Identification of Residues at the α and ε Subunit Interfaces Mediating Species Selectivity of Waglerin-1 for Nicotinic Acetylcholine Receptors*

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Waglerin-1 (Wtx-1) is a 22-amino acid peptide that is a competitive antagonist of the muscle nicotinic receptor (nAChR). We find that Wtx-1 binds 2100-fold more tightly to the α-ε than to the α-δ binding site interface of the mouse nAChR. Moreover, Wtx-1 binds 100-fold more tightly to the α-ε interface from mouse nAChR than that from rat or human sources. Site-directed mutagenesis of residues differing in the extracellular domains of rat and mouse ε subunits indicates that residues 59 and 115 mediate the species difference in Wtx-1 affinity. Mutation of residues 59 (Asp in mouse, Glu in rat ε) and 115 (Tyr in mouse, Ser in rat ε) converts Wtx-1 affinity for the α-ε interface of one species to that of the other species. Studies of different mutations at position 59 indicate both steric and electrostatic contributions to Wtx-1 affinity, whereas at position 115, both aromatic and polar groups contribute to affinity. The human nAChR also has lower affinity for Wtx-1 than mouse nAChR, but unlike rat nAChR, residues in both α and ε subunits mediate the affinity difference. In human nAChR, polar residues (Ser-187 and Thr-189) confer low affinity, whereas in mouse nAChR aromatic residues (Trp-187 and Phe-189) confer high affinity. The overall results show that non-conserved residues at the nAChR binding site, although not crucial for activation by ACh, govern the potency of neuromuscular toxins.

The muscle nicotinic receptor (1–3) contains five polypeptide subunits arranged with radial symmetry around a central pore. Two copies of α and one each of β, δ, and γ (in the embryonic receptor form) or ε (in the adult form) (4) are arranged around the central pore in the counterclockwise order: α-γ/ε-α-δ-β as established from the crystal structure determination of the acetylcholine binding protein from the freshwater snail Lymnaea stagnalis (5, 6). Each of the five subunits contains between 445 and 497 amino acids with residues 1 through ~210 forming the extracellular ligand binding domain. Each receptor subunit has up to three N-linked glycosylation sites and four transmembrane spans, giving the pentamer a molecular mass of nearly 300 kDa with 20 membrane spans. The binding sites for agonists and competitive antagonists are found at interfaces of the α-δ and α-ε (or α-γ) subunits of the receptor. Full activation of the nAChR requires simultaneous binding of two agonist molecules, but antagonists block activation by occupying only one of the two sites.

Snakes of the Elapidae and Hydrophidae families are notorious for producing toxins that target nicotinic receptors (7). These small proteins of 57–80 amino acids are commonly called “3-fingered” snake toxins for their characteristic three loop topology, with each of the three fingers extending from a core “knuckle” region consisting of four conserved disulfide bonds. Three-fingered toxins such as α-bungarotoxin have been used to probe the nAChR for over 30 years (8). On the other hand, the Viperidae family of snakes does not make 3-fingered toxins and were generally believed not to confer toxicity by targeting nicotinic receptors. Wagler’s pit viper, Tropidolaemus wagleri, is unique among Viperids for morphological reasons as well as for the unique components of its venom. Four related peptides, whose sequence differences are boldfaced, have been isolated from the venom of this species, all of which cause paralysis by neuromuscular blockade (9–12) and are selective for the adult form of the receptor (13).

Wtx-1: NH₂–GGKPDLRPCHPPCHYIPKPR–COOH
Wtx-2: NH₂–GGKPDLRPCYPPCHYIPKPR–COOH
Wtx-3: NH₂–SLGGKPDLRPCCHYPCHYIPKPR–COOH
Wtx-4: NH₂–SLGGKPDLRPCYPPCHYIPKPR–COOH

Structure 1

The solved NMR structures for Waglerin-1 (14, 15) show there is a single intramolecular disulfide between the two cysteines. The present work was inspired by observations of C. Y. Lee and colleagues who nearly forty years ago showed that α-bungarotoxin irreversibly blocks neuromuscular transmission (16). Their more recent studies indicate a remarkable difference in waglerin toxicity between mice and rats (17). Mice were paralyzed by a 0.5 μg intravenous injection in as little as 5 min; rats were completely resistant to 20 times the mouse lethal dose. A similar species selectivity was noted in isolated phrenic nerve-hemidiaphragm preparations, with over a 40-fold difference in sensitivity (17). Waglerin-1 also has no effect when

1 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; 125I-α-BgTx, 125I-α-bungarotoxin; Wtx-1, Waglerin-1; HEK, human embryonic kidney; AChBP, acetylcholine binding protein.
applied to a chicken biventer cervicis muscle-nerve preparation (18). For obvious reasons, similar toxicity data are not available for human.

Here we show that sequence differences between mouse, rat, and human nAChRs account for the specificity of Wtx-1. The identified residues are located in regions of the three-dimensional structure known to contribute to the nAChR binding site. Because the Wtx-1 binding profile for each species can be reconstructed by mutating only these key residues, the species selectivity is not likely due to global structural changes. Rather, the key residues likely interact directly with Wtx-1 or affect its bound orientation. The overall results show how non-conserved residues at the nAChR binding site interface govern species specificity of competitive antagonists.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Waglerin-1—The crude peptides, synthesized by the American Peptide Company (San Jose, CA) or Synpep (Dublin, CA) were dissolved to 0.8 mg/ml in 30 mM Tris-HCl, pH 8.2–8.5, sterile-filtered and left overnight at room temperature to form the single intramolecular disulfide. After disulfide bond cyclization, 0.1% trifluoroacetic acid was added to the solution to stop cyclization and prevent the formation of intermolecular disulfides. A 2-ml injection of the peptide solution was loaded onto a 5-ml high performance liquid chromatography sample loop and purified on a 10-250-mm semi-preparative C18 column (Vydac) and eluted using a 0.1% trifluoroacetic acid solution to stop cyclization and 0.1% trifluoroacetic acid70% acetonitrile solvent system changing at 1% for Solvent B every 3 min. Cyclized peptide elutes from the column 1–2 min earlier in the gradient than uncyclized peptides or dimerized peptides formed by intermolecular disulfide formation. Fractions containing the purified peptide were pooled, frozen, and lyophilized. Representative samples from different lots were checked for purity and correct mass by matrix-assisted laser desorption or ion-spray mass spectrometry.

Mutagenesis of nAChR Subunits—Cloned cDNAs for mouse α (19), β (20), γ (21), δ (22), and ε (24); rat α, β, δ, ε, and γ (gifts from A. Engel) were ligated into the mammalian expression vector pRcRc/HisB (Invitrogen or Qiagen) or by cesium chloride ultracentrifugation. The wild-type ε subunits were cDNA from different lots were checked for purity and correct mass by matrix-assisted laser desorption or ion-spray mass spectrometry.

Results

Species Specificity of Waglerin and the Effect of Replacing the ε Subunit—We measured Wtx-1 binding by competition against the initial rate of 125I-α-BgtTx binding to intact HEK cells transfected with cDNAs encoding adult nAChR from mouse or rat. Wtx-1 binds to mouse nAChR with two distinct dissociation constants differing by 2100-fold, whereas it binds to rat nAChR with dissociation constants differing by only 80-fold (Fig. 1). Because full activation of the nAChR requires simultaneous binding of two agonist molecules, and antagonists block activation by occupying only one of the two sites, the very different dissociation constants at one site underlie the species specificity of Wtx-1 in vivo.

To determine which nAChR subunits mediate Wtx-1 selectivity between mouse and rat nAChRs, we substituted the...
Delineation of Residues on the ε Subunit Governing Interspecies Affinity Differences—Of the 218 amino acids forming the extracellular domain responsible for ligand recognition, only 10 differ between mouse and rat ε subunits. To determine which of the 10 residues mediates species specificity, we constructed chimeric ε subunits from rat and mouse using a common AIII restriction site near the codon for amino acid 83. The isolated restriction fragments were ligated to make chimeric subunits, designated εαεεr and εεεεs, roughly bisecting the extracellular domain. When combined with mouse α, β, and δ subunits, each chimera produced Wtx-1 affinities intermediate between those for wild-type mouse and rat (Fig. 2). The intermediate affinities indicate at least two determinants of Wtx-1 selectivity, one N-terminal and one C-terminal to the junction at position 83. Of the four N-terminal residue differences, only positions 59 and 61 are near the ligand recognition site (1), whereas positions 67 and 76 are in or near the main immunogenic region removed from the binding site (30). Of the six C-terminal residues, positions 168 and 115 are near the ligand recognition site. Candidate residues C-terminal to 115 account for selectivity between mouse and rat nAChRs. Consequently, combining the rat double mutant εεεεs with the mouse ε subunit at positions 59 and 115 suggests that residues at positions 59 and 115 mediate the interaction at residue 59. The intermediate affinities suggest that residues at positions 59 and 115 account for selectivity between mouse and rat nAChRs.

To determine which residues mediate species selectivity, we worked with the mouse ε subunit and mutant all four N-terminal residue differences and one C-terminal difference. Of the four N-terminal mutations, only εD59E reduced affinity to that observed for the εαεεεr chimera (Fig. 3 and Table I). In the C-terminal region, εY115S reduced affinity to that observed for the εεεεs chimera (Fig. 3). These intermediate Wtx-1 affinities suggest that residues at positions 59 and 115 account for selectivity between mouse and rat nAChRs.

To determine whether residues at positions 59 and 115 together confer Wtx-1 selectivity, we constructed the corresponding double mutations in mouse and rat ε subunits. The mouse double mutant εD59E/Y115S reduces Wtx-1 affinity to within 2-fold of the wild-type rat ε subunit. Conversely, the rat double mutant εE59D/S115Y increases Wtx-1 affinity to within 2-fold of the wild-type mouse ε subunit (Fig. 3 and Table I). Thus species selectivity of Wtx-1 can be closely mimicked by substituting residues at positions 59 and 115 of the ε subunit.

Once the primary determinants of Waglerin affinity were identified, we characterized how each receptor determinant interacts with Wtx-1. Working with the mouse ε subunit, we mutated Asp-59 to Ala (the corresponding residue in mouse δ), Asn (to remove the negative charge), and Gln (the corresponding residue of mouse γ). The resulting rank order of affinity for mouse ε 59 residues is Asp = Ala > Asn ≥ Gln > Glu (Table I). Removing the charge does not in itself significantly decrease affinity (no change, 3-fold, or 5-fold for the Ala, Asn, and Gln substitutions, respectively). Changing the Asp to a Glu, which increases the length of the side chain by one methylene group, causes the largest decrease. These observations indicate that the interaction at residue 59 is not purely electrostatic, but instead is mediated by a combination of electrostatic and other stabilizing forces within a confined space. Steric occlusion incurred by addition of a single methylene in the side chain may alter the position of the charge or the orientation of bound Waglerin. In the rat ε mutants tested, the affinities followed a similar rank order, Asp > Asn > Gln > Glu (Table I), further showing that both electrostatic forces and steric constraints mediate the interaction at residue 59.

Position 115 of the ε subunit of both rat and mouse nAChR was mutated to Phe and Ser to determine the role of the aromatic ring in governing affinity for Wtx-1. For both rat and mouse ε subunits, the rank order of Wtx-1 affinity was Tyr > Phe > Ser at position 115, indicating that both the aromatic ring and the hydroxyl group contribute to high affinity binding of Wtx-1 at the α-ε interface.

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2 B. E. Molles, unpublished observations.
Determinants of Waglerin Affinity in the Human Receptor—We next compared Wtx-1 binding to human nAChR with that for mouse nAChR. Wtx-1 binds to human nAChR with two distinct affinities, but compared with mouse nAChR, it shows 70-fold lower affinity at the α-ε site and 10-fold lower affinity at the α-δ site (Fig. 4). To determine whether the ε subunit contributes to the low affinity of human nAChR, we coexpressed mouse α, β, and δ subunits with human ε. The resulting hybrid nAChRs bind Wtx-1 with 14-fold lower affinity at the α-ε site compared with the all mouse reference, indicating that some of the lower affinity for human nAChR originates in the ε subunit. Although no human ε mutants were tested, the human ε, like rat, has a Ser at residue 115, a residue shown to mediate reduced affinity of rat ε. On the other hand, human ε contains Asp at position 59, like mouse, which promotes high affinity for the nAChR. Thus, Ser-115 in human ε should be a primary determinant for the human- mouse difference in affinity.

To determine whether the α subunit contributes to low affinity of human nAChR, we cotransfected human α with mouse β, ε, and δ subunits. The resulting hybrid nAChRs bind Wtx-1 with 10-fold lower affinity at both binding sites compared with the all mouse reference, indicating that the α subunit is also responsible for the low affinity of human nAChR. We therefore constructed chimeric α subunits composed of mouse and human sequences to identify residues responsible for the mouse/human affinity difference. Residues 1–124 of the mouse α subunit were joined with residues 125–496 of human α, and residues 1–172 of human were joined to 173–496 of the mouse α subunit. When mouse sequence occupies positions 1–124 of the α subunit, Wtx-1 binding coincides with that of the wild-type human α subunit (Fig. 4B), indicating that residues C-terminal to residue 124 affect affinity. On the other hand, when human sequence occupies positions 1–172, Wtx-1 binding coincides with that of the all mouse reference, indicating that residues C-terminal to position 172 affect affinity. The mouse-human α subunit chimeras, therefore, indicate that the determinants of the affinity difference are C-terminal to position 172 in the α subunit.

Between position 172 and the first transmembrane span at position 210, four residues differ between the mouse and human wild-type α subunits: positions 181, 187, 189, and 195. We therefore generated point mutations at each of these positions in both mouse and human subunits, mutating each residue to that in the other species. Each mutant α subunit was cotransfected with mouse wild-type β, δ, and ε subunits, and Wtx-1 binding was measured. For mutations at positions 181 and 195, virtually no change in affinity is observed in either mouse or human α subunits (Table II). For positions 187 and 189, a more complex interplay of the two residues is evident. The mouse αW187S mutation decreases Wtx-1 affinity only 2-fold at both sites, and the corresponding human mutant (human αS187W) increases affinity 2- and 4-fold (Fig. 5, A and B). At position 189, the mouse mutation (αE189T) reduces affinity to that for human α, but the human mutant (αT189F) increases affinity only 2-fold at each site (Fig. 5, A and B). Changing both residues simultaneously in the human αS187WT189F double mutant gives nearly identical affinities to the wild-type mouse α at both sites. However, the mouse αW187SF189T double mutant decreases affinity 3-fold at each site, less than the αF189T single mutant alone (Table II).

Careful inspection of the data in Fig. 5 reveals that a Wtx-1 determinant in the α subunit also affects the rate of α-BgTx association. As is evident from the plateau in the curves in Fig. 5, nAChRs containing the human α subunit typically have a lower apparent proportion of α-ε binding sites. This most likely results from a disparity between 1251-α-BgTx association rates at the α-ε site compared with the α-δ site, where association is faster at the α-δ site. Our results clearly show that the 187 position is critical to determining this difference in association rates. The presence of Ser at position 187, whether in the wild-type human α, in the mouse αW187S mutant or the mouse αW187SF189T double mutant, leads to a lower apparent ratio of α-ε to α-δ sites. Conversely, the presence of Trp in the wild-type mouse, the human αS187W mutant, or the human αS187WT189F double mutant, leads to a 1:1 ratio of α-ε to α-δ sites (Fig. 5B). Thus the residue at 187 position governs the relative rate of α-BgTx association at the α-ε and α-δ sites.
nAChR binding site structure to agonist and competitive antagonist binding. The subunit interface is designed to bind ACh with low affinity in the resting state and to bind ACh much more tightly when in the open channel and desensitized states. This state dependence of the nAChR binding site implies a very delicate design, which is mediated by conserved residues that do not tolerate mutation. On the other hand, there are numerous non-conserved residues at the binding site that readily tolerate mutation as far as activation by ACh is concerned. However, variations in these residues profoundly affect the potency of a variety of neurotoxins found in nature, including d-tubocurarine, α-conotoxins, α-neurotoxins, and waglerins. Nature, therefore, takes advantage of these benign variations in protein structure in designing neurotoxins.

In the present study, Waglerin-1 was used to explore the nature of the nAChR binding sites by examining differences in affinity found among the adult forms of the nAChR cloned from mouse, rat, and human. Positions 59 and 115 of the ε subunit mediate the nearly 100-fold difference in Waglerin affinity at the αε site between the mouse and rat orthologs of the adult nAChR. These residue positions have been found in previous work to be of primary or secondary importance for the interaction of the nAChR with other ligands. The ϵAsp-59 position is one of two determinants for the higher affinity of dimethyl d-tubocurarine for the αε interface of the receptor (33). This position is proximal to residue Trp-55, which is affinity labeled by d-tubocurarine (34) and nicotine (35) in the Torpedo γ subunit, and is located in Segment E of the nAChR binding site. The ε position 115 is flanked by residue 111, which regulates selectivity of α-conotoxin MI for the αδ over the αγ site (31), and positions 116 and 117, which mediate selectivity of the αγ site for dimethyl d-tubocurarine (26). These residues belong to Segment F of the binding site. εTyr-115 contributes to efficient surface expression of nAChR in COS cells formed from the rat ε subunit (36). The rat ε subunit nAChR expressed in COS cells transfected with nAChR α, β, and δ subunits from either mouse or rat yields a receptor that has a 10-fold lower cell surface expression than receptors made with the mouse ε subunits. In our system, in which HEK293 instead of COS cells are employed, this mutation gave ~5-fold lower expression (data not shown). The influence of this residue in both subunit assembly and ligand specificity supports its location at a subunit interface.

**Interaction of Waglerin with Residues 59 and 115 of the ε Subunit**

Substitution of a series of side chains indicated in both the mouse and rat ε subunits, the smaller side chain at position 59 tended to confer the higher affinity. The largest difference in affinity was observed when either the mouse or rat ε subunit was lengthened from Asp to Glu or vice versa. This suggests that the negative charge at this position is beneficial only if the side chain is of a certain length and can be counterbalanced by steric repulsion. Mutations of both the rat and mouse subunits to residues related to Tyr-115 in mouse reveal a rank order of affinity, Tyr ≈ Phe > Ser. Because a phenyl moiety is of primary importance to the interaction of Wtx-1 with the nAChR, a cation–π interaction between a positively charged moiety of the Waglerin peptide and the aromatic ring of εTyr-115 is a likely stabilizing interaction. The presence of the hydroxyl group can potentially allow for a secondary hydrogen bond with the positively charged group (37). Thus, removal of the hydroxyl group in the mouse εY115F mutant could decrease affinity slightly due to the loss of hydrogen bonding or to enhanced electronegativity from the aromatic hydroxyl moiety.
Interaction of Waglerin-1 with Residues 187 and 189 of the \( \alpha \) Subunit

The present work has additionally identified the residues of mouse- and human-derived \( \alpha \) subunits that mediate a difference in affinity between nAChR of the two species. The human ortholog of the adult nAChR, like the rat, has nearly a 100-fold lower affinity for Waglerin-1 compared with the mouse. However, at least half of the free energy difference results from residues 187 and 189 of the \( \alpha \) subunit rather than the \( \epsilon \) subunit. Positions 187 and 189 are in Segment C of the \( \alpha \) subunit, a region that has been extensively studied by mutagenesis and affinity labeling studies. Residues 187 and 189 are altered in mongoose and cobra to form glycosylation sites at Asn-187 (mongoose) and Asn-189 (cobra), conferring resistance to \( \alpha \)-bungarotoxin in these animals (38).

Analysis of \( \epsilon \), \( \alpha \), \( \delta \), and \( \gamma \) Subunit Residues That Differ between Species

\( \epsilon \) Subunit Variations—The mouse and rat \( \epsilon \) subunits were extensively mutated to determine the source of the 120-fold difference in affinity between the \( \alpha-\epsilon \) sites formed by subunits cloned from the respective species. The human \( \epsilon \) subunit, when cotransfected with mouse \( \alpha, \beta, \delta \) subunits, also produces a 14-lower affinity at the \( \alpha-\epsilon \) interface than the mouse \( \epsilon \) subunit. Of the 28 extracellular domain positions tested, three are in binding site segments (Fig. 5). The mouse \( \epsilon \) has a Tyr at position 115; mutating this position to a Ser (found in both human and rat) reduces affinity by 9-fold, nearly matching the reduced affinity at the \( \alpha-\epsilon \) site found for receptors made from human \( \epsilon \) cotransfected with mouse \( \alpha, \beta, \delta \) subunits. Thus the reduced \( \alpha-\epsilon \) site affinity conferred by the human \( \epsilon \) subunit is probably due to the same residue 115 that is responsible for the mouse-rat difference in affinity.

\( \alpha \) Subunit Variations—Extensive modification of the human and mouse \( \alpha \) subunits revealed residues 187 and 189 affected affinity for Waglerin-1. The \( \alpha \) subunit of the rat receptor has three residues that differ from mouse \( \alpha \). Of these three, only a difference at 195 falls in a known binding site segment (Fig. 6A). This position in the \( \alpha \) subunit was modified in both the mouse (Thr at 195) and human (Asp at 195) forms of the receptor. There was no change in affinity for these two mutants, and it is thus expected that the Asn-195 present in the rat \( \alpha \) would also not change affinity.

\( \delta \) Subunit Species Variations—Mouse and human \( \delta \) subunits differ by 39 residues. Although individual mutations in the \( \delta \) subunit were not examined, all of the reduced affinity observed at the human \( \alpha-\delta \) site can most likely be explained by residues that differ in the \( \alpha \) subunits, not in the \( \delta \) subunits. The \( \alpha-\delta \) site
The human receptor has 10-fold lower affinity for Waglerin-1, as do receptors formed from the human α2βδε subunit cotransfected with mouse α1βδε, indicating that differences in the α2βδε site affinities of mouse and human receptors are purely due to the α2 subunits.

Waglerin-1 also binds to the mouse α2βδε interface binding site with 4-fold higher affinity than the rat α2βδε. There are 13 residues that differ between the mouse and rat α2βδε subunits. A likely candidate for this 4-fold difference in affinity is position 113. The corresponding position in the mouse α2, Tyr-111, mediates a 4-fold reduction in affinity when mutated to Ser (data not shown). Mouse and human α2βδε subunits both have a Tyr at this position, whereas rat has a Ser, suggesting that the 4-fold reduced affinity for the rat α2βδε site is due to the presence of a Ser residue instead of the Tyr.

In the present study, Waglerin-1 is shown to have a nearly 100-fold higher affinity for the mouse αβδε nAChR than both the rat and human αβδε nAChR orthologs. This large difference in affinity is undoubtedly responsible for the observed difference in lethal activity of the peptide when injected into an intact mouse or rat as well as the difference in activities in isolated muscle preparations from the respective species (17).

It is interesting to note that α-conotoxin SI also has a similar species selectivity in that it has significantly lower activities for mammal-derived receptors than it does for receptors from fish (39–41). An analysis of the residues differing between the α-conotoxin and waglerin peptides may indicate which receptor residues mediate preferential selectivity for both peptide families.

Recently, a structure of a soluble acetylcholine binding protein was solved and shown to be homologous to the extracellular domain of the nAChR, with the closest residue identity to the α7 receptor (5). Both of these proteins assemble to form homopentamers. A 7- to 12-residue insertion between positions 166 and 175 (α2 numbering) is found for the α2δε, α2εδ, and α2εε subunits that is not found in the binding protein. However, this region is not a determinant of the species differences seen in Waglerin-1 specificity. Hence, the binding protein template provides a useful framework for describing the spatial relationships of their residues (Fig. 7). As is evident, the involved residues form a cluster well within the binding surface of a 22-amino acid peptide. This information, when combined with other approaches such as γ to ε residue substitutions and mutant cycle analysis, should ultimately yield a description of the interact-

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**Fig. 6. Sequence alignment of mouse, rat, and human α and ε subunits.** Residues diverging in at least one of the sequences are shown in **boldface**. Binding site **Segments A**, B, C, D, E, F, and G as determined from chemical labeling, mutagenesis, and proximity in the crystal structure of the acetylcholine binding protein are **boxed**, with key residue positions **numbered**. **A**, the α187 and α189 positions mediating the difference in affinity for the mouse and rat ε subunits are **highlighted**. Note that the human ε subunit, like the rat, has a Ser at position 115 that mediates lower affinity Waglerin-1 binding in the rat. **Dashed boxes** indicate additional mutated residues. The ε subunit is truncated 10 residues N-terminal to the first transmembrane region.
peptide, contrary to the observations made in mice. Although no published reports document the effects of the *T. wagleri* venom in human beings, anecdotal human envenomation evidence has suggested that human nAChR may be similarly resistant to the toxin (43). Our binding assays performed on the human form of the receptor provide a molecular mechanism to explain these anecdotes.

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**REFERENCES**

1. Corringer, P. J., Le Novère, N., and Changeux, J. P. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 431–458
2. Arias, H. R. (2000) Neurochem. Int. 36, 595–645
3. Katin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
4. Reynolds, J. A., and Karlin, A. (1978) Biochemistry 17, 2035–2038
5. Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van Der Oost, J., Smith, A. B., and Siama, T. K. (2001) Nature 411, 269–276
6. Smit, A. B., Syed, N. I., Schaap, D., van Minnen, J., Klumperman, J., Kitis, K. S., Lodder, H., van der Schors, R. C., van Elk, R., Sorgeredrager, B., Brejc, K., Sixma, T. K., and Geraerts, W. P. (2001) Nature 411, 261–268
7. Endo, T., and Tamiya, N. (1993) in Soothe Toxins (Harvey, A. L., ed) pp. 165–222, Pergamon Press, New York
8. Changeux, J. P., Kasai, M., and Lee, C. Y. (1970) Proc. Natl. Acad. Sci. U. S. A. 67, 1241–1247
9. Aiken, S. P., Sellin, L. C., Schmidt, J. J., Weinstein, S. A., and McArdle, J. J. (1992) Pharmcol. Toxicol. 70, 459–462
10. Weinstein, S. A., Schmidt, J. J., Bernheimer, A. W., and Smith, L. A. (1991) Toxicol 39, 227–236
11. Schmidt, J. J., Weinstein, S. A., and Smith, L. A. (1992) Toxicol 30, 1027–1036
12. Schmidt, J. J., and Weinstein, S. A. (1995) Toxicol 33, 1043–1049
13. McRill, J. L., Lenz, T. L., Witzemann, V., Schwarz, H., Weinstein, S. A., and Schmidt, J. J. (1999) J. Pharmacol. Exp. Ther. 289, 543–550
14. Sellin, L. C., Mattila, K., Annila, A., Schmidt, J. J., McArdle, J. J., Hyvonen, M., Rantala, T. T., and Kivistö, T. (1996) Biophys. J. 70, 3–13
15. Chuang, L. C., Yu, H. M., Chen, C., Huang, T. H., Wu, S. H., and Wang, K. T. (1996) Biochim. Biophys. Acta 1292, 145–155
16. Chang, C. C., and Lee, C. Y. (1983) Arch. Int. Pharmacodynam. 144, 241–257
17. Lin, W. W., Smith, L. A., and Lee, C. Y. (1995) Toxicol 35, 111–114
18. Tan, N. H., and Tan, C. S. (1989) Toxicol 27, 349–357
19. Isenberg, K. E., Mudd, J., Shah, V., and Merlie, J. P. (1986) Nucleic Acids Res. 14, 5111
20. Buonanno, A., Mudd, J., and Merlie, J. P. (1989) J. Biol. Chem. 264, 7611–7616
21. Yu, L., LaPolla, R. J., and Davidson, N. (1986) Nucleic Acids Res. 14, 5559–5565
22. LaPolla, R. J., Mayne, K. M., and Davidson, N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7970–7974
23. Gardner, P. D. (1990) Nucleic Acids Res. 18, 6714
24. Wittneben, V., Stein, E., Sugiyama, N., Ristenpart, T., Koenen, M., Kues, W., Criedo, M., Hofmann, M., and Sakmann, B. (1996) Eur. J. Biochem. 244, 437–448
25. Schoepfer, R., Luther, M., and Lindstrom, J. (1988) FEBS Lett. 236, 235–240
26. Sine, S. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 9436–9440
27. Lee, B. S., Gunn, R. B., and Keptis, R. R. (1991) J. Biol. Chem. 266, 11448–11454
28. Sine, S., and Taylor, P. (1979) J. Biol. Chem. 254, 3315–3325
29. Taylor, P., Osaka, H., Molles, B. E., Sugiyama, N., Marchet, P., Ackermann, E. J., Malany, S., McArdle, J. J., Sine, S. M., and Tsigelny, I. (1998) J. Physiol. (Paris) 92, 79–83
30. Tzartos, S. J., Cung, M. T., Demange, P., Loutrari, H., Mamalaki, A., Marraud, M., Papadouli, I., Sakarellos, C., and Tsikaris, V. (1991) Mol. Neurobiol. 5, 1–29
31. Sine, S. M., Kreienkamp, H. J., Bren, N., Maeda, R., and Taylor, P. (1995) Neuron 15, 205–211
32. Osaka, H., Malany, S., Kanter, J. R., Sine, S. M., and Taylor, P. (1999) J. Biol. Chem. 274, 9581–9586
33. Bren, N., and Sine, S. M. (1997) J. Biol. Chem. 272, 30793–30798
34. Chiara, D. C., and Cohen, J. B. (1997) J. Biol. Chem. 272, 32940–32950
35. Chiara, D. C., Middelton, R. E., and Cohen, J. B. (1998) FEBS Lett. 423, 223–226
36. Gu, Y., Camacho, P., Gardner, P., and Hall, Z. W. (1991) Neuron 6, 879–887
37. Gallivan, J. P., and Dougherty, D. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9439–9440
38. Kreienkamp, H. J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994) J. Biol. Chem. 269, 8108–8114
39. Zafaralla, G. C., Ramlo, C., Gray, W. R., Karlstrom, R., Olivera, B. M., and Cruz, L. J. (1994) Biochemistry 27, 7102–7105
40. Groebe, D. R., and Abramson, S. N. (1995) J. Biol. Chem. 270, 281–286
41. Groebe, D. R., Gray, W. R., and Abramson, S. N. (1997) Biochemistry 36, 6469–6474
42. Barcham, D., Odavia, M., Kechva, E., and Fuchs, S. (1995) Biochemistry 34, 9172–9176
43. Reid, H., Thean, P., and Martin, W. (1963) Br. Med. J. I, 992

![Fig. 7. Location of residues determining the species differences in Waglerin-1 affinity modeled on the crystal structure of the acetylcholine binding protein (AChBP). Residues αTyr-187, αPhe-189, αAsp-59, and εTyr-115 are shown in their aligned positions of the AChBP crystal structure (5), a protein homologous to the extracellular domain of the nAChR. Coordinates for Waglerin-1 are adapted from averaged NMR structures (15). The colors for Waglerin are: green, α-carbon chain ribbon; red, oxygen; yellow, disulfide bond; blue, nitrogen. The interface of two subunits of the AChBP is shown viewed from its outer curvature. The connecting sequence to the first transmembrane span is located at the bottom of the figure. Red denotes α-helical and turquoise β-sheeted sheet structures. The figure demonstrates that the receptor residues are within a distance that can be spanned by the 22-amino acid Waglerin-1 peptide.](image-url)