Voltage-sensitive sodium channels play a crucial role in the transmission of electrical signals in excitable cells (1, 2). Several laboratories have used toxins that alter channel function to advance our understanding of sodium channel biology (3, 4). These toxins have been classified by Catterall (4) as binding to one of five different receptor sites on the channel with consequent effects on either activation, inactivation, or ion conductance. Of particular interest, primarily because of their potential to advance our understanding of sodium channel biology (3, 4), are toxins that delay sodium channel inactivation, resulting in enhanced sodium influx and ultimately giving rise to an increase in the force of contraction (5–8). The classical drugs used to treat heart failure are the cardiac glycosides ouabain and digoxin, which are potent inhibitors of the Na⁺/K⁺-ATPase, and the β-adrenoreceptor agonists dopamine and dobutamine (9–11). The life-threatening toxicity associated with digoxin and the decreased effectiveness of the β-agonists as heart failure progresses underscore the importance of identifying novel agents displaying cardiotonic activity (11).

Sea anemones are a rich source of biologically active polypeptides with diverse pharmacological activities. While most anemone toxins were isolated as neurotoxins, Anthopleurin A and B (ApA and ApB) obtained from the sea anemone Anthopleura xanthogrammica were originally isolated based on their displaying cardiotonic activity (8, 12). In isolated cardiac muscle, ApA is more potent than digoxin, being effective at nanomolar (nM) concentrations (13). More importantly, studies using anesthetized dogs demonstrate that this activity is not associated with adverse effects on heart rate or blood pressure (13, 14). ApB is even more potent than ApA, displaying its maximal inotropic activity at 0.3 nM (8). While their antigenticity and lack of oral activity in animals preclude the use of these toxins as drugs in their naturally occurring forms, understanding the molecular interactions between these toxins and the sodium channel could form the basis for rational design of new drugs displaying enhanced cardiotonic activity. It is essential that the approach taken combine available structural information with functional studies in order to define regions of the molecule that contribute either to high affinity or selectivity for the cardiac channel.

ApA and ApB are naturally occurring homologs that differ in only 7 out of 49 residues and are cross-linked by three disulfide bonds (8, 10). Ion flux studies have established that ApB exhibits nanomolar affinity for both the cardiac and neuronal isoforms of the sodium channel, while ApA binds much less tightly to the latter (15). Under voltage clamp conditions, both ApA and ApB bind preferentially to the cardiac channel, and ApB binding affinity is increased 100-fold in comparison with the values obtained by ion flux (16). In contrast, ApB binding to the neuronal channel differs by only about 4-fold in these two assays. These results indicate that ApB binds both to the open and closed conformations of the channel and greatly prefers binding to the closed channel over other states.

This laboratory has previously cloned a synthetic gene for toxins that delay sodium channel inactivation, resulting in enhanced sodium influx and ultimately giving rise to an increase in the force of contraction (5–8). The classical drugs used to treat heart failure are the cardiac glycosides ouabain and digoxin, which are potent inhibitors of the Na⁺/K⁺-ATPase, and the β-adrenoreceptor agonists dopamine and dobutamine (9–11). The life-threatening toxicity associated with digoxin and the decreased effectiveness of the β-agonists as heart failure progresses underscore the importance of identifying novel agents displaying cardiotonic activity (11).

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ApB and produced the recombinant protein using a bacterial expression system (17, 18). Using site-directed mutagenesis, we were able to specifically target residues and determine their contribution to activity by measuring sodium uptake in tissue culture cells. These studies have emphasized cationic residues that are either unique to ApB or conserved among anemone toxins (15, 19). Studies on the unique cationic residues in ApB indicated that the polar side chain of Arg-12 is important in activity (15). In contrast, mutating the conserved positively charged residues Arg-14 and Lys-48 establishes that these residues play a smaller role in activity (19). A set of three mutant toxins containing pairwise replacements of the cationic side chains were not completely inactivated although their apparent affinities were significantly diminished raising the possibility of compensatory effects upon replacement of single cationic sites (16). Although our previous studies have identified residues which contribute 1–2 kcal/mol of binding energy, it is clear that additional sites of interaction remain unidentified (15).

The solution structures of both ApA and ApB have recently been solved by multidimensional NMR and reveal a four-stranded anti-parallel β-sheet structure common to both polypeptides (20, 21). In a recently published model, the cationic side chains of Arg-12, Arg-14, and Lys-49 of ApB are located close together, and the solution structure, while not unambiguous, is generally consistent with this model (16, 21). The three-dimensional structures of homologous scorpion toxins that interact with the sodium channel and affect either activation or inactivation are highly similar to each other although unrelated to those of anemone toxins (22–24). A notable conserved structural similarity among scorpion toxins is a surface-exposed hydrophobic region (22, 23, 25). Based on sequence analogies and chemical modification studies, Foncalilla-Camps et al. (26) proposed that this hydrophobic region is either directly involved in channel binding or helps align other residues that are important for both binding and specificity. Although ApB lacks an analogous surface hydrophobic face, a number of its hydrophobic side chains are at least partially exposed, including a subset of those found in proximity to the cationic cluster mentioned above (8, 16, 21). The present study was initiated in order to ascertain the extent to which these exposed hydrophobic residues in ApB might participate in the toxin-channel interaction. Sequence comparisons among homologous toxins and three-dimensional structural information lead us to target Ile-43 and Leu-18 to determine their role in toxin activity. Our results indicate that only highly conservative substitutions, resulting in modest changes in affinity, are tolerated for Ile-43, a residue which is distal to the proposed cationic cluster. In striking contrast, Leu-18, a proximal residue, contributes significantly to the high affinity of ApB for both isoforms of the sodium channel, thus further delineating the binding surface presented by these toxins. This study represents the first direct demonstration that hydrophobic residues play an essential role in sodium channel toxin function.

**Experimental Procedures**

**Reagents and Enzymes**

The highest grade chemicals commercially available were used in all experiments. Restriction endonucleases and modification enzymes were obtained from Life Technologies, Inc. The radiochemicals [32P]dATP and 22NaCl were purchased from DuPont NEN. The Sequenase kit from U. S. Biochemical Corp. was used to sequence all mutant constructs. Staphylococcal protease (V8 protease) was acquired from Sigma. Tissue culture media was obtained from J RH Scientific and Fisher Scientific while the fetal calf serum was from Hyclone and United Biochemicals Inc.

**DNA Methodology**

Well established protocols for bacterial transformation, plasmid isolation, and cloning techniques were used (27). The expression vector pMG2, which encodes the synthetic gene for ApB fused to the 3’ end of the gene for the bacteriophage gene-9 protein under control of the T7 promoter, was used to express high levels of mutant and wild-type proteins (18). The plasmid pMG7, designed to encode a polyglutamic acid sequence at the 5’ end of the gene for ApB, was constructed using PCR technology. The sense strand primer contained the polyglutamate encoding sequence, 5’-CCCTCTGAATTCCAGGAGAGTGAGAGGGGGG-3’, and was paired with an antisense primer recognizing the 3’ end of the gene. The resulting PCR product was then cloned into pMG2 (18) to generate the plasmid pMG7. This vector was further engineered to introduce a polylinker at the downstream end by converting the EcoRI site of pMG7 to an SstI site. Amplification using the antisense primer 5’-CGCTTCTAAGAGCTCTTATTATT-3’ and a sense strand primer that recognized the 3’ end of gene-9. The product was digested with EcoRI and SstI and cloned into pER9 (15) to generate the plasmid pKB13. Mutations were introduced by PCR amplification of the ApB gene encoded in pKB13 using primers BD-21 and KB-56 for the Leu-18 mutations and BD-13, BD-15, BD-16, BD-17, and KB-56 for the Ile-43 replacements (Table I). Each reaction included 30 repetitive cycles: an initial melting step (94°C, 1 min), an annealing step (2 min), and elongation (72°C, 2 min), with annealing temperatures based on the melting temperatures of the primers. PCR products encoding the Leu-18 or Ile-43 mutants were digested with appropriate restriction enzymes and gel-purified prior to ligation into pKB13. Ligation reactions were transformed into JM109 or XL1-Blue cells to obtain the desired clones pKB17 (L18A/L18V/L18F/L18I), pKB11 (I43G), and pKB12 (I43L/I43V/I43A/I43F). These bacterial strains were used routinely for plasmid propagation. All constructs were sequenced by dideoxynucleotide chain termination (28) using the methodology outlined by U. S. Biochemical Corp.

**Expression and Purification of Mutant Proteins**

The expression plasmid pKB13, which encodes a synthetic gene for ApB fused to the 3’ end of the bacteriophage gene-9 protein, was used to express large quantities of fusion protein (18). The mutant plasmids pKB17 (L18A/L18V), pKB11 (I43G), and pKB12 (I43L/I43V/I43A/I43F) were transformed into the Escherichia coli expression strain BL21(DE3). Mutant fusion proteins were purified using anion exchange chromatography, reoxidized in the presence of glutathione (GSSG: GSSG, 1 μm:0.2 μm), and cleaved with staphylococcal protease to obtain intact toxin. Final purification to homogeneity was achieved by reverse-phase HPLC on a C4 column.

**Cell Cultures**

Cell lines expressing either the neuronal or cardiac isoform of the sodium channel were used for functional characterization of mutant toxins. The murine neuroblastoma cell line (N1E-115) was a generous gift from Dr. Marshall Nirenberg (NHLBI, National Institutes of Health). Cells were grown for 3 days in 24-well tissue culture dishes in 90% Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum and 10% unstimulated penicillin-streptomycin in a humid atmosphere containing 10% CO₂ at 37°C. After the cells reached confluence, they were maintained in medium containing 1.5% fetal calf serum and 1.5% demethyl sulfoxide for 48 h to enhance adherence to the plates prior to uptake assays. Dr. Laurie Donahue (Health Sciences Center, Texas Tech University) kindly provided us with a rat peripheral neurotumor cell line (RT4-B) which expresses predominantly the tetrodotoxin-resistant cardiac myotonic skeletal isoform of the sodium channel (29, 30). These cells were grown under similar conditions, but since they adhered readily to the plates, maintenance in dimethyl sulfoxide/solvent was not required and the cells were assayed when confluent.

**Analytical Methods**

Prior to amino acid analyses, samples of the mutant proteins were hydrolyzed with HCl in vacuo for 22 h at 110°C. Hydrolysates were derivatized with phenylisothiocyanate and analyzed on a Picotag column. The secondary structures of the proteins were analyzed by circular dichroism spectropolarimetry using a Jasco J-710 spectropolarimeter. Samples were prepared in 5 mM sodium phosphate buffer (pH 6.8), and their far UV spectra were compared by a least squares fit to a composite structure based on the proteins of known secondary structure: myoglobin, lysozyme, ribonuclease A, papain, cytochrome c, hemoglobin, α-chymotrypsinogen, trypsin, and horse liver alcohol dehydrogenase.
The restriction enzymes combined within the primer sequences are underlined, and the codons mutated are highlighted in bold in the primer sequences.

### Table I

| Primer | Strand | Sequence | Mutants obtained |
|--------|--------|----------|------------------|
| BD-13  | Sense  | 5′-AGCCGATGCTCCGAAC (G/T) (G/A/T/C) (G/T) GGCTG-3′ | 143G |
| BD-15  | Sense  | 5′-GAAAGCCATGCTCCGAAC (G/T) CCGGTG-3′ | 143A |
| BD-16  | Sense  | 5′-GAAAGCCATGCTCCGAAC (G/T) CCGGTG-3′ | 143L |
| BD-17  | Sense  | 5′-GAAAGCCATGCTCCGAAC (G/T) CCGGTG-3′ | 143F/143V |
| BD-21  | Sense  | 5′-CTGATGGCCAGCAAGTTGCTG-3′ | L18A/L18B/L18F |
| KB-56  | Antisense | 5′-AACCTTGGG CTTGAGC-3′ | HindIII/PstI |

### Functional Characterization of ApB Mutants by Ion Flux

Assessment of toxin function is done by measuring veratridine-dependent sodium uptake in both the N1E-115 and RT4-B cell lines under conditions which have been previously described in detail (15, 31). RT4-B cells were assayed when confluent (day 4), whereas the N1E-115 cells were differentiated when confluent and assayed 48 h later. Cells were preincubated in sodium-free binding solutions containing 20 cells were differentiated when confluent and assayed 48 h later. Cells RT4-B cellswere assayed when confluent (day 4), whereasthe N1E-115 conditions which have been previously described in detail (15, 31).

### RESULTS

#### Identification and Characterization of the ApB Mutants—Using PCR methodology we have generated a set of mutants at Leu-18 and Ile-43. Primers BD-21 and KB-56 were used to obtain the Leu-18 mutants, and cassette mutagenesis was used to replace the wild-type ApB sequence in Ap-B/HindIII cleaved pBK13 with the PCR product containing the Leu-18 mutations, generating plasmids pBK17. Several replacements, including L18A/L18B/L18F, were identified by sequencing. In a similar manner we created the plasmids pBK11 (I43G) and pBK12 (I43L/I43A/I43F/I43V) that encoded replacements for Ile-43. Dideoxy sequencing confirmed the presence of the desired substitutions in the resulting plasmids pBK17, pBK11, and pBK12 and the absence of spontaneous mutations. The plasmids pBK17 (L18A/L18B), pBK11 (I43G), and pBK12 (I43L/I43A/I43F/I43V) were used in subsequent investigations to ascertain the importance of these sites in anemone toxin activity.

#### Expression and Isolation of Mutant Toxins—The L18A/L18B and the I43L/I43A/I43G/I43F mutants were all cloned into the expression plasmid pBK13 in which recombinant ApB protein is expressed as a fusion protein with the bacteriophage T7 gene-9 protein (15, 16). Typical yields for wild-type ApB range from 3–4 mg per 500 mg of fusion protein. Consistently higher yields of 5–6 mg per 500 mg of fusion protein were obtained for the L18A mutation, while the yields of L18B were lower and varied between different protein preparations. We attribute this decreased yield to the lower specific activity of the batch of staphylococcal protease used for L18B. For the conservative substitutions I43L and I43V, we were able to isolate normal amounts of intact toxin. However, further truncation of the side chain to I43A or I43G resulted in the absence of an identifiable ApB-like HPLC peak after staphylococcal protease digestion, despite isolation of normal quantities of fusion protein. Several modifications of the reoxidation protocol including using dithiothreitol as a reducing agent early in the isolation procedure, diluting the protein to 0.1 mg/ml prior to oxidation or maintaining the sample under nitrogen prior to oxidation failed to yield the I43A toxin. While a small split peak migrating at the same position as wild-type ApB on the HPLC...
in RT4-B cells, with 672- and 320-fold decreases in apparent
ability of the ApB mutants to stimulate 22Na uptake
shown). Guanidine chloride at temperatures up to 80°C (data not
determined changes, the compositions closely resemble that of
wild type ApB (Table II), although low levels of glutamic acid
residues/mol) were observed for some samples (see “Discussion”). Secondary structures and thermal stabilities of the mu-
tant proteins were assessed by circular dichroism. The far UV
#1 residue/mol) were observed for some samples (see “Discus-
acid analysis of the L18A/L18V and I43L/I43V proteins verified
determined by Cleland (32), and the points represent the experimental data. The
equation \( K_{0.5} \) (nm) obtained are: ApB (22 ± 3 and L18A (7897 ± 985),
L18V (5087 ± 1751), I43L (52 ± 9), and I43V (173 ± 25). Based on these
data, the maximal levels of uptake detected for the mutants relative to
ApB were L18A (0.7), L18V (0.4), I43L (1.1), and I43V (1.0).
affinity for the wild-type ApB for the neuronal and cardiac isoforms of the sodium channel.
ApB alone does not induce sodium uptake in these cells, and, therefore, subsaturating quantities of veratridine are added to
induce the conducting state of the channel. Addition of ApB,
which increases the open probability of the channel by causing
delayed inactivation, thus results in a dose-dependent increase
in sodium uptake. Representative dose-response curves and the
derived kinetic constants are shown in Figs. 1 and 2. The data
obtained for all the mutants in both cell lines are con-
trasted in the bar graph depicted in Fig. 3.

The apparent binding affinities of wild-type ApB for the
neuronal and cardiac isoforms of the sodium channel, as esti-
mated by ion flux measurements, are 22 nM and 9 nM, respec-
tively (15, 18, 19). Thus, the apparent neuronal \( K_{0.5} \) for the
L18A mutant (7.9 µM) represents at least a 359-fold reduction in
affinity. Similarly, a 231-fold reduction is observed for L18V,
displaying a neuronal \( K_{0.5} \) of 5.1 µM. The same trend is evident in
RT4-B cells, with 672- and 320-fold decreases in apparent
affinity for the L18A and L18V toxins, respectively. Compari-
sion of affinities in the two cell types yields a discrimination
index for L18A of 0.5, indicating that its ability to preferen-
tially bind to the cardiac channel is compromised. L18V also
has a reduced discrimination index of 0.7. The highest velocity
observed for the L18A/L18V mutants is between 40 and 70% of
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Although the affinity of ApB for the cardiac channel is substanti-
ally lower than for the neuronal isoform, the uptake values obtained for I43V were only 2-fold different from those of wild-type toxin. Thus, in both assay systems, L18A displays a lesser preference for the cardiac channel than does wild-type toxin.

**DISCUSSION**

This study targets hydrophobic residues in the toxin ApB by site-directed mutagenesis to establish their role in activity. Previous experiments demonstrated the importance of a cationic cluster including Arg-12, Arg-14, and Lys-49 to toxin activity (15, 16, 19). Using a model structure of ApB described by Khera et al. (16), we have identified residues based on proximity to this basic region and assessed their contribution to biological activity. In this model, the CD1 carbon of Leu-18 is within 10 Å of the NH1 and NH2 of Arg-14 while Ile-43 is clearly on the opposite face of the molecule. Selecting Ile-43, distal to the cluster, and Leu-18, a residue proximal to the cationic region, has enabled us to determine the nature of the potential intermolecular contacts made by two distinct surfaces of the toxin.

Using a PCR-based approach with a wobble containing primer, we generated two panels of mutants, of which a subset was expressed and characterized. Only highly conservative substitutions to leucine or valine were tolerated at position 43. We interpret these results as supporting the hypothesis that Ile-43 is involved in ApB folding, consistent with our model suggesting that it packs tightly against the Phe-24 and Tyr-25 side chains (16). We suggest that the I43A and I43G mutants disrupt the resulting hydrophobic region, preventing the protein from folding to a form allowing correct pairing of the three disulfide bonds. Since the ApB coordinates are not accessible in the Brookhaven data base, we are presently unable to verify this prediction.

Structural characterization of the mutants includes amino acid analysis and circular dichroism studies. While the amino acid compositions are overall in good agreement with that of wild-type ApB, the glutamic acid content unexpectedly ranged from 0.6–1.1 residues per mol. Previously, we attributed this to a system artifact (16). Recently, however, characterization by mass spectrometry and N-terminal sequencing of ApB expressed from pKB13 revealed the presence of two forms, one having a residue of glutamate at the amino terminus.2 This extra glutamate is found only in ApB expressed from pKB13, in which the toxin sequence is preceded by five consecutive glutamates. Glu-ApB, like an N-terminally extended form we characterized earlier (18), is functionally identical with the wild-type toxin. Furthermore, all the mutants retained as their predominant secondary structural motif the β-sheet as assessed by circular dichroism. Thermal denaturation profiles for all mutants were essentially unchanged, confirming the structural stability of the toxins.

We have made two replacements to evaluate the contribution of the hydrophobic residue Leu-18. Substitution with alanine represents a side chain truncation in which all interactions made by atoms beyond the β-carbon are removed. Replacement with valine should restore some of the hydrophobicity. Characterization of both mutants by ion flux demonstrates a pivotal

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2 R. A. Byrd, personal communication.
role for Leu-18 in toxin activity as exemplified by the several hundredfold (231–672-fold) loss in activity observed in both model systems analyzed. By comparison, double neutralization mutants in the cationic cluster reduce apparent affinities by a maximum of 72-fold (16).

The estimated maximal level of uptake obtained for the Leu-18 mutants is only 40–70% of that seen at saturating levels of ApB. Either we have not saturated ApB binding sites even at concentrations of 10–25 μM mutant toxin, or the ability of the bound mutants to stabilize the open conformation is compromised. Based on the results depicted in Figs. 1 and 2, we favor the former explanation, which suggests that the Kd values are even higher than those presented here. Because the range of concentrations we are able to assay is restricted by the amount of protein we can produce, we are unable to confirm this prediction. Nonetheless, the key role of Leu-18 is amply supported by the data presented.

In order to more closely assess the role of Leu-18 in activity, we assayed the most severely impaired mutant, L18A, by whole cell patch clamp. There are important differences in the two assay systems used. Under ion flux conditions, the channel is maintained in the open conformation due to the presence of subsaturating concentrations of the alkaloid veratridine. In contrast, in the electrophysiological assays, the cells are maintained in the open conformation due to the presence of the bound mutants to stabilize the open conformation is compromised. Based on the results depicted in Figs. 1 and 2, we favor the former explanation, which suggests that the Kd values are even higher than those presented here. Because the range of concentrations we are able to assay is restricted by the amount of protein we can produce, we are unable to confirm this prediction. Nonetheless, the key role of Leu-18 is amply supported by the data presented.

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Crucial Role of Leu-18 of Anthopleurin B

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