ω-Agatoxins: Novel Calcium Channel Antagonists of Two Subtypes from Funnel Web Spider (Agelenopsis aperta) Venom*

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A new series of polypeptide presynaptic antagonists ("ω-agatoxins") was purified from venom of the funnel web spider Agelenopsis aperta. Physiological data indicate that all of these peptides are antagonists of voltage-sensitive calcium channels. Although all three ω-agatoxins (Aga) described here (ω-Aga-IA, ω-Aga-IB, and ω-Aga-IIA) block insect neuromuscular transmission presynaptically, biochemical data permit their subclassification as Type I and Type II toxins. Type I toxins (ω-Aga-IA and -IB) are 7.5 kDa, have closely related amino acid sequences, and exhibit characteristic tryptophan-like UV absorbance spectra. Complete Edman sequencing of ω-Aga-IA reveals it to be a 66-amino acid polypeptide containing 9 cysteines and 5 tryptophan residues. ω-Aga-IIA, a Type II toxin, is 11 kDa, shows limited amino acid sequence similarity to the Type I toxins, and exhibits mixed tryptophan- and tyrosine-like absorbance. Nanomolar concentrations of ω-Aga-IIA inhibit the specific binding of 125I-labeled ω-conotoxin GVIA to chick synaptosomal membranes while ω-Aga-IA and -IB have no effect under identical conditions. The ω-agatoxins thus are defined as two subtypes of neuronal calcium channel toxins with different structural characteristics and calcium channel binding specificities.

The funnel web spider Agelenopsis aperta immobilizes its prey by injection of a potent paralytic venom. Paralysis is associated with three distinct effects of the venom on insect neuromuscular transmission: postsynaptic antagonism, presynaptic activation, and presynaptic antagonism. Each of these actions is associated with a separate class of neurotoxins, the α-, ω-, and ω-agatoxines (1–4). The ω-agatoxines, a family of low molecular weight acylpolyamines, reversibly suppress excitatory junctional potentials (EJPs) in insect muscle by blocking the glutamate-sensitive receptor channel complex (2). The α- and ω-agatoxines are polypeptides which affect presynaptic voltage-sensitive ion channels. The ω-agatoxines induce repetitive activity in motor neurons and increase the rate of transmitter release (2); their chemical characteristics (3) and physiological actions are similar to scorpion toxins, which modify sodium channel kinetics in insect axons (5, 6). A third class of neurotoxins in Agelenopsis venom, the ω-agatoxines, produce long lasting suppression of neurotransmitter release from presynaptic stores. The actions of the ω-agatoxins are consistent with antagonism of voltage-sensitive calcium channels (4).

We report here the isolation and biochemical characterization of three ω-agatoxins from Agelenopsis venom. While all three toxins show functionally similar presynaptic antagonism of insect neuromuscular transmission, biochemical criteria indicate that they fall into two distinct groups.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Neuromuscular Block by ω-Agatoxines—RPLC fractionation of crude A. aperta venom yielded a series of polypeptides ("ω-agatoxins") which block neuromuscular transmission in housefly body wall muscle. Three of these peptides, ω-Aga-IA, ω-Aga-IB, and ω-Aga-IIA, were identified based on their ability to produce long lasting suppression of neurally evoked EJPs without affecting ionophoretic glutamate potentials (IPs; Fig. 1, A and B). Whereas EJPs depend on the sequential steps of endogenous neurotransmitter release and postsynaptic receptor action, IPs result from artificial application of glutamate and thus are not dependent on endogenous transmitter release. Since the IP remains unchanged after EJP block by the ω-agatoxines, toxin action cannot be explained by disruption of glutamate signaling at the postsynaptic membrane. Instead, block of the EJP may involve reduction in the amount of neurotransmitter released by nerve stimulation. Detailed electrophysiological analyses of neuromuscular antagonism by ω-Aga-IA indeed demonstrate its ability to suppress transmitter release by blocking voltage-activated calcium channels (4). This mode of action is clearly distinguishable from those of the α- and ω-agatoxines previously isolated from A. aperta venom (2). For example, neuromuscular antagonism by the α-agatoxin AG460 results in suppression of both EJPs and IPs (Fig. 1C), an effect that reflects postsynaptic action at the glutamate-sensitive receptor channel complex. The μ-agatoxines, represented by μ-Aga-I, induce repetitive activity in motor units without inhibiting transmission (Fig. 1D).

Neuromuscular antagonism by the ω-agatoxins is long lasting, as opposed to the reversible block caused by the α-
**Synaptosomal Membranes**—Evidence that \( \omega \)-agatoxins reduce transmitter release by blocking neuronal calcium channels led us to investigate their possible effect on specific \( \omega -CgTx \) binding to chick synaptosomal membranes (8, 15). Membranes were preincubated with each \( \omega \)-agatoxin prior to their exposure to \( \omega -CgTx \). Prior exposure of membranes to 0.2 or 0.5 \( \mu M \) \( \omega -Aga-IIIa \) virtually abolishes specific binding of \( \beta ^{125}I-\omega -CgTx \) (Table I); the IC\(_{50} \) for this inhibition is approximately 10 nM. In contrast, pre-exposure of membranes to \( \omega -Aga-IA \) or \( \omega -Aga-IB \) (0.5-1.0 \( \mu M \)) has no effect on \( \beta ^{125}I-\omega -CgTx \) binding under identical conditions. These binding data demonstrate a clear inhibition of \( \beta ^{125}I-CgTx \) binding by \( \omega -Aga-IIIa \) but not by \( \omega -Aga-IA \) or -IB.

**Purification of \( \omega \)-Agatoxins**—Crude venom was thawed, mixed thoroughly, and dissolved in 1% trifluoroacetic acid. Initial fractionations were performed with a Brownlee RP-8 column using a linear 0.50%/min solvent gradient of acetonitrile/water (0-60%) in constant 0.1% trifluoroacetic acid. Under these conditions, the \( \omega \)-agatoxins elute immediately following the \( \mu \)-agatoxins (Fig. 3). In this initial fractionation, \( \omega -Aga-IA \) and -IB co-elute at about 28% acetonitrile.
acetonitrile, while \(\omega\)-Aga-IIA elutes later as part of a larger peak at about 32% acetonitrile. \(\omega\)-Aga-IA and -IB are characterized by distinctive tryptophan-like absorbance spectra (250–310 nm), while the absorbance of \(\omega\)-Aga-IIA reflects mixed aromatic character (Fig. 6).

Two additional chromatographic steps were necessary for purification of each \(\omega\)-agatoxin to homogeneity. \(\omega\)-Aga-IA and \(\omega\)-Aga-IB were further resolved by chromatography with a Vydac C18 wide pore semipreparative column using a 0.3%/min gradient of \(n\)-propyl alcohol/water (20–30%) in constant 1% trifluoroacetic acid at a flow rate of 2.0 ml/min (Fig. 4A). Final purification of each toxin to remove minor contaminants was accomplished with a Vydac C18 column using a 0.2%/min linear gradient from 40–50% acetonitrile/water in constant 0.1% heptfluorobutyric acid at a 1.0 ml/min flow rate (not shown). \(\omega\)-Aga-IIA was separated from a larger peak using a Brownlee RP8 analytical column and a 0.3%/min \(n\)-propyl alcohol/water gradient (20–30%) in constant 1% trifluoroacetic acid at 1.0 ml/min (Fig. 4B). Final purification of \(\omega\)-Aga-IIA was accomplished using the Vydac C4 analytical column and a 0.5%/min acetonitrile/water gradient (35–45%) in constant 0.1% heptfluorobutyric acid. Purity of each toxin was confirmed by sequence analyses and the presence of a single band on SDS-PAGE electrophoresis.

Crude Agelesion venom contains an average value \((n = 3)\) of 128 mg/ml total protein (12.8% w/v). Purifications of 50-ml batches of crude venom resulted in the following yields of \(\omega\)-agatoxins relative to total protein in the venom: \(\omega\)-Aga-IA, 0.6%; \(\omega\)-Aga-IB, 0.12%; and \(\omega\)-Aga-IIA, 0.12%. Based on molecular weights determined from SDS-PAGE electrophoresis, and amino acid composition analysis, crude venom concentrations of each \(\omega\)-agatoxin are: \(\omega\)-Aga-IA, 100–150 \(\mu\)M; \(\omega\)-Aga-IB and -IIA, 5–20 \(\mu\)M.

Characterization of \(\omega\)-Agatoxins—Each RPLC-purified \(\omega\)-agatoxin appeared as a single band upon SDS-PAGE electrophoresis (Fig. 5). Estimated masses are 7.5 kDa for \(\omega\)-Aga-IA and -IB and 11 kDa for \(\omega\)-Aga-IIA. Amino acid analyses of reduced pyridylethylated \(\omega\)-Aga-IA and -IB (Table II) show them to contain 9 cysteines. Total cysteine content of \(\omega\)-Aga-IIA was not determined by amino acid composition analysis, but NH2-terminal amino acid sequencing (see below) indicates that 6 of the first 28 residues are cysteines. Tryptophan analysis of mercaptoethanesulfonic acid hydrolysates yielded 5 mol/mol for \(\omega\)-Aga-IA and \(\omega\)-Aga-IB and 3 mol/mol for \(\omega\)-Aga-IIA. \(\omega\)-Aga-IA and \(\omega\)-Aga-IB contain little or no tyrosine, while \(\omega\)-Aga-IIA contains 7 mol/mol. These data explain UV absorbance spectra indicating an essentially pure tryptophan-like character for \(\omega\)-Aga-IA and -IB (Fig. 6A) but a mixed trp/tyr character for \(\omega\)-Aga-IIA (Fig. 6B).

Sequence Determination of \(\omega\)-Aga-IA—Edman sequencing of a 400-pmol sample of reduced pyridylethylated \(\omega\)-Aga-IA yielded assignments of amino acid residues 1–50 (Fig. 7). Further sequencing of the molecule was facilitated by the occurrence of methionine at position 46. Cleavage of the peptide with cyanogen bromide produced two major fragments whose amino acid compositions (Table III) provided confirmation of the NH2-terminal sequence (1–46) and a COOH-terminal fragment for sequencing of residues 51–66. Sequencing of 300 pmol of the COOH-terminal CNBr fragment yielded residues 47–66. The first 4 amino acids of the COOH-terminal fragment were in agreement with positions 47–50.

**Fig. 4.** Secondary purifications of \(\omega\)-agatoxins. A, \(\omega\)-Aga-IB is separated from \(\omega\)-Aga-IA using a Vydac C18 wide pore semipreparative column at a flow rate of 2.0 ml/min and a linear 0.5%/min propyl alcohol-water gradient (dotted line) in constant 1.0% trifluoroacetic acid. \(\omega\)-Aga-IA is typically 5–10-fold more abundant than \(\omega\)-Aga-IB in the crude venom. B, separation of \(\omega\)-Aga-IA using a Brownlee C4 wide pore column and a linear 0.5%/min propyl alcohol-water gradient (dotted line) in constant 1.0% trifluoroacetic acid run at 1.0%/min. \(\omega\)-Aga-IIA emerges as a minor peak and occurs at about the same abundance as \(\omega\)-Aga-IB in the crude venom.

**Fig. 5.** Tricine SDS-PAGE electrophoresis of RPLC-purified \(\omega\)-agatoxins; sizes of the reduced denatured toxins were interpolated from a standard curve of RF versus molecular weight of a series of standard proteins. Each \(\omega\)-agatoxin was taken up in a mixture of 0.4 M Tris, 4% SDS, 20% glycerol, 30 mM EDTA, 14% 2-mercaptoethanol and boiled for 10 min prior to loading on the gel. Approximate masses of the three \(\omega\)-agatoxins, which migrated as single bands, are: A, \(\omega\)-Aga-IA, 7.5 kDa; B, \(\omega\)-Aga-IIA, 11.0; and C, \(\omega\)-Aga-IB, 7.5 kDa. The \(\omega\)-Aga-IIA sample shown in lane C was taken from the second RPLC purification step (shown in Fig. 4B) at which point a minor contaminant running at about 7.0 kDa was present on the gel. Final purification of \(\omega\)-Aga-IIA using 0.5%/min acetonitrile/water gradient (35–45%) in constant 0.1% HFBA removed this contaminant, which proved to be devoid of biological activity.
Fig. 9. NH₂-terminal amino acid sequences of the ω-agatoxins. A, residues 1-36 are shown for ω-Aga-IA and -IB and 1-25 for ω-Aga-IIA. Comparison of ω-Aga-IA and -IB residues 1-36 reveals 72% correspondence between all amino acids and identical placement of cysteine residues at positions 10, 17, 19, 26, and 28. Considerably lower correspondence (43%) is observed between ω-Aga-IIA or -IB and ω-Aga-IIA. Nonetheless, substantial correspondence of cysteine positions (10, 17, 26, and 28) is observed between ω-Aga-IIA and ω-Aga-IA. B, alignment of ω-agatoxin and ω-agatoxin (3) sequences reveals noticeable correspondence of the underlined amino acid residues at positions 19, 20, 21, 22, 25, 26, 27, 33, and 35.

Previously assigned from the NH₂-terminal sequence. The complete sequence assignment of ω-Aga-IA (Fig. 7) was verified by amino acid composition and mass spectrometric analysis of trypsin digestion fragments.

Nine of the expected 11 fragments from trypsin digestion of reduced and pyridylethylated ω-Aga-IA were isolated by RPLC. The amino acid compositions of these fragments confirmed the assignments made from Edman sequencing (Table IV). Although trypsin fragments were not analyzed directly for tryptophan composition, fragments 23-25, 28-31, 32-44, and 60-66 exhibited characteristic tryptophan-like UV absorbance spectra virtually identical to that of the free acid. Furthermore, the magnitude of 280-nm absorbance of fragment 60-66 on a molar basis was twice that of the other trypsin fragments, an observation consistent with the presence of two tryptophan residues. All other fragments (3-18, 19-22, 26-27, 48-53, and 54-59) were devoid of tryptophan-like absorbance.

Fast atom bombardment mass spectrometry analysis of fragments 3-18, 32-44, and 60-66 yielded expected protonated molecular ions (MH⁺) of 1802, 1705, and 876, respectively. High resolution analysis of the COOH-terminal heptapeptide (60-66) yielded MH⁺ of 876.351, which corresponds to the predicted value of 876.389 for the free acid A-E-W-G-L-N-W-OH, as opposed to the mass of 875.405 predicted for the amilorilated heptapeptide. Positive ion daughter spectra derived from the COOH-terminal heptapeptide (Fig. 8) confirmed the results of Edman sequencing.

Disulfide Bonding—Determination of multiple cysteine residues in ω-Aga-IA suggests the presence of disulfide bridging. We found that the biological activity of the toxin remains unchanged following 10 min of boiling but that reduction of the toxin using β-mercaptoethanol followed by RPLC purification results in complete loss of biological activity (block of the EJP) at concentrations up to 100 nM, a concentration 25 times in excess of the EC₅₀ for EJP block by native ω-Aga-IA (4). Disulfide bonding thus appears to be essential for the biological activity of ω-Aga-IA and by analogy, ω-Aga-IB and -IIA.

NH₂-terminal Sequence Comparison of ω-Aga-IA, ω-Aga-IB, and ω-Aga-IIA—Comparison of NH₂-terminal amino acid sequences indicates a high degree of similarity between ω-Aga-IA and -IB, and substantial differences between these peptides and ω-Aga-IIA (Fig. 9A). Residues 1-36 of ω-Aga-IA and -IB show 72% overall similarity and identical placement of cysteines at positions 10, 17, 19, 26, and 28. The NH₂ terminus of ω-Aga-IIA shows only 43% correspondence to ω-Aga-IA or -IB. However, cysteine residues at positions 2, 9, 18, and 21 of ω-Aga-IIA and positions 10, 17, 26, and 28 of ω-Aga-IA and -IB are spaced identically (Fig. 9A). This suggests that some disulfide bridges may be conserved in the ω-agatoxins.

The NH₂-terminal sequences of the ω-agatoxins show considerable correspondence with those of the μ-agatoxins (3). Alignment of ω-agatoxins and μ-agatoxins as shown in Fig. 9B reveals corresponding amino acids at positions 19, 20, 21, 22, 25, 26, 27, 33, and 35.

DISCUSSION

The ω-agatoxins are the third functionally distinct class of synaptic toxins to be isolated from A. aperta venom.Acting as presynaptic antagonists, they reduce the amount of transmitter released from nerve terminals, an effect observed as suppression of EJPs but not IPSPs. In contrast, the ω-agatoxins are antagonists of the glutamate receptor channel complex, leading to suppression of both EJP and IPSP. These two classes of toxin are distinguished from the μ-agatoxins, which have excitatory rather than antagonistic effects on neuromuscular transmission (2). The three classes of synaptic toxins occurring in A. aperta venom thus are defined as postsynaptic antagonists (α-agatoxins), presynaptic activators (μ-agatoxins), and presynaptic antagonists (ω-agatoxins).

Several lines of evidence suggest that the ω-agatoxins suppress transmitter release by blocking voltage-sensitive calcium channels. We have provided detailed evidence (4) demonstrating that ω-Aga-IA blocks voltage-sensitive calcium channels in motor nerve terminals and neuronal cell bodies in insects (4). ω-Aga-IA also suppresses high threshold calcium currents in rat dorsal root ganglia (17) and blocks transmission at the frog cutaneous pectoris nerve-muscle junction (4), indicating that it interacts with calcium channels in both vertebrate and invertebrate nervous tissue. Because of its physiological and biochemical similarities to ω-Aga-IA, ω-Aga-IB also appears likely to function as a calcium channel antagonist. ω-Aga-IIA likewise blocks insect neuromuscular transmission presynaptically and in addition blocks the binding of ω-CgTx to chick synaptosomal membranes. Since ω-CgTx binds to a high affinity site affecting voltage-sensitive calcium channel antagonists from Spider Venom
Presynaptic Calcium Channel Antagonists from Spider Venom

### Table V

<table>
<thead>
<tr>
<th>w-Agatoxin</th>
<th>Molecular mass</th>
<th>UV spectrum</th>
<th>Inhibition of w-CgTx binding in crude venom</th>
<th>Yield</th>
<th>% total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>w-Aga-IA</td>
<td>7.5 Trp</td>
<td>-</td>
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<td>0.60</td>
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</tr>
<tr>
<td>w-Aga-IB</td>
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<td>-</td>
<td>20</td>
<td>0.12</td>
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</tr>
<tr>
<td>w-Aga-IIA</td>
<td>11 Trp + Tyr</td>
<td>+</td>
<td>20</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

calcium channels in this tissue (8), inhibition of its binding indicates that w-Aga-IIA may block calcium channel antagonists in the avian central nervous system. Recent data showing that w-Aga-IIA blocks the voltage-dependent influx of $^{45}$Ca into chick brain synaptosomes supports this hypothesis.

The combined evidence thus shows that while all three w-agatoxins interact with neuronal calcium channels in both insect and vertebrate nervous tissue, their biochemical and physiological properties are distinct (Table V). We therefore subclassify them as Type I and Type II w-agatoxins based on differences in molecular weight, amino acid sequence, and ability to inhibit w-CgTx binding. The Type I toxins (w-Aga-IA and -IB) are of similar size (7.5 kDa), show high sequence similarity, and do not inhibit w-CgTx binding to chick synaptosomal membranes. Since w-Aga-IA interacts with calcium channels in both invertebrate and vertebrate nerve membranes and does not affect the binding of w-CgTx, Type I toxins may define a new calcium channel binding site distinct from that of w-CgTx. However, unlike w-CgTx, w-Aga-IIA blocks insect neuromuscular transmission suggesting that it may have a novel specificity of its own. Further characterization of the biochemical and physiological specificities of Type I and Type II toxins is needed. For example, it is not known whether inhibition of w-CgTx binding by w-Aga-IIA is competitive or noncompetitive. Precedence for noncompetitive inhibition of w-CgTx binding by spider venom constituent(s) has been reported by Feigenbaum et al. (20) using crude venom from Plectreurys tristis spiders.

Constituents of Hololena curta (21) and P. tristis (22) spider venoms also cause presynaptic antagonism at the insect neuromuscular junction, but these substances appear to be inactive in vertebrate tissue. Hololena toxin, a polypeptide dimer of apparent mass 15 kDa and Plectreurys toxins of approximately 7 kDa block neuromuscular transmission in Drosophila larvae, apparently via a direct action on voltage-sensitive calcium channels. Since no amino acid sequence data are available for Hololena or Plectreurys toxins, their relationship to the w-agatoxins is unclear at this time. Semipurified fractions from Agelenopsis venom affect calcium channels in vertebrate tissue (23, 24), but details regarding the chemical characteristics and physiological activities of these toxins have not yet appeared.

Complete amino acid sequencing of w-Aga-IA shows it to be an unblocked peptide of 66 amino acids including 9 cysteines. The presence of 5 tryptophan residues and low tyrosine content accounts for the strong indole-like absorbance exhibited by this toxin as well as w-Aga-IA, which is similar in size and NH$_2$-terminal sequence (75%, with 100% correspondence in the spacing of cysteine residues). Only limited sequence similarity exists between Type I toxins (w-Aga-IA and -IB) and w-Aga-IIA (43%), although higher correspondence is observed in the placement of cysteine residues (97%) suggesting that a particular pattern of disulfide bridges may be an important determinant of biological activity. Some degree of sequence similarity is apparent in the placement of cysteine residues between the w-agatoxins and $\mu$-agatoxins (3). This is intriguing since the $\mu$-agatoxins interact with sodium rather than calcium channels (2).

In summary, the w-agatoxins show a spectrum of activity affecting both invertebrate and vertebrate nervous tissue. All three w-agatoxins block insect neuromuscular transmission; w-Aga-IA also appears to block calcium channels in rat sensory neurons (17) and frog motor neurons (4). w-Aga-IIA, but not Type I toxins, inhibits w-CgTx binding in chick synaptosomal membranes. If the apparent specificities for Type I and Type II toxins relate to different classes of calcium channels, the w-agatoxins may prove useful as specific ligands for channel characterization and classification. Further structural and functional analyses of Type I and II w-agatoxins are currently in progress.

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REFERENCES


Presynaptic Calcium Channel Antagonists from Spider Venom

**EXPERIMENTAL PROCEDURES**

**Materials**: Adult male argiope aurantia venom was provided by Spider Plains, Black Canyon, AZ. The venom was collected and pooled from individual spiders by a steel needle technique which removes contaminant from gut peridochloro. Cyanogen bromide (0.1% w/v), picryl, trifluoroacetate (TFA) and N-acetylpropionamide (NAPA) were purchased from Pierce, EMD, and Sigma-Aldrich. Blue 2850 from Sigma, SDS, Try, His, Lys, avidin, bovine serum albumin (BSA), BSA buffer, betaine buffer, TMEED, and amphotericin B were from Bio-Rad, protein molecular weight standards for SDS electrophoresis were from Bethesda Research Laboratories. 125I-labeled protein 32P-labeled protein 866, were also purchased from NEN. Acrylamide gel was from Bio-Rad. Acrylamide was from ICN. X-ray film was from Kodak.

**Procedures**: The solutions were analyzed on 12% (v/v) acrylamide gels containing 0.1% (v/v) SDS at pH 8.8. The gel was electrophoresed at 30 mA per gel for 1.5 h at 250 V, followed by staining with Coomassie blue. The proteins were visualized as blue bands. The bands were excised from the gel and subjected to excitation in ethanolic solution.

**Results**: A total of 12 bands were detected in the venom. The bands were excised and subjected to analysis.

**Discussion**: Presynaptic calcium channel antagonists from spider venom have been identified. The venom contains a number of proteins that inhibit the release of neurotransmitters. The mechanisms of action of these proteins are under investigation.
Figure 6: UV absorption spectra of α-Aga IA, IB, and IIA. α-Aga IA and IB within甲状amid cyclotides (channels which are distinct from the α-Aga IIA. These are shown to have significant absorption bands at 260 and 300 nm. The spectrum on the right is compared to 272, 279 and 280 for free tryptophan, which of α-Aga IA lacks a tryptophan residue at 277. The peak shoulder is 190 nm. The peak absorbance for each substance is normalized to 100%.
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