Affinity of Native κ-Bungarotoxin and Site-Directed Mutants for the Muscle Nicotinic Acetylcholine Receptor†

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ABSTRACT: κ-Bungarotoxin (κ-bgt) is a 66-residue peptide originally purified from snake venom that acts as an antagonist at certain acetylcholine receptors. It is one of four homologous κ-neurotoxins that are distinguished from the structurally related α-neurotoxins by their ability to block the α_3-subunit-containing neuronal nicotinic acetylcholine receptor (nAChR). It has been reported that venom-purified κ-bgt also displays some affinity for the α_1-subunit-containing muscle nAChR to which the α-neurotoxins bind with high affinity. Here we report the effects of particular mutations on the ability of recombinant κ-bgt to block the binding of ^{125}I-α-bgt to nAChRs found in fetal mouse muscle and chick skeletal muscle. While the replacement of a proline residue found in all κ-neurotoxins with an alanine (P-42-A) has relatively little effect, the introduction of a lysine, which is found in 90% of active α-neurotoxins at the same position (P-42-K), eliminates muscle receptor affinity at the concentrations tested. In contrast, the replacement of a glutamine in κ-bgt with a tryptophan found in all active α-neurotoxins (Q-32-W) increases the affinity of κ-bgt for the muscle receptor. When the arginine residue found in all active α- and κ-neurotoxins is replaced by an alanine (R-40-A), the ability of κ-bgt to block the muscle receptor is reduced to undetectable levels. The affinity of recombinant κ-bgt for the muscle receptor is also shown to be 1–2 orders of magnitude lower than that of venom-purified κ-bgt (IC_{50} = 10 μM and 150 nM, respectively). We conclude that commercially available venom-purified κ-bgt contains pharmacologically significant amounts of an α-neurotoxin, probably α-bgt, that is found in the same venom.

The venoms of poisonous snakes in the elapid and hydrophid families contain a variety of toxic compounds that are defined according to their different modes of action. A major class of toxins, which includes the curare-mimetic postsynaptic neurotoxins and the cytotoxins (also called cardiotoxins), are small proteins (7–8 kDa) that are related by strong sequence and structural homology (Endo & Tamiya, 1991). While the cytotoxins act nonspecifically to cause persistent depolarization of tissues, the postsynaptic neurotoxins, of which over ninety have been isolated and sequenced, bind with high affinity and specificity to the vertebrate nicotinic acetylcholine receptor (nAChR) embedded in the postsynaptic membranes of neuronal and skeletal muscle tissues. In this way, the postsynaptic neurotoxins prevent both the binding of the natural neurotransmitter, acetylcholine (ACh), and the proper functioning of the nAChR as a ligand-gated transmembrane ion channel.

The postsynaptic neurotoxins are further classified as either α-neurotoxins or κ-neurotoxins according to their affinities for different nAChR subtypes. The α-neurotoxins are known to bind with high affinity ($K_d \leq 1$ nM) to the α_1-subunit-containing nAChR found at the mammalian neuromuscular junction and with comparable affinity (K_d = 1 nM) to an α_7-subunit-containing receptor recently identified in neuronal tissues (Clarke, 1992; Vijayaraghavan et al., 1992). The more recently identified κ-neurotoxins, of which four are known (Chiappinelli et al., 1990), bind with high affinity (K_d = 5–10 nM) to an α_3-containing neuronal nAChR and with lower affinity (1 μM) to an α_4-subunit-containing neuronal nAChR (Chiappinelli, 1992). The α-neurotoxins have no apparent affinity for nAChRs lacking α_1- or α_7-subunits. In contrast, the κ-neurotoxins have been reported to bind to the α_1-containing muscle receptor, albeit with relatively low affinities (130 nM–1 μM) as compared to the α-neurotoxins (Loring et al., 1984, 1986; Wolf et al., 1988).

The three-dimensional structures of the α- and κ-neurotoxins have been shown to be fundamentally similar by X-ray crystallography (Low et al., 1976; Walkinshaw et al., 1980; Agard & Stroud, 1982; Love & Stroud, 1986; Low & Corfield, 1986; Corfield et al., 1989; Betzel et al., 1991; Sacchettini et al., 1992; Dewan et al., 1994) and NMR spectroscopy (Endo et al., 1981; Inagaki et al., 1981, 1985; Hider et al., 1982; Basus et al., 1988; Basus & Scheek, 1988; Yu et al., 1990; Oswald et al., 1991; Sutcliffe et al., 1992; Zinn-Justin et al., 1992). The toxins consist of three polypeptide loops and contain four or five conserved disulfide bonds. The major element of secondary structure shared by all is a triple-stranded, antiparallel β-sheet consisting of both strands of the second loop and one strand from the third loop.

It is likely that the general receptor-binding abilities of the toxins are dependent on conserved residues common to both toxin families, while their abilities to distinguish among nAChR subtypes are due to consistent differences between the families. A comparison of the primary structures of the toxins suggests which amino acid residues might be functionally

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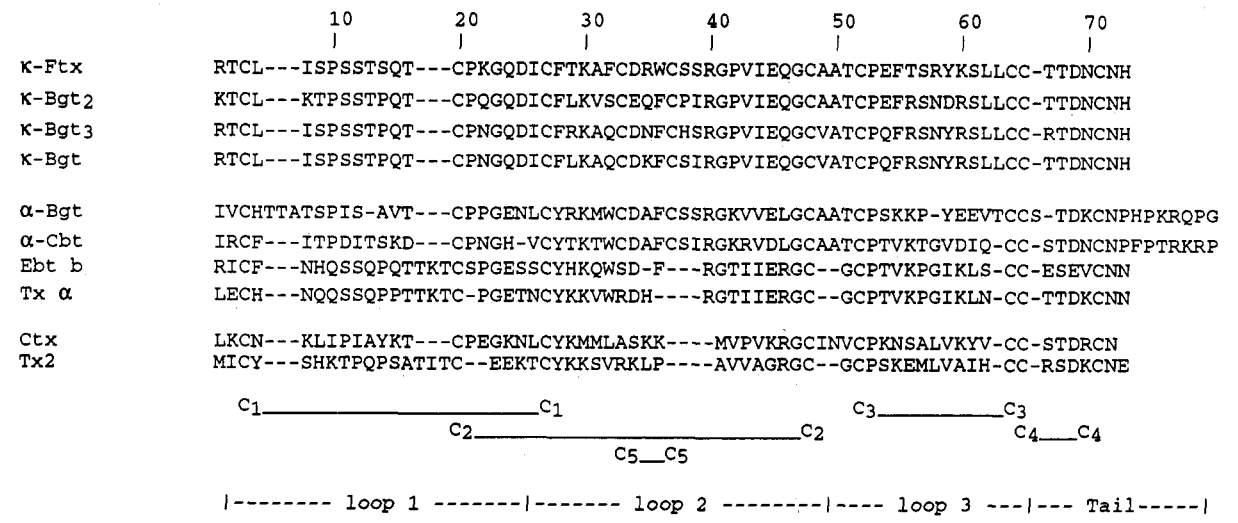

FIGURE 1: Sequence homology among representative curare-mimetic neurotoxins and cytotoxins. κ-Bungarotoxin, κ_2-bungarotoxin, and κ_3-bungarotoxin (κ-Bgt, κ-Bgt_2, and κ-Bgt_3) from *Bungarus multicinctus* and κ-flavitoxin (κ-Ftx) from *Bungarus flaviceps* are the four κ-neurotoxins. α-Bungarotoxin (α-Bgt) from *Bungarus multicinctus* and α-cobratoxin (α-Cbt) from *Naja naja siamensis* are long α-neurotoxins. Erabutoxin *b* (Ebt b) from *Laticauda semifasciata* and toxin α (Tx α) from *Naja nigricollis* are short α-neurotoxins. Cardiotoxin $V^{II}4$ (Ctx) from *Naja mossambica mossambica* and toxin 2 (Tx2) from *Dendroaspis angusticeps* are cytotoxins. The positions of the invariant cysteines and native disulfide bonds are indicated. The sequence segments corresponding to the three structural loops are also shown.

important. In addition to residues that are thought necessary for maintaining toxin structure, including eight or ten highly conserved cysteines that form the disulfide bonds in the toxins' native conformations, four other residues stand out for potential functional significance (the numbering of residues is based on Figure 1). A tryptophan residue found in all α-neurotoxins (Trp-32) and a lysine residue found in 90% of α-neurotoxins (Lys-42) are not present in any κ-neurotoxin, while a conserved proline residue (Pro-42) is found only in the κ-neurotoxins. Most striking is the presence in 100% of active α– and κ–neurotoxins of an arginine residue at position 40 (Arg-40).

During the past 25 years, the α-neurotoxins have been studied in order to elucidate the details of toxin–receptor contact. Until recently, this research has relied primarily on the specific chemical modifications of toxin residues and to a limited extent on the use of synthetic peptides corresponding to native and mutant toxin sequences (Endo & Tamiya, 1991). The results of these studies depend upon the particular toxin assayed, but have consistently implicated several residues in receptor binding including the highly conserved Trp-32 and Arg-40.

The most direct data concerning functionally important residues within intact proteins have come from the recent cloning, expression, and mutagenesis of the α-neurotoxins, erabutoxin *a* (Ducancel et al., 1989; Boyot et al., 1990; Pillet et al., 1993) and α-bgt (Rosenthal et al., 1994), and the κ-neurotoxin, κ-bgt (Fiordalisi et al., 1991, 1992, 1994). These studies have involved the production and analysis of selected mutants and have illustrated the importance of some highly conserved residues. In the present work, the binding of recombinantly-expressed κ-bgt and selected mutants to the α_1-subunit-containing neuromuscular nAChR found in chick skeletal muscle is analyzed. The contributions to muscle receptor affinity of residues conserved within the α-neurotoxin and κ-neurotoxin families as well as residues conserved across both families were chosen for study. This work complements the earlier study in which the effects of these same mutations on κ-bgt affinity for the α_3-containing neuronal nAChR were studied (Fiordalisi et al., 1994). We also report significant differences between the affinities of recombinant and venom-derived κ-bgt for the muscle receptor and suggest an explanation for this observation.

MATERIALS AND METHODS

Preparation of Toxins. Venom-derived κ-bgt was purchased from Biotoxins (St. Cloud, FL). No contaminating proteins were detectable in venom-derived κ-bgt by standard automated sequence and compositional analyses. Recombinant κ-bgt was expressed in *Escherichia coli* and isolated as previously reported (Fiordalisi et al., 1994). Mutant toxins were characterized by automated sequence and compositional analyses, mass spectrometry, and CD spectroscopy as previously reported (Fiordalisi et al., 1994).

Iodination of α-Bgt. α-Bgt was iodinated as previously described (Wolf et al., 1988). Briefly, 1.25 nmol of α-bgt was combined with 1 mCi of sodium [^{125}I]iodide in 250 mM sodium phosphate, pH 7.4, in glass tubes previously coated with 4.3 μg of Iodogen reagent (Pierce Chemical Co.). The reaction proceeded for 20 min at 22 °C, after which free iodine was separated from iodinated toxin by passing the reaction over a 10 mL Sephadex G10 column. The incorporation of ^{125}I was confirmed using a Beckman 8000 γ counter. ^{125}I was incorporated into α-bgt to a final concentration of 724 Ci/mmol. Two 1 mL fractions containing 65% and 35%, respectively, of the radiolabeled α-bgt were collected from the Sephadex column. Dilution assays showed that the more concentrated of the two fractions had a final protein concentration of 800 μM. This sample was used as the stock of ^{125}I-α-bgt for the subsequent assays.

Quail Fibroblast-Expressed Muscle Receptor Assays. The QT-6 quail fibroblast cell line expressing the mouse fetal muscle nAChR was produced as previously described (Phillips et al., 1991). Cells were grown in complete growth medium containing Earle's balanced salt solution (EBSS, Sigma Chemical, St. Louis, MO) with 10% (v/v) tryptose phosphate broth (TPB, GIBCO, Grand Islands, NY), 5% fetal bovine serum (FBS, Hyclone, Logan, UT), 1% dimethyl sulfoxide, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified 5% CO_2–95% air atmosphere.

Cells were grown at 37 °C in 15 mm plates until confluent (2–3 days) and then treated with 3.5 mM butyrate for 1 day prior to use. All steps of the binding assays were performed at 22 °C. Cells were washed three times with 300 μL of

EBSS buffered with 10 mM HEPES, pH 7.3, and supplemented with 120 mM glucose, 0.2% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (this solution is referred to as EBSS+). Cells were then preincubated with various concentrations of venom-derived κ-bgt or recombinant mutants for 15 min in 300 μL of EBSS+. After the preincubation period, cells were washed quickly three times with EBSS+ and then exposed to 300 μL of 10 nM ^{125}I-α-bgt in EBSS+ for 5 min. Radiolabel was removed, and the cells were washed three times with EBSS+. Cells were removed by repeatedly washing 300 μL of 0.1 N NaOH over the bottom of the plate, followed by a 300 μL wash with EBSS+. The washes for each plate were then pooled, and receptor-bound radiolabel was counted in a TM-analytic 1191 γ counter. Total binding was determined in the absence of added toxin during the preincubation period, and nonspecific binding was detemined in the presence of 1 μM unlabeled α-bgt during the preincubation. All assays were performed in duplicate.

Chick Skeletal Muscle Receptor Assays. Fourteen-day embryonic chicks were sacrificed by decapitation. Thigh muscle tissue was removed and homogenized with a glass-on-glass homogenizer in 2.3 times (w/v) chick HEPES tyrode (CHT) buffer, pH 7.2, containing 10 mM HEPES, 150 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$, and 2 mM $MgCl_2$. The homogenate was centrifuged at 16000*g* for 10 min. The supernatant was discarded, and the pellet was resuspended in the original volume of CHT. Muscle homogenate (9 μL) was preincubated for 2 h in the presence of various concentrations of recombinant native or mutant κ-bgts in a total volume of 25.7 μL. Controls were identical except for the absence of κ-toxins in both the total binding and nonspecific binding controls and the presence of 1 μM α-bgt in the nonspecific control. All reactions were performed in duplicate and at 22 °C. After preincubation, 4.3 μL of 70 nM ^{125}I-α-bgt was added to each reaction and diluted after 7 min with 300 μL of cold CHT. The reactions were centrifuged for 10 min at 16000*g* and the supernatants discarded. Pellets were counted in a Beckman 8000 γ counter, washed with an additional 300 μL of CHT, centrifuged, and recounted after removal of the supernatant. This procedure was performed at least three times for each recombinant construct tested. Data were analyzed by GraphPAD-InPlot software or SigmaPlot.

While many small molecules that bind to the nAChR can reach equilibrium within minutes, postsynaptic neurotoxins are much larger molecules that are thought to bind to the receptor through several contact points. The complexity of this interaction may contribute to the need for long equilibration periods (2–3 h). During the 2-h preincubation period, the recombinant toxins develop an equilibrium between their free and receptor-bound states that is dependent upon their affinities for the receptor. Assayed over a range of concentrations, a particular recombinant toxin will generate a dose–response curve from which the concentration of toxin that blocks 50% of specific ^{125}I-α-bgt binding to the muscle receptor (inhibitory concentration-50% or IC_{50}) can be determined. The IC_{50}s can be used to compare directly the relative affinities of the recombinant toxins. Direct calculation of dissociation constants (K_d) from the data is accomplished by the method of Colquhoun (Colquhoun & Rang, 1976). This method defines *r* as the ratio of specific ^{125}I-α-bgt binding in the absence of recombinant toxin to specific binding in the presence of recombinant toxin. $r - 1$ is plotted on the abscissa vs toxin concentration on the ordinant. When both axes are logarithmic, linear regression analysis produces a line that crosses the *x*-axis at $r - 1 = 10^0$ at the K_d of the toxin.

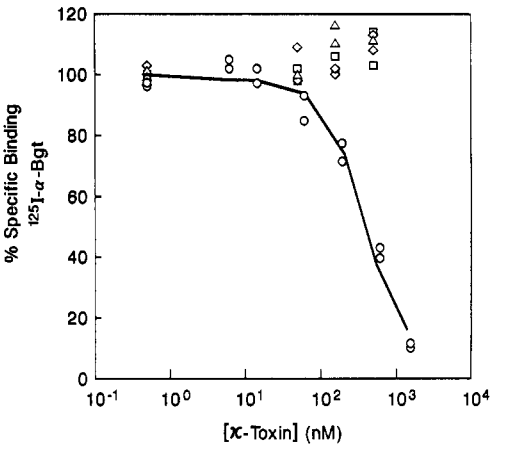

FIGURE 2: Inhibition of ^{125}I-α-bgt binding to muscle nAChRs expressed on QT-6 cells by venom-derived κ-bgt and recombinant mutants. Venom-derived κ-bgt, ○; Q-32-W, △; R-40-A, ◇; P-42-K, □.

RESULTS AND DISCUSSION

Quail Fibroblast-Expressed Muscle Receptor Assays. Initial studies involving the muscle receptor utilized those mutants originally generated for the investigation of κ-bgt's interaction with the chick ciliary ganglion receptor. Because insufficient recombinant κ-bgt was available for the mouse muscle receptor studies, a base-line dose–response curve for the native toxin was generated with venom-derived κ-bgt (Figure 2) whose composition, sequence, concentration, and purity were confirmed by standard protein sequencing, compositional, and mass spectrometry analyses. The IC_{50} for κ-bgt binding to the muscle receptor obtained from this curve is 430 nM. This concentration is expressed using a molecular mass of 14.5 kDa since it is known that κ-bgt exists in solution as a noncovalent dimer (Chiappinelli & Lee, 1985; Chiappinelli & Wolf, 1989; Oswald et al., 1991; Fiordalisi & Grant, 1994). Various concentrations of κ-bgt mutants (P-42-K, Q-32-W, and R-40-A) were assayed under identical conditions and compared to the native κ-bgt dose–response curve (Figure 2). At the concentrations tested, none of the mutants demonstrated any affinity for the muscle receptor as shown by their inability to block ^{125}I-α-bgt binding to the receptor. The replacement with alanine of Arg-40, perfectly conserved in both α- and κ-neurotoxins and implicated in receptor binding by earlier studies (Pillet et al., 1993; Fiordalisi et al., 1994), might be expected to produce this result. However, the inability of P-42-K and Q-32-W to bind to the muscle receptor at these concentrations was surprising, given the fact that Q-32-W showed full activity and P-42-K showed only partially reduced blocking activity at the α_3-containing nAChR found in the chick ciliary ganglion (Fiordalisi et al., 1994). Both of these mutations involved the introduction into κ-bgt of residues thought to be crucial for the muscle receptor binding of α-neurotoxins, and it was thought that they might even increase the muscle receptor affinity of κ-bgt. Instead, they apparently eliminate the affinity of κ-bgt for the muscle receptor at concentrations up to 1 μM.

In order to confirm the applicability of a dose–response curve generated using venom-derived κ-bgt to the analysis of recombinant mutants, a direct comparison of muscle receptor affinity between venom-derived and recombinant κ-bgt was performed at a single concentration equal to the K_d calculated using venom-derived κ-bgt (430 nM). A range of concentrations could not be assayed due to the small amount of recombinant κ-bgt available at the time. As shown in Table

Table 1: Inhibition of ^{125}I-α-Bungarotoxin Binding to Muscle nAChRs Expressed in QT-6 Cells by Venom-Derived and Recombinant κ-Bungarotoxin

	toxin concentration (nM)	% specific binding (^{125}I-α-bgt)
vd-κ-bgt	430	63
r-κ-bgt	430	100

1, κ-bgt isolated from whole venom blocked 37% of ^{125}I-α-bgt binding to QT-6-expressed muscle receptors while recombinant κ-bgt produced no measurable block at the same concentration and under the same conditions. The possibility that the sample of recombinant κ-bgt used was inactive due to improper folding or other factors was eliminated by demonstrating its ability to block the neuronal receptor in the chick ciliary ganglion with a potency equal to that of venom-derived κ-bgt (Fiordalisi et al., 1994).

The discrepancy between the potency of the commercially available venom-derived κ-bgt and recombinant κ-bgt that exhibited full activity on the chick ciliary ganglia made it necessary to produce sufficient quantities of recombinant κ-bgt before evaluation of the mutants could continue. A chick skeletal muscle assay, which is performed in smaller volumes (see Materials and Methods), facilitated these studies by requiring significantly less recombinant toxin for complete analyses.

Chick Skeletal Muscle Assays. The results with this assay indicate that κ-bgt does have affinity for the muscle receptor but that it is 1–2 orders of magnitude lower than previously reported. This is illustrated directly in Figure 3 in which are shown the muscle receptor dose–response curves corresponding to α-bgt (IC_{50} = 0.7 nM), venom-derived κ-bgt (IC_{50} = 150 nM), and recombinant κ-bgt (IC_{50} = 10 μM). The dose–response curve for venom-derived κ-bgt (IC_{50} = 150 nM) determined here is not consistent with that produced using the QT-6-expressed muscle receptor (IC_{50} = 430 nM, Figure 1) nor with that reported previously for *Torpedo* receptor (IC_{50} = 1 μM) (Loring et al., 1986). These observations, however, are consistent with contamination of the venom-derived κ-bgt by α-bgt. This possibility is supported by the fact that these two toxins are found in the same venom where α-bgt represents 15% of dry venom weight and κ-bgt represents less than 0.5%. Moreover, they are highly homologous and might copurify to some degree. This type of cross-contamination has been previously reported and led, in fact, to the initial identification of κ-bgt as a contaminant in some commercially available lots of α-bgt (Chiappinelli & Zigmond, 1978; Chiappinelli et al., 1981; Chiappinelli, 1983; Loring et al., 1984). Referring to Figure 2, if we assume a contamination level of just 0.2%, then the concentration of α-bgt that produced a 50% block of the muscle receptor would be 0.86 nM. This value is consistent with the known K_d of α-bgt at the muscle receptor (0.5–1 nM) and would be beyond the ability of most commonly used chemical and physical techniques to detect. Furthermore, a comparison of the affinities reported for the binding of κ-bgt to the muscle receptor from other tissues also shows a range of values. While the variability in these affinities might be a consequence of the identity of the receptors used or differences in assay conditions, it might also result from different levels of α-bgt contamination in the κ-bgt preparations used. This evidence strongly suggests that the commercially available venom-derived κ-bgt used in this and other studies might have been contaminated by pharmacologically significant amounts of α-bgt. In order to confirm this hypothesis directly, venom-derived κ-bgt that is shown by binding assays

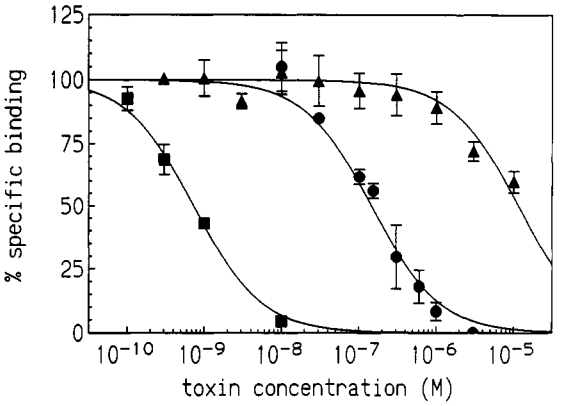

FIGURE 3: Inhibition of ^{125}I-α-bgt binding to chick skeletal muscle by recombinant κ-bgt, venom-derived κ-bgt, and α-bgt. Recombinant κ-bgt, ▲; venom-derived κ-bgt, ●; α-bgt, ■. The data are expressed as means ± SEM at each point (n = 3–4).

Table 2: IC_{50} and K_d Values for the Binding of α-Bungarotoxin, Venom-Derived κ-Bungarotoxin, Recombinant κ-Bungarotoxin, and Recombinant Mutants to Chick Ciliary Ganglion and Chick Skeletal Muscle nAChRs

	neuronal nAChR (α_3-containing)	muscle nAChR [$(\alpha_1)_2\beta\gamma\delta$]		
toxin	IC_{50} (μM)	IC_{50} (μM)	log K_d ± SD	K_d (μM)
α-bgt	>10	0.0007	−9.2	0.0006
vd-κ-bgt	0.1	0.15	−6.8	0.16
r-κ-bgt	0.12	11.4	−5.0 ± 0.19	10
r-κ-bgt Q-32-W	0.05	1.4	−5.64 ± 0.226	2.3
r-κ-bgt R-40-A	>10	>10	–[a]	–[a]
r-κ-bgt P-42-K	1.6	>10	–[b]	–[b]
r-κ-bgt P-42-A	0.05	10	−4.22 ± 0.385	60

[a] No binding was observed at 10 μM. [b] No binding was observed at 3 μM.

to have significant muscle receptor blocking activity must be further purified and separated into active and inactive fractions whose distinct identities could then be confirmed. Unfortunately, the single commercial source of κ-bgt stopped supplying this product several years ago.

Four κ-bgt mutants were also assayed in chick skeletal muscle at concentrations ranging from 1 pM to 10 μM. The R-40-A and P-42-K mutants showed no ability to block the binding of ^{125}I-α-bgt to the muscle receptor at concentrations of 10 and 3 μM, respectively, as compared to a 50% block produced by the nonmutant at 10 μM (Table 2). Since the arginine at position 40 is invariant among all α- and κ-neurotoxins and has been implicated by several studies in receptor binding, it is not surprising that its removal from the toxin greatly reduces receptor affinity. These results are identical to those obtained for this mutant in the chick ciliary ganglion assay in which the R-40-A mutant had no detectable affinity for the neuronal receptor (Fiordalisi et al., 1994). Together, these results clearly indicate a general role for Arg-40 in the interaction of both α- and κ-neurotoxins to the α_1- and α_3-containing nAChRs, respectively, and in the cross-reactivity of κ-bgt with the α_1-containing receptor. It has been suggested that this residue mimics the quarternary ammonium group of the acetylcholine molecule, binding to the receptor in the same location.

Interpretation of the data for the P-42-K mutant is less clear. This position is always occupied by a prolyl residue in κ-neurotoxins, and it was thought that the introduction into κ-bgt of a residue found at this position in most α-neurotoxins might give this mutant greater affinity for the muscle receptor

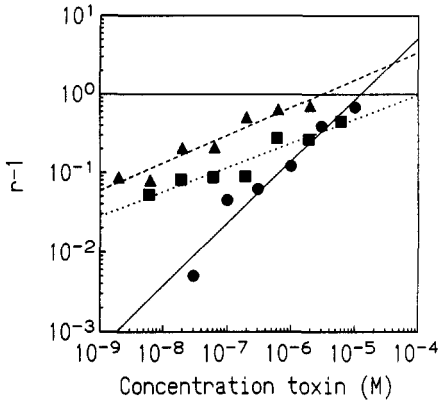

FIGURE 4: Direct determination of dissociation constants for recombinant native κ-bgt, ●; Q-32-W, ▲; and P-42-A, ■, at the chick skeletal muscle nAChR.

than the nonmutant κ-bgt. Instead, a lysine at this position prevents the binding of κ-bgt to the muscle receptor at concentrations as high as 3 μM. Similar results were obtained for this mutant in the chick ciliary ganglion assay, in which P-42-K showed a significantly decreased ability to block nicotinic synaptic transmission (Fiordalisi et al., 1994). The natural presence of a small, uncharged side chain, such as that of proline, at position 42 in all κ-neurotoxins suggests that the structure of the α_3-containing neuronal receptor is physically unable to tolerate a large side chain, such as that of lysine, and that the inability of P-42-K to block the neuronal receptor is probably due to steric or charge interference by the lysine side chain. Since this residue is normally present in the α-neurotoxins, the muscle receptor must be able to physically accommodate its side chain if it is in the same orientation and position relative to the rest of the toxin molecule. Further, many studies have indicated that Lys-42 plays a significant and active role in the binding of α-neurotoxins to the muscle receptor. Since its presence in κ-bgt is not tolerated by the muscle receptor, it may be concluded that the three-dimensional structures of α-bgt and κ-bgt are sufficiently different at this position that the orientation of the Lys-42 side chain no longer corresponds to that in α-bgt and interferes with binding when placed in κ-bgt.

Three of the recombinant toxins (nonmutant, Q-32-W, and P-42-A) were shown to bind to the neuromuscular nAChR with a range of affinities. An analysis of these data by the method of Colquhoun et al. (1976) is shown in Figure 4. The direct determination of dissociation constants by this method indicates that the affinity of P-42-A for the muscle receptor is lower than that of the native toxin. This is in contrast to the activity of this same mutant at the α_3-containing neuronal nAChR found in the chick ciliary ganglion, for which the P-42-A mutant showed an activity equal to that of the nonmutant toxin at all concentrations tested.

Like the P-42-K mutation, the Q-32-W mutation introduces a residue into κ-bgt that is highly conserved in active α-neurotoxins. As shown in Figure 4, the binding of the Q-32-W mutant suggests that the introduction into κ-bgt of a tryptophan found invariant among active α-neurotoxins is not only tolerated by the muscle receptor, but may actually increase the affinity of the toxin for the muscle receptor. The dissociation constant of the Q-32-W mutant [K_d(Q-32-W) = 2.3 μM] is approaching an order of magnitude lower than that of the nonmutant toxin [K_d(nonmutant) = 10 μM] and supports a role for Trp-32 in the muscle receptor affinity of the α-neurotoxins. Results obtained with this mutant in κ-bgt using the chick ciliary ganglion assay indicated that the Q-32-W substitution did not interfere with toxin activity. The replacement of Trp-32 with either a histidine residue or a phenylalanine residue has been shown to reduce the affinity of erabutoxin *a* (an α-neurotoxin) for the muscle receptor by 1–2 orders of magnitude (Pillet et al., 1993). Tryptophan has the largest side-chain group of the 20 common amino acids and yet it is accommodated by the structures of both receptor subtypes. This is not surprising in the muscle receptor considering the perfectly conserved nature of Trp-32 in the high affinity α-neurotoxins. However, the ability of this mutant to bind with full affinity to the neuronal receptor indicates that a non-native tryptophan can be physically accommodated by this receptor. The difference between the receptor-binding characteristics of the P-42-K and Q-32-W mutants may have less to do with the particular substitutions made than with potential differences in the conformations of α- and κ-bgts themselves at these positions. Conformational differences may orient Lys-42 differently in each toxin, preventing the same receptors from binding both.

The interpretation of the muscle receptor assay data is based on the fact that the recombinant κ-neurotoxins produced for this work are properly folded and accurately reflect the native affinity of κ-bgt for the muscle receptor. Conclusive evidence that the recombinant toxins used in these studies had attained their native conformations was the demonstration that three of the five tested (nonmutant κ-bgt, Q-32-W, and P-42-A) displayed an ability to block the neuronal nAChR in the chick ciliary ganglion with an affinity indistinguishable from that of venom-derived κ-bgt and that the CD profiles of all five recombinant molecules were essentially the same as that of native κ-bgt (Fiordalisi et al., 1994). Moreover, analytical ultracentrifugation confirmed that all formed dimers in solution, consistent with properly folded κ-bgt (Fiordalisi, 1994).

By comparing the abilities of venom-derived and recombinant κ-bgt to inhibit the binding of ^{125}I-α-bgt to the muscle nAChR, we have shown that the affinity of κ-bgt for the muscle nAChR is lower than previously believed. The evidence presented here strongly supports the hypothesis that the presence of low levels of α-bgt in the commercially available, venom-derived κ-bgt samples used in this and earlier studies may be responsible for the observed muscle receptor block. However, the data confirm that κ-bgt does have some affinity for the muscle receptor and that this affinity is affected by certain mutations. The data confirm the importance to receptor binding of residues highly conserved in either the α-neurotoxins (Trp-32), the κ-neurotoxins (Pro-42), or both (Arg-40) and suggest that structural differences between the toxin families may play a role in their respective receptor specificities. These results, along with those previously reported for activity at the chick ciliary ganglion receptor (Fiordalisi et al., 1994), indicate that the basis for receptor specificity is likely to be more complex than the presence or absence of just a few conserved residues. Possible determinants which are under investigation include the effect of the overall polypeptide fold on side-chain orientation, the cumulative effect of multiple residues, and the dimerization of the κ-neurotoxins.

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REFERENCES

Agard, D. A., & Stroud, R. M. (1982) *Acta Crystallogr. A38*, 186–194.

Basus, V. J., & Scheek, R. M. (1988) *Biochemistry 27*, 2772–2775.

Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry 27*, 2763–2771.

Betzel, C., Lange, G., Pal, G. P., Wilson, K. S., Maelicke, A., & Saenger, W. (1991) *J. Biol. Chem. 266*, 21530–21536.

Boyot, P., Pillet, L., Ducancel, F., Boulain, J., Tremeau, O., & Menez, A. (1990) *FEBS Lett. 266*, 87–90.

Chiappinelli, V. A. (1983) *Brain Res. 277*, 9–21.

Chiappinelli, V. A. (1992) *Recent Advances in Toxinology Research* (Gopalakrishnakone, P, & Tan, C. K., Eds.) pp 103–120, National University of Singapore, Singapore.

Chiappinelli, V. A., & Zigmond, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2999–3003.

Chiappinelli, V. A., & Lee, J. C. (1985) *J. Biol. Chem. 260*, 6182–6186.

Chiappinelli, V. A., & Wolf, K. M. (1989) *Biochemistry 28*, 8543–8547.

Chiappinelli, V. A., Cohen, J. B., & Zigmond, R. E. (1981) *Brain Res. 211*, 107–126.

Chiappinelli, V. A., Wolf, K. M., Grant, G., & Chen, S. (1990) *Brain Res. 309*, 237–248.

Clarke, P. B. S. (1992) *Trends Pharmacol. Sci. 13*, 407–413.

Colquhoun, D., & Rang, H. P. (1976) *Mol. Pharmacol. 12*, 519–535.

Corfield, P. W. R., Lee, T., & Low, B. W. (1989) *J. Biol. Chem. 264*, 9239–9242.

Dewan, J. C., Grant, G. A., & Sacchettini, J. C. (1994) *Biochemistry* (in press).

Ducancel, F., Boulain, J.-C., Tremeau, O., & Menez, A. (1989) *Protein Eng. 3*, 139–143.

Endo, T., & Tamiya, N. (1991) *Snake Toxins* (Harvey, A. L., Eds.) pp 165–222, Pergamon Press, New York.

Endo, T., Inagaki, F., Hayashi, K., & Miyazawa, T. (1981) *Eur. J. Biochem. 120*, 117–124.

Fiordalisi, J. J. (1994) Doctoral Dissertation, Washington University School of Medicine, St. Louis, MO.

Fiordalisi, J. J., & Grant, G. A. (1994) *Techniques in Protein Chemistry V* (Crabb, J., Ed.) pp 269–274, Academic Press, San Diego.

Fiordalisi, J. J., Fetter, C. H., TenHarmsel, A., Gigowski, R., Chiappinelli, V. A., & Grant, G. A. (1991) *Biochemistry 30*, 10337–10343.

Fiordalisi, J. J., Gigowski, R. L., & Grant, G. A. (1992) *Protein Expression Purif. 2*, 282–289.

Fiordalisi, J. J., Al-Rabiee, R., Chiappinelli, V. A., & Grant, G. A. (1994) *Biochemistry 33*, 3872–3877.

Hider, R. C., Drake, A. F., Inagaki, F., Williams, R. J. P., Endo, T., & Miyazawa, T. (1982) *J. Mol. Biol. 158*, 275–291.

Inagaki, F., Tamiya, N., Miyazawa, T., & Williams, R. J. P. (1981) *FEBS Lett. 118*, 621–625.

Inagaki, F., Hider, R. C., Hodges, S. J., & Drake, A. F. (1985) *J. Mol.Biol. 183*, 575–590.

Loring, R. H., Chiappinelli, V. A., Zigmond, R. E., & Cohen, J. B. (1984) *Neuroscience 11*, 989–999.

Loring, R. H., Andrews, D., Lane, W., & Zigmond, R. E. (1986) *Brain Res. 385*, 30–37.

Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37–46.

Low, B. W., & Corfield, W. R. (1986) *Eur. J. Biochem. 161*, 579–587.

Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D., & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2991–2994.

Oswald, R. E., Sutcliffe, M. J., Bamberger, M., Loring, R. H., Braswell, E., & Dobson, C. M. (1991) *Biochemistry 30*, 4901–4909.

Phillips, W. D., Kopta, C., Blount, P., Gardner, P. D., Steinbach, J. H., & Merlie, J. P. (1991) *Science 251*, 568–570.

Pillet, L., Tremeau, O., Ducancel, F., Drevet, P., Zinn-Justin, S., Pinkasfeld, S., Boulain, J.-C., & Menez, A. (1993) *J. Biol. Chem. 268*, 909–916.

Rosenthal, J. A., Hsu, S. H., Schneider, D., Gentile, L. N., Messier, N. J., Vaslet, C. A., & Hawrot, E. (1994) *J. Biol. Chem. 269*, 11178–11185.

Sacchettini, J. C., Patel, S., Scapin, G., Fiordalisi, J. J., & Grant, G. A. (1992) *J. Mol. Biol. 226*, 559–562.

Sutcliffe, M. J., Dobson, C. M., & Oswald, R. E. (1992) *Biochemistry 31*, 2962–2970.

Vijayaraghavan, S., Pugh, P. C., Zhang, Z., Rathouz, M. M., & Berg, D. (1992) *Neuron 8*, 353–362.

Walkinshaw, M. D., Saenger, W., & Maelicke, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2400–2404.

Wolf, K. M., Ciarleglio, A., & Chiappinelli, V. A. (1988) *Brain Res. 439*, 249–258.

Yu, C., Lee, C., Chuang, L., Shei, Y., & Wang, C. Y. (1990) *Eur. J. Biochem. 193*, 789–799.

Zinn-Justin, S., Roumestand, C., Gilquin, B., Bontems, F., Menez, A., & Toma, F. (1992) *Biochemistry 31*, 11335–11347.