Lycotoxins, Antimicrobial Peptides from Venom of the Wolf Spider Lycosa carolinensis*

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Two peptide toxins with antimicrobial activity, lycotoxins I and II, were identified from venom of the wolf spider Lycosa carolinensis (Araneae: Lycosidae) by virtue of their abilities to reduce ion and voltage gradients across membranes. Both peptides were purified to homogeneity by reversed-phase liquid chromatography and determined to have the following primary structures by Edman microsequencing: IWLTALKFLGKHAA-KHLAKQQLSKL-NH2 for lycotoxin I and KIKWFKTMK-SIAKFIAKEQMKKHLGGE-OH for lycotoxin II. The predicted secondary structures of the lycotoxins display amphipathic α-helix character typical of antimicrobial pore-forming peptides. Antimicrobial assays showed that both lycotoxins potently inhibit the growth of bacteria (Escherichia coli) and yeast (Candida glabrata) at micromolar concentrations. To verify its hypothesized pore-forming activity, lycotoxin I was synthesized and shown to promote efflux of Ca2+ from synaptosomes, to cause hemolysis of erythrocytes, and to dissipate voltage gradients across muscle membrane. The lycotoxins may play a dual role in spider-prey interaction, functioning both in the prey capture strategy as well as to protect the spider from potentially infectious organisms arising from prey ingestion. Spider venoms may represent a potentially new source of novel antimicrobial agents with important medical applications.

Most spiders are voracious predators, employing paralytic venoms to immobilize or kill their prey. The majority of known spider venom toxins attack the nervous system, causing disruption of impulse conduction and/or synaptic transmission through actions on ion channels (1–3) and exocytosis proteins (4, 5). Alternatively, many spider venoms cause cell membrane disruption and consequent tissue necrosis through enzymatic actions (6–8).

The wolf spider Lycosa carolinensis (Araneae: Lycosidae) is an omnivorous hunting spider with a widespread distribution in the United States. The spider hides underground during the day, but leaves its burrow during the night in search of prey. The effects of wolf spider bites on humans are not considered to be severe (9), and relatively little is known with regard to the chemical composition of L. carolinensis venom. Previous studies involving related species indicate the presence of neuroactive constituents (10).

Here we characterize two peptide toxins from L. carolinensis venom that are both neuroactive and antimicrobial. Named here as “lycotoxins,” they appear from their amphipathic character and physiological actions to function as pore formers to increase membrane permeability and to effect lysis of both prokaryotic and eukaryotic cells.

EXPERIMENTAL PROCEDURES

Toxin Purification—Wolf spiders (Lycosa carolinensis Araneae: Lycosidae) were collected from Yavapai County, AZ and held in individual containers in the Department of Entomology, University of California, Riverside, CA. Whole venom was obtained through an electrical milking technique and held at −80 °C until processed. Prior to fractionation by reversed-phase liquid chromatography, venom was dissolved in 0.1% aqueous trifluoroacetic acid and fractionated with a Brownlee wide-pore C8 column (4.6 × 150 mm) using a linear gradient of aqueous acetonitrile (40% to 100%) in constant 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Subsequent purification of biologically active peaks involved variation of organic modifier (acetonitrile or n-propyl alcohol), ion-pairing agent (trifluoroacetic acid or heptfluorobutyric acid), or column (Brownlee C8 or Vydac C4).

Preparation of Synaptosomes—Synaptosomes were prepared from the whole brains of 14–30-day-old Sprague-Dawley rats as described previously (11) and resuspended in “calcium-free” saline (145 mM NaCl, 5 mM KCl, 1.4 mM MgCl2, 1.2 mM NaH2PO4, 10 mM glucose, 20 mM HEPES, adjusted to pH 7.4 with Tris base).

Synaptosomal [3H]Ca2+ Measurements—In most experiments, 45 μl of resuspended synaptosomes (~180 μg of membrane proteins) was incubated with 5 μl of test fraction or deionized water (control) for a specified period of time. To this was added 0.5 μl [3H]Ca2+ in 50 μl of either “high potassium” saline (same as calcium-free saline but containing 137 mM KCl and 1.0 mM CaCl2) or “low potassium” saline (similar to calcium-free saline but containing 1.0 mM CaCl2). Following a 3-s incubation, the reaction was stopped by addition of 200 μl of termination solution (30 mM EGTA, 120 mM NaCl, and 5 mM KCl, adjusted to pH 7.6 with Tris base). Extrasynaptosomal [3H]Ca2+ was removed by rapid filtration with washing buffer (145 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1.4 mM MgCl2, and 20 mM HEPES at pH 7.4) using a Skatron cell harvester. Filters were immersed in 4 ml of Beckman Ready Safe scintillation fluid and subjected to scintillation counting.

Mass Spectrometry, Amino Acid Composition, and Sequencing Analyses—All analyses were performed at the Biotechnology Instrumentation Facility, University of California, Riverside. Matrix-assisted laser desorption time-of-flight mass analyses were performed with a Finnigan Lasermat instrument. Amino acid composition and sequencing analyses were performed as described previously (12).

Peptide Synthesis—Synthetic lycotoxin I was prepared by the Sussex Center for Neuroscience, University of Sussex, Sussex, United Kingdom, on an Applied Biosystems Model 432A Synergy automated peptide synthesizer. The peptide was prepared by solid-phase synthesis using Fmoc (N-(9-fluorenylmethoxycarbonyl) N-terminal protection of amino acids, and amino acids were activated to form an active ester by 2-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. Peptide cleavage from the resin and side chain deprotection were achieved using trifluoroacetic acid and the scavengers thioisole and ethanedithiol (100 μl each). Cleavage was conducted for 3 h at room temperature, followed by extraction of the peptide using methyl- t-butyl ether to separate scavengers and reaction by-products. The peptide was

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FIG. 1. Isolation of lycotoxins from venom of the wolf spider L. carolinensis. A, whole venom (25 μl) was fractionated on a Brownlee C₈ column (300 Å; 4.6 × 250 mm) using a linear gradient of acetonitrile/water (0.5%/min) in constant 0.1% trifluoroacetic acid. Elution of lycotoxins I and II is indicated by arrows. B, shown is the elution profile of lycotoxin I using a linear gradient of n-propyl alcohol/water (0.33%/min) in constant 0.5% trifluoroacetic acid (same column as described for A). C, shown is the elution profile of 7.5 nmol of native lycotoxin I using a Vydac C₈ column (300 Å; 4.6 × 250 mm) and an acetonitrile/water gradient (0.25%/min) in constant 0.1% heptafluorobutyric acid. D, shown is the coelution of native lycotoxin I (4 nmol) and synthetic lycotoxins I and II using the same conditions as described for C. mAU, milli-absorbance units.

lyophilized and purified by preparative RPLC¹ using an Aquapore Octyl Prep 10 cartridge column (10 × 250 mm) at 5 ml/min with a gradient of 0–60% solvent B (solvent A = 5% CH₃CN and 0.1% trifluoroacetic acid; solvent B = 60% CH₃CN and 0.08% trifluoroacetic acid).

Antimicrobial Assays—Magainin B (13), a synthetic peptide analog related to magainin 2 isolated from Xenopus laevis (14), was a generous gift from Dr. Hao-Chia Chen (National Institutes of Health, Bethesda, MD). Escherichia coli D31 (CGSC 5165) was kindly provided by Dr. B. J. Bachmann (E. coli Genetic Stock Center, Department of Biology, Yale University). E. coli DH5 was from Dr. F. M. Sladek (Department of Entomology, University of California, Riverside). Both yeasts Candida glabrata (ATCC 2001) and Candida albicans (clinical isolate) were from Dr. R. Mehra (Department of Entomology, University of California, Riverside).

Lycotoxin I, lycotoxin II, and magainin B were tested for antimicrobial activity against one Gram-positive bacteria (Bacillus thuringiensis subsp. israelensis), two Gram-negative bacteria (E. coli D31 and DH5), and two fungi (C. glabrata ATCC 2001 and C. albicans) by plate growth inhibition assay on thin agar as described below. The E. coli strains and B. thuringiensis were grown in liquid LB broth (1% Bacto-Tryptone, 0.5% Bacto-yeast extract, and 1% NaCl in H₂O) (15) to exponential phase with an A₆₀₀ of 0.8, representing 10⁷ colony-forming units/ml. Bacteria were added to warm (~45 °C) 0.7% agar in LB broth at a ratio of 1 μl of bacteria/ml of medium. Then, 8 or 35 ml of the bacteria-agarose/LB mixture was poured over the bottom of a 1.5% agarose/LB plate in a 100 × 20- or 150 × 20-mm dish. 5 μl of magainin B or lycotoxins of various concentrations was then applied to the solidified plate surface as discrete drops. After incubation at 37 °C overnight, the effects of magainin B and lycotoxins were recorded as the clear spots in the bacterial lawn.

The antimicrobial activities of lycotoxins and magainin B on E. coli D31 in liquid culture were determined in 96-well plates in a final volume of 100 μl as follows. 10 μl of different concentrations of peptides was added to 90 μl of LB broth containing the inoculate of E. coli D31 adjusted to 10⁶ to 10⁷ colony-forming units/ml. After 7 h of incubation at 37 °C, inhibition of growth was determined by measuring the absorbance at 570 nm. Each minimal inhibitory concentration was determined from two independent experiments performed in duplicate.

C. albicans and C. glabrata were grown overnight in YTD medium (1% yeast extract, 2% Tryptone, and 2% glucose) at 37 °C, giving an A₆₀₀ of ~0.9–1.0 (representing 10⁹ colony-forming units/ml). 100 μl of the yeast was diluted in 10 ml of sterilized water. 500 μl of diluted yeast

FIG. 2. Effects of lycotoxins I and II on synaptosomal ⁴⁵Ca²⁺ levels under depolarized or resting conditions. Synaptosomes were exposed to 10 μM lycotoxin I (LyC I) or II (LyC II) for 30 min prior to simultaneous addition of ⁴⁵Ca²⁺ and either high or low potassium. Lycotoxins were omitted in respective control experiments. Histogram values are the average of three experiments, and error bars are S.E. Further details are given under “Experimental Procedures.” White bars, depolarized conditions; shaded bars, resting conditions.

| Amino acid | Lycotoxin I | Lycotoxin II |
|-----------|-------------|-------------|
| Thr       | 1.2 (1)     | 1.5 (1)     |
| Ser       | 1.3 (1)     | 1.5 (1)     |
| Glx       | 2.3 (2)     | 4.1 (3)     |
| Gly       | 1.3 (1)     | 2.7 (2)     |
| Ala       | 4.3 (4)     | 2.7 (2)     |
| Ile       | 1.2 (1)     | 4.4 (3)     |
| Leu       | 6.4 (6)     | 1.3 (1)     |
| Phe       | 1.2 (1)     | 2.6 (2)     |
| Lys       | 3.7 (5)     | 2.6 (8)     |
| His       | 1.3 (2)     | 1.6 (1)     |
| Trp       | 1.0 (1)     | 1.0 (1)     |
| Met       | 0.1 (0)     | 3.0 (2)     |

¹ The abbreviations used are: RPLC, reversed-phase liquid chromatography; PBS, phosphate-buffered saline; EJP, excitatory junctional potential.

² The presence of Trp was based on UV absorption spectrum.

Table I

Amino acid composition of native lycotoxins I and II

| Amino acid | Lycotoxin I | Lycotoxin II |
|-----------|-------------|-------------|
| Thr       | 1.2 (1)     | 1.5 (1)     |
| Ser       | 1.3 (1)     | 1.5 (1)     |
| Glx       | 2.3 (2)     | 4.1 (3)     |
| Gly       | 1.3 (1)     | 2.7 (2)     |
| Ala       | 4.3 (4)     | 2.7 (2)     |
| Ile       | 1.2 (1)     | 4.4 (3)     |
| Leu       | 6.4 (6)     | 1.3 (1)     |
| Phe       | 1.2 (1)     | 2.6 (2)     |
| Lys       | 3.7 (5)     | 2.6 (8)     |
| His       | 1.3 (2)     | 1.6 (1)     |
| Trp       | 1.0 (1)     | 1.0 (1)     |
| Met       | 0.1 (0)     | 3.0 (2)     |
Antimicrobial Peptide Lycotoxins

was plated evenly on each 150 × 20-mm agarose plate of YTD medium. 5 μl of magainin B or lycotoxins of various concentrations was then applied to the solidified plate as discrete drops. After incubation at 37 °C overnight, the effects of magainin B and lycotoxins were recorded as the clear spots in the yeast lawn.

Hemolysis Assay—Hemolytic activity of both lycotoxin I and magainin B was assayed with heparinized red blood cells from rabbit (Oryctolagus cuniculus) rinsed three times in PBS (50 mM NaH2PO4 and 150 mM NaCl, pH 7.0) by centrifugation for 5 min at 4310 g. The supernatant was then incubated at room temperature for 1 h in deionized water (positive control), in PBS (blank), or with synthetic lycotoxin I or magainin B at various concentrations (6.25–200 μM) in PBS. The samples were centrifuged at 11,640 × g for 3 min. The supernatant was separated from the pellet, and its absorbance was measured at 570 nm. Zero hemolysis (blank) and 100% hemolysis controls were determined as described under “Experimental Procedures.” Two fractions were identified as described previously (16).

Neuromuscular Pharmacology—Lycotoxin I was assayed on the body wall muscles of prepupal house flies (Musca domestica, NAIDM strain) using the intracellular electrophysiological recording methods described previously (16).

RESULTS

Isolation of Lycotoxins—In the process of screening spider venoms for calcium channel antagonism, we found that venom of the wolf spider L. carolinensis (1:1000 dilution) disrupts 45Ca2+ ion flux in depolarized rat brain synaptosomes. As we demonstrate below, this effect results from dissipation of chemical and electrical gradients across membranes rather than from calcium channel antagonism. To further characterize this biological activity, we fractionated the venom by reversed-phase liquid chromatography using the protocols described under “Experimental Procedures.” Two fractions were identified and named lycotoxins I and II (Fig. 1A). Each lycotoxin was purified in subsequent steps to homogeneity (Fig. 1, B and C).

Calcium flux assays used to follow the purification of the lycotoxins are shown in Fig. 2. Depolarization of rat synaptosomes by elevating the external potassium concentration increases synaptosomal 45Ca2+ levels by almost 2–4-fold, due to opening of voltage-dependent calcium channels. Depolarization-induced 45Ca2+ uptake was completely inhibited following pre-exposure to lycotoxin I (10 μM) or lycotoxin II (10 μM) (Fig. 2). Both lycotoxins (10 μM) also depleted basal 45Ca2+ levels in the absence of potassium depolarization (Fig. 2). Taken together, these results suggest that the lycotoxins interfere with the ability of synaptosomes to sequester Ca2+.

Amino Acid and Mass Analyses—Quantitative amino acid composition analysis of each lycotoxin gave the results shown in Table I; the presence of tryptophan was deduced from on-line UV absorption spectra of lycotoxins (data not shown). Amino acid composition analysis allowed us to determine the concentrations of lycotoxins in the crude venom to be ~5 mM. Preliminary mass analysis (matrix-assisted laser desorption time-of-

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**Table II**

Sequence analysis of lycotoxin I

| Cycle | Amino acid | Yield* |
|-------|------------|--------|
| 1     | Met        | 31     |
| 2     | Thr        | 17     |
| 3     | Thr        | 15     |
| 4     | Thr        | 14     |
| 5     | Lys        | 7      |
| 6     | Lys        | 4      |
| 7     | Lys        | 3      |
| 8     | Lys        | 2      |
| 9     | Lys        | 1      |
| 10    | Lys        | 1      |

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**Table III**

Sequence analysis of lycotoxin II

| Cycle | Amino acid | Yield* |
|-------|------------|--------|
| 1     | Lys        | 41     |
| 2     | Lys        | 37     |
| 3     | Lys        | 33     |
| 4     | Lys        | 30     |
| 5     | Lys        | 27     |
| 6     | Lys        | 24     |
| 7     | Lys        | 21     |
| 8     | Lys        | 18     |

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* Picomole yield was corrected for background and lag.
flight) gave molecular mass values of 2843 for lycotoxin I and 3204 for lycotoxin II. Based on molecular mass values for each toxin, the lycotoxins were estimated to contain 25–28 amino acid residues, including tryptophan.

**Primary Structures of Lycotoxins**—The primary structures of the lycotoxins were determined by automated Edman degradation. The amino acid sequence of lycotoxin I was identified as IWLTALKFLGKHAAKHKLAKQQLSKL-NH₂ (Table II). The sequence of lycotoxin II was determined to be KIKWFKTMKSIAKFLQAKQMKKHLOGGE-OH (Table III). The deduced sequences are in good agreement with the results from amino acid composition analyses (Table I). The C-terminal amidation of lycotoxin I was assigned by high resolution electrospray mass spectrometry. The mass of the native peptide was [MH]⁺ = 2843.5, which is virtually identical to the mass of 2843.5 predicted for the C-terminal amidated peptide, whereas that predicted for the free acid is 2844.5. Mass analysis of lycotoxin II yielded [MH]⁺ = 3206.0, compared with the predicted values of 3205.8 for the free acid form and 3204.8 for the amidated form. From this comparison, we concluded that lycotoxin II occurs as the free acid.

To confirm the chemical identity of lycotoxin I and to provide sufficient quantities for further biological experiments, the peptide was prepared synthetically. Co-injection of native lycotoxin I (4 nmol) and synthetic lycotoxin I (3 nmol) gave a single peak on reversed-phase liquid chromatography (Fig. 1D); integration of this peak indicated the presence of both substances as the peak area was proportional to the total amount of the two materials tested. This confirmed that native lycotoxin I and synthetic lycotoxin I have identical RPLC elution profiles. Additionally, native and synthetic peptides were compared and found to be indistinguishable in their ability to deplete 45Ca²⁺ levels in rat synaptosomes (Fig. 3). Logistic curves fitted to the data showed that synthetic lycotoxin I had an EC₅₀ of 5.0 ± 0.13 μM as compared with native lycotoxin I with an EC₅₀ of 4.5 ± 0.14 μM. Taken together, these results show that synthetic lycotoxin I is chemically and biologically indistinguishable from the native peptide.

**Amphipathic α-Helical Character of Lycotoxins**—The primary structures of lycotoxins are characterized by lysine re-
Antimicrobial Peptide Lycotoxins—A}ntimicrobial Activity of Lycotoxins—The lycotoxins show potent antimicrobial activity against both prokaryotic and eukaryotic cells, as demonstrated by classical zone inhibition assays. White dots represent inhibition of cell growth at sites of toxin application. Rows A–C, Gram-negative bacteria (E. coli strain DH5); rows D–F, Gram-positive bacteria (B. thuringiensis subsp. israelensis); rows G–I, yeast (C. glabrata). Magainin B was applied in rows A, D, and G; lycotoxin I was applied in rows B, E, and H; and lycotoxin II was applied in rows C, F, and I. Toxin concentrations (micromolar) are shown at the top of each column.

Lycotoxins show antimicrobial activity against both prokaryotic and eukaryotic cells, as demonstrated by classical zone inhibition assays. White dots represent inhibition of cell growth at sites of toxin application. Rows A–C, Gram-negative bacteria (E. coli strain DH5); rows D–F, Gram-positive bacteria (B. thuringiensis subsp. israelensis); rows G–I, yeast (C. glabrata). Magainin B was applied in rows A, D, and G; lycotoxin I was applied in rows B, E, and H; and lycotoxin II was applied in rows C, F, and I. Toxin concentrations (micromolar) are shown at the top of each column.

peaks occurring every fourth or fifth position. A GenBank™ search resulted in the identification of several peptides showing a similar lysine motif, including adenoregulin, a peptide from the skin secretion of the frog Phyllomedusa bicolor (17), and dermaseptins, from insect hemolymph (14, 18) (Fig. 4A). Magainins, dermaseptins, and adenoregulin are all antimicrobial pore-forming peptides with characteristic amphipathic α-helical secondary structures (14, 17, 18). Interestingly, the predicted secondary structures of lycotoxins I and II show that the majority of their amino acids occur in an α-helix conformation according to Chou-Fasman (19) and Garnier-Osguthorpe-Robson (20) principles. When plotted as α-helical wheels, the majority of the hydrophobic and hydrophilic amino acid residues occur on opposite sides of the helix (Fig. 4, B and C). For lycotoxin I, for instance, six charged or hydrophilic residues occur on one side of the cylindrical surface, and eight hydrophobic residues are arranged on the opposite site. Except for the C-terminal lysine (lysine 24), all positively charged lysine residues are on the hydrophilic side. For lycotoxin II, six lysines and the C-terminal glutamate occur on the hydrophilic side, whereas eight hydrophobic residues and two lysines occur on the predicted hydrophobic side. This suggests that both lycotoxins I and II may be configured as amphipathic α-helices spanning large portions of the peptides. In fact, such amphipathic α-helical secondary structure has been predicted for many other peptides including PGLa, XPF, and bombinins from frog skin and cecropins from insects (21, 22). The finding that lycotoxins are significantly conserved with antimicrobial peptides in both the primary and secondary structures led to experiments designed to test them for antimicrobial activity.

Antimicrobial Activity of Lycotoxins—The lycotoxins show potent antimicrobial activity against both prokaryotic and eukaryotic cells in plate growth inhibition assays. Against E. coli strain DH5 (Fig. 5, rows A–C), lycotoxin II was most active, showing inhibitory activity at concentrations as low as 40 μM as compared with 60–80 μM for magainin B and 80–150 μM for lycotoxin I. The most sensitive to lycotoxin I was a Gram-positive bacterial species, B. thuringiensis subsp. israelensis (Fig. 5, row E), for which the minimal inhibitory concentration of lycotoxin I was 5 μM (lowest concentration tested); the minimal inhibitory concentration for magainin B and lycotoxin II was 60 μM (Fig. 5, rows D and F). Against Gram-negative E. coli strain D31 (23), lycotoxins I and II were similarly active in the 10–20 μM range (Table IV). Finally, lycotoxins I and II inhibited growth of a yeast species, C. albicans, at 40 μM, whereas twice that concentration was required for observable activity with magainin B (Fig. 5, rows G–I). A summary of the results from plate growth inhibition assays is shown in Table IV. In general, the lycotoxins were more active as antimicrobial agents than magainin B.

When tested in a liquid growth inhibition assay (Fig. 6), the antimicrobial actions of lycotoxin I, lycotoxin II, and magainin B appeared very abruptly at submicromolar concentrations (−0.6–0.7 μM). When applied jointly, the effects of lycotoxins I and II were additive, suggesting that they have little or no synergistic action. The slope factors for these inhibition curves were in excess of 25; the steep relationship between concentration and lytic activity indicates a phase change in the membrane that most likely involves a high degree of cooperativity between toxin molecules and is characteristic of peptides for which aggregation confers antimicrobial activity (24).

Lysis of Erythrocytes—Pore-forming peptides such as the magainins lyse erythrocytes at high micromolar concentrations (13). Given the sequence similarities between the lycotoxins and magainins, we compared lycotoxin I and magainin B for the ability to lyse rabbit erythrocytes (Fig. 7). Neither lycotoxin I nor magainin B showed detectable hemolytic activity at concentrations below 30 μM. However, both peptides showed significant hemolytic activity at concentrations above 100 μM, with lycotoxin I being more active. For instance, at 200 μM, 55% hemolysis was observed for lycotoxin I ((A_{Lycotoxin}/A_{Hb}) × 100%) and 35% for magainin B. These results provide further evidence for the pore-forming activity of lycotoxin I.

Lycotoxin Dissipates Ion Gradients—To further characterize the effects of lycotoxins on ion gradients, we performed several experiments using synaptosomes preloaded with 45Ca2+ (Fig. 8). Synaptosomes (~180 μg of membrane protein) accumulated a maximum of 65 pmol of 45Ca2+ within 10 min (Fig. 8, Treatments 1 and 3). Note that Treatment 2, which involved exposure to 46Ca2+ and cobalt simultaneously, blocked 45Ca2+ accumulation, whereas addition of cobalt after a 10-min delay (Treatment 3) permitted accumulation to the control level. Cobalt is known to antagonize both inward and outward 45Ca2+ movements due to inhibition of calcium channels and the Na+/Ca2+ exchanger (25).

Addition of synthetic lycotoxin I at time 0 (Fig. 8, Treatment 4) or after a 15-min preloading period (Treatment 5) reduced control 45Ca2+ accumulation by 80%. The result of Treatment 4 suggests that the toxin prevents 45Ca2+ sequestration, whereas the result of Treatment 5 indicates that the toxin promotes 45Ca2+ efflux from preloaded synaptosomes. If preloaded synaptosomes were treated sequentially with cobalt followed by lycotoxin I, the control response was reduced 50%. Taken together, these results suggest that lycotoxin I dissipates 45Ca2+ gradients in preloaded synaptosomes.

Lycotoxin I Dissipates Membrane Electrochemical Potential—Since insects are the natural prey targeted by wolf spiders, we tested the effects of lycotoxin I on insect musculature. Specifically, we examined the effects of the toxin on muscle membrane potentials. A pore-forming activity of lycotoxin would be manifested as a diminution of the resting membrane.

![Image](image-url)
potential. Intracellular recordings were made from body wall muscles 6A and 7A of larval third instar house flies (M. domestica). The resting potential ($E_{\text{rest}}$) and amplitude of the neurally evoked excitatory junctional potential (EJP) were recorded and plotted every 4 s (Fig. 9). Brief exposure to synthetic lycotoxin I (1 $\mu M$) caused a slight but reversible depolarization of the $E_{\text{rest}}$ and a corresponding decrease in the amplitude of the EJP. When 3 $\mu M$ lycotoxin I was applied, the $E_{\text{rest}}$ depolarized from −60 to 0 mV. At the same time, the EJP was completely blocked. These results show that lycotoxin I dissipates the muscle cell resting potential in this preparation.

**DISCUSSION**

We have identified and characterized the lycotoxins, two broad-spectrum antimicrobial peptides that occur in venom of the wolf spider L. carolinensis. To our knowledge, this is the first report documenting the occurrence of antimicrobial peptides in spider venom. The lycotoxins occur at concentrations of 1–5 mM in whole L. carolinensis venom, which is in the range previously reported for agatoxins occurring in funnel web spider (Agelenopsis aperta) venom (12, 26–28). Such high concentrations suggest that the lycotoxins play an important role in the biochemical strategy used by the spider to capture insect prey. We observed a complete loss of cell membrane potential and block of neuromuscular transmission in insect body wall muscles by lycotoxin I (3 $\mu M$). Furthermore, the lycotoxins (2–20 $\mu M$) caused efflux of calcium ions from rat brain synaposomes. These results indicate that the lycotoxins dissipate ion and voltage gradients across the membranes of excitable cells and likely contribute to paralysis of envenomated prey.

The utility of the lycotoxins in prey capture is complemented by their antimicrobial activity, which may serve as a defense against infectious organisms arising from prey ingestion. Precedent for this concept can be found in reports of magainins and defensins expressed by epithelial cells in gastric and intestinal mucosa (29–31), where they may have multiple functions, including defense against microbial infection and regulation of symbiotic gut flora (32–35). The mode of action of the lycotoxins therefore may serve two functions for the wolf spider: as a paralytic agent used for prey capture as well as a defense against infectious microbes arising from prey ingestion.

Lycotoxins I and II are cationic peptides containing 25 and 27 amino acids, respectively. They share 52% sequence similarity with each other. Theoretical predictions of their secondary structures suggest the formation of a cationic amphipathic $\alpha$-helix in which lysine residues are clustered on the face of the cylindrical surface. This type of secondary structure is characteristic of magainins, dermaseptins, and adenoregulin. Indeed, lycotoxin I shows 60% sequence similarity to magainin B and 48% similarity to adenoregulin. Lycotoxin II shows 45% similarity to magainin B and 48% similarity to adenoregulin. The

**TABLE IV**

| Organisms                          | Range of tested conc. (minimum to maximum) | Minimum effective conc$^a$ |
|------------------------------------|---------------------------------------------|-----------------------------|
|                                    |                                             | Lycotoxin I | Lycotoxin II | Magainin B |
| E. coli D31                        | $10.0–150.0$                                | $10.0–20.0$ | $<10.0$     | $20.0–30.0$  |
| E. coli DH5                        | $40.0–200.0$                                | $80.0–150.0$ | $<40.0$     | $50.0–60.0$  |
| B. thuringiensis subsp. israelensis| $5.0–150.0$                                 | $<5.0$       | $10.0–60.0$ | $10.0–60.0$  |
| C. glabrata ATCC 2001              | $100.0–500.0$                               | $100.0–150.0$ | $100.0–150.0$ | $150.0–200.0$ |
| C. albicans clinical isolate       | $25.0–200.0$                                | $100.0–200.0$ | ND$^b$      | ND          |

$^a$ Minimum effective concentrations were determined as the range between the highest noneffective concentration and the lowest effective concentration. The concentration here applies to the toxin solution applied to plates.

$^b$ ND, no detectable effects in the test concentration range.

**FIG. 6.** Antimicrobial activity of lycotoxin I, lycotoxin II, and magainin B using an E. coli strain D31 suspension assay. The combined action of lycotoxins I and II is shown (■). Inhibition by each toxin was determined after a 7-h incubation. The control condition involved addition of toxin-free LB broth.

**FIG. 7.** Hemolytic activity of lycotoxin I compared with that of magainin B. Release of hemoglobin was determined by measuring the absorbance at 570 nm. Rabbit erythrocytes were incubated with increasing concentrations of lycotoxin I (○) or magainin B (●) dissolved in PBS for 1 h. The positive control for cell lysis was determined by addition of water (△). PBS was used as a blank for the absorbance measurement.
antimicrobial actions of the lycotoxins are also consistent with this group of amphipathic α-helical peptides, which also includes PGLa, CPF, and XPF (36, 37).

Natural antimicrobial peptides with amphipathic α-helical structures (38) have been shown to disrupt biological membranes through pore formation (39–45) or, in some cases, through destabilization of membrane phospholipid packing (46). These peptides form channels by self-aggregation of peptide monomers, whereby hydrophilic residues on one side of the helix face inward and hydrophobic residues on the opposite side of the helix interact with fatty acid side chains of the lipid bilayer.

At least four lines of evidence presented in this paper support the hypothesis that lycotoxins behave as pore-forming peptides. 1) They inhibit the growth of bacteria at a characteristic critical concentration, an indicator of self-aggregation of the peptide; 2) cause 45Ca2+ efflux from rat brain synaptosomes; 3) induce hemolysis of erythrocytes; and 4) dissipate the electrochemical potential across muscle membrane. Experiments in which lycotoxins showed antimicrobial activity were conducted in parallel with magainin B, which showed similar potency in some of the assays. Of particular interest are the data on lysis of E. coli strain D31, in which both lycotoxins I and II and magainin B showed extremely steep concentration response curves (Fig. 6). In contrast, these agents lysed red blood cells at much higher concentrations with parallel but far less steep concentration curves (Fig. 7). These data indicate that the lycotoxins and magainin B operate according to similar but probably distinct mechanisms in each system. Finally, the amino acid sequences of lycotoxin I and magainin B are similar but probably distinct mechanisms in each system.

Although the lycotoxins share significant sequence similarity with magainin B, different specificities were observed for each peptide. Lycotoxin I was more effective against the Gram-positive bacteria B. thuringiensis and yeast than magainin B. Magainin B (10 μM) also showed no effect on synaptosomal Ca2+ efflux (data not shown), whereas both lycotoxins caused 45Ca2+ efflux from synaptosomes.

The lycotoxins define a new functional subclass of spider venom toxins based on their amino acid sequences and antimicrobial activity. As with the magainins, we believe that the lycotoxins have the potential to stimulate development of new useful therapeutic agents (47, 48). The rapid development of antibiotic-resistant bacteria points to the ongoing need for novel antimicrobial agents (38). Our findings suggest that spider venoms represent a potentially widespread source of novel antimicrobial agents that may have important medical implications.

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