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A neutral amino acid change in segment IIS4 dramatically alters the gating properties of the voltage-dependent sodium channel

(ion channel/site-directed mutagenesis/voltage clamp/*Xenopus* oocyte)

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ABSTRACT **Sodium channels encoded by the rat IIA cDNA clone [Auld, V. J., Goldin, A. L., Krafte, D. S., Marshall, J., Dunn, J., Catterall, W. A., Lester, H. A., Davidson, N. & Dunn, R. J. (1988) *Neuron* 1, 449–461] differ at seven amino acid residues from those encoded by the rat II cDNA [Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. (1986) *Nature (London)* 320, 188–192]. When expressed in *Xenopus* oocytes, rat IIA channels display a current–voltage relationship that is shifted 20–25 mV in the depolarizing direction relative to channels expressed from rat II cDNA or rat brain poly(A)$^+$ mRNA. By modifying each variant residue in rat IIA to the corresponding residue in rat II, we demonstrate that a single Phe → Leu substitution at position 860 in the S4 segment of domain II is sufficient to shift the current–voltage relationship to that observed for channels expressed from rat brain poly(A)$^+$ RNA or rat II cDNA. Rat genomic DNA encodes leucine but not phenylalanine at position 860, indicating that the phenylalanine at this position in rat IIA cDNA likely results from reverse transcriptase error.**

Voltage-dependent sodium channels mediate the main component of the inward current during action potential propagation by excitable tissues. Purified sodium channels from various sources have in common a 260-kDa glycoprotein α subunit that forms the transmembrane ion pore (1). Although the channels isolated from eel electroplax channel contain only this α subunit, mammalian sodium channels have smaller associated subunits. Rat brain sodium channels copurify with two smaller subunits termed β1 (36 kDa) and β2 (33 kDa) (2), and the rabbit skeletal muscle sodium channel copurifies with one β subunit (38 kDa) (3). The functional significance of these β subunits has not been determined. Oocyte expression experiments indicate that a low molecular weight brain protein, possibly a β subunit, though not essential for α subunit function, contributes to rapid sodium channel inactivation under the expression conditions tested (4, 5).

The cDNAs encoding the α subunits of sodium channels from eel electroplax (6), rat brain (rat I, II, IIA, and III) (4, 7, 8), rat skeletal muscle (9), and *Drosophila* (10, 11) have been cloned. The primary sequences indicate that each α subunit contains four internal homology domains, each of which has been predicted to contain either six (7) or eight transmembrane segments (12, 13). When injected into frog oocytes, the α subunits from rat brain and skeletal muscle form functional sodium channels (4, 9, 14, 15), although the inactivation properties of the expressed channels are altered (4, 9). With cloning and expression of several α subunits achieved, research now centers on studies to identify structural elements that function in the critical channel properties of activation, inactivation, and ion selectivity.

Each of the four sodium channel homology domains contains a motif, termed S4, which consists of a 20-residue segment containing positive charges at every third position interspersed with largely nonpolar residues. This S4 motif has been postulated to form a critical element of the voltage sensor (1, 7). The cDNAs encoding voltage-dependent calcium and potassium channels have also been cloned (16, 17). Within both of these sequences is a region of striking similarity to the sodium channel S4 motif, supporting the idea that S4 is a critical element in the voltage-dependent channel mechanism. Further support has been provided by mutations introduced into the positive residues of S4, which alter the voltage dependence of channel activation (18).

In previous studies we have constructed a sodium channel α subunit cDNA (rat IIA) from seven overlapping partial cDNA clones (4). Translation of rat IIA RNA in *Xenopus* oocytes produced channels with a current–voltage (*I–V*) relationship that was shifted 20–25 mV in the depolarizing direction, inactivated at a slower rate, and decreased more rapidly during repetitive stimulation (unpublished data) than did brain poly(A)$^+$ RNA-induced currents. Coinjection of low molecular weight rat brain RNA increased the rate of macroscopic inactivation to levels comparable to the poly(A)$^+$ RNA-induced channels but had no effect on the shift in the *I–V* relationship.

In contrast to our results with rat IIA channels, channels produced in oocytes from rat II RNA displayed a *I–V* relationship that was virtually identical to that for channels expressed from poly(A)$^+$ RNA (15). This result suggested that structural differences between rat IIA and rat II were responsible for the observed differences in channel activation. In the present study we have used specific mutagenesis of the rat IIA cDNA to identify a single residue that causes this difference.

MATERIALS AND METHODS

Sequencing. The complete sodium channel cDNA from pVA200 was restricted with various enzymes (*Sau*3A, *Hin*fI, *Bst*NI, *Mae* II, *Hae* III), and the resulting fragments were subcloned into pGEM7-Z (Promega Biotec). Single-stranded DNA was produced and sequenced using Sequenase (United States Biochemical) (19).

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Abbreviations: TTX, tetrodotoxin; *I–V*, current–voltage.

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Site-Directed Mutagenesis. Small [≈1 kilobase (kb)] regions of sodium channel cDNA covering the nucleotide to be altered were subcloned into pGEM7-Z. A modified version of the *dut*$^-$, *ung*$^-$ procedure outlined by Kunkel and colleagues (20, 21) was used with 20-base-pair (bp) oligonucleotides containing a single base pair mismatch. The resulting colonies were screened by radiolabeled oligonucleotide hybridization and sequenced. The modified fragment was then inserted into the full-length sodium channel cDNA (pVA200).

Expression of α Subunit mRNA in *Xenopus* Oocytes. Functional channel mRNAs were generated by linearizing each construct with *Cla* I and transcribing *in vitro* with T7 RNA polymerase as described (4, 22). Individual oocytes were injected with 7 ng of each RNA except for M860, for which 0.7 ng was injected. Seventy nanograms of poly(A)$^+$ RNA and 7 ng of RNA from the original construct (pVA200) were injected as controls. After 2 days at 20°C the oocytes were analyzed using a two-electrode voltage clamp as described (4, 5). The recording solution consisted of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.5). Passive and capacitive components were eliminated by subtraction of equivalent records obtained in the presence of 400 nM tetrodotoxin (TTX).

RESULTS

Two Closely Related Sodium Channel Sequences. We have previously reported that the DNA sequence of the seven cDNAs used to construct the full-length rat IIA cDNA indicated only 36 nucleotide differences from the sequence reported for rat II. Most of these differences are in third codon positions, and in total they result in only six amino acid substitutions between the two predicted proteins (4). We have now resequenced the full-length rat IIA construct, pVA200. The results confirm the presence of the 36 nucleotide differences and have uncovered a previously undetected nucleotide difference at position 2580. This additional variant nucleotide encodes a phenylalanine in rat IIA at amino acid position 860 in the S4 segment of homology domain II where the rat II sequence encodes leucine. All seven variant amino acids are listed in Table 1. We have used RNase protection and polymerase chain reaction (PCR) amplification methods to examine the possibility that some of these residues may have arisen from cDNA cloning artifacts (data not shown). RNase protection experiments on rat brain RNA indicated that the 144 nucleotides (nucleotides 565–708 in ref. 4) that contain 22 of the total of 36 variant nucleotides found in the rat IIA sequence, including the codons for aspartate positions 189 and 209, were present in the *in vivo* rat IIA transcripts. On the other hand, PCR amplification and sequencing of the genomic DNA around nucleotide position 2580 clearly showed that the phenylalanine residue encoded here was due to an artifact of the cloning procedures. Leucine was encoded by the seven independent genomic sequences determined.

Table 1. Summary of the amino acid differences between rat IIA and rat II

Nucleotide position	Amino acid position	Amino acid change		Position in structure model*
		Rat IIA	Rat II	
565	189	Asp	Asn	Domain I, S3
625	209	Asp	Asn	Domain I, S3
1613	538	Arg	Gln	Interdomain I, II
1736	579	Ser	Arg	Interdomain I, II
2580	860	Phe	Leu	Domain II, S4
3185	1062	Asp	Gly	Interdomain II, III
4066	1356	Leu	Val	Domain III, S5

*Ref. 7.

We have not ruled out the possibility of cDNA artifacts in the remaining four variant nucleotides that cause changes in the protein sequence (1613, 1736, 3185, and 4066).

Site-Directed Mutagenesis. To determine which residues were responsible for the altered gating properties of rat IIA, the codon for each of the variant amino acids was altered through site-directed mutagenesis. The seven variant amino acids were changed to the corresponding residues in rat II (Table 1). Site-directed mutagenesis was carried out using the *dut*$^-$, *ung*$^-$ system (20) with oligonucleotides containing single base pair mismatches. Each altered subclone was sequenced and then placed back into the sodium channel construct pVA200. The final constructs were tested by restriction analysis to detect any rearrangements or abnormalities. Further manipulations were carried out to produce an α subunit that was altered at six of the seven sites (189, 209, 538, 1062, and 1356).

Expression of the Modified Sodium Channel Constructs. Individual constructs were linearized and RNA synthesis was carried out *in vitro* using T7 RNA polymerase (4). Injection of RNA transcribed from the sodium channel constructs with altered residues at positions 189, 209, 538, 579, 1062, and 1356 independently and combined (pM123456) resulted in currents that exhibited similar depolarizing shifts in the *I–V* relationships as was observed for pVA200 RNA-induced currents (Fig. 1). On the other hand, the single amino acid change at position 860 (Phe → Leu) resulted in sodium currents with a *I–V* relationship that peaks at −5 mV, indistinguishable from that induced by rat brain poly(A)$^+$ RNA (Fig. 2). In addition to restoration of the normal *I–V* relationship, this single amino acid substitution also dramatically increased the macroscopic sodium current in the injected oocytes. Currents approximately as large as those resulting from injection of 7 ng of pVA200 RNA were observed following injection of only 0.7 ng of pM860. When corrected for the shift in maximal conductance of M860 relative to VA200 (ca. −25 mV), these results imply an increase of at least 5-fold in numbers of functional channels expressed per unit amount of RNA.

As we have shown previously (4), macroscopic inactivation of rat IIA α subunit sodium channels is markedly slower than that of channels expressed from rat brain poly(A)$^+$ RNA (Fig. 3). Sodium channels with each of the altered residues shown in Table 1 also exhibited slower macroscopic inactivation (data not shown). In particular, sodium channels with the Phe → Leu substitution at amino acid position 860 demonstrated slow inactivation compared to rat brain poly(A)$^+$ RNA-induced channels (Fig. 3). This confirms that the specific amino acid substitution that restored the normal *I–V* curve did not affect the rate of macroscopic inactivation at the levels of expression studied. In addition, the steady-state voltage dependence of inactivation is similar for sodium channels resulting from injection of pVA200 RNA or pM860 RNA, with the single amino acid substitution. The voltage for half-maximal inactivation was between −30 and −35 mV in both cases (data not shown). Thus the single mutation shifting the peak of the *I–V* curve did not affect either the rate of inactivation or the steady-state voltage dependence of inactivation of the sodium channels.

DISCUSSION

Present models of the sodium channel predict that the S4 segment plays a critical role in the voltage-dependent conformational changes that underlie channel gating. In support of this idea, analogous sequences to S4 have been found in calcium and potassium voltage-activated channels. The unique feature of all S4-like sequences is the presence of four to eight positive amino acids spaced at three-residue intervals along a putative α-helical segment. It has been proposed that the S4 helix spans the membrane, with an orientation such

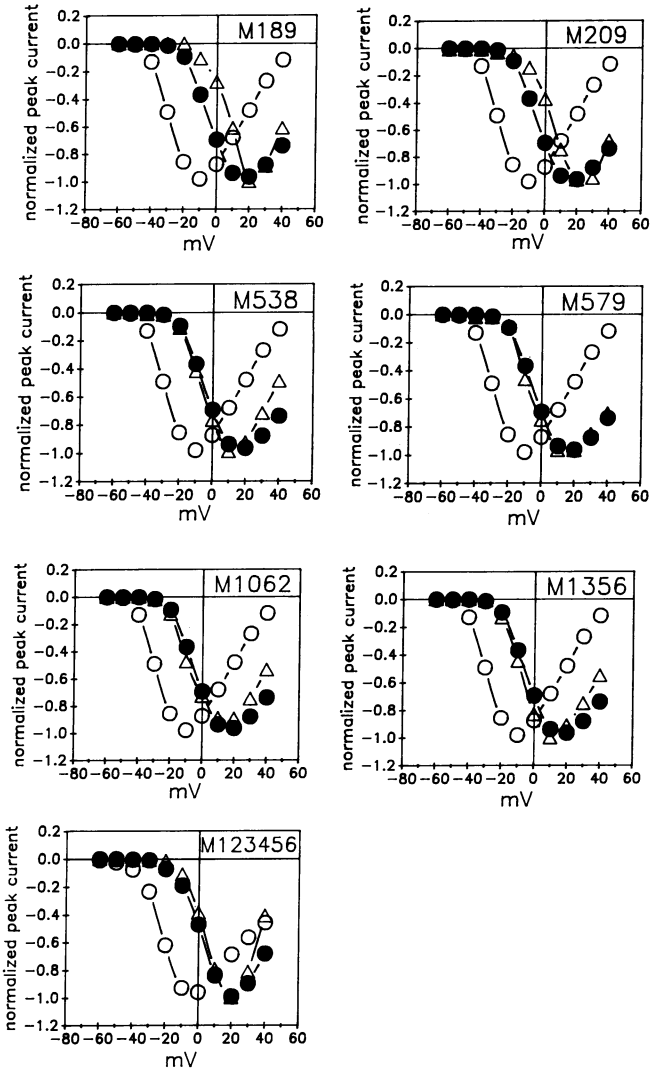

FIG. 1. *I–V* curves of currents following injection of RNA from mutated Na^+ channels. Oocytes were injected with 70 ng of rat brain poly(A)$^+$ RNA (○), 7 ng of *in vitro* transcribed RNA from the rat IIA VA200 clone (●), or 7 ng of *in vitro* transcribed RNA from the mutant channel constructs (△). The oocytes were held at −100 mV, and currents were elicited by 12-ms voltage steps from −90 mV to +50 mV in 10-mV increments. The normalized peak inward current during each depolarizing step was plotted versus the test potential. Individual mutants are indicated by the amino acid position that has been altered (see Table 1). M123456 represents RNA from one clone containing the six mutations shown separately in the figure.

that membrane depolarization causes a rotational twist of the S4 helix moving the positive charges during channel gating (1, 7). Recently, this proposal has been supported by the results of site-specific mutagenesis of the rat brain sodium channel (18). When positively charged residues in the S4 segments were replaced by neutral or negatively charged residues, activation was shifted along the voltage axis. Furthermore, such mutations of residues in the S4 segment of domain I reduced the slope of the voltage dependence curve, indicating direct involvement of these positive charges in gating. Although detailed mechanisms for channel gating must await determination of channel structure, the evidence for direct involvement of the positive charges in the S4 segments in the response to depolarization is compelling.

The present study demonstrates that alteration of a single neutral residue, leucine-860, in the S4 sequence of domain II results in sodium channels with a shift in the *I–V* relationship of 20–25 mV toward more positive potentials. We believe that this shift arises from a change in the voltage dependence of

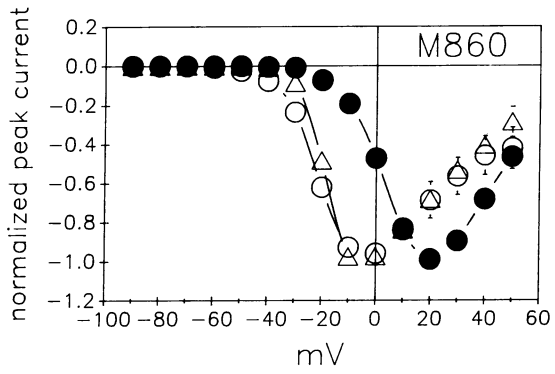

FIG. 2. *I–V* curve of currents following injection of mutant 860 RNA. Oocytes were injected with 70 ng of rat brain poly(A)$^+$ RNA (○), 7 ng of *in vitro* transcribed RNA from the rat IIA VA200 clone (●), or 0.7 ng of *in vitro* transcribed RNA from the M860 clone (△). Na^+ currents were recorded and *I–V* curves were plotted as described for Fig. 1. The data represent the mean ± SEM for at least six oocytes for each sample.

the probability of channel activation rather than from artifacts in electrophysiological recording from oocytes because (*i*) channels produced by rat II, rat IIA, and poly(A)$^+$ RNA have similar single channel conductances and linear single channel *I–V* relationships between −50 mV and 0 mV (5, 23),

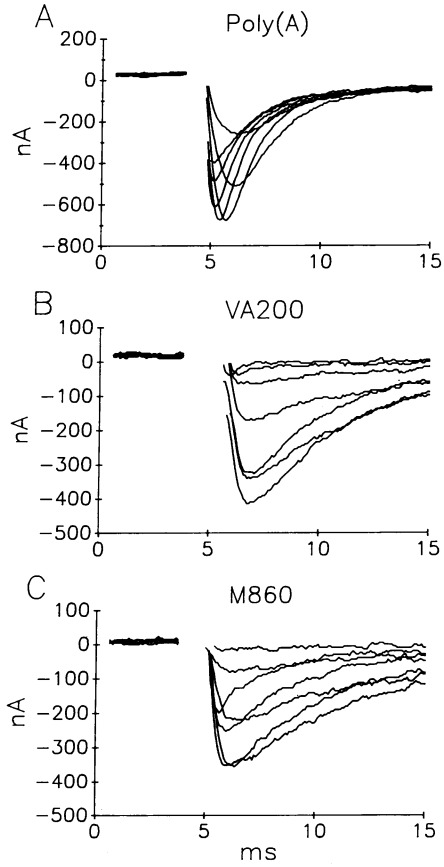

FIG. 3. Na^+ currents in *Xenopus* oocytes following RNA injection. Oocytes were injected with 70 ng of rat brain poly(A)$^+$ RNA (*A*), 1.4 ng of *in vitro* transcribed RNA from the rat IIA VA200 clone (*B*), or 0.14 ng of *in vitro* transcribed RNA from the M860 clone (*C*). Currents were elicited from a holding potential of −100 mV as described in the legend to Fig. 1. Resistive and capacitive currents have been eliminated by subtracting the current during application of 400 nM TTX. Only the traces for test pulses from −30 mV to +30 mV are shown.

(*ii*) sodium channels produced by M860 and VA200 give currents with similar time courses, and (*iii*) comparisons were made for current amplitudes differing by <2-fold, whereas *I–V* relationships were little affected by 10-fold changes in level of expression.

The 20- to 25-mV shift in activation between VA200 and M860 is due to the alteration of a single neutral amino acid residue, leucine-860, in the S4 sequence of domain II (Fig. 4). This shift is approximately equivalent to the shift in activation observed by Stuhmer *et al.* (18) from the combined neutralization of the two positive charges, 859 and 862, that are on each side of the leucine-860. We have considered several possible explanations for the dramatic effect of the Leu → Phe substitution.

One possibility is that the substitution of the larger, aromatic phenylalanine at site 860 disrupts the interactions of lysine-859 and lysine-862 with other elements of the voltage-sensing apparatus. The leucine residue could be permissive for the critical interactions of the charges, but not directly involved in the process. The insertion of the larger, aromatic phenylalanine side chain may shift the S4 helix within the channel tertiary structure, physically obstruct the movement of the S4 segment, or create additional stable hydrophobic interactions with surrounding residues. On the other hand, the fact that phenylalanine residues do occur in the S4 segments of domains I and II and that phenylalanine and leucine occur at equivalent positions in IIS4 when comparing rat, eel, and *Drosophila* sequences (Fig. 4) supports the idea that phenylalanine is not necessarily disruptive to the S4 structure.

A second possibility is that leucine-860 participates directly in the interactions that are critical for voltage activation. Our observation that alteration of leucine 860 causes a dramatic change in gating behavior suggests that leucine 860 is involved in a critical interaction with one or more adjacent transmembrane helices. As shown in Fig. 4, leucine-860 is conserved in all S4 segments in domain II sequenced to date. McCormack *et al.* (24) have made the intriguing observation that, in the cloned K^+ channels and Na^+ channels, the S4 segment C termini and flanking regions have sequences that resemble the leucine-zipper motif. The Leu → Phe alteration in rat IIA alters one of the leucine elements of this potential zipper. Disruption of this structure by substitution with phenylalanine could inhibit the conformational changes or interactions required for channel opening.

In the work of Stuhmer *et al.* (18), mutations that neutralize positive charges at the C-terminal side of S4 in domain I resulted in a positive shift in channel activation, whereas neutralizations on the N-terminal side resulted in a negative shift. These authors suggest that these modifications have changed the electric field at the voltage-sensing structures. Our results indicate that shifts in the peak of the *I–V* curve as severe as those resulting from removal of positive charges can occur by modifying a neutral residue in the C-terminal side of S4. Thus the structure of the S4 segment and its interactions with adjacent elements of the channel are probable factors in the observed voltage shifts, and these effects are as important as are possible changes in the local electric field.

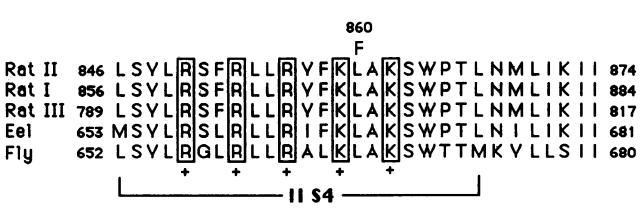

FIG. 4. Diagram of the S4 segment of domain II. Primary sequences for segment IIS4 from rat I (7), rat II (7), rat III (8), eel (6), and *Drosophila* (10) Na^+ channel α subunits are compared.

Two other mutations analyzed in the present study alter charged residues that may be at the membrane surface. These alterations, aspartic acid-189 and aspartic acid-209, alter charged residues on opposite sides of transmembrane segment S3 (4). Models of the sodium channel propose that the S3 segment may line the channel pore (12). As shown in Fig. 1, addition of a negative charge to either the intracellular side, aspartic acid-189, or to the extracellular side, aspartic acid-209, of S3 has no effect on the voltage dependence of activation. Although two channel cDNAs have been cloned with either aspartic acid (rat IIA) or asparagine (rat II) at positions 189 and 208, oocyte expression studies do not indicate a functional difference due to these amino acids.

The studies reported here show that a neutral mutation in the putative voltage-sensing S4 segment of the sodium channel has profound effects on the voltage gating properties and on the number of functional channels. Understanding the mechanisms of these effects will require the development of more detailed structural models for channel structure, particularly around the S4 motif, but our results indicate that the leucine residue at position 860 has a special role to play in channel assembly and function.

We find it a refreshing lesson in understanding how science sometimes progresses to note that a fortuitous mutation that would not have readily been conceived by *a priori* reasoning sheds significant light on the relationship between sequence and function for this complex channel.

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