The Role of Exposed Tryptophan Residues in the Activity of the Cardiotonic Polypeptide Anthopleurin B*

Belinda L. Dias-Kadambi, Kelly A. Combs‡, Chester L. Drum§, Dorothy A. Hanck¶, and Kenneth M. Blumenthal‡

From the ‡Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267–0524 and ¶Departments of Medicine and of the Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

Scorpion and sea anemone venoms contain several polypeptides that delay inactivation of voltage-sensitive sodium channels via interaction with a common site. In this report, we target exposed hydrophobic residues at positions 33 and 45 of anthopleurin B (ApB) by polymerase chain reaction mutagenesis to ascertain their contribution to toxin activity. Nonconservative replacements are not permitted at position 33, indicating that Trp-33 may play an important structural role. Strikingly, the relatively conservative substitution of Trp-33 by phenylalanine results in major reductions in binding affinity for both the cardiac and neuronal channel isoforms as measured by ion flux, whereas substitution with tyrosine is tolerated and exhibits near wild-type affinities, suggesting that either the ability to form a hydrogen bond or the amphiphilic nature of the side chain are important at this position. Electrophysiological analysis of W33F indicates that its diminished affinity is primarily due to a decreased association rate. Analysis of a panel of mutants at Trp-45 shows only modest changes in apparent binding affinity for both channel isoforms but significant effects on \( V_{\text{max}} \). In neuronal channels, the maximal levels of uptake for W45A/S/F are about 50% those seen with ApB. This effect is also observed for W45A and W45S in the cardiac model, wherein W45F is normal. These results suggest that a hydrophobic contact is involved in toxin-induced stabilization of the open conformation of the cardiac sodium channel. We conclude that Trp-33 contributes significantly to apparent affinity, whereas Trp-45 does not appear to affect binding \emph{per se}. Furthermore, W33F is the first ApB mutant that displays a significantly altered association rate and may prove to be a useful probe of the channel binding site.

Sea anemone venoms contain a diverse array of polypeptide toxins that delay inactivation of voltage-sensitive sodium channels in excitable cells. Of particular interest, because they exhibit potent cardiotonic activity, are anthopleurin A (ApA) and anthopleurin B (ApB), isolated from the anemone \emph{Anthopleura xanthogrammica}. Both ApA and ApB are basic polypeptides with 49 amino acid residues that are cross-linked by three disulfide bonds (Norton et al., 1976; Norton, 1981; Norton, 1991). They are naturally occurring homologs related by seven sequence substitutions and a net charge difference of +2, with ApB being more cationic (Norton, 1981). Ion flux assays reveal that ApB is 18-fold more active than ApA on the neuronal channel, whereas both toxins display similar affinity, in the nanomolar range, for the cardiac isoform. Electrophysiological assays also identify ApA as having greater affinity for cardiac than neuronal channels, whereas ApB has an even higher affinity for cardiac channels than predicted from flux and retains the ability to differentiate between channel isoforms (Khra et al., 1995).

The positive inotropic activities of ApA and ApB are associated with their ability to prolong the conducting state of the cardiac channel resulting in increased sodium influx and, therefore, to an increase in the force of contraction (Norton, 1991). A comparative analysis of the cardiotonic activity of these toxins in rat cardiac cells demonstrates that ApB is more potent than ApA (Renault et al., 1986). The cardiac glycoside digoxin, which inhibits the Na\(^+\),K\(^-\)ATPase, is currently used to treat heart failure in spite of the high levels of toxicity associated with its prolonged use (Doggrell et al., 1994). ApA is more effective than digoxin in augmenting the force of contraction, and its activity in anesthetized dogs is not associated with an increase in heart rate or blood pressure (Scriabine et al., 1979; Gross et al., 1985). Although the usefulness of these natural toxins as substitutes for the currently available therapeutics is limited, they clearly display high sodium channel affinity, resulting in potent cardiotonic activity. Therefore, an understanding of the molecular basis for their interaction with the sodium channel will provide valuable information for the design of novel synthetic drugs having enhanced positive inotropic activity.

In addition to the anemone toxins, \(\alpha\)-scorpion toxins also delay membrane repolarization in excitable cells (Catterall, 1977; Strichartz et al., 1987). The \(\alpha\)-scorpion toxin, \emph{Leiurus quinquestratus} toxin V, and the anemone toxin, \emph{Anemonia sulcata} toxin II, compete for a common binding site on the rat brain sodium channel (Catterall and Beress, 1978) that has been localized to the S5-S6 loop of domains I and IV using site-specific antibodies (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989). It is intriguing that these two unique classes of polypeptides, which exhibit no interclass sequence similarities, bind with high affinity to the sodium channel, delaying its inactivation (Catterall, 1977; Strichartz et al., 1987; Vincent et al., 1980; Frelin et al., 1984).

The three-dimensional structures of homologous scorpion toxins which affect either activation or inactivation of the so-
dium channel are highly related. A common motif documented is an exposed hydrophobic region that includes many conserved residues thought to be essential for activity (Fontecilla-Camps et al., 1982; El Ayeb et al., 1986; Fontecilla-Camps et al., 1988; Darbon et al., 1991). Fontecilla-Camps et al. (1981) proposed that despite the diverse pharmacological activities of the different scorpion toxins, this conserved face might be involved in a direct binding interaction with the sodium channel. Alternatively, this extensive hydrophobic contact could serve to orient other conserved residues that in fact confer the specificity and high affinity of the toxin for the channel (Fontecilla-Camps et al., 1981).

Because of the functional similarity among the anemone and scorpion toxins and the proposed importance of the hydrophobic face of the latter proteins, we set about analyzing the contribution of surface hydrophobic residues of ApB to activity. The solution structures of both ApA and ApB are essentially identical, with a core structure consisting of a four-stranded antiparallel β-sheet connected by three loops, the first of which is the longest and least well defined (Monks et al., 1995; Pallaghy et al., 1995). All three tryptophan residues are highly conserved among anemone toxins that interact with the mammalian sodium channel (Norton, 1991). Although these toxins lack the extended hydrophobic face seen in scorpion toxins, the biophysical evidence that the tryptophan residues of ApA are partially or fully solvent-exposed is quite strong. Proton chemical shifts for Trp-45 of ApA are similar to those observed in small peptides, consistent with this residue being solvent-exposed, whereas the shifts observed for Trp-23 and Trp-33 are indicative of mutual interaction between these side chains (Gooley et al., 1986). The ApA solution structure confirms the close proximity of the aromatic rings of Trp-23 and Trp-33. However, their functional role in ApA is unclear, since complete chemical modification with 2-nitrophenylsulfonyl chloride was reported to yield a product that displayed “full or partial activity” (Newcomb et al., 1980).

Our laboratory has cloned a synthetic gene for ApB and developed a bacterial expression system capable of producing recombinant toxin (Gallagher and Blumenthal, 1992). Site-directed mutagenesis of ApB has identified a cluster of functionally important cationic residues including Arg-12, Arg-14, and Lys-49 (Gallagher and Blumenthal, 1994; Khera and Blumenthal, 1994; Khera et al., 1995). Very recently, we have demonstrated that Leu-18, which is adjacent to this cationic cluster, makes a greater contribution to binding affinity than any of the cationic side chains identified previously (Dias-Kadambi et al., 1996). These studies represent initial advances toward rational drug design based on these cardiotonic polypeptides. In this report, we have taken a similar approach to evaluating the role of Trp-45 and Trp-33 of ApB in binding to either the neuronal or cardiac isoforms of the voltage-sensitive sodium channel. Relatively modest changes in apparent binding affinity for both channel isoforms were detected for each Trp-45 substitution. The W33Y mutant also displayed a similarly moderate reduction in apparent affinity for both channels. In striking contrast, the apparent binding affinity of W33F for the neuronal and cardiac channels was severely compromised (50- and 30-fold, respectively), implicating Trp-33, and primarily its amphiphilic character, in the interaction of ApB with the sodium channel.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Reagents**

Taq DNA polymerase, DNA ligase, and all restriction enzymes were obtained from Life Technologies, Inc. Deoxyribonuclease triphosphates used in the PCR reactions were products of Pharmacia LKB Biotechnology Inc. DNase, RNase, protease inhibitors, ouabain, and veratridine were all purchased from Sigma. Lysozyme and Sequenase were obtained from U. S. Biochemical Corp., and [32P]dATP were purchased from DuPont NEN. Staphylococcal protease (Staphylococcus aureus, strain V8) was from ICN, fetal calf serum from either United Biochemical Inc. or Hyclone Laboratories, and Dulbecco’s modified Eagle’s medium from JRH Biosciences and Mediatech. All other reagents were of the highest quality available and purchased from well established commercial sources.

**Cell Cultures**

Murine neuroblastoma cells (NIE-115) that express the neuronal isoform of the sodium channel were a kind gift from Dr. Marshall Nirenberg (National Heart, Lung, and Blood Institute, National Institutes of Health). The RT4-B cell line (Donahue et al., 1991; Zeng et al., 1995) was generously provided by Dr. Laurie Donahue (Texas Tech University, Health Sciences Center). This rat neurotumor line expresses predominantly the TTX-resistant sodium channel isoform designated SKM2, which is structurally identical to the cardiac channel. Both cell lines were maintained in 90% Dulbecco’s modified Eagle’s medium (JRH Biosciences and Mediatech, respectively) containing 10% fetal calf serum (Hyclone Laboratories and U. S. Biochemical Corp., respectively) and 110 units per ml each of penicillin and streptomycin at 37 °C in a humid atmosphere containing 10% CO₂.

**Plasmodia and Bacterial Strains**

The expression vectors pMG2 and pKB13, which encode the synthetic gene for ApB fused to the 3’ end of the gene for the bacteriophage T7Gene-9 protein under control of the T7 promoter, were used to express high levels of mutant and wild-type proteins (Gallagher and Blumenthal, 1992). pKB13 encodes a pentaglutamate sequence at the 5’ end of the ApB gene, designed to enhance cleavability with staphylococcal protease (Dias-Kadambi et al., 1996). The Escherichia coli strains JM109 and XL1 Blue were used for plasmid propagation, whereas the BL21(DE3) strain was used to express high levels of the fusion protein (Gallagher and Blumenthal, 1992).

**DNA Methods**

All the DNA methodologies, including bacterial transformation, plasmid isolation, and cloning techniques were based on established procedures (Maniatis et al., 1982). PCR experiments were carried out as outlined by Perkin-Elmer with a representative PCR cycle that included an initial melting step (94 °C, 1 min), an annealing step (1 min), followed by polymerization (72 °C, 2 min) and was repeated for 30 cycles. The annealing temperature was adjusted depending on the melting temperatures of the individual primers. Primers typically contained a wobble codon in order to generate a panel of mutants, and the PCR products with these degenerate primers were digested with a restriction enzyme that cleaves on polyacrylamide gels, and cloned into the expression vectors pMG2 or pKB13 (Gallagher and Blumenthal, 1992, Dias-Kadambi et al., 1996). Double-stranded dideoxynucleotide sequencing was performed on all constructs (Sanger et al., 1980), using methodology outlined by the supplier.

**Expression and Isolation of Active Toxin**

Mutant toxins were expressed and purified as described previously (Gallagher and Blumenthal, 1992). Following anion exchange chromatography on DE52, disulfide bonds were oxidized using redox couples of glutathione, and the fusion protein was hydrolyzed with staphylococcal protease to generate intact active toxin. Final purification was achieved using reverse phase high performance liquid chromatography. Liberation of ApB from the pKB13 construct, containing a pentaglutamate sequence, was significantly more efficient than from the plasmids described previously (Dias-Kadambi et al., 1996).

**Analytical Methods**

Amino acid analysis of wild-type and mutant toxins was performed using standard protocols. Proteins were hydrolyzed in vacuo for 22 h at 110 °C, derivatized using phenylisothiocyanate, and analyzed by high performance liquid chromatography on a Pico Tag column. The secondary structures of these proteins were estimated from circular dichroism spectra recorded on a Jasco J-710 spectropolarimeter. Thermal denaturation studies were performed from 20—80 °C in 5 mM phosphate buffer (pH 6.8) containing 1.5 mM guanidine HCl. The spectral data were compared with a least squares fit of a reference spectrum containing the secondary structural data for the known proteins myoglobin, lysozyme, ribonuclease A, papain, cytochrome c, hemoglobin, α-chymotrypsin, trypsin, and horse liver alcohol dehydrogenase.
Role of Exposed Trp Residues in Activity of Anthopleurin B

Functional Characterization of the Mutant Proteins by Ion Flux

The ability of ApB to enhance sodium uptake was tested in two cell lines that express either the neuronal (N1E-115) or cardiac (RT4-B) isoform of the sodium channel. N1E-115 cells were seeded at a density of 15,000 cells per cm² in 24-well tissue culture plates (Linbro) and grown in medium (Dulbecco’s modified Eagle’s medium) containing 10% fetal calf serum for 3 days at 37°C as described above. On day 3, growth medium was replaced with differentiation medium (containing 1.5% fetal calf serum and 1.5% dimethyl sulfoxide), and cultures were assayed on day 4. The RT4-B cells were seeded and grown under the same conditions as used for N1E-115 cells. Since the former cells did not require any differentiation, they were typically assayed on day 4. Sodium uptake experiments were performed in the presence of substrate (20 μM) levels of the poor agonist veratridine as described previously (Schweitz et al., 1981; Gallagher and Blumenthal, 1992).

Absolute uptake values were expressed in terms of nmol per min per mg cell protein, corrected for basal uptake due to veratridine alone. The maximal levels of uptake for the mutants were normalized to those at saturating levels of ApB (500 nM) and 20 μM veratridine. The experimental data shown were obtained from multiple experiments and fitted with a single hyperbolic function as described by Cleland (1979), yielding the kinetic constants Vₘₐₓ and Kᵥₐₚ.

Functional Characterization by Electrophysiological Methods

Solutions—For both cell types, the control bath solution consisted of 70 mM NaCl, 70 mM CsCl, 1 or 2 mM CaCl₂, 0 or 1 mM MgCl₂, and 10 mM HEPES (pH 7.4). All toxin solutions were made in the bath solution, containing 0.5% bovine serum albumin and the specified concentration of toxin. Concentration of cells were assayed on day 5. The RT4-B cells were seeded and grown under the same conditions as used for N1E-115 cells. Since the former cells did not require any differentiation, they were typically assayed on day 4. Sodium uptake experiments were performed in the presence of substrate (20 μM) levels of the poor agonist veratridine as described above. Absolute uptake values were expressed in terms of nmol per min per mg cell protein, corrected for basal uptake due to veratridine alone. The maximal levels of uptake for the mutants were normalized to those at saturating levels of ApB (500 nM) and 20 μM veratridine. The experimental data shown were obtained from multiple experiments and fitted with a single hyperbolic function as described by Cleland (1979), yielding the kinetic constants Vₘₐₓ and Kᵥₐₚ.

Recording Protocol—Recordings were made using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Voltage protocols were executed on a 486DX-2–50 computer running CLAMPPEX 6.0.1 (Axon). Data were filtered at 5 kHz with a 4-pole Bessel filter and digitized at 50 kHz (12-bit resolution). Pipette resistances ranged from 700 kΩ to 1.1 MΩ. Suction was used to gain electrical access to the cell, which was then voltage-clamped to ~140 (RT4-B) or ~120 mV (N1E-115). A current–voltage relationship was recorded and a steady-state inactivation protocol performed. In every case, the holding potential used was sufficient to hyperpolarize to ensure complete availability of INa. Voltage control was determined by examination of the time to peak of the whole-cell current and the slope factor of the conductance transform. In order to facilitate solution changes on the single-second time scale, we used a two-chambered apparatus. To measure the time course of modification, cells were depolarized for 10 ms every 1–2 s (depending on the rate of modification). In fitting the data, the first two points of a 1-Hz record train and the first point of a 0.5-Hz record train were excluded. Separate current voltage and steady-state inactivation protocols were taken after the modification in order to assay for continued voltage control. In order to observe dissociation of the toxin, 10-ms depolarizations were begun again at a rate of 1 or 0.5 Hz, and the cell moved back to the control bath.

Modification rates were determined by taking an average of the current between 7.6 and 8 ms. For both channel isoforms, all the unmodified current had decayed before this window. Thus, by averaging the current 7.6–8.0 ms after depolarization, the measured current was ensured to be directly proportional to the number of modified channels in the cell. The averages were plotted against time, and a single exponential curve fit to the data using a least squares minimization routine included with the Origin 3.5.2 software (Microcal Software, Northampton, MA). Unmodification time constants were determined similarly. Because unmodification (toxin dissociation) is a first order process, k₉ was simply the inverse of the unmodification time constant. The on-rate (k₈) was determined through the relationship shown in Equation 1:

\[ k₈ = \frac{(h₉₀ - h₉₅)}{[T]} \]  

(1)

where h₉₀ was the inverse of the fitted τ of modification time course and [T] the toxin concentration.

RESULTS

Generation of Trp-45 and Trp-33 Mutants—PCR mutagenesis has been used to target Trp-45 and Trp-33 to determine whether these surface hydrophobic residues are involved in toxin activity. The Trp-45 mutants were obtained using the primers BD-10, which contains a wobble at the Trp-45 position, and KB-11, which recognizes the 5’ end of the ApB gene in the vector pMG2 (Gallagher and Blumenthal, 1992) (Table I). The PCR product obtained, encoding the entire ApB gene, was digested with EcoRI and shuttled as a cassette into EcoRI-cut pMG2. Several mutants including W45S/A/V/H/L were identified using this approach. In addition to these mutants, we wished to characterize a more conservative substitution W45F that was not obtained using wobble PCR. W45F was obtained by PCR amplification with primers BD-12 and KB-56 followed by digestion with NcoI and SstI. The wild-type ApB sequence in pKB13 was replaced with the resulting PCR product via cassette mutagenesis to obtain the W45F replacement. Similarly, a panel of mutants at position 33 including W33F/A/S/V was constructed in the sense orientation. The plasmids pKB9 (W45F), pKB12 (W45F), pKB13 (W33F/A/S), and pAC11 (W33Y) were used for further analyses.

Expression of Mutant Toxins—Mutants W45S and W45A were cloned into the expression plasmid pMG2, and toxins

| Primer | Strand | Sequence | Mutants obtained |
|--------|--------|----------|------------------|
| BD-10  | Antisense | 5'−CTTGGCAGACCATGCTTCACTCTTACAGCTGCGGATC−3' | W45S/A/V/H/L |
| BD-12  | Sense | 5'−AGGCGGATGTCGGACACATCGGC−3' | W45F |
| BD-18  | Antisense | 5'−TTCGGACATGGGCTTTACAGTTTG | W33/S/A/V |
| BD-19  | Antisense | 5'−TTCGGACATGGGCTTTACAGTTTG | W33F |
| AC-2   | Antisense | 5'−CTTGGCATCAGGCGCTTTACAGTTTG | W33Y |
| KB-11  | Sense | 5'−CTATGGCTGACAGTCG−3' | NcoI |
| KB-56  | Antisense | 5'−AACGGTGGGGCGGTACGG−3' | NcoI |

Analysis—Modification rates were determined by taking an average of the current between 7.6 and 8 ms. For both channel isoforms, all the unmodified current had decayed before this window. Thus, by averaging the current 7.6–8.0 ms after depolarization, the measured current was ensured to be directly proportional to the number of modified channels in the cell. The averages were plotted against time, and a single exponential curve fit to the data using a least squares minimization routine included with the Origin 3.5.2 software (Microcal Software, Northampton, MA). Unmodification time constants were determined similarly. Because unmodification (toxin dissociation) is a first order process, k₉ was simply the inverse of the unmodification time constant. The on-rate (k₈) was determined through the relationship shown in Equation 1:

\[ k₈ = \frac{(h₉₀ - h₉₅)}{[T]} \]  

(1)

where h₉₀ was the inverse of the fitted τ of modification time course and [T] the toxin concentration.
HCl. Compared with ApB, no discernible differences in the temperature from 20 to 80°C in the presence of 1.5M guanidine their secondary structures were recorded as a function of tem-
peratures, to investigate the thermal stabilities of the mutants, were structurally indistinguishable from wild-type ApB. Addi-
tionally, to determine the thermal stabilities of the Trp-33 or Trp-45 mutants were characterized further as described below.

Structural Characterization of the Mutant Toxins—Amino acid analysis of the mutant proteins confirms the presence of the substituted amino acid residue, and spectrophotometric determination of the tryptophan contents is consistent with the loss of a tryptophan residue in each of the mutants analyzed (Table II). With the exception of an additional 0.6–1.9 glutamic acid residues per mol observed in some of the mutants, the remaining compositions of the mutants mirrored that of wild-type toxin. To precisely determine their glutamic acid contents, W45S/A/F and W33Y were further analyzed by MALDI-TOF mass spectrometry, which confirmed the addition of a single glutamic acid residue in the W33Y and W45F proteins, both of which were expressed from the vector pKB13 containing the pentaglutamate staphylococcal protease recognition sequence (data not shown). Glu-ApB, similar to an N-terminally extended form we characterized earlier, is functionally identical to the natural toxin (Gallagher and Blumenthal, 1992). Circular dichroism spectra were obtained for each of the mutants in order to assess their secondary structures and structural stabilities. Like wild-type ApB, the predominant structural motif observed in all of the mutants is β-sheet, with contents ranging from 44 to 65% (data not shown). W45A, in which tryptophan is substituted with a β-strand breaker, shows a small decrease in β-sheet, whereas all other mutants were structurally indistinguishable from wild-type ApB. Additionally, to investigate the thermal stabilities of the mutants, their secondary structures were recorded as a function of temperature from 20 to 80 °C in the presence of 1.5 M guanidine HCl. Compared with ApB, no discernible differences in the thermal stabilities of the Trp-33 or Trp-45 mutants were detected.

Functional Characterization of the Mutant Toxins by Ion Flux—The activities of toxin variants can be estimated by measuring their abilities to synergistically enhance veratridine-dependent sodium uptake in excitable cells (Gallagher and Blumenthal, 1992). Anemone toxins such as ApB, as well as scorpion toxins, exemplified by Leiurus toxin V, prolong but do not induce the conducting state of the channel (Catterall, 1977; Gallagher and Blumenthal, 1992). Thus, in the presence of subsaturating quantities of a poor agonist such as veratridine,

Not corrected for partial degradation of serine and threonine during acid hydrolysis; numbers in parentheses represent summation of the known sequence of ApB. ND, not determined.

| Amino acid | ApB | W33F | W33Y | W45F | W45S | W45A |
|------------|-----|------|------|------|------|------|
| Aspartic acid | 4.7 (5) | 5.0 (5) | 5.0 (5) | 5.3 (5) | 5.0 (5) | 5.0 (5) |
| Glutamic acid | 0.6 (0) | 0.8 (0) | 0.5 (0) | 1.9 (0) | 0.2 (0) | 0.6 (0) |
| Cysteine | 4.0 (6) | ND | 3.6 (6) | ND | ND | ND |
| Serine | 3.6 (4) | 3.8 (4) | 3.9 (4) | 3.8 (4) | 4.8 (5) | 4.1 (4) |
| Glycine | 8.0 (8) | 8.2 (8) | 7.9 (8) | 8.2 (8) | 8.3 (8) | 7.7 (8) |
| Histidine | 1.7 (2) | 1.7 (2) | 2.0 (2) | 2.0 (2) | 2.0 (2) | 1.9 (2) |
| Threonine | 1.3 (1) | 1.1 (1) | 1.0 (1) | 1.1 (1) | 1.1 (1) | 1.3 (1) |
| Alanine | 1.4 (1) | 1.2 (1) | 1.1 (1) | 1.3 (1) | 1.2 (1) | 2.2 (2) |
| Arginine | 2.1 (2) | 2.0 (2) | 2.1 (2) | 2.2 (2) | 2.1 (2) | 2.6 (2) |
| Proline | 5.9 (6) | 6.2 (6) | 6.5 (6) | 7.6 (6) | 6.4 (6) | 6.7 (6) |
| Tyrosine | 0.9 (1) | 1.0 (1) | 1.6 (2) | 1.1 (1) | 1.0 (1) | 1.0 (1) |
| Valine | 1.4 (1) | 1.2 (1) | 1.1 (1) | 1.2 (1) | 1.1 (1) | 1.1 (1) |
| Methionine | 0.1 (0) | 0.1 (0) | 0.1 (0) | 0.1 (0) | 0.1 (0) | 0 (0) |
| Isoleucine | 2.3 (2) | 2.0 (2) | 2.0 (2) | 2.2 (2) | 2.2 (2) | 1.9 (2) |
| Leucine | 3.2 (3) | 3.1 (3) | 3.2 (3) | 3.4 (3) | 3.1 (3) | 2.9 (3) |
| Phenylalanine | 1.3 (1) | 1.9 (2) | 1.1 (1) | 2.3 (2) | 1.0 (1) | 0.9 (1) |
| Lysine | 2.9 (3) | 2.9 (3) | 2.8 (3) | 2.5 (3) | 3.0 (3) | 2.6 (3) |
| Tryptophan | 4.0 (6) | 3.6 (6) | 3.8 (6) | 4.1 (6) | 4.2 (6) | 4.6 (6) |
| M<sub>r</sub> (by MALDI) | 5273.8 | 5235.0 | 5250.6 | 5236.1 | 5174.9 | 5159.3 |

<sup>a</sup> Determined as cysteic acid after performic acid oxidation.
<sup>b</sup> Determined spectrophotometrically.

![Fig. 1. Veratridine-dependent Na<sup>+</sup> uptake by N1E-115 cells.](http://www.jbc.org/)
sodium uptake in both the N1E-115 and RT4-B lines, expressing the tetrodotoxin TTX-sensitive neuronal and TTX-resistant cardiac channel isoforms, respectively, is enhanced by ApB in a concentration-dependent manner. Effects of a given mutation on toxin affinity are then reflected as changes in the \( K_{0.5} \) value for sodium uptake, whereas alterations in the ability of a mutant to stabilize the open conformation of the channel are manifested in the \( V_{\text{max}} \) term.

Dose-response curves for wild-type ApB and the mutants in the N1E-115 line are compared in Fig. 1, and the relevant kinetic constants are summarized in Table III. In contrast to the high apparent affinity of wild-type ApB for the neuronal channel (22 nm), the \( K_{0.5} \) for the W33F mutant is 1.1 \( \mu \)M, representing a 50-fold reduction in affinity. Only a 5-fold reduction in apparent affinity is observed for W33Y in this cell line. Because only the W33F mutant displays a substantial decrease in affinity, a significant portion of the binding energy contributed by position 33 must be due to either the electronegative indole nitrogen or the tyrosine hydroxyl group. Position 45 is far more plastic since each substitution tested here results in a small but significant loss of apparent binding affinity, ranging from about 4-fold for W45A to just over 7-fold for W45S.

Fig. 2 depicts a parallel analysis carried out in the RT4-B line. Similar to the results described above, the \( K_{0.5} \) for W33F is 279 nm corresponding to a 30-fold loss in apparent binding affinity for the cardiac channel compared with wild-type ApB (9 nm). In contrast, W33Y and the three Trp-45 mutants analyzed result in losses of apparent affinity ranging from 3- to 8-fold (Table III).

In addition to requiring that the toxin bind to the channel, the assay requires that it stabilize the channel in its open conformation, and this ability manifests itself in the \( V_{\text{max}} \) of sodium uptake. As shown in Figs. 1 and 2, and summarized in Table III, both W33F and W33Y display maximal levels of uptake comparable with those for wild-type ApB in both channel types. However, in the neuronal model, the relative \( V_{\text{max}} \) of all Trp-45 mutants is significantly reduced, ranging from 40 to 60% that of wild-type ApB. This result raises the possibility that an interaction between Trp-45 and the neuronal sodium channel may be important in stabilizing the open conformation of the latter molecule induced by veratridine. Interestingly, while W45A and W45S are similarly impaired in the RT4-B line, the W45F toxin displays a normal \( V_{\text{max}} \) in this model. The implications of these results will be discussed below.

Functional Characterization of W33F, W45F, and W45A by Electrophysiology—The most interesting mutants, W33F, W45F, and W45A, were also analyzed by voltage clamp. When compared with previous electrophysiological determinations of the \( K_{0} \) for wild-type ApB (Khera et al., 1995), W33F exhibits a 266-fold decreased affinity for the cardiac channel and a 102-fold decreased affinity for the neuronal isoform (Table IV). These decreases in affinity are caused primarily by reductions in association rate (148-fold for cardiac and 31-fold for neuronal channels). The mutant toxin retains a 22-fold preference for the cardioid channel as compared with the 57-fold preference exhibited by wild type. The preference of W33F for the cardiac over the neuronal isoform is determined primarily by a 65-fold difference in off-rate, as compared with a 3-fold difference in the calculated on-rate.

Electrophysiological determinations of toxin \( K_{D} \) for W45F are qualitatively similar to those determined by sodium flux. W45F shows an 11-fold decreased affinity for the cardiac channel and a 3.7-fold decreased affinity for the neuronal channel (Table IV). The ordering of toxin preference for the cardiac over the neuronal isoform is determined primarily by a 21-fold difference in its off-rate. Similar to W33F, the mutant toxin shows a 19-fold preference for the cardiac over the neuronal isoform as compared with a 57-fold preference exhibited by the wild-type toxin.

To confirm that the diminished \( V_{\text{max}} \) seen for the W45 mutants is not an artifact of the ion flux assay, we compared the abilities of the W45A and wild-type toxins to augment persistent current in RT4B cells. For these experiments temperature was increased to 29 °C in order to more closely mimic conditions of the flux assay. Following exposure to 20 \( \mu \)M veratridine, cells were depolarized to a variety of test potentials from a holding potential of –150 mV, resulting in the appearance of a long-lasting current across the entire voltage range examined. Cells were then exposed to wild-type ApB or W45A at approximately 10 times their respective \( K_{D} \) values. The response to

### Table III

| Toxin | \( K_{0.5} \), neuronal | \( K_{0.5} \), cardiac | \( V_{\text{max}} \), mutant/ApB | \( V_{\text{max}} \), mutant/ApB |
|-------|-----------------|-----------------|----------------|----------------|
| ApB   | 22 ± 3          | 9 ± 3           | 1              | 1              |
| W33F  | 108 ± 253       | 279 ± 33        | 1              | 0.9            |
| W33Y  | 116 ± 28        | 51 ± 15         | 1.1            | 1.0            |
| W45F  | 108 ± 32        | 39 ± 5          | 0.6            | 0.9            |
| W45S  | 158 ± 28        | 30 ± 4          | 0.6            | 0.5            |
| W45A  | 97 ± 20         | 69 ± 9          | 0.4            | 0.4            |

**FIG. 2.** Veratridine-dependent \( ^{22}\text{Na} \) uptake by RT4B cells. Dose-response curves for ApB (●), W45A (○), W45S (○), W45F (■), W33F (■), and W33Y (□) were determined as in Fig. 1. These data have been corrected for basal uptake due to veratridine.
Role of Exposed Trp Residues in Activity of Anthopleurin B

**TABLE IV**

| Toxin   | ApB | W33F | W45F | W45A |
|---------|-----|------|------|------|
| Cardiac | 1.4 ± 0.4 (n = 3) | 0.0094 ± 0.0047 (n = 6) | 0.47 ± 0.2 (n = 8) | 1.72 ± 0.62 (n = 3) |
| Neuronal| 0.93 ± 0.12 (n = 5) | 0.03 ± 0.007 (n = 5) | 0.54 ± 0.11 (n = 11) | 0.35 ± 0.063 (n = 6) |

**DISCUSSION**

Our current understanding of functionally important residues in ApB has come primarily from mutagenesis studies. Analysis of single and double mutants at cationic sites in ApB has implicated Arg-12, Arg-14, and Lys-49 in toxin affinity and channel isoform selectivity, and a model structure of ApB indicates that these residues are clustered together, forming a significant contact region (Gallagher and Blumenthal, 1994; Khera and Blumenthal, 1994; Khera et al., 1995). Furthermore, we have found that Leu-18, an aliphatic residue in the vicinity of the cationic cluster, is a major determinant of high affinity binding of ApB to both isoforms of the sodium channel, providing the first direct evidence for the role of hydrophobic residues in toxin activity (Dias-Kadambi et al., 1996). A general trend emerging is that the region around the cationic cluster appears to contribute significantly to the toxin-channel interaction.

In work in other laboratories on scorpion toxins implicates surface hydrophobic residues in their binding to the sodium channel (Fontecilla-Camps et al., 1982; Fontecilla-Camps et al., 1988; El Ayeb et al., 1986; Darbon et al., 1991). Since studies on Leiurus and Anemonia toxins demonstrate that they compete in a single channel binding site, it thus seemed reasonable that a subset of the hydrophobic residues of ApB might also be involved in its binding to the sodium channel (Catterall and Beress, 1978). Our results with Leu-18 mutants of ApB corroborate this concept, and we have therefore extended our investigation in the direction of hydrophobic residues by focusing on exposed aromatic side chains of ApB.

The solution structures of ApA and ApB reveal that the side chain of Trp-45 is fully exposed in both homologs while tryptophans at positions 23 and 33 are shielded. Photochemically induced nuclear polarization (Photo-CIDNP) experiments indicate that Trp-33 and Trp-45 are accessible to a water-soluble dye and therefore solvent-exposed, whereas Trp-23 is by this criterion partially shielded (Norton et al., 1986). Although the degree of exposure of each individual tryptophan is not precisely known, there is sufficient evidence to suggest that both toxin is qualitatively similar in the two groups. Peak currents are decreased and the current at 400 ms markedly increased (Fig. 3A). When the toxin + veratridine currents are normalized to those obtained in the presence of veratridine alone (Fig. 3B), it is apparent the degree of augmentation seen for W45A is only 70% that seen with ApB. Thus, qualitatively similar mutant-specific effects on channel are observed both at the ion flux and single cell level. However, since these effects are only seen in the presence of veratridine, they may be ascribable to channel conformations found uniquely in the ternary (i.e. channel-veratridine-ApB) complex. That the effects in the two systems are not quantitatively identical may be related to the different temperatures at which the two experiments are performed (29 °C for electrophysiology versus 37 °C for ion flux), since the effects of veratridine are known to be strongly temperature-dependent (Tanaka et al., 1983).

![Fig. 3. Differences in augmentation of persistent current by ApB and W45A in the presence of veratridine.](http://www.jbc.org/)

In contrast to Trp-33, only 3–8-fold changes in K_d are observed for all the replacements assayed at Trp-45, including alanine, serine, and phenylalanine. Given the nonconservative nature of some of these substitutions, and the retention of
appreciable binding affinity, we conclude that Trp-45 does not provide determinants essential for ApB binding. Structural models of ApB (Fig. 4) are also consistent with these two residues playing quite distinct roles, since Trp-33 is proximal and Trp-45 distal to the cationic cluster that we have previously shown to be important for activity.

While our data are consistent with Trp-45 playing at most a modest role in binding affinity, an intriguing observation emerges when $V_{\text{max}}$ values for these mutants are compared with that of wild-type ApB. Replacement of Trp-45 with alanine, serine, or phenylalanine causes a significant diminution of $V_{\text{max}}$ in N1E-115 cells, whereas only the first two replacements yield this result in the cardiac (RT4-B) model. For W45A, $V_{\text{max}}$ is decreased to a significantly lower level than that seen with natural ApA, which itself displays an uptake $V_{\text{max}}$ only 65% that of natural or recombinant ApB (Gallagher and Blumenthal, 1992).

Veratridine both decreases the ion selectivity of the sodium channel and modifies its gating kinetics, indicating coupling between the selectivity filter and gating apparatus (Frelin et al., 1981). It is therefore possible that veratridine treatment also alters the ability of both naturally occurring ApA and a subset of our ApB mutants to stabilize the open conformation of the channel. Moreover, the striking restoration of full cardiac efficacy seen with the W45F toxin strongly suggests that a hydrophobic moiety at this position contributes to stabilization of the open conformation of the cardiac sodium channel. Although the physiological consequences of the reduced $V_{\text{max}}$ are not completely clear, analysis of toxins like ApA and the Trp-45 mutants may prove useful in the characterization of open channel conformations.

Electrophysiological analysis of the most severely impaired mutant, W33F, is particularly interesting because in this case the loss in binding affinity is caused by a reduced on-rate. As proposed for the interaction of $\mu$-conotoxin with the sodium channel, certain residues may affect the approach and docking of the toxin to its acceptor site, whereas others are critical to the strength of association once the initial binding event has occurred (Becker et al., 1992). By these criteria, Trp-33 belongs in the former category and is the only ApB mutant characterized thus far to do so. Because no alterations in the overall conformation of W33F are detected by circular dichroism, it is unlikely that incorrect protein folding accounts for the striking changes in association rates observed. However, this does not rule out the possibility of local perturbations within the vicinity of W33F and below the detection level of spectropolarimetry accounting for the reduced on-rates. This mutant might thus be useful as a direct probe of toxin interaction with its binding site.

The results described herein, including retention of appreciable toxin activity with W33F and the significant loss of binding with W33F, are indicative either of Trp-33 forming a critical intermolecular hydrogen bond with the sodium channel or its intramolecular stabilization of a high affinity conformation within the toxin binding region. The energies of hydrogen bonds have been previously estimated to be between 0.5 and 1.5 kcal per mol (Fersht et al., 1985). The decrease in apparent affinity observed for W33F correlates with a reduction in apparent binding affinity ranging from 2.0 to 2.3 kcal per mol for both channel isoforms. This could be consistent with the loss of a hydrogen bond, particularly if minor rearrangements in the local environment also occur. Alternatively, and consistent with the proximity of Trp-33 to the cationic cluster (Fig. 4), the indole group might interact with the guanidinium group of Arg-12 or Arg-14, maintaining a high affinity conformation. This mechanism, involving stabilization of positively charged groups via interactions with electron-rich $\pi$ systems of aromatic side chains, has previously been suggested for the binding of acetylcholine to its receptor (Unwin, 1993). Similarly, tyrosine residues have been implicated in the high affinity binding of tetraethylammonium ions with the potassium channel (MacKinnon and Yellen, 1990) and the guanidinium to toxins tetrodotoxin and saxitoxin with the sodium channel (Satin et al., 1992). Analysis of a variety of biological systems indicates that the order of the effect observed for cation-$\pi$ interactions is $\text{Trp} > \text{Tyr} > \text{Phe}$ (Dougherty, 1996). Our results are analogous to this scenario wherein significant binding is retained when the electron-rich tryptophan and tyrosine side chains are present whereas binding is reduced although measurable upon substitution with phenylalanine.

It is evident from this study that hydrophobic residues like Trp-33 are major determinants of ApB activity. This residue influences a critical parameter for drug design, the rate of association of the toxin with its receptor, and might provide valuable insights regarding the toxin binding site. Although Trp-45 does not affect binding per se, it may affect the stabilization of the open conformation of the cardiac channel. Examination of additional mutations in the vicinity of these residues,

FIG. 4. Relative positioning of Trp-33, Trp-45, and residues comprising the cationic cluster. The structure shown is a stereo view based on a model developed by Khera et al. (1995), with the side chains of Arg-12, Arg-14, Trp-33, Trp-45, and Lys-49 indicated and the peptide backbone highlighted as a stranded ribbon. Note that while Trp-33 is closely juxtaposed to the cluster, Trp-45 is located on the reverse side of the molecule, and in this view is partially obscured by the backbone ribbon.
combined with high resolution structural analysis, should provide definitive answers to questions regarding these two different facets of toxin activity.

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REFERENCES
Becker, S., Prusak-Sochaczewski, E., Zamponi, G., Beck-Sickinger, A. G., Gordon, R. D. & French, R. J. (1992) Biochemistry 31, 8229–8238
Catterall, W. A. (1990) J. Biol. Chem. 265, 8660–8668
Caterall, W. A. & Beress, L. (1978) J. Biol. Chem. 253, 7393–7396
Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
Dabren, H., Weber, C. & Braun, W. (1991) Biochemistry 30, 1836–1845
Dias-Kadambi, B. L., Drum, C. L., Hanck, D. A. & Blumenthal, K. M. (1996) J. Biol. Chem. 271, 9422–9428
Doggrell, S., Hoey, A. & Brown, L. (1994) Clin. Exp. Pharmacol. Physiol. 21, S3–S45
Donahue, M. L., Schaller, K. & Sueoka, N. (1991) Dev. Biol. 147, 415–424
Dougherty, D. A. (1996) Science 270, 161–170
El Ayeb, M., Darbon, H., Bahraoui, E. M., Vargas, O. & Rochat, H. (1986) Eur. J. Pharmacol. 103–138
Fersht, A. R., Shi, J., Knill-Jones, J., Lowe, D. M., Varghese, M. Y. & Wolpert, L. (1985) Nature 314, 235–238
Ferré, J. P., Balerna, M., Wang, G. (1987) Annu. Rev. Neurosci. 10, 237–267
Frelin, C., Vigne, P., Schweitz, H. & Lazdunski, M. (1984) Mol. Pharmacol. 26, 70–74
Gallagher, M. J. & Blumenthal, K. M. (1992) J. Biol. Chem. 267, 13958–13963
Gallagher, M. J. & Blumenthal, K. M. (1994) J. Biol. Chem. 269, 254–259
Gosley, P. R., Blunt, J. W., Beress, L., Norton, T. R. & Norton, R. S. (1986) J. Biol. Chem. 261, 1536–1541
Gross, G. J., Warttierv, D. C., Hardman, H. F. & Shibata, S. (1985) Eur. J. Pharmacol. 101, 437–442
Khera, P. K. & Blumenthal, K. M. (1994) J. Biol. Chem. 269, 921–925
Khera, P. K., Benzinger, R. G., Lipkind, G., Drum, C. L., Hanck, D. A. & Blumenthal, K. M. (1995) Biochemistry 34, 8533–8541
MacKinnon, R. & Yellen, G. (1990) Science 250, 276–279
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
Mons, S. A., Pallaghy, P. K., Scanlon, M. J. & Norton, R. S. (1995) Structure 3, 791–803
Newcomb, R., Yasunobu, K. T., Seriguchi, D. & Norton, T. R. (1980) in Frontiers in Protein Chemistry (Lui, D. T., Mamiya, G. & Yasunobu, K. T., eds) pp. 539–550, Elsevier-North Holland, New York
Norton, T. R. (1981) Fed. Proc. 40, 21–25
Norton, R. S. (1991) Toxicon 29, 1551–1584
Norton, R. S., Shibata, S., Kashiwagi, M., & Bentley, J. (1996) J. Pharmacol. Sci. 65, 1368–1374
Norton, R. S., Beress, L., Stob, S., Boelens, R. & Kapteijn, R. (1986) Eur. J. Biochem. 157, 343–346
Pallaghy, P. K., Scanlon, M. J., Monks, S. A. & Norton, R. S. (1995) Biochemistry 34, 3782–3794
Renaud, J. F., Fosset, M., Schweitz, H. & Lazdunski, M. (1986) Eur. J. Pharmacol. 120, 161–170
Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. (1980) J. Mol. Biol. 143, 161–176
Satin, J., Kyle, J. W., Chen, M., Bell, P., Crihbs, L. L., Fozard, H. A. & Rogart, R. B. (1992) Science 256, 1202–1205
Schweitz, H., Vincent, J., Barhanin, J.-P., Frelin, C., Linden, G., Hugues, M. & Lazdunski, M. (1991) Biochemistry 30, 5245–5252
Scriabine, A., Van Arman, C. G., Morgan, G., Morris, A. A., Bennett, C. D., & Boudiar, N. (1979) J. Cardiovasc. Pharmacol. 1, 571–583
Strichartz, G., Rando, T. & Wang, G. (1987) J. Neurosci. 7, 1571–1579
Tanaka, J. C., Eccleston, J. F. & Barchi, R. L. (1983) J. Biol. Chem. 258, 1531–1536
Thomsen, W. J. & Catterall, W. A. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 8742–8746
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Belinda L. Dias-Kadambi, Kelly A. Combs, Chester L. Drum, Dorothy A. Hanck and Kenneth M. Blumenthal

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