

# Post-translational Amino Acid Isomerization

A FUNCTIONALLY IMPORTANT D-AMINO ACID IN AN EXCITATORY PEPTIDE\*

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The post-translational modification of an L- to a D-amino acid has been documented in relatively few gene products, mostly in small peptides under 10 amino acids in length. In this report, we demonstrate that a 46-amino acid polypeptide toxin has one D-phenylalanine at position 44, and that the epimerization from an L-Phe to a D-Phe has a dramatic effect on the excitatory effects of the peptide. In one electrophysiological assay carried out, the D-Phe-containing peptide was extremely potent, whereas the unmodified polypeptide had no biological activity, demonstrating that the chirality of the post-translationally modified amino acid is functionally significant. The peptide toxin analyzed, r11a, belongs to the I-gene superfamily of conotoxins that has four disulfide cross-links. The D-Phe in r11a is at the third amino acid from the C terminus, the same relative position from the C-terminal end as the D-amino acid in  $\omega$ -agatoxin TK from a spider, an unrelated peptide. Thus, although post-translational amino acid isomerization appears to have no strong specificity for the chemical nature of the amino acid side chain, the few peptides where this modification has been established suggest that there may be preferential sites for isomerization to a D-amino acid.

A remarkable post-translational modification of polypeptides synthesized on ribosomes is the conversion of a standard L-amino acid into the corresponding D-isomer (1, 2). Most cases of post-translational isomerization to D-amino acids have been documented in small peptides from vertebrate, arthropod, and molluscan systems. This post-translational modification does not appear to be specific for a particular amino acid; a variety of different amino acids have been post-translationally converted to the D-form. The diverse biological systems in which D-amino acid-containing gene products have been characterized, and the apparently unrelated sequence contexts in which these post-translationally modified amino acids occur raises the question of whether such a post-translational modification can be predicted.

A difficulty in critically assessing the natural distribution of the post-translational isomerization of amino acids is that the larger the gene product, the more difficult it becomes to ascer-

tain the presence of a modified amino acid. In small peptides, the effect of converting a single L- to a D-amino acid can cause a significant difference in chromatographic and other properties; however, as the polypeptide gets larger, converting one amino acid residue to the corresponding D-isomer results in more subtle global changes. Furthermore, in contrast to all other post-translational modifications, isomerization to a D-amino acid is not detectable by any of the standard techniques used in proteomics, such as mass spectrometry. In order to be able to predict when the post-translational conversion to a D-amino acid might occur, the identification of more *bona fide* cases of natural polypeptides having a D-amino acid is required.

The first larger peptidic gene product in which a D-amino acid was identified was  $\omega$ -agatoxin TK from the funnel spider *Agelenopsis aperta*: a single D-serine was identified toward the C-terminal end (3). The only other larger peptide with a D-amino acid, also from an arthropod system, was the crustacean hyperglycemic hormone, where the amino acid modified is toward the N-terminal end (4–6). There is no similarity at all that can be detected between the sequence context of the post-translational isomerization in these two cases.

Among peptides that have been modified from different sources such as frog skin, molluscan nervous tissue, or platypus venom, the D-amino acid is most often located at the second position from the N terminus (7–14). However, in the contryphans, a group of octapeptides from the venom of *Conus*, the D-amino acid is found at the fourth amino acid residue (15–17). In both the frog peptides and in the contryphans, the position within the peptide seems to be the dominant determinant of whether a change in chirality from an L-amino acid to a D-amino acid will occur, rather than the type of amino acid at that position.

In this work, we describe the presence of a D-amino acid in a member of a recently characterized group of conotoxins, the I-superfamily (18). This conotoxin, r11a, is only the third example found of a larger polypeptidic gene product, which has definitively been shown to have undergone this unusual post-translational modification. We have synthesized both the L- and the D-amino acid-containing isomers of conotoxin r11a, and demonstrate that the presence of the modified amino acid causes a marked change in biological activity.

## EXPERIMENTAL PROCEDURES

*Purification of Conotoxin r11a from Venom*—Crude venom extract was prepared as described earlier (15). The extract was loaded onto a Vydac C<sub>18</sub> semipreparative HPLC<sup>1</sup> column and eluted at 5 ml/min with

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<sup>1</sup> The abbreviations used are: HPLC, high performance liquid chromatography; MTBE, methyl-tert-butyl ether; MALDI, matrix-assisted laser desorption ionization; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA, N,N-diisopropylethylamine; i.c., intracranially; MS, mass spectrometry.

a gradient of solvent A (0.1% trifluoroacetic acid) and solvent B<sub>90</sub> (0.085% trifluoroacetic acid in 90% acetonitrile). Further purification was done on a Vydac C<sub>18</sub> analytical column at a flow rate of 1 ml/min.

**Mass Spectrometry**—Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained using a Voyager DE STR mass spectrometer, courtesy of the Mass Spectrometry and Proteomic Core Facility, University of Utah. Alternatively, MALDI spectra were obtained using a Bruker REFLEX time-of-flight mass spectrometer (Bruker Daltonics) fitted with a gridless reflectron, an N2 laser, and a 100 MHz digitizer, courtesy of the Salk Institute for Biological Studies (La Jolla, CA).

**Peptide Synthesis**—Peptides were synthesized on solid support by an automated peptide synthesizer using *N*-Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-protected amino acids, HBTU and DIEA (19), courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility, University of Utah. All cysteine residues were trityl-protected. The coupling time was 1 h. Peptide cleavage/deprotection was accomplished with reagent K (82.5% trifluoroacetic acid, 5% phenol, 5% H<sub>2</sub>O, 5% thioanisole, 2.5% 1,2-ethanedithiol) for 2 h at room temperature. Soluble crude peptides were precipitated with cold MTBE and centrifuged. The pellet was washed with MTBE, centrifuged again, then dissolved in 25% acetonitrile in 0.1% trifluoroacetic acid, and lyophilized. The linear peptides were purified on a Vydac C<sub>18</sub> semipreparative HPLC column.

**Oxidative Folding**—Oxidative folding of peptides was done with 0.1 M Tris-HCl, pH 8.7, containing 1 mM EDTA, 1 mM GSSG, and 1 mM GSH at room temperature for 3 h. The reaction was initiated by adding the linear synthetic peptide to a final concentration of 20 μM, then quenched by adding formic acid to a final concentration of 8%. The oxidized form was purified on a Vydac C<sub>18</sub> HPLC column with a gradient of 15–60% solvent B (0.1% trifluoroacetic acid in 90% acetonitrile) for 40 min.

**Reduction and Alkylation**—Two nanomoles of peptide were dissolved in 40 μl of 50% acetonitrile in 0.1% trifluoroacetic acid, and reduced by addition of 10 μl of reduction buffer (0.5 M Tris acetate, pH 8.0, containing 5 mM EDTA and 100 mM dithiothreitol) and incubation at 65 °C for 30 min. The peptide was then alkylated with 40 μl of 0.5 M iodoacetic acid in 0.5 M Tris acetate, pH 8.0, containing 5 mM EDTA in the dark at room temperature for 30 min. The reaction mixture was applied to a Vydac C<sub>18</sub> analytical HPLC column and eluted with 30% solvent B for 15 min followed by a gradient of 30–55% solvent B for 25 min.

**Digestion with Endoproteinase Asp-N**—Both natural and synthetic conotoxin r11a were digested with endoproteinase Asp-N (Roche Applied Science) to generate fragments. Two nanomoles of the reduced and alkylated peptide were dissolved in 4 μl of 30% acetonitrile and added to 36 μl of endoproteinase Asp-N solution (2.8 μg/ml in 50 mM Tris-HCl, pH 7.5) to yield an enzyme/substrate ratio of 1:100 (w/w). After 2 h of incubation at 35 °C, the reaction was stopped by addition of 40 μl of 0.1 M EDTA. The digestion products were separated on Vydac C<sub>18</sub> analytical HPLC column using a gradient of 5–50% solvent B for 45 min. The digestion products were analyzed by MALDI mass spectrometry.

**Digestion with Chymotrypsin**—Natural and synthetic conotoxin r11a were digested with sequencing-grade chymotrypsin (Roche Applied Science). Two nmol of alkylated peptide were dissolved in 4 μl of 30% acetonitrile and added to 34 μl of chymotrypsin solution (5.6 μg/ml in 50 mM Tris-HCl, pH 7.5) to yield an enzyme/substrate ratio of 1:50 (w/w). After 12 h of incubation at room temperature, the digestion products were separated on a Vydac C<sub>18</sub> analytical HPLC column, using a gradient as for endoproteinase Asp-N digestion products, and then analyzed by MALDI mass spectrometry. Additionally, peptide fragments were sequenced using an Applied Biosystems Model 492 Sequenator, courtesy of Dr. Robert Schackmann (DNA/Peptide Facility, University of Utah).

**Bioassay**—Conotoxins were dissolved in normal saline solution (NSS) and injected using a 29-gauge insulin syringe. Swiss Webster mice (15 days old) were injected intracranially (i.c.) with 20 μl of the conotoxin solution as described previously (20). Control animals were similarly injected with NSS. Proper animal care and use protocols were followed in accordance with the guidelines set by the University of Utah Institutional Animal Care and Use Committee.

**Electrophysiology**—The *cutaneous pectoris* muscle preparation of *Rana pipiens* was used as described previously (18). All experiments were performed on a single batch of summer frogs purchased from Charles D. Sullivan (Nashville, TN). Briefly, the recording/stimulating chamber was made from a silicone elastomer, Sylgard (Dow Corning), and consisted of a rectangular trough (about 1 mm × 15 mm × 4 mm deep) and an adjacent series of four cylindrical wells (4 mm diameter × 4 mm deep). The different compartments were separated by 1-mm-wide partitions. A mini-muscle preparation (see Ref. 21) was placed in the

trough, and the attached motor nerve was draped into the adjacent wells, with the proximal (severed) end of the nerve in the well farthest from the trough. Portions of the nerve overlying the partition, and therefore exposed to air, were covered with Vaseline. A pair of stimulating electrodes spanned the two wells furthest from the trough, and of these two wells, that closest the trough also contained a ground electrode. Pulses, 0.1 ms in duration and 6 V in amplitude, and applied via a stimulation isolation unit, were used to stimulate the motor nerve at a frequency of 2/min. To record the activity of the muscle, a pair of recording electrodes was located in the trough, with one electrode near the middle and the other at one end. Activity of the nerve was recorded with a second pair of electrodes located in the two wells closest to the trough. Each set of recording electrodes were connected to a differential A/C amplifier, and the signal bandpass filtered (1 Hz to 3 kHz) and digitized at a sampling frequency of 10 kHz. This arrangement allowed the electrical activity from nerve and muscle to be recorded simultaneously. Homemade software programmed in LabVIEW (National Instruments, Austin, TX) was used for data acquisition. All electrodes were 0.01 diameter stainless steel wires. Solutions in all compartments were static and refreshed periodically by manual replacement. Peptides were dissolved in frog Ringer's containing 0.1 mg/ml bovine serum albumin. To apply peptide, the fluid in the muscle-containing trough was removed and replaced with peptide at the indicated concentration. The solution was stirred by aspirating the solution into the tip of a pipettor, then discharging it back out into the trough, for 20 cycles. During this solution exchange and stirring, the inputs of the recording amplifiers were grounded for protection. The entire process took between 1.5 and 2 min and accounts for the hiatus in the records in Fig. 4. All recordings were performed at room temperature (~21 °C).

## RESULTS

**Non-identity of Synthetic Conotoxin (All L Version) and Natural Conotoxin r11a: Preliminary Evidence Suggesting the Presence of a D-Amino Acid**—The excitatory conotoxin r11a which belongs to the I-superfamily is a relatively large conotoxin (46 amino acids) with four disulfide bonds, purified from the venom of the piscivorous species *Conus radiatus*. The purification of the natural conotoxin used in the studies described below was carried out by a modification of the method described previously (18). *C. radiatus* venom has at least 18 different I-superfamily peptides; all that have been purified from venom are excitatory. A major challenge in purification is the separation of one isoform from another. As shown in Fig. 1, peptide r11a was purified to homogeneity, but this required four reverse-phase HPLC runs to separate the peptide from contaminating isoforms.

The linear peptide with all L-amino acids was chemically synthesized and folded, and the resultant synthetic peptide was shown to elicit excitatory biological activity. However, a biochemical characterization of folded synthetic peptide revealed that it did not co-elute with the natural peptide. When the natural peptide was reduced, the linear synthetic peptide and the linear natural peptide also did not co-elute. However, the mass value of the synthetic material was identical to that of the natural peptide. A preliminary functional characterization indicated that the synthetic peptide, though biologically active, was not as potent as natural r11a. Together, these initial data raised the possibility that a D-amino acid residue might be present in the natural conotoxin r11a.

In order to further explore this possibility, both the reduced and alkylated synthetic (all L version) and natural peptides were digested with endoproteinase Asp-N as described under "Experimental Procedures." After digestion with the endoproteinase, the products were analyzed by HPLC. Three digestion products were identified by mass spectrometry. These were the predicted end digestion products of endoproteinase Asp-N (Fig. 2A): an N-terminal heptapeptide (P2), a nonapeptide from residues 8 to 16 (P1) and a large C-terminal fragment from residues 17 to 46 (P3). The two smaller peptides from synthetic and natural r11a were identical in their elution properties on HPLC, but there was a small shift between the synthetic and

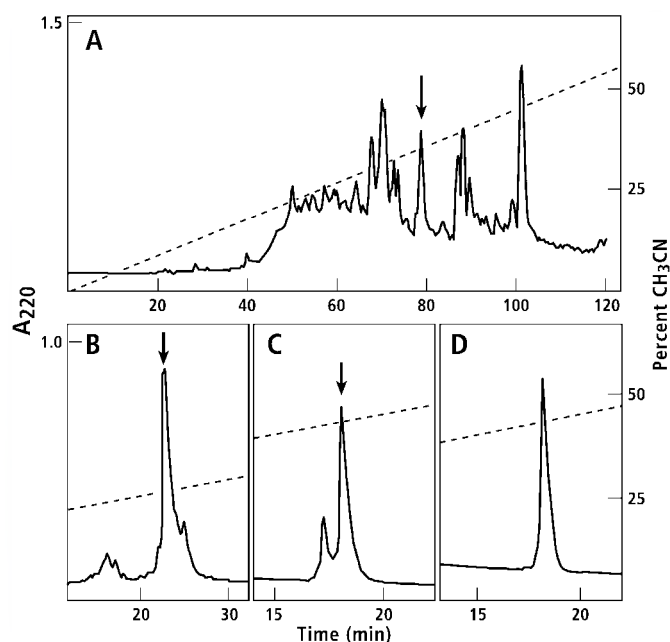


FIG. 1. Purification of natural conotoxin r11a. **A**, fractionation of crude extract from *C. radiatus* venom on a  $C_{18}$  semipreparative HPLC column eluted with a gradient of 0–60% solvent  $B_{90}$  (0.085% trifluoroacetic acid in 90% acetonitrile) over 120 min at a flow rate of 5 ml/min. The arrow indicates the fraction with r11a, and the dotted line indicates the acetonitrile gradient. **B**, elution of the fraction marked by an arrow in **A** using a  $C_{18}$  analytical column with a gradient of 20–40%  $B_{90}$  over 50 min at 1 ml/min flow rate. **C**, further purification of the fraction indicated by an arrow in **B** using a gradient of 30–55%  $B_{90}$  over 25 min at 1 ml/min. **D**, purification of the fraction marked by the arrow in **C** using the same gradient and flow rate.

natural peptide for P3, the large C-terminal product. This indicated that any D-amino acid(s) were likely to be between amino acids 17 and 46.

The reduced and alkylated natural and synthetic linear peptides yielded different products upon digestion with chymotrypsin (Fig. 2B). For the synthetic peptide, four different digestion products were obtained. A combination of mass spectrometry and Edman sequencing was used to identify the cleavage products. This analysis revealed that chymotrypsin cleavage after Trp<sup>33</sup> and Phe<sup>44</sup> had occurred on the synthetic peptide, but that the digestion at these loci was incomplete. Thus, two of the predicted digestion products, the fragments from amino acids 1 to 33 (P4) and 34 to 44 (P5) were obtained, as well as two incomplete digestion products (1–44 (P7) and 34–46 (P6)). However, when a similar digestion of the natural peptide was carried out, a strikingly different pattern was obtained. No digestion after Phe<sup>44</sup> was detected, and only fragments from 1 to 33 and a new fragment whose sequence and mass value were consistent with AA 34 to 46 (P6, dashed line) were obtained. However, although the mass of the peptide was consistent with residues 34–46, this peptide fragment did not co-elute with the corresponding fragment from the synthetic r11a. These results are consistent with the presence of a D-amino acid in the interval from amino acids 34 to 46.

We reasoned that if residue 44 were in fact a D-amino acid, the failure of chymotrypsin to cleave after Phe<sup>44</sup> in the natural peptide would be explained. Thus, together, the data led us to the specific working hypothesis that natural conotoxin r11a differs in having a D-phenylalanine residue at position 44, instead of the L-Phe in the synthetic peptide.

**Direct Verification of the Presence of a D-Amino Acid in r11a by Chemical Synthesis**—The hypothesis that r11a contains a D-phenylalanine residue at position 44 was directly tested by

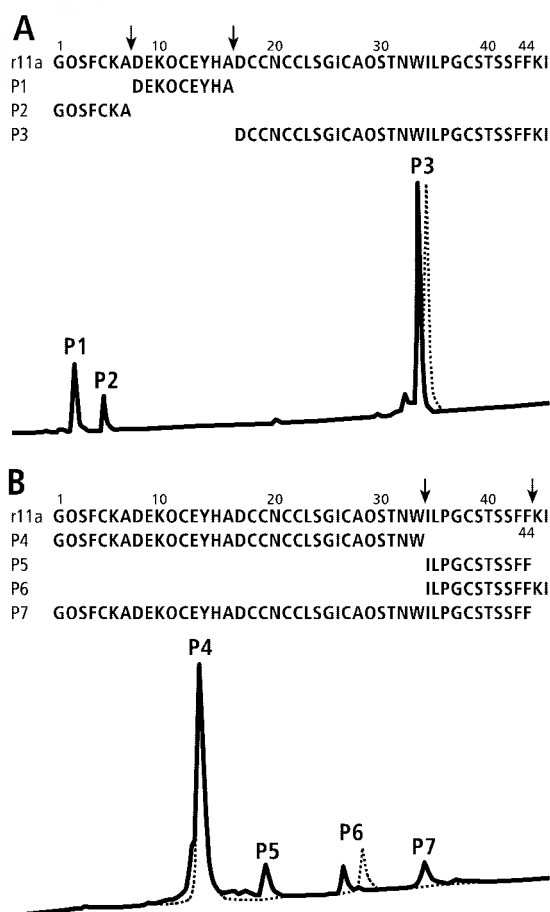


FIG. 2. Digestion of synthetic [L-Phe<sup>44</sup>]r11a and natural r11a, as described under “Experimental Procedures.” Peaks denoted by solid lines and dashed lines are products from synthetic and natural r11a, respectively. Arrows indicate cleavage sites by the enzymes. **A**, end products of digestion with endoproteinase Asp-N. **B**, end products of digestion with chymotrypsin.

chemical synthesis. Peptide P6 with amino acids from 34 to 46 was chemically synthesized with both L-Phe<sup>44</sup> and D-Phe<sup>44</sup>, when these synthetic peptides were chromatographed with the corresponding chymotrypsin fragment from natural r11a, only the D-Phe<sup>44</sup> peptide co-eluted, and the L-Phe<sup>44</sup> peptide was resolved from the natural 34–46 fragment.

These results led us to synthesize the entire peptide r11a as described under “Experimental Procedures.” An all L version and a D-Phe<sup>44</sup> version of the r11a primary sequence were synthesized, and both peptides were folded. As is shown in Fig. 3, only the D-Phe<sup>44</sup>-containing conotoxin r11a co-eluted with the natural peptide. This provides direct biochemical evidence that Phe<sup>44</sup> is post-translationally modified from an L- to a D-amino acid in the natural conotoxin. Thus, the correct amino acid sequence of the conotoxin r11a is: GOSFCKADEKOCEYHADCCNCLSGICAOSTNWILPGCSTSSFFKI, where F is D-phenylalanine. We refer to the D-Phe<sup>44</sup> version of the peptide simply as r11a and the all L version as [L-Phe<sup>44</sup>]r11a.

**Biological Activity of Synthetic Peptides r11a and [L-Phe<sup>44</sup>]r11a**—A functional comparison of the folded r11a analogs with different chirality at position 44 was carried out both by an electrophysiological characterization with an *in vitro* nerve-muscle preparation from frog, as well as by using an *in vivo* behavioral assay on mice. Unlike either natural or synthetic r11a, both of which induce repetitive activity in the amphibian nerve-muscle preparation at concentrations as low as 0.1  $\mu$ M (see below), no repetitive activity was seen with [L-Phe<sup>44</sup>]r11a when tested at concentrations up to 25  $\mu$ M. Thus,

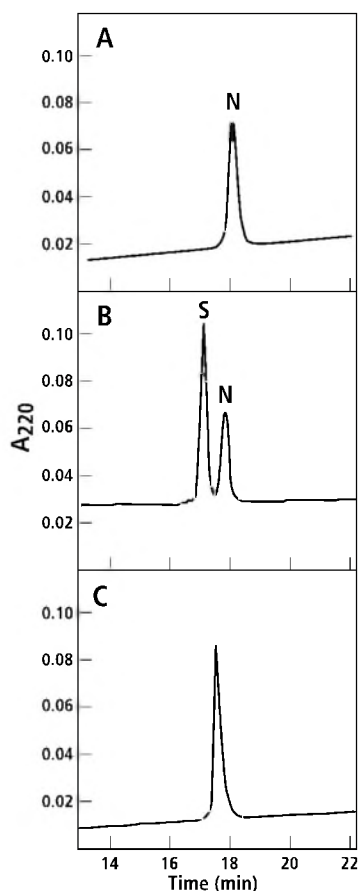


FIG. 3. A, HPLC chromatograph of natural r11a (0.7 nmol). B, HPLC analysis of a mixture of 1 nmol of synthetic [ $L$ -Phe $^{14}$ ]r11a and 0.7 nmol of natural r11a; the peaks are indicated by S and N, respectively. C, co-elution of natural (0.5 nmol) and synthetic (0.5 nmol) conotoxin r11a (with D-Phe $^{14}$ ). The elutions were done using a C $_{18}$  analytical column with a gradient of 30–55% B $_{90}$  over 25 min at 1 ml/min flow rate.

using the *in vitro* frog nerve-muscle preparation, the natural D-Phe form of the peptide is >250-fold more potent than the L-Phe form. However, when a whole animal behavioral assay (with 15-day-old mice) was used to assess the D-Phe and L-Phe forms of r11a, a smaller differential (10–20-fold) was observed in potency of the D-Phe and L-Phe r11a analogs. This estimate is based on comparing the dose in which hyperactivity symptoms but no death occurred (5 versus 100 pmol for the D- and L-Phe forms), as well as the doses in which marked hyperactivity was followed by death some minutes later (20 versus 250 pmol for the D- and L-Phe forms). Thus, using two very different assays, the natural D-Phe r11a peptide is significantly more potent than the L-Phe r11a analog.

**Comparison of the Activity of Native and Synthetic Peptides r11a**—The ability of the natural r11a to elicit trains of action potentials in an isolated nerve-muscle preparation from frog was previously reported (18). Normally, action potentials are not seen in skeletal muscle unless the nerve is stimulated; that is, spontaneous action potentials are not seen. Furthermore, a single stimulus applied to the motor nerve usually produces only a single action potential in the muscle. However, in the presence of I-superfamily conotoxins both evoked and spontaneous trains of action potentials are observed. This is the case for both natural and synthetic r11a. The effects produced by the peptides were comparable in several respects: 1) On-rate. At a concentration of 1  $\mu$ M, both peptides induced repetitive activity within a minute of their application. However, at lower concentrations, the first signs of repetitive activity took several

minutes to appear (see Fig. 4 for examples). At 100 nM, the mean ( $\pm$ S.D.) induction time in three trials with each peptide was  $3.8 \pm 0.8$  min for the natural peptide and  $4.9 \pm 2.2$  min for the synthetic peptide. Thus, within experimental error, the peptides have the same on-rate. 2) Off-rate. The repetitive activity induced by both peptides persisted for >18 h following their washout. Thus, the effect of both peptides is relatively irreversible. 3) When the peptide-induced activities are simultaneously recorded in nerve and muscle, it was observed that each action potential in the muscle is preceded by an action potential in the nerve. This was originally reported for the native peptide (18) and is also seen with the synthetic peptide (Fig. 5). This indicates that with both natural and synthetic peptides, repetitive activity is primarily induced in the motor nerve and only secondarily in the muscle. That is, the peptide induces repetitive activity in the motor nerve, and this activity is conveyed to the muscle via synaptic transmission. As would be expected, when synaptic transmission is blocked by exposing the preparation to 5  $\mu$ M d-tubocurarine, an acetylcholine receptor-antagonist, repetitive activity in the muscle, but not in the nerve, is blocked (not illustrated).

#### DISCUSSION

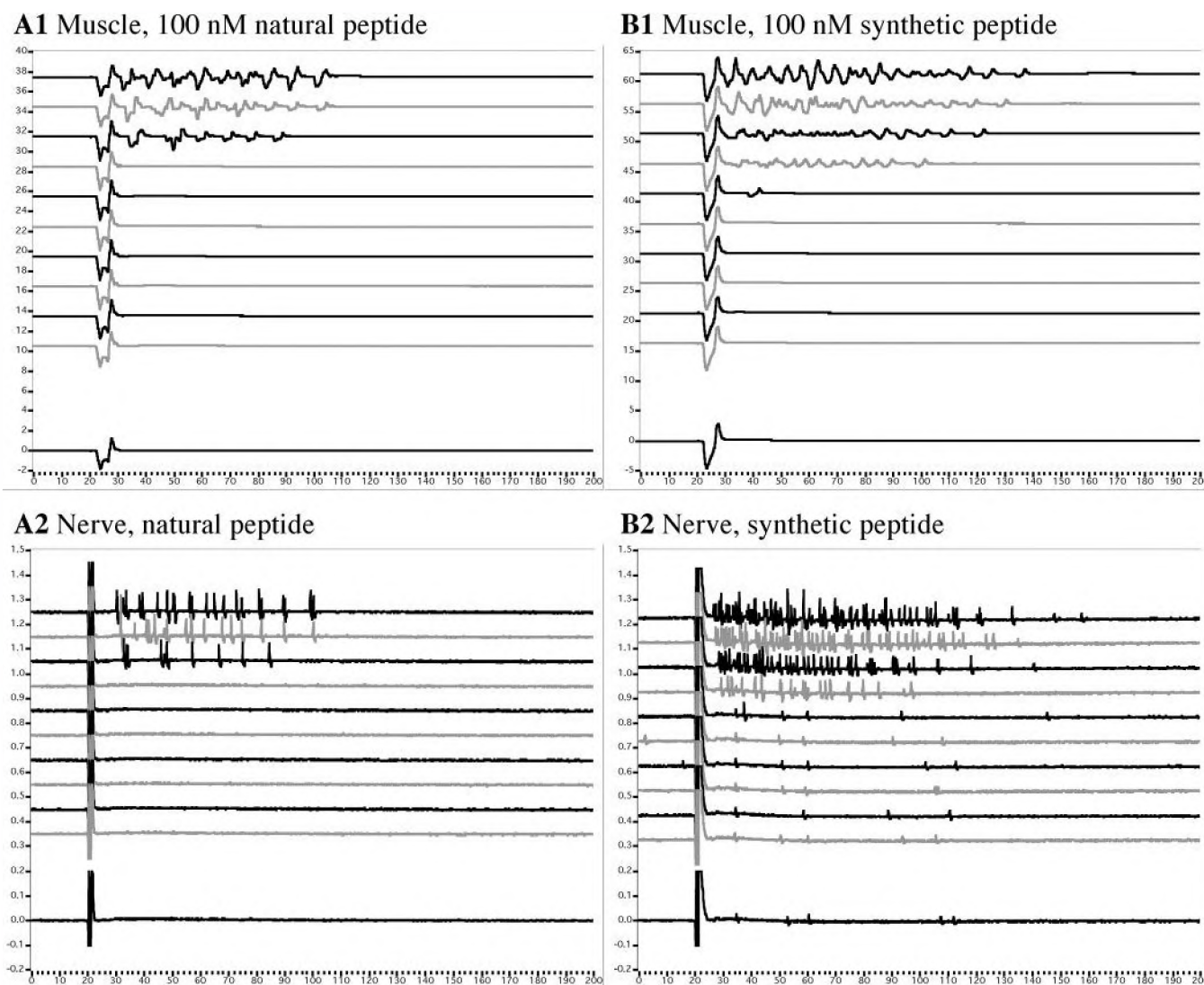
The studies above have demonstrated the presence of an unusual post-translationally modified amino acid, D-phenylalanine, at amino acids 44 of r11a, a conotoxin with eight Cys residues and 46 amino acids. This is the first multiple disulfide-bonded conotoxin shown to contain a D-amino acid. The post-translational modification is important for optimal biological activity of the toxin.

D-Amino acids have a sporadic distribution in a number of biological systems. These have been characterized mostly in smaller peptides (for a comprehensive review on D-amino acids see Ref. 2), most notably those produced by frog skin, as well as by various invertebrate and mammalian sources. D-amino acids were described previously in the contryphans, a family of small peptides, which are widely distributed across the genus *Conus* (15–17). These small peptides (typically, eight amino acids) have a single disulfide bond; most have either a D-tryptophan or D-leucine residue (Table I). The contryphans were notable biochemically because of the slow equilibration between two conformational states exhibited by many of these peptides; the presence of a D-Trp residue was required for the slow conformational equilibrium.

The notable feature of the I-conotoxin superfamily, to which conotoxin r11a characterized above belongs, is the large number of different molecular isoforms differing in their primary sequences that can be present in a single venom. Conotoxin r11a is from the venom of *C. radiatus*, which is believed to be fish-hunting species. More than 20 distinct I-superfamily conotoxins, each significantly different in sequence, are expressed by *C. radiatus*. However, these share different extents of homology to the D-phenylalanine-containing conotoxin r11a characterized in this report. The modified phenylalanine residue is conserved in a subset of several I-superfamily conotoxins from *C. radiatus* and other *Conus* species, strongly suggesting that at least the members of this subset of I-superfamily conotoxins undergo the post-translational isomerization at this locus. The amino acids preceding the D-Phe residue are part of a conserved sequence motif, —GCSTSSFF—(where F is the D-Phe residue) (18).<sup>2</sup>

We have demonstrated that the D-Phe residue is important for the biological activity of conotoxin r11a. If an L-Phe, instead of a D-Phe is present, the peptide is significantly less active as an excitotoxin. A comparison of native and synthetic D-Phe-containing peptides was carried out. Since the effects of both

<sup>2</sup> M. Watkins and B. Olivera, unpublished results.



**FIG. 4. Comparison of natural versus synthetic r11a.** Responses were obtained simultaneously from muscle and nerve every 30 s, except during solution change. Successive responses are shown from *bottom to top*, before (*lowest trace*) and during (*remaining traces*) exposure to 100 nM of native r11a (*panels A1 and A2*) or synthetic r11a (*B1 and B2*). The gap between the lowest trace and the one immediately above it reflects the time it took to apply the peptide (see "Experimental Procedures"). Vertical and horizontal units are mV and ms, respectively. Responses from nerve (*panels A2 and B2*) are shown at high amplification; thus, the repetitive action potentials, which are from individual axons, are clearly seen at the expense of truncating the evoked compound action potential. The latter is seen in each panel immediately following the nerve stimulus, which was applied after 20 ms from start of each trace. To enhance readability, every other trace is shown in *gray*. The first trace to show repetitive activity occurred after about 4 min for the natural peptide and after about 3 min for the synthetic peptide. The small action potentials in panel B2, evident in all traces, represent sensory stretch receptor activity (see Ref. 18); note that the peptide-induced action potentials are larger, and these large action potentials are correlated with corresponding events in the muscle (see also Fig. 5).

native and synthetic peptides are relatively irreversible, equilibrium dose-response measurements could not be obtained. Instead, as an index of toxin potency, the time interval between the administration of peptide and the first observation of repetitive activity (the induction time) was used. Higher concentrations of peptide had shorter induction times. At the same concentration, native and synthetic peptides had similar induction times. Furthermore, both peptides targeted channels in the nerve, rather than the muscle. Thus, both natural and synthetic peptides have comparable potencies and specificities. These functional features, along with their chemical characteristics, lead us to conclude that the natural and synthetic r11a are identical.

Although all of the I-superfamily peptides characterized so far appear to be excitotoxins, and several have been shown to target K channels in animals as diverse as mammals and stem chordates (22), it remains to be established which of the I-superfamily members (that all share the same Class 11 Cys

pattern found in r11a) also contain D-amino acids. The discovery of a D-amino acid in conotoxin r11a introduces a cautionary note into using standard genetic/proteomic methods to investigate conotoxins: although the sequence of r11a and other I-superfamily conotoxins can be readily derived by PCR/cloning, and the number of hydroxylated Pro residues can be deduced by mass spectrometry, the presence of a D-amino acid would have eluded both of these methods.

We note that although the sequence of conotoxin r11a has no apparent homology to the  $\omega$ -Aga-TK spider toxin, and the amino acids modified are different (Phe *versus* Ser), the locus of modification is the same distance from the C terminus (see Table I, A). In conotoxin r11a, which has 46 residues, it is a Phe residue at amino acid 44; in  $\omega$ -Aga-TK, which has 48 residues, it is a Ser residue at amino acid 46. The characterization of a D-amino acid in conotoxin r11a raises the possibility that the third amino acid from the C terminus may be a favored site. This prediction is suggested by the discovery that another

## A Natural peptide

## B Synthetic peptide

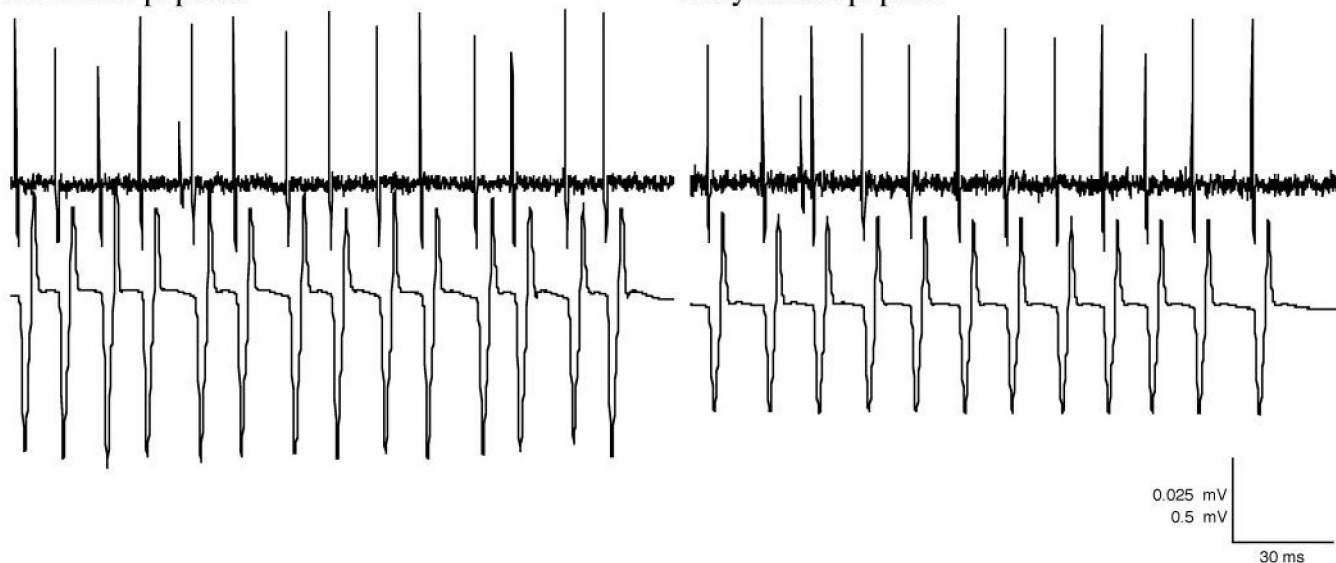


FIG. 5. Repetitive responses recorded simultaneously in nerve and muscle. Repetitive action potentials induced by 100 nM natural (A) and synthetic (B) peptides. Note that for both panels, each action potential in the muscle recording (lower trace) has a corresponding action potential that just precedes it in the nerve recording (upper trace). Calibration scale of 0.025 mV is for nerve, and 0.5 mV is for muscle.

TABLE I  
Peptides containing D-amino acids

A. Peptides with a D-amino acid at position 3 from the C terminus			
Peptide	Sequence	Source	Ref.
$\omega$ -Agatoxin TK	EDNCIAEDYGKCTWGGTKCCRRPCRCMSMIGTNCECTPRLIMEGLSFA <sup>a</sup>	<i>Agelenopsis aperta</i>	(3)
r11a	GOSFCKADEKCOCEYHADCCNCLSGICAOSTNWILPGCSTSSFFKI <sup>a,b,c</sup>	<i>C. radiatus</i>	This work
Achatin-I	GFAD <sup>a,c</sup>	<i>Achatina fulica</i>	(8)
B. Peptides with a D-amino acid at position 2 from the N terminus			
Peptide	Sequence	Source	Ref.
Fulicin	FNEFV <sup>a,d</sup>	<i>Achatina fulica</i>	(11)
Achatin-I	GFAD <sup>a,c</sup>	<i>Achatina fulica</i>	(8)
Mytilus-FFRF amide	ALAGDHFFRF <sup>a,d</sup>	<i>Mytilus edulis</i>	(12)
Dermorphin	YAFGYPS <sup>a,d</sup>	<i>Phyllomedusa sauvagei</i>	(7)
Met-deltorphin	YMFHLM <sup>a,d</sup>	<i>Phyllomedusa sauvagei</i>	(9, 10)
Platypus peptide	LLHDHPNPRKYKPAKGLSKGCFGLKLDRIGSTSGLGC <sup>a</sup>	<i>Ornithorhynchus anatinus</i>	(13, 14)
C. Peptides with a D-amino acid at position 4 from the N terminus			
Peptide	Sequence	Source	Ref.
Contryphan-R	GCOWEPWC <sup>a,b,d</sup>	<i>C. radiatus</i>	(15)
Contryphan-Sm	GCOWQPWC <sup>a,b,d</sup>	<i>Conus stercusmuscarum</i>	(16)
Leu-Contryphan-P	GCVLLPWC <sup>a,c</sup>	<i>Conus purpurascens</i>	(17)

<sup>a</sup> The underlined letter indicates D-isomers.

<sup>b</sup> O, 4-*trans*-hydroxyproline.

<sup>c</sup> C-terminal free acid.

<sup>d</sup> C-terminal amidation.

I-superfamily conotoxin, with a Leu residue at the third position from the C terminus, has a D-Leu in the native peptide.<sup>3</sup> It is noteworthy that in Table I, A–C, examples of peptides from different phyla are shown (*Agelenopsis* is a spider and *Conus* is a mollusc; *Achatina* and *Mytilus* are molluscs; *Phyllomedusa* is a frog; *Ornithorhynchus* is a platypus). This broad phylogenetic distribution, combined with the preferential loci of the amino acids modified, suggests that this post-translational isomerization, which occurs in many animal taxa, may be carried out by enzymatic systems that may be specific for a particular amino acid locus rather than the chemical nature of the amino acid isomerized.

A summary of the natural occurrence of D-amino acids in

polypeptides is shown in Table I. The evidence that D-amino acids found in most polypeptides are formed post-translationally has been reviewed (2). In many cases, mRNA encoding a precursor for the peptide containing the D-amino acid was identified. (The first peptide where post-translational modification was definitely established was dermorphin in 1987 by Gunther Kreil's laboratory (23).) The codon specifying the standard L-amino acid is found in the mRNA sequence at the locus for the corresponding D-amino acid, suggesting conventional translation by ribosomes, followed by post-translational isomerization. Enzymatic activities have been purified both from spider venoms (24) and from frog skin secretions<sup>4</sup> that specifically convert one L-amino acid to the D-amino acid in the

<sup>3</sup> O. Buczek and E. Jimenez, manuscript in preparation.

<sup>4</sup> A. Jilek and G. Kreil, personal communication.

peptide precursor. The D-amino acids found in conotoxins are therefore presumed to be produced post-translationally. In most cases, the relevant cDNA clones encoding these conotoxins with D-amino acids have been identified.

It is noteworthy that *C. radiatus*, the organism used for the present study, is the first in which two post-translationally modified polypeptides have been characterized that differ in their D-amino acids (Phe in the present peptide, Trp in contryphan-R), as well as in the loci in which these D-amino acids are found on the polypeptide chain. This raises the possibility that different enzymatic activities catalyze the isomerization from L- to D-amino acids in the two *C. radiatus* peptides; whether these are related to the factors identified in the spider and frog systems remains to be established.

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