces calcium entry in human erythrocytes.

Alternatively, calcium may directly control the catalytic activity of phospholipase-D by binding to some region of this enzyme, which may contribute to an increase in lipid catalysis. The catalytic region of brown spider phospholipase-D is a metal coordinated domain that is dependent on magnesium [Murakami et al., 2005, 2006]. Calcium and magnesium belong to the same chemical family of alkaline earth metals. However, no changes in the measurement of phospholipase-D activity (using SM as substrate) were observed in the presence of different increasing doses of calcium. Therefore, the idea that phospholipase-D-induced hemolysis is controlled by direct binding of calcium, stimulating catalysis activation in the enzyme, was refused. Together with the above mentioned results, our data show that brown spider phospholipase-D-induced hemolysis is dependent on an extracellular influx of calcium because the absence of smooth endoplasmic reticulum and mitochondria in erythrocytes (two organelles related with intracellular store of calcium) prevents the erythrocyte from discharging intracellular stores of this ion.

To understand whether calcium influx into erythrocytes following recombinant phospholipase-D treatment was a consequence of a leakage of membrane of cells or as consequence of the activation of calcium channel, we analyzed toxin-induced hemolysis in the presence of different ion channel blockers or with different calcium channel inhibitors. A decrease of percentage of hemolysis after toxin treatment was observed just in the presence of ruthenium red (an unspecific calcium channel inhibitor) and nifedipine (selective calcium channel L-type blocker). No changes were observed in toxin-induced hemolysis in the presence of other inhibitors such as procaine (sodium channel blocker), flunarizine (calcium channel T-type blocker), or clotrimazole (specific inhibitor of Ca^{2+}-activated K^{+} channels "Gardos-type"). Based on the results reported herein, we can conclude that the influx of extracellular calcium into human erythrocytes after recombinant phospholipase-D exposure is channel mediated rather than as a consequence of leakage promoted by enzymes acting on erythrocyte plasma integrity. Additionally, the experiments showing that toxin-induced hemolysis occurs just after calcium up take and appears at least 2 h following toxin treatment corroborate this hypothesis. Finally, the fact that nifedipine and verapamil (two calcium channel L-type inhibitors) blocked toxin-induced hemolysis in a dose-dependent manner, strengthened the hypothesis that calcium influx occurs through a L-type channel and plays a role in hemolysis.

Interestingly, the experiments performed using higher doses of nifedipine or verapamil (calcium L-type channel blockers) showed that the inhibition of hemolysis after recombinant toxin exposure although clear, was partial. These observations suggest that mechanisms other than calcium influx can contribute to hemolysis. It has been reported that lipid mediators such as those produced following phospholipase-D treatment of cells (ceramide 1-phosphate or its metabolites and LPA), can activate several intracellular signaling cascades that may also contribute to hemolysis. For instance, these bioactive lipids interact with G-protein-coupled receptors that activate oncogenes such as *Rho* and *Ras*, PLC, PI3K, and adenylyl cyclase, which may contribute to hemolysis [Spiegel, 2003; Anliker and Chun, 2004].

In conclusion, we have shown herein that the metabolism of membrane phospholipids such as SM and LPC and the influx of calcium mediated by a L-type channel in human erythrocytes treated with brown spider venom recombinant phospholipase-D is involved in hemolysis.

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