Site-Directed Mutagenesis of κ-Bungarotoxin: Implications for Neuronal Receptor Specificity†

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ABSTRACT: Postsynaptic polypeptide neurotoxins isolated from the venoms of elapid and hydrophid snakes exhibit the ability to bind selectively to and inhibit different types of receptors that function in nerve signal transmission. On the basis of their amino acid sequences and three-dimensional structures, these neurotoxins are clearly related, but nothing is yet known about the basis for their physiological receptor specificity. In this report, site-directed mutants of κ-bungarotoxin, produced by an *Escherichia coli* expression system, are tested to determine the function of selected amino acid side chains in the interaction between toxin and receptor. Highly conserved residues at the bottom of the second loop (a region that has been shown to be a major point of contact with the receptor), particularly those residues at the junction between the β-sheet and the end of the loop, were selected. The results demonstrate that a single amino acid substitution of the invariant arginine residue (Arg-40 to Ala-40) renders the toxin unable to inhibit nerve transmission in the chick ciliary ganglion up to a concentration of 10 μM. Significantly, the results also show that conversion to alanine of the nearby proline residue (Pro-42) found to be invariant in all κ-neurotoxins, but not found in any potent α-neurotoxin, produces a toxin with full inhibitory capacity. However, the introduction of a lysine residue at this position (P-42-K), like that found in α-bungarotoxin, reduces activity significantly. Thus, in contrast to the α-neurotoxins, there seems to be a requirement for short uncharged side chains, such as proline or alanine, at this position in κ-neurotoxins, but the specific presence of the imino acid proline in κ-bungarotoxin is not strictly required and thus does not affect the orientation of the critical arginine side chain. These results suggest that this residue is at the interface of the toxin–receptor complex. The only residue always found in α-neurotoxins and never found in κ-neurotoxins is Trp-32. It has long been hypothesized to form the basis for receptor specificity between these two families of postsynaptic neurotoxins. Placement of a tryptophan residue in κ-bungarotoxin at the position where it is found as an invariant residue in all active α-neurotoxins also had no effect on κ-bungarotoxin's ability to block nerve transmission in the ciliary ganglia. Thus, the neuronal receptor is fully capable of accommodating a bulky hydrophobic side chain at this position, and it does not contribute to the specificity of the toxin for the neuronal receptor. However, the absence of Trp-32 in κ-neurotoxins may contribute to their low affinity for the muscle receptor, since it has been demonstrated that this residue probably plays a role in the interaction of α-neurotoxins with muscle receptors.

Postsynaptic polypeptide neurotoxins found in the venom of elapid and hydrophid snakes are selective antagonists of nicotinic acetylcholine receptors (Endo & Tamiya, 1991; Chiappinelli, 1991, 1993). On the basis of their selective binding to, and inhibition of, different subclasses of acetylcholine receptors, two distinct families have been recognized. These are the α- and κ-neurotoxins, which display specificity for the muscle end-plate receptor or neuronal receptors such as those found in the chick ciliary ganglion, respectively. Very little cross-reactivity is seen between the α- and κ-neurotoxins and their respective receptors. The amino acid sequences of these neurotoxins clearly show that they are related, but the basis of their distinct physiological selectivities is not understood. Furthermore, X-ray (Bourne et al., 1985; Betzel et al., 1991) and NMR (Basus et al., 1988; Basus & Scheek, 1988; Oswald et al., 1991) investigations indicate that the overall three-dimensional structures of the α- and κ-neurotoxins are similar. These toxins have proven to be essential reagents for the classification of receptor subtypes and in some instances for receptor isolation. A clearer understanding of the molecular basis for their selectivity offers the potential to develop new pharmaceutical agents that may be more selective than those currently available, as well as other beneficial products such as new biodegradable insecticides (Chiappinelli, 1993).

According to a recent review by Endo and Tamiya (1991), 88 different, but related, active venom-derived postsynaptic toxins from elapid and hydrophid snakes have been isolated and sequenced. Of these, 84 are α-toxins and 4 are κ-toxins on the basis of their receptor specificity. The α-neurotoxins can be further defined as long or short α-neurotoxins. Short α-neurotoxins contain 60–62 amino acid residues and four disulfide bridges in common positions (Figure 1). Long α-neurotoxins usually consist of 70–74 amino acid residues and have a fifth disulfide bond in addition to the four found in short neurotoxins. Short and long α-neurotoxins are also distinguished by conserved sequence deletions and additions relative to one another. All of the κ-neurotoxins identified

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               10        20        30        40        50        60        70
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α-neurotoxins
         *                *      *    +       **     **   **          **    *
α-Bgt  IVCHTTATSPIS-AVT---CPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVT-CCSTDKCNPHPKRQPG
α-Cbt  IRCF---ITPDITSKD---CPNGH-VCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQ-CCSTDNCNPFPTRKRP
LS III RECY---LNPHDTQT----CPSGQEICYVKSWCNAWCSSRGKVLEFGCAATCPSVNTGTEIK-CCSADKCNTYP
Nno I  ITCY---KTPIPITSET--CAPGQNLCYTKTWCDAWCGSRGKVIELGCAATCPTVESYQDIK-CCSTDDCNPHPKQKRP
As b   LSCY---LGYKHSQT----CPPGENVCFVKTWCDGFCNTRGERIIMGCAATCPSVNTGTEIK-CCSADKCNTYP
Nm 394 KRCY---RTPDLKSQT---CPPGEDLCYTKKWCDAWCTSRGKVIELGCVATCPKVKPYEQIT-CCSTDNCNPHPKMKP
Ebt b  RICF---NHQSSQPQTTKTCSPGESSCYHKQWSD-F---RGTIIERGC--GCPTVKPGIKLS-CCESEVCNN
NmmIII LNCH---NQMSAQPPTTTRCSRWETNCYKKRWRDH----RGYKTERGC--GCPTVKKGIQLH-CCTSDNCNN
Tx a   LECH---NQQSSQPPTTKTC-PGETNCYKKVWRDH----RGTIIERGC--GCPTVKPGIKLN-CCTTDKCNN

κ-neurotoxins
         *#               *      *            **#   #**   ** #     #  #**    * #
κ-Bgt  RTCL---ISPSSTPQT---CPNGQDICFLKAQCDKFCSIRGPVIEQGCVATCPQFRSNYRSLLCCTTDNCNH
κ-Ftx  RTCL---ISPSSTSQT---CPKGQDICFTKAFCDRWCSSRGPVIEQGCAATCPEFTSRYKSLLCCTTDNCNH
κ-Bgt2 KTCL---KTPSSTPQT---CPQGQDICFLKVSCEQFCPIRGPVIEQGCAATCPEFRSNDRSLLCCTTDNCNH
κ-Bgt3 RTCL---ISPSSTPQT---CPNGQDICFRKAQCDNFCHSRGPVIEQGCVATCPQFRSNYRSLLCCRTDNCNH

         C1______________________C1                       C3__________C3
                          C2__________________________C2               C4___C4
                                       C5__C5

       |-------- loop 1 -------|------- loop 2 --------|---- loop 3 ---|--- Tail-----|
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FIGURE 1: Sequence homology among α- and κ-neurotoxins. Members of each group are shown to highlight the conserved similarities and differences among groups. Those residues that are invariant in all α- and κ- neurotoxins are indicated with an asterisk (*). Those residues found in all α-neurotoxins but no κ-neurotoxins are indicated with a cross (+), and those residues found in all κ-neurotoxins but no α-neurotoxins are indicated with a number sign (#). α-Bungarotoxin from *Bungarus multicinctus* (α-Bgt), α-cobratoxin from *Naja naja siamensis* (α-Cbt), toxin LS III from *Laticauda semifasciata* (LS III), neurotoxin I from *Naja naja oxiana* (Nno I), toxin *b* from *Astrotia stokesii* (As b), toxin 3.9.4 from *Naja melanoleuca* (Nm 394), erabutoxin *b* from *Laticauda semifasciata* (Ebt b), toxin III from *Naja mossambica mossambica* (NmmIII), and toxin *a* from *Naja nigricollis* (Tx a) are the α-neurotoxins shown. κ-Bungarotoxin from *Bungarus multicinctus*, Taiwan (κ-Bgt), κ-flavitoxin from *Bungarus flaviceps* (κ-Ftx), and κ-bungarotoxins 2 and 3 from *Bungarus multicinctus*, China (κ-Bgt_2 and κ-Bgt_3) are the κ-neurotoxins shown.The numbering is according to position in the figure and takes into account gaps introduced to optimize homology. The disulfide bridges and elements of structure are indicated below the sequences.

consist of 66 amino acid residues and, on the basis of amino acid sequence alignments, contain the five disulfide bonds found in long α-neurotoxins. The κ-neurotoxins also contain the amino acid deletions in the vicinity of positions 16-20 and amino acid additions between positions 36 and 40, which are characteristic of long α-neurotoxins. Moreover, the κ-neurotoxins display a unique single-residue insertion in the vicinity of position 62.

All of the structures that have been determined for both the α- and κ-neurotoxins are similar, consisting of three large main-chain loops bound by four disulfide bonds at one end. The long neurotoxins contain an additional disulfide bridge at the opposite end of the middle loop. The two strands of the central loop and the second or C-terminal strand of the third loop constitute a twisted, antiparallel β-sheet (see Figure 1).

Because of the similarity in their structures, the variants among these toxins can be viewed as natural mutants and they form a basis upon which candidate residues for site-specific mutagenesis can be chosen for the purpose of unraveling the basis for the different specificities seen for the two families. In addition, numerous chemical modification studies [reviewed in Endo and Tamiya (1991) and Chiappinelli (1993)] and a recent site-directed mutation study (Pillet et al., 1993) provide additional evidence for the role of specific residues within the α-neurotoxins. These include, in addition to the conserved cysteine residues, Trp-32 and Arg-40. These residues are located toward the bottom of the middle loop of the α-neurotoxins, just below the limits of the β-sheet (see Figure 2), where their side chains point in the same direction into the solvent (Bourne et al., 1985; Betzel et al., 1991; Basus et al., 1988; Basus & Scheek, 1988). Some investigators have proposed that the arginine side chain mimics the positive charge on the acetylcholine molecule (Karlsson et al., 1973; Martin et al., 1983), and it is generally believed that this area provides a major contact region with the acetylcholine receptor.

An inspection of the homology presented in Endo & Tamiya (1991) and represented on a more limited basis in Figure 1 shows that the pattern of residue conservation among the α- and κ-neurotoxins can be placed in three groups. These are as follows: (1) those residues absolutely conserved in all active neurotoxins, both α and κ; (2) those residues found in all κ-neurotoxins but in no α-neurotoxins; and (3) those residues found in all α-neurotoxins but in no κ-neurotoxins. Those residues conserved among all active toxins are considered good candidates as residues important to the overall interaction of these toxins with the nicotinic receptors. Those residues unique to one family or the other are considered as potential determinants of their differential selectivity for receptor subclasses, which has been observed between the α- and κ-neurotoxins. Residues that fall into each of the three aforementioned categories are found at the bottom of the central loop, where a substantial interaction with the receptor probably takes place. This report describes the results of selective site-directed mutations of κ-bungarotoxin and demonstrates that two highly conserved residues in particular that have long been thought to possibly be responsible for the differential specificity do not, in fact, affect the activity of κ-bungarotoxin for the chick ciliary ganglion receptor.

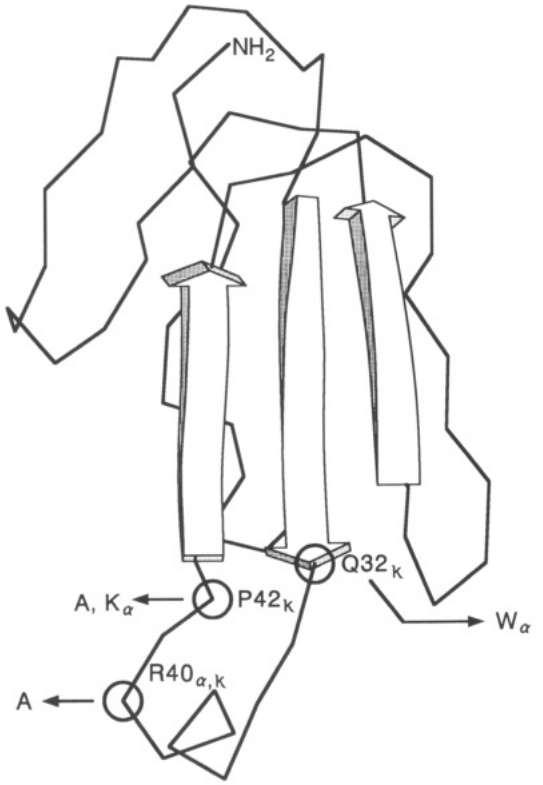

FIGURE 2: Representative structure of snake venom postsynaptic neurotoxins. The general relationship of the three-stranded β-sheet with the bottom of the middle loop is shown, as well as the position of the mutated residues at the junction between these two regions. Residues conserved in either α- or κ-neurotoxins are designated with the appropriate subscript, and the mutations to the κ-bungarotoxin sequence are shown with arrows. The amino terminus of the polypeptide is indicated (NH_2). The carboxy terminus is behind the bottom end (head of the arrow) of the middle strand of the β-sheet.

MATERIALS AND METHODS

Venom-purified κ-bungarotoxin was obtained from Biotoxins Inc. (St. Cloud, FL) and verified by sequence and composition analysis. The expression vector pEZZ 18 and IgG Sepharose 6FF were from Pharmacia.

Expression and Purification of Native and Mutant κ-Bungarotoxins. Native and mutant neurotoxins were produced as fusion proteins in *Escherichia coli* by a variation of the method described previously (Fiordalisi et al., 1991). The identity of the carrier protein in the fusion construct was changed to the IgG-binding domain of protein A by splicing the gene for κ-bungarotoxin into the pEZZ 18 gene fusion vector (Lowenadler et al., 1987). The resulting construct produced a fusion protein consisting of the protein A signal sequence and two synthetic "Z" domains, which are based on the IgG-binding domain of protein A, followed by κ-bungarotoxin. As previously described (Fiordalisi et al., 1991), a methionine was placed just prior to the first residue of κ-bungarotoxin, so that the toxin protein could be cleaved from the fusion protein with CNBr. The resulting construct is referred to as pEZK.

Cells were grown in either a New Brunswick BioFlo III (5 L) or a Braun Biostat B (5 L) high-density fermentor. Cells were harvested by centrifugation, and the contents of the periplasmic space were retained as previously described (Nilsson & Abrahamsen, 1990; Randall & Hardy, 1986). Fusion protein was isolated by affinity chromatography on IgG Sepharose 6FF (Lowenadler et al., 1987). The fusion protein was eluted from the column with acidic pH buffers, as described by the manufacturer, and immediately lyophilized. The isolated fusion protein was subjected to CNBr cleavage as previously described (Fiordalisi et al., 1992). The cleaved material was chromatographed with a Waters model 600 HPLC on a Beckman SEC-2000 gel filtration column first, followed by reverse-phase chromatography on a Vydac C4 (5 μm, 4.6 × 250 mm) column. Sodium phosphate (25 mM, pH 8) was used for the gel filtration buffer, and a linear gradient of acetonitrile in 0.1% trifluoroacetic acid was used for the reverse-phase chromatography. Recombinant κ-bungarotoxin was identified by Western blot analysis, with rabbit anti-κ-bungarotoxin antisera (Fiordalisi et al., 1992), after each column step, and the appropriate pools were made.

Site-Directed Mutagenesis. Mutagenesis was performed by the cassette method utilizing silent restriction sites engineered into the gene for κ-bungarotoxin (Fiordalisi et al., 1991). The DNA sequences of the mutant genes were verified by Sanger dideoxy sequencing. Mutagenic oligonucleotides were synthesized on Applied Biosystems 380A, 380B, or 394 DNA synthesizers according to the manufacturer's protocols in the Washington University Protein Chemistry Laboratory.

Characterization of the Mutant Proteins. The mutant proteins were characterized by automated sequence analysis on Applied Biosystems Model 470A or 477A protein sequencers, by amino acid compositional analysis on a Beckman 6300 analyzer, and by electrospray ionization mass spectrometry on a Vestec Model 201 single quadrupole mass spectrometer. Dichroic spectra were determined on a Jasco J-600 spectropolarimeter. Protein concentration was determined by amino acid analysis on a Beckman 6300 amino acid analyzer. Samples were hydrolyzed in the gas phase with 6 N HCl in evacuated, sealed glass tubes for 1 h at 160 °C. Hydrolysates were dried on a Savant Speed-Vac, redissolved in 0.05 N HCl, and analyzed.

Assay for Biological Activity. Biological activity was determined by assessment of the neurotoxin's ability to block nerve transmission in chick ciliary ganglia, as previously described (Chiappinelli et al., 1990; Fiordalisi et al., 1991). The degree of blockage (decrease in the amplitude of the action potential) was determined at regular intervals after exposure to the mutant toxins. These data were analyzed by linear regression programs, and the potency of the toxin was judged by the blockage in nerve transmission that resulted after a 30-min exposure of the ganglia to the neurotoxin. The viability of the ganglia during the experiments was verified by the recovery of impulse propagation after removal of the toxin and washing with buffer. Consistent with that previously reported (Chiappinelli et al., 1990), recovery was generally observed within 1–4 h after washing began, depending on the degree of blockage prior to washing.

RESULTS AND DISCUSSION

Choice of Mutants. The sequences of the proteins constituting the postsynaptic neurotoxin family (Endo & Tamiya, 1991; Danse & Garnier, 1990; Grant & Chiapinelli, 1985; Grant et al., 1988; Chiappinelli et al., 1990) reveal a pattern of conserved residues, which may play a common role in α- and κ-neurotoxin-receptor interactions. In contrast, certain other residues, which are conserved in one toxin family but not the other, may be responsible for the differential interaction of the toxins with receptor subclasses. Residue conservation, as well as the results from chemical modification studies and a recent report on the site-directed mutagenesis of erabutoxin (Pillet et al., 1993), was used as the basis for selecting a limited number of candidate residues for these studies. The residues

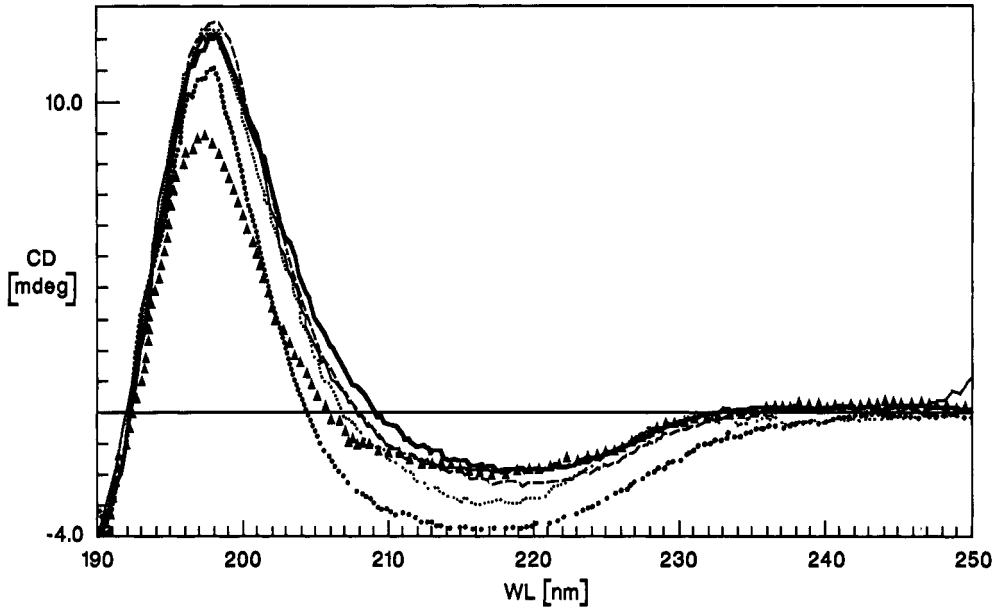

FIGURE 3: Circular dichroism spectra of native and mutant κ-bungarotoxins: native venom-purified κ-bungarotoxin, thick solid line; native recombinant κ-bungarotoxin, dashed line; R-40A, thin solid line; P-42-A, triangles; Q-32-W, squares; P-42-K, dotted line.

to be tested were chosen carefully because of the difficulty in obtaining sufficient amounts of properly folded κ-bungarotoxin from the available expression systems (Fiordalisi et al., 1992) and the protein requirements of the chick ciliary ganglion assay, which forced the number of mutations attempted for this investigation to be limited.

The only residue that appears in all active α-neurotoxins but never appears in any κ-neurotoxin is Trp-32. Since no active variants exist that do not contain this residue, it has long been the subject of chemical modification studies. However, the results have been mixed, with some investigations indicating a role for Trp-32 in toxicity and others indicating no role for Trp-32 [reviewed in Endo and Tamiya (1991)]. The recent report by Pillet et al. (1991) indicates that changing Trp-32 in erabutoxin to a histidine residue or a phenylalanine residue results in 8.6- and 67-fold decreases in binding to a muscle-type nicotinic acetylcholine receptor, respectively. Thus, Trp-32 appears to be important in expressing neurotoxicity. Because of this, and because it is the only completely conserved residue in α-neurotoxins that is never found in κ-neurotoxins, it was selected as a subject of this investigation. Specifically, the question to be asked was as follows: Does the insertion of a tryptophan residue into κ-bungarotoxin at this position decrease its neurotoxicity for the chick ciliary ganglia neuronal receptor?

In contrast to the single residue (Trp-32) that is found in all active α-toxins and no κ-toxins, several residues are found in all active κ-toxins and no α-toxins. This is probably partly due to the fact that only four κ-neurotoxins have so far been identified and sequenced. Those residues found in all κ-toxins but no α-toxins are Leu-4, Pro-42, Gln-46, Phe-55, Ser-60, Leu-62, and His-71. Of these, the residue that has received the most attention is Pro-42 because of its proximity to Arg-40, which is conserved in all active neurotoxins and is the one residue that plays the most significant role in neurotoxicity (Yang et al., 1974) and receptor binding (Pillet et al., 1993). Because of the often cited role of proline residues in altering the conformation or direction of polypeptide chains in proteins, and because Pro-42 occupies a key position at the junction between the β-sheet at the lower end of the middle loop, Pro-42 has long been considered the most obvious candidate for conferring the different specificity of the κ-neurotoxins. Thus, Pro-42 was also chosen as a subject of mutagenesis for this investigation. The question to be asked was as follows: does the removal of Pro-42 from κ-bungarotoxin diminish its ability to inhibit nerve transmission in the chick ciliary ganglion? Finally, Arg-40 was chosen as a mutagenesis site, since although it has been shown to be very important in α-neurotoxins, no direct evidence for its importance in κ-neurotoxins was yet available. Thus, it was important to confirm its role in κ-neurotoxins, particularly within the context of the nearby Pro-42 mutations.

Preparation of Mutants. The fusion protein produced from pEZK is transported to the periplasmic space during expression under control of the protein A signal sequence. Purification consists of harvesting the contents of the periplasmic space by osmotic shock (Nilsson & Abrahamsen, 1990; Randall & Hardy, 1986) and isolating the fusion protein on an IgG affinity column (Lowenadler et al., 1987). After elution from the column, the neurotoxin is cleaved from the fusion protein with CNBr, as previously described. The neurotoxin is then isolated from the digest by HPLC gel filtration and reverse-phase steps. As previously reported (Fiordalisi et al., 1992), not all of the κ-bungarotoxin produced in this way is active. A significant proportion of the protein is not properly folded and results in inactive species. Properly folded, biologically active κ-bungarotoxin is separated from all inactive species during reverse-phase chromatography. The integrity of the isolated toxins is verified by CD analysis (Figure 3) and electrospray mass spectrometry. The properly folded toxin has a characteristic CD spectrum that reflects its β-sheet content. Improperly folded toxin has a CD spectrum that more closely resembles that of a random coil (Fiordalisi et al., 1992). Therefore, it is possible to detect contamination by improperly folded toxin by its effect on the CD spectrum. The mass of each mutant was determined to be within 1–2 mass units of the calculated mass.

Assay for Biological Activity on Chick Ciliary Ganglia. In attempting to unravel the structure–function relationships of postsynaptic neurotoxins and the basis of their interactions with the nicotinic acetylcholine receptors, the most relevant parameter is their effect on chemical-based nerve transmission. For this purpose, the chick ciliary ganglion assay was chosen. This assay was crucial to the discovery of the neuronal nicotinic

receptor and to the recognition of the specific action of κ-bungarotoxin. It is, thus, the benchmark of κ-bungarotoxin or κ-bungarotoxin-like activity.

Binding assays such as those developed for the α-neurotoxins, while more quantitative than the ganglion assay, only measure binding and not inhibition or enhancement of nerve transmission. Until it is conclusively demonstrated that the particular binding phenomenon observed is directly and proportionately linked to modulation of nerve transmission, such binding assays are difficult to interpret and, therefore, are of limited benefit. This relationship has not yet been firmly established for κ-bungarotoxin and its biological targets. Further, it has been demonstrated that neuronal tissue appears to contain different types of extrasynaptic toxin receptors, which would interfere with the interpretation of binding data. In any case, a convenient binding assay for κ-bungarotoxin has not yet been developed because of a lack of a plentiful source of homogeneous κ-bungarotoxin-specific receptor and because the exact subunit composition of this receptor is still unclear.

Although it is not possible to derive association constants and determine off-rates with the chick ciliary ganglion assay, it is reproducible and provides an accurate measure of potency in terms of the concentration of native or mutant toxin required to produce a blockage with respect to time. The relative heights of the impulse propagated from the ciliary receptor are derived from oscilloscope tracings and plotted vs the time of exposure to the neurotoxin. Exposure of the ganglion to a particular concentration of toxin is allowed to proceed for an appropriate period of time, after which the toxin is washed out with a continuous flow of buffer. The ganglion continues to be monitored until recovery of the nerve impulse is seen. Since the amplitude of the impulse at any particular concentration of toxin is initially dependent on the length of exposure, the degree of inhibition seen after 30 min is measured. The 30-min interval was chosen since it was found to reproducibly reflect the degree of inhibition and was a convenient period of measurement given the time constraints dictated by ganglion viability. The data are corrected for any drop in impulse height that may be due to stopping buffer flow during incubation with the toxin by determining the slope of the inhibition curve with linear regression analysis. Complete blockage, which occurs at or prior to 30 min of incubation, is considered as 100% inhibition of nerve transmission. The percent inhibition is then plotted vs the concentration of toxin. Each assay is performed on a fresh ganglion, and all of the assays that make up a particular curve are conducted within a few days of each other. Our experience has shown that the response to a particular toxin is completely consistent from one ganglion to another. For example, equivalent concentrations of a particular neurotoxin, whether from the same or different preparations, when assayed months apart, produce points that consistently fall on the same curve. Moreover, the assays are always conducted such that the recovery of nerve transmission upon the removal of toxin is assessed. This demonstrates that the ganglion has remained viable throughout the experiment, gives a qualitative assessment of the off-rate (i.e., rapid or slow recovery of transmission), and allows one to judge whether or not the blockage appears consistent with receptor binding.

Biological Activity of Mutant κ-Bungarotoxins. The results of the chick ciliary ganglia bioassays on the native and mutant recombinant κ-bungarotoxins are presented in Figure 4. Toxin concentrations are expressed in terms of the dimer because of the observation that κ-bungarotoxin is prevalently a dimer in solution at physiologically active concentrations (Chiappinelli & Lee, 1985; Fiordalisi & Grant, 1994) and is most probably its active form. Venom-derived κ-bungarotoxin (data not shown) produces a curve that is indistinguishable from that of recombinant native κ-bungarotoxin in Figure 4. Thus, both venom-purified and recombinant native κ-bungarotoxin produce inhibition of nerve transmission with 50% blockage at approximately 100 nM concentration. The Pro-42-Lys mutant is capable of yielding complete blockage at 2 μM but is unable to block nerve transmission at 200 nM, a concentration at which native κ-bungarotoxin produces complete inhibition. The concentration at which 50% blockage is seen for Pro-42-Lys is approximately 1600 nM. Thus, this single amino acid change produces a toxin with decreased potency in the chick ciliary ganglion assay relative to native κ-bungarotoxin. However, changing Pro-42 to an alanine residue (Pro-42-Ala) results in a fully active toxin. This suggests that the presence of the proline is not crucial for κ-bungarotoxin activity and does not significantly alter the orientation of the Arg-40 side chain, but that this position is less tolerant of a charged, bulky side chain, like the Lys found in α-bungarotoxin.

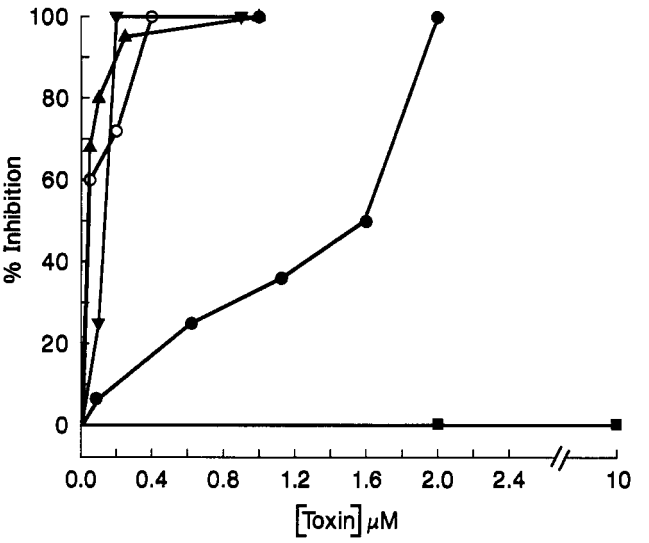

FIGURE 4: Degree of inhibition of nerve transmission in chick ciliary ganglia as a function of toxin concentration. Each plot was generated from a series of chick ciliary ganglion assays conducted with the indicated recombinant protein: native recombinant κ-bungarotoxin, ▼; Q-32-W, ▲; P-42-A, ○; P-42-K, ●; R-40-A, ■.

More striking is the observation that the Arg-40-Ala mutant is inactive up to 10 μM toxin. This observation suggests that Arg-40 is essential for activity, since replacing it with alanine eliminates inhibitory activity. It has been suggested that this residue may mimic the quaternary ammonium group of acetylcholine, interacting with the same region of the receptor. This observation also supports the hypothesis that it is likely that the primary receptor interaction of both families (α and κ) is due to the conserved residues they have in common, such as Arg-40. Mutations of this arginyl residue in erabutoxin *a* (Pillet et al., 1993) also had a profound effect on its ability to bind to *Torpedo marmurata* acetylcholine receptors. However, this is the first demonstration of the importance of this residue in a κ-neurotoxin.

Perhaps most surprising is the observation that when tryptophan is placed in κ-bungarotoxin at the position where it is found to be invariant in α-neurotoxins, but never appears in κ-neurotoxins (Figure 1, position 32), the resulting toxin is fully able to block the ganglion receptor. Thus, although it never appears naturally in a κ-neurotoxin, the presence of

tryptophan at this position does not disrupt the toxin's interaction with its receptor. Apparently, the neuronal receptor is capable of tolerating a large hydrophobic side chain at this position. Site-directed mutagenesis studies in erabutoxin *a* (Pillet et al., 1993) suggest that this tryptophan residue is more important in the binding of α-neurotoxins to muscle receptors. Mutations of this tryptophanyl residue to either a histidine or a phenylalanine decreased the K_d of the toxin by approximately 9- and 70-fold, respectively. Thus, although the presence of a tryptophan at position 32 in κ-bungarotoxin does not affect its interaction with ciliary ganglia receptors, its normal absence in κ-bungarotoxin may be responsible, at least in part, for κ-bungarotoxin's low affinity for the muscle receptor.

Since the determination of the amino acid sequences of the κ-neurotoxins, much speculation has focused on the presence of Pro-42 and the absence of a Trp at position 32 as the basis for their receptor specificity. This report presents evidence that neither the lack of the proline residue nor the presence of the tryptophan appears to have any effect on the activity of κ-bungarotoxin at the chick ciliary ganglia receptor. The tryptophan at position 32 has long been thought to be crucial for receptor binding in the α-neurotoxins. While these data do not refute that hypothesis, the significant finding is that the tryptophan has no effect in the case of κ-bungarotoxin and the ciliary ganglia receptor. The lack of this tryptophan residue in κ-bungarotoxin may play at least a partial role in the very low affinity of κ-bungarotoxin for the muscle receptor. Pro-42 also is not a requirement for κ-bungarotoxin activity. All that seems to be required at this normally conserved position is the presence of a neutral amino acid residue with a relatively short side chain. These results suggest that this residue is at the interface of the toxin–receptor complex, but more work is necessary to distinguish the relative contributions of charge and bulk at this position.

The complete lack of activity, even at very high concentrations, produced by the alteration of the Arg-40 side chain clearly shows that single amino acid substitutions can have dramatic effects in these neurotoxins. The studies of Pillet et al. (1993) with erabutoxin have led to the conclusion that binding of α-neurotoxins to their receptors is most likely dependent on multiple contacts. This conclusion was reached because no single mutation completely abolished binding, although some, such as the arginyl residue corresponding to Arg-40 in the present study, reduced it by approximately 300-fold. Since an appropriate binding assay for κ-bungarotoxin is not readily available, it is not known whether the Arg-40-Ala mutant still binds to its receptor through other points of contact. It is likely that it still does, albeit to a much lower extent, as seen in the erabutoxin studies. This is the first demonstration that Arg-40 also plays an important role in the neurotoxicity of κ-neurotoxins.

The finding that the two major conserved residue differences (at positions 32 and 42, Figures 1 and 2) between α- and κ-bungarotoxins do not account for their profound difference in receptor specificity is significant in itself, because it ends the speculation that has surrounded these residues ever since the sequence of the first κ-toxin was determined. It is possible that the difference in specificity is a function of the cumulative contributions of a number of residues. Unfortunately, a comparison of the available primary and three-dimensional structures of the relevant proteins does not yield any additional compelling candidates. However, there is one additional striking difference between the α- and κ-neurotoxin families that may be a potential candidate. This is the ability of the κ-neurotoxins to form dimers in solution, while the α-neurotoxins do not. The dissociation constant for κ-bungarotoxin, which is approximately 2.7×10^{-8} M (Fiordalisi & Grant, 1994), indicates that the toxin largely exists as a dimer at physiologically active concentrations ($1–2 \times 10^{-7}$ M). It is possible that this dimerization may have a significant effect on the toxin's interaction with its receptor. Recent NMR data (Oswald et al., 1991) suggest that the dimer interface may involve residues at positions 55–62 in Figure 1. This is an area of relatively low homology between the α- and κ-neurotoxins and one which could be effectively probed by site-directed mutagenesis. This is the subject of ongoing efforts.

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